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Development of Molecular Biological Methods to Analyze Bacterial Species Diversity in Freshwater and Soil Ecosystems

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A new method was developed for the rapid analysis of diverse bacterial species in the natural environment. Our method is based on PCR-single-strands-conformation polymorphism (PCR-SSCP) and selective isolation technique of single-stranded DNA. Variable V3 fragments of 16S rDNA were amplified by PCR with bacterial 16S rDNA primers, where one of the primers was biotinylated at the 5'-end. The biotinylated strands of the PCR products were selectively isolated by using streptavidin paramagnetic particles and a magnetic stand, to prevent SSCP analysis producing heteroduplexes from heterogeneous DNA samples. The selected strands were separated by electrophoresis on a polyacrylamide gel, and detected by silver staining. Analysis of PCR products from 8 bacterial strains demonstrated their characteristic DNA band patterns. In addition, changes in the structure of the bacterial community and species diversity in the microcosm treated with phenol could be monitored. After 3 weeks of incubation, phenol and its intermediate, 2-hydroxy-muconic-semialdehyde, were degraded by indigenous bacteria. These dominating bacterial populations were identified as strong bands on an SSCP gel. Therefore, this study provides useful tools for microbial community analysis of natural habitats.

Key words: PCR, SSCP, 16S rRNA, diversity analysis, phenol-degrading bacteria

Analysis of changes in the composition of a community can be used to characterize its response to stress or environmental changes. Species abundance and diversity measurements are sensitive indicators of environmental conditions (2). However, only small fractions of bacteria in natural environments can be isolated and characterized, thus the number of cultured bacteria is fewer than 10% of the bacteria found in nature and only about 5,000 species of bacteria have been accurately described (1, 24). There are many difficulties in understanding the structure of bacterial communities in natural ecosystems using present culture methods with selective media (4, 25).

Various molecular biological methods were used to reveal species composition of bacterial populations without a cultivation-dependent technique. A detailed view of the composition of a community can be obtained by sampling the nucleic acid population from a habitat in order to determine the abundance of phylogenetically meaningful sequences, particularly of 16S rDNA. Many of those attempts have been performed intensively with cloning and sequencing of 16S rDNAs (3, 9, 14, 19). In spite of many applications, the sequencing methods are inadequate for ecological studies as such methods require much time and effort. Hence, these methods can not provide an immediate overview of the structure of whole bacterial assemblages. The technique of generating a pattern from sequence variants with phylogenetic meaning is desirable from different points of views (7, 11, 15). The electrophoretic pattern yields a representation of the community as a whole or a section of it, defined by the selected primers. With such techniques, it is possible to generate and compare characteristic products or patterns from both cultivated isolates and directly extracted microbial community DNA (10).

Our procedure is based on PCR, single-strands-conformation polymorphism (SSCP), and selective isolation technique of single-stranded DNA. The SSCP analysis is a simple and effective method for the detection of minor sequence changes in DNA amplified by PCR (22). Under non-denaturing conditions, single-stranded DNA has a folded structure which is determined by intramolecular interactions and its nucleotide sequence. The electrophoretic mobility of DNA in a gel is dependent not only on its size and molecular weight, but also on its topology (27). Therefore, in SSCP analysis, DNA fragments that have equal size, but different sequences can be separated into different bands by polyacrylamide gel electrophoresis due to the different mobility of their folded structure (6).

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Since no GC clamp primers, gradient gels, or specific apparatus are required, SSCP is potentially more simple and straightforward than denaturing gradient gel electrophoresis (DGGE) or thermal gradient gel electrophoresis (TGGE) analysis. The PCR-SSCP technique was proposed as a simple and efficient method in studying bacterial communities (7). However, few disadvantages can be noticed in SSCP analysis. These include the high rate of reannealing of DNA strands after initial denaturation during electrophoresis (21), and the presence of more than one band from one population due to the different shape of two complementary single-stranded DNA and the formation of heteroduplex DNA with similar sequences of single-stranded DNA from others (7, 20).

The purpose of this work was to develop a molecular method to analyze bacterial species diversity in aquatic and soil ecosystems. The specific variable region in 16S rRNA genes was amplified, and single-stranded DNA was isolated and analyzed by the SSCP technique. Furthermore, the monitored results of the microcosm added with phenol showed that this procedure is applicable to the assessment of bacterial community structure in freshwater and soil ecosystems.

