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Nonradioactive Method To Study Genetic Profiles of Natural Bacterial Communities by PCR–Single-Strand-Conformation Polymorphism

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We describe a new method for studying the structure and diversity of bacterial communities in the natural ecosystem. Our approach is based on single-strand-conformation polymorphism (SSCP) analysis of PCR products of 16S rRNA genes from complex bacterial populations. A pair of eubacterial universal primers for amplification of the variable V3 region were designed from the 16S rRNA sequences of 1,262 bacterial strains. The PCR conditions were optimized by using genomic DNAs from five gram-positive and seven gram-negative strains. The SSCP analysis of the PCR products demonstrated that a bacterial strain generated its characteristic band pattern and that other strains generated other band patterns, so that the relative diversity in bacterial communities could be measured. In addition, this method was sensitive enough to detect a bacterial population that made up less than 1.5% of a bacterial community. The distinctive differences between bacterial populations were observed in an oligotrophic lake and a eutrophic pond in a field study. The method presented here, using combined PCR amplification and SSCP pattern analyses of 16S rRNA genes, provides a useful tool to study bacterial community structures in various ecosystems.

Although the study of the structure and function of bacterial communities is crucial to an understanding of the dynamics and stability of the natural ecosystem, the limited methodology available at present hardly makes it possible to study species composition and diversity of whole bacterial communities. Only small fractions of bacteria in natural environments can be isolated and characterized (37); fewer than 10% of the bacteria found in nature have been cultured, and only 5,000 species of bacteria have been accurately described (1). There are many problems in using the present culture methods with selective media to study the structure of bacterial communities in natural ecosystems (7, 35). Various molecular biological methods have been used to attempt to determine the species composition of bacterial populations without enrichment culture. Many of those attempts involved cloning and sequencing of the 16S rRNAs (3, 4, 32). The results from various marine ecosystems support the notion that novel lineages of proteobacteria are one of the most abundant members of bacterioplankton communities in subtropical oceans (5, 22, 36).

In spite of their many other applications, there are practical problems in using molecular biological methods for ecological studies. Because significantly large numbers of samples must be analyzed in the surveys assessing temporal and spatial dynamics of bacterial populations in a natural ecosystem, the methods used must be more rapid and simpler. The sequencing methods are inadequate for this purpose because they require a lot of time and effort and hence cannot provide an immediate overview on the structure of whole bacterial assemblages. The DNA hybridization method with a specific oligonucleotide probe is useful for a specific population as well as for dominant groups (2, 9, 28). However, it also needs as much time and effort as sequencing methods, since many parallel probes and experiments for each sample are required to analyze the complex community structure in natural ecosystems. To avoid these problems, restriction fragment length polymorphism patterns of 16S rRNA and the size variation of the spacer region between 16S and 23S rRNA were proposed as a new basis for a more simple and efficient method (12, 18, 21). However, the restriction fragment length polymorphism analysis, which is based on the size difference of digested fragments, has limited resolution in identifying a specific phylogenetic group within a complex community, since it cannot utilize sequence information other than restriction sites. In the case of spacer polymorphism analysis, the drawback is the fact that more than one kind of PCR product with different sizes can result from a single organism. For example, there are two kinds of spacers in *E. coli*. The *E. coli* genome is known to contain seven *rrn* loci (13, 14). In four of them, the spacer region contains a single $tRNA^{Glu}$ gene. The other three loci have two tRNA genes in this spacer region: $tRNA^{Ile}$ and $tRNA^{Ala}$. Another restriction in applying these methods is that known data on restriction fragment length polymorphism patterns and spacer regions are insufficient. Recently, a novel approach which can give better profiles of bacterial community structure was suggested by Muyzer et al. (23). They used the denaturing gradient gel electrophoresis method, which separates PCR products by the difference of nucleotide sequence in melting domains. However, some practical disadvantages remain. To generate the GC clamp indispensable for the stability of transitional molecules, a relatively long primer must be used, and it may cause artifacts in the annealing step of the PCR. In addition, the results of denaturing gradient gel electrophoresis may be affected by the heteroduplex molecules produced during PCR. The mismatched base pairs contained in heteroduplexes render them inherently less stable under the denaturing conditions of denaturing gradient gel electrophoresis (31).

Our procedure is based on PCR and single-strand-conformation polymorphism (SSCP). This method has been used primarily to detect known or novel polymorphisms and mutations in human genes (26, 27). Under nondenaturing conditions, single-stranded DNA has a folded structure which is determined by intramolecular interactions and its nucleotide

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sequence. The electrophoretic mobility of the DNA in a gel is dependent not only on its length and molecular weight but also on its shape (39). Therefore, in SSCP analysis, DNA fragments with the same size but different sequences can be separated into different bands in polyacrylamide gel electrophoresis because of the different mobilities of their folded structure (11).

The purpose of this work was to develop a new method for studying the structure and diversity of bacterial communities in an aquatic ecosystem. The PCR amplification of specific variable regions in 16S rRNA genes was optimized, and the amplified products were separated by SSCP-heteroduplex analysis. Comparison of distinct band patterns with a laser beam densitometer showed that this procedure is applicable to the assessment of bacterial community structure in various aquatic ecosystems.

MATERIALS AND METHODS

Bacteria. *Bacillus megaterium* KCTC 3007, *Bacillus subtilis* KCTC 1021, *Micrococcus luteus* KCTC 1056, *Nocardioides simplex* KCTC 9106, *Staphylococcus aureus* KCTC 1916, *Acinetobacter calcoaceticus* KCTC 2357, *Enterobacter pyrinus* KCTC 2520, *Escherichia coli* KCTC 1039, *Helicobacter pylori* KCTC 2691, *Pseudomonas cepacia* KCTC 2475, *Zoogloea ramigera* KCTC 2531, and *Zymomonas mobilis* KCTC 1534 were obtained from the Korean Collection for Type Cultures (KCTC). After 24 h of cultivation under the culture conditions recommended, the cells were harvested by centrifugation and bacterial DNA was extracted.

Nucleic acid extraction. A modification of the procedure described by Rochelle et al. was used in extracting DNA from bacteria (30). The bacterial cell pellet, resuspended in 300 μl of lysozyme solution (0.15 M NaCl, 0.1 M EDTA [pH 8.0] 15 mg of lysozyme ml^{-1}), was incubated at 37°C for 1 h with mixing by inversion every 15 min. The sample was cooled on ice. After addition of 300 μl of SDS buffer (0.1 M NaCl, 0.5 M Tris-HCl [pH 8.0], 4% sodium dodecyl sulfate [SDS]), the sample was incubated in an ice bath for 10 min and then let stand at 55°C for 10 min. The freezing-thawing process was repeated three times. Bacterial genomic DNA was extracted and purified from cell lysates by two sequential phenol-chloroform extractions and an isopropanol precipitation. The DNA pellet was washed with 70% ethanol and resuspended in sterile TE buffer. These DNA preparations were stored at −20°C and used as template DNAs in the subsequent PCR.

PCR. To design the eubacterial universal primers for amplification of 16S rDNA fragments, the database of eubacterial 16S rRNA sequences (EMBL) was analyzed with a computer program designed for this study (24). PCR amplification was performed with a DNA thermal cycler (Perkin-Elmer Co.) under the following conditions: 94°C for 5 min; 30 cycles of denaturation at 94°C for 1.5 min, annealing at 62°C for 1.5 min, and