Materials and Methods

Bacterial strains and culture

The bacterial strains listed in the table 1 were obtained from the Korean Collection for Type Cultures (KCTC). After 24h of cultivation in recommended culture media and conditions, the bacterial colonies were harvested, placed into microtubes with sterile toothpicks, and their genomic DNAs were extracted.

Design of microcosm experiments

The water sample for the microcosm experiment was obtained from the surface layer of a small stream in Cheongju city (Korea), where the discharge from a waste-water treatment plant and fresh water of the upper stream were mixed together. A sample was collected in a sterile bottle (9 l) and stored in a cooler. In the laboratory, the water sample was prefiltered with filter paper (Whatman No. 2) and then divided equally into 4 one-liter-glass-bottles. Two replicates of experimental treatments were performed by adding phenol solution (final concentration of 200 ppm). The experimental and control samples were shaken (120 rpm) in the dark at 27°C (*in situ* temperature) for 3 weeks. The degradation of phenol and its intermediate, 2-hydroxy-muconic-semialdehyde, were indicated by a decrease in absorbance at 270 and 375 nm, respectively, and measured spectrophotometrically. After the desired incubation, bacterial cells were filtered by a sterile Sterivex-GV filter unit (pore size 0.22 μm, Millipore, U.S.A.) and stored at -70°C. In order to isolate the dominant phenol-degrading bacteria from the incubated samples, 0.5 ml of the sample was spread onto agar plates containing MM2 medium and 200 ppm of phenol. The plates were incubated at 25°C for 7 days. The colonies that appeared on one plate were picked and then restreaked onto new plates. This purification procedure was repeated several times.

Table 1. Standard strains used to evaluate the PCR-SSCP analysis method

Strains	KCTC[a] No.	Size[b] of PCR product (bp)
Staphylococcus aureus	KCTC 1916	204
Acinetobacter calcoaceticus	KCTC 2357	205
Bacillus subtilis	KCTC 1021	205
Bacillus megaterium	KCTC 3007	204
Escherichia coli	KCTC 1039	204
Nocardioides simplex	KCTC 9106	184
Micrococcus luteus	KCTC 1056	184
Zoogloea ramigera	KCTC 2531	179

[a]KCTC: Korean Collection for Type Cultures

[b]The expected size of products was calculated from sequences of the ribosomal database project (RDP).

Nucleic acid extraction

The bacterial genomic DNAs of standard strains and samples of microcosm were prepared using the protocol as described by Rhochelle *et al.* (18). The bacterial cell pellet, resuspended in 300 μl of lysozyme solution (0.15 M NaCl, 0.1 M EDTA, pH 8.0, lysozyme 15 mg · ml^{-1}), was incubated for 1 h at 37°C while gently mixing the solution at 15 min intervals. The sample was cooled on ice. After adding 300 μl of SDS buffer (0.1 M NaCl, 0.5 M Tris-HCl, pH 8.0, SDS 4%), the sample was incubated in an ice bath for 10 min, and then stood for 10 min at 55°C. This freezing-thawing process was repeated three times. Bacterial genomic DNA was extracted and purified from cell lysates by two sequential phenol-chloroform extractions and isopropanol precipitation. The DNA pellet was washed with 70% ethanol and resuspended in a sterile TE buffer. These DNA preparations were stored at -20°C and used as template DNAs in the subsequent PCR.

PCR amplification of 16S rDNA fragments

The fragments of 16S rDNA were amplified from genomic DNA by PCR using two bacterial 16S rDNA primers SRV3-1 (5'-CGGYCCAGACTCCTACGGG-3'; *Escherichia coli* nucleotides 330 to 348) and SRV3-2 (5'-TTACCGCGGCTGCTGGCAC-3'; *E. coli* nucleotides 515 to 533) (7). Primer SRV3-2 was biotinylated at the 5'-end to selectively separate single-stranded DNA from the PCR products. The reaction mixtures contained 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 0.1 μM of each oligonucleotide primer, and 2.0 units of *Taq* DNA polymerase (Bioneer, Korea) at a final

volume of 100 μl. DNA templates were added at a final concentration of 1 ng · l^{-1}. PCR amplification was performed with a DNA thermal cycler (MJ Research, U.S.A.) under the following conditions: first for 5 min at 94°C, followed by 30 cycles (denaturation for 1.5 min at 94°C, annealing for 1.5 min at 62°C, extension for 2.0 min at 72°C), and finally for 20 min at 72°C. The PCR products were purified with DNA PrepMate (Bioneer, Korea) and electrophoresed in a 2.0% agarose gel.

Isolation of the biotinylated strands

The single strands with a biotinylated primer were selectively isolated from the PCR products by using Streptavidin MagneSphere paramagnetic particles (SA-PMP) and a magnetic separation stand (Promega, U.S.A.). PCR products were gently mixed with an equal volume of SA-PMP solution washed with 0.5× SSC buffer. After incubation at room temperature for 10 min, SA-PMP and PCR products were captured by the magnetic stand and washed with 0.1× SSC buffer three times. To denature PCR products and separate biotinylated strands, the sample was soaked in 0.2 M NaOH solution and incubated for 10 min at room temperature. After washing once again with 0.2 M NaOH, the samples were mixed with 25% NH_4OH solution and incubated at 65°C for 15 min to separate single-stranded DNA (ssDNA) from SA-PMP. Ammonia was removed by using a vacuum microcentrifuge and the obtained ssDNA pellet was resuspended in TE buffer.

SSCP pattern analysis.

The modified method of SSCP pattern analysis was used for the separation of PCR amplified products (8). After mixing with an equal volume of loading buffer (95% formamide, 10 mM NaOH, 20 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol FF), the DNA sample was heated for 3 min at 95°C, and then was cooled to room temperature before loading. The samples were electrophoresed in a 6% acrylamide-bis (49:1) gel (glycerol 10%) by using 0.6× TBE buffer for 4 h at 1000 volts. After electrophoresis, the acrylamide gel was silver stained by incubating in 0.15% silver nitrate solution for 10 min, rinsing with distilled water, developing with a reagent (1.5% NaOH, 0.01% sodium borohydride, 0.15% formaldehyde) for 5~10 min, washing with distilled water, fixing with 0.75% sodium carbonate solution for 10 min, and rinsing again with distilled water. The silver stained gel was scanned and analyzed by using the HP-6200C scanner (Hewlett Packard, U.S.A.) and Imager II system (Bioneer, Korea).

Results and Discussion

PCR amplification of the 16S rDNA fragments

In order to amplify the 16S rDNAs of microbial communities, universal primers were selected. When the primers of SRV3-1 and SRV3-2 were analyzed using the Probe Match program (the number of permitted mismatches was 0) from the ribosomal database project (RDP), 6,248 matches for SRV3-1 and 10,235 matches for SRV3-2 were found from the sequences of the bacteria domain (12). Amplified 16S rDNA fragments in different bacterial species with these primers correspond to positions 330 to 533 in *E. coli*, which include the V3 region (16). Eight bacterial strains that have various taxonomic positions were used to determine optimal PCR conditions. These bacterial 16S rRNA sequences were listed in the RDP database and the expected sizes of PCR products ranged from 179 bp to 205 bp (Table 1). The result of agarose electrophoresis with the PCR products showed that variable V3 regions of 16S rDNA were amplified successfully without any byproducts and these primers could be applied to the PCR of complex populations (Fig. 1).

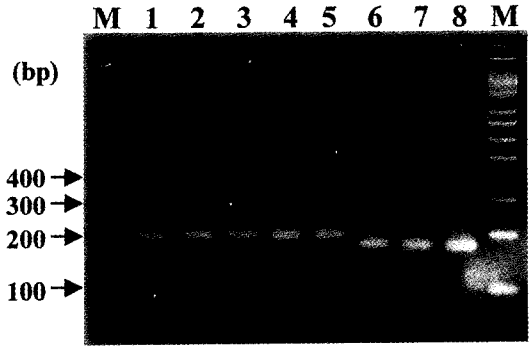

Fig. 1. Agarose gel electrophoresis of the PCR products amplified from standard strains. Lanes : M, size marker; 1, *S. aureus*; 2, *A. calcoaceticus*; 3, *B. subtilis*; 4, *B.megaterium*; 5, *E. coli*; 6, *N. simplex*; 7, *M.luteus*; 8, *Z. ramigera*.

SSCP analysis with single-stranded DNAs

A PCR product from a single strain showed two bands corresponding to two single-stranded DNAs (ssDNA) separated from double-stranded DNA (dsDNA) (Fig. 2, lanes 1 and 2). Since the mobilities of ssDNAs and heteroduplex DNAs were slower than that of dsDNAs, the band of renatured dsDNAs was observed on the bottom of the gel (data not shown). In addition, dsDNA and ssDNA were individually distinguishable by their stained color. The color of dsDNA was dark-brown, while that of ssDNA was red-brown. When a sample containing PCR products of two strains applied to SSCP analysis, two additional dark bands, which were not identified from individual experiments in a single strain, were also observed in the upper area (Fig. 2, lane 3). According to the previous report (7), these new bands were dsDNAs of heteroduplexes generated from the complementary strands of each strain. Although the positions of heteroduplex DNA bands are related to the size and sequence homology

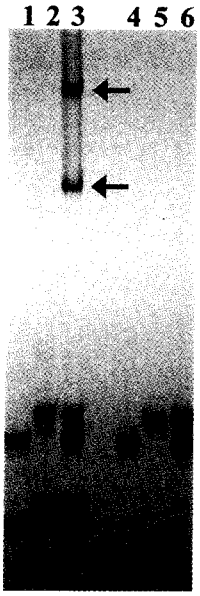

Fig. 2. SSCP analysis and selective isolation of biotinylated ssDNAs from the PCR products. Lanes: 1, PCR product of *B. subtilis*; 2, PCR product of *A. calcoaceticus*; 3, mixture of PCR products of *B. subtilis* and *A. calcoaceticus*; 4, isolated ssDNA of *B. subtilis*; 5, isolated ssDNA of *A. calcoaceticus*; 6, mixture of isolated ssDNAs of *B. subtilis* and *A. calcoaceticus*. Heteroduplex DNAs formed from complementary strands of two strains are marked with arrows.

of DNAs of two strains, and provide information about bacterial communities to some extent, the heteroduplex bands can not represent a single strain but the combination of two strains and may make complex banding patterns, which are difficult to interpret, especially in the analysis of natural populations. In addition, the high rate of reannealing of DNA strands after initial denaturation during electrophoresis generated obscure results and required high concentrations of DNAs.

Due to these problems, improved methods are needed which do not produce the heteroduplex bands. This is achieved by SSCP analysis with single strands of the PCR products. In order to select specific single strands from the mixed samples, biotinylated SRV3-2 primer was applied to PCR amplification. Only the biotinylated single strand could bind to the paramagnetic particles which were coated with streptavidin. After denaturation with 0.2 M NaOH solution, paramagnetic particles with a single biotinylated strand were isolated by a magnetic stand. With these modified methods, only ssDNAs were observed in lanes that were loaded with PCR products of two strains (Fig. 2, lanes 4, 5 and 6).

Another procedure for selective preparation of ssDNAs from PCR products was proposed by Schwieger and Tebbe (20), where the phosphorylated DNA strand was removed by lambda exonuclease digestion. They suspected that phosphorylation would be a less drastic modification of a primer than biotinylation, as judged by the

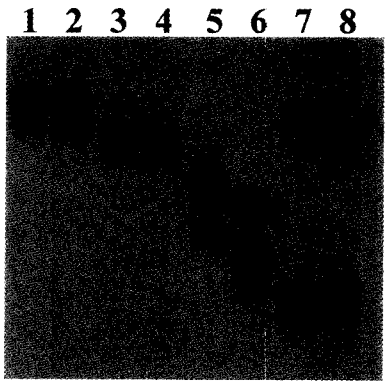

Fig. 3. Characteristic SSCP patterns of ssDNAs generated from standard strains. Lanes: 1, *S. aureus*; 2, *A. calcoaceticus*; 3, *B. subtilis*; 4, *B. megaterium*; 5, *E. coli*; 6, *N. simplex*; 7, *M. luteus*; 8, *Z. ramigera*.

molecule size, and therefore it would interfere less with DNA polymerase during PCR amplification. However, we assumed that physical separation by magnetic force had less artifacts than removal by an enzyme reaction. The enzyme reaction may be influenced by conditions such as pH, temperature and complex PCR products. When considering denaturing gradient gel electrophoresis (DGGE), where primers with long GC-rich sequences (approximately 40-mers) are used (15), we believe that a biotinylated primer may have less bias in PCR.

SSCP analysis with the standard strains

To evaluate the potential of SSCP for community analysis, PCR products from 8 bacterial strains were analyzed. Since ssDNAs of similar lengths were distinguished from each other by different mobilities based on secondary structures, SSCP analysis with single isolated strands (using magnetic stand) demonstrated species-specific band patterns, which were distinct and highly reproducible genetic fingerprints (Fig. 3). However, the ssDNA of a strain frequently produced double bands on a high resolution gel. These bands, with different electrophoretic mobilities, were reproducible with repeated experiments. After treatment with a Klenow fragment, the upper band was less apparent or disappeared (data not shown). It is possible that the PCR products, a mixture of blunt-end and single-A overhangs, were responsible for the formation of the observed double bands.

Secondary structure in 16S rRNA is greatly conserved (12, 16). It seems that only limited sequence changes can cause detectable structural changes of ssDNA and separate the bands in the complex sample. However, our PCR products contain 450 loops/stem region of V3 fragment, which is one of the variable regions that showed great variations among the genera and species (5, 7). Furthermore, it was found that because of its high resolving power, polyacrylamide gel electrophoresis could distinguish most conformational changes caused by subtle

sequence differences such as one base substitution in several-hundred-base fragments (22). If PCR products of the strains belonging to the same group have identical shape, they will not produce distinguishable bands through the SSCP analysis. For example, *A. calcoaceticus* and *E. coli* belong to the proteobacteria gamma group, and *M. luteus* and *N. simplex* are clustered to the high G+C gram-positive bacteria. However, the location of the DNA bands of these bacteria differ from each other (Fig. 3, lanes 2, 5, 6 and 7). Therefore, the SSCP technique can be used to separate 16S rDNA fragments which are amplified from samples of complex bacterial populations.

SSCP analysis with the mixed sample

The performance and sensitivity of our molecular biological methods were evaluated by analyzing PCR products from samples with variable ratios of three strains: *A. calcoaceticus*, *B. megaterium* and *N. simplex*. The genomic DNA of *B. megaterium* was serially diluted fourfold in a mixture of template DNAs of three strains, while those of *A. calcoaceticus* and *N. simplex* were kept constant. As shown in Fig. 4, different intensities of DNA bands amplified from *B. megaterium* were observed in the SSCP profile. The bands of *B. megaterium* were detected in samples serially diluted up to 4^{-5}. This high sensitivity will be useful in analysing minor populations in complex communities.

The abundance of distinct ssDNAs within the sample represents the community composition. The bands of the pattern are not directly interpretable by phylogenetic terms. Their fingerprints, however, represent the frequency of distribution of different sequences in a sample. Variation in such fingerprints due to induced variations in community composition may be easily observed. The degree of pattern change may be quantified to some extent for ranking purposes. Finally, band isolation and sequencing is one straightforward possibility to investigate induced changes in detail with respect to responsive community members.

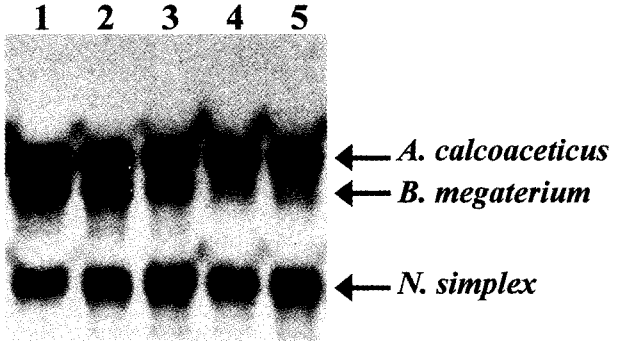

Fig. 4. PCR-SSCP analysis of genomic DNA mixtures of three strains in various ratios. The genomic DNA of *B.megaterium* was serially diluted four-fold in the mixture of template DNAs of three strains, while those of *A. calcoaceticus* and *N. simplex* were kept constant.

Analysis of bacterial communities in microcosm

The microcosms for the control and phenol treatment were incubated for 3 weeks. The degradation of phenol was monitored by a spectrophotometer (Fig. 5). 2-Hydroxymuconicsemialdehyde, an intermediate of phenol degradation, was yellow after 1 day of incubation and showed maximal optical densities at 2 days. After 3 weeks of incubation, most of the phenol and 2-hydroxymuconicsemialdehyde were eliminated. The average rate of phenol degradation was 4.6 %·day^{-1} for 3 weeks. It is likely that discharge of wastewater contains many bacteria which have potential to degrade phenol and 2-hydroxymuconicsemialdehyde. Phenol degradation and production of 2-hydroxymuconicsemialdehyde were not observed in sterile water which was added with phenol and incubated as the microcosm. The bacterial populations were harvested from the microcosm on three occasions (beginning of incubation, 2 weeks, and 3 weeks). The genomic DNA was extracted and analyzed by the selective PCR-SSCP technique in this study. Many distinctive bands in the separation patterns were detected. These bands were most likely derived from many different bacterial species constituting microbial communities (Fig. 6). Although the same protocols were used for DNA extraction, PCR, single strand isolation, and SSCP pattern analysis, the banding patterns of the microcosm treated with phenol differed from the controls. A dramatic change in the community was observed in the treated sample after 2 weeks, where about 75% of added phenol was degraded (Fig. 6, lane 4). At least 7 intensive bands from the dominant populations, which may degrade phenol or 2-hydroxy-muconicsemialdehyde, could be identified, while the control sample showed more complex and diverse banding patterns. However, after 3 weeks the phenol was used up by the bacteria and biodiversity of the community was restored

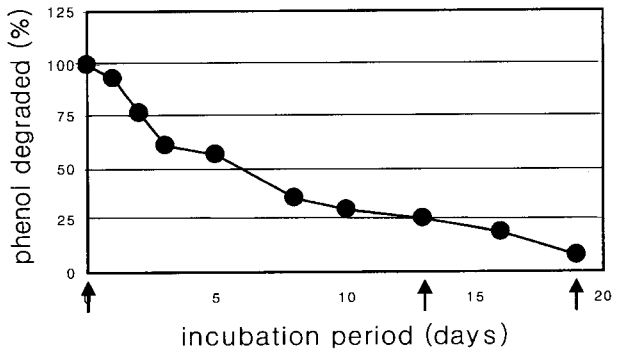

Fig. 5. Degradation of phenol by indigenous bacteria in the microcosm. DNA extractions for PCR-SSCP analysis were marked as arrows on the X-axis.

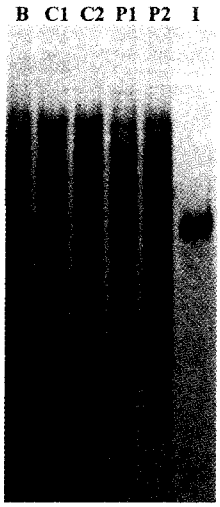

Fig. 6. PCR-SSCP analysis of bacterial communities in the microcosm and the isolated strain. Lanes : B, initial stage of the experiments; C1, control sample incubated for two weeks; C2, control sample incubated for three weeks; P1, sample added with phenol incubated for two weeks; P2, sample added with phenol incubated for three weeks; I, phenol-degrading strain isolated from P1.

(Fig. 6, lane 5).

The phenol-degrading heterotrophic bacteria were also isolated from the sample with phenol after 2 weeks of incubation. We obtained 10 strains from one agar plate, but all of them showed the same SSCP bands (data not shown). We suspect that these strains are the same strains having close phylogenetic relations. Thus, it appears that the culture technique failed to isolate bacteria, which adapted to changes in the environment and generated 7 predominant bands through SSCP analysis, because of intrinsic selectivity (Fig. 6, lanes P1 and I). The results indicate the existence of uncultivated bacteria which degrade phenol and 2-hydroxy-muconicsemialdehyde in the aquatic ecosystem. Such results from activated sludge were reported by Watanabe *et al.* (26) and correlates with the opinion that the fractions of cultured bacteria are fewer than 10% of microorganisms found in the natural environment (1), and reports of viable, but nonculturable (VBNC) cases in many bacterial strains (13, 17). Although there are biases in molecular ecological methods, these appear to be less limiting than those of culture-based analyses. Thus it appears that the rRNA approach, together with other molecular techniques, bears great potential for analysis of microbial diversities in the natural environment.

In conclusion, the modified method described here provides an immediate display of whole members of a complex bacterial population. It is less time-consuming, less laborious and offers several advantages over previous approaches. In contrast to DGGE, larger gels can easily be applied to an increase in the resolution of the gel. Rapid and detailed information can be obtained by reamplification and sequencing of separated bands in the gel. Future developments may further enhance the performance of the PCR-SSCP approach. Therefore, our methods consisting of direct DNA extraction, amplification of 16S rDNA fragment with PCR, selective isolation of ssDNA, and SSCP pattern analysis is useful in studying temporal and spatial changes in bacterial community structures existing in freshwater and soil ecosystems.

Acknowledgments

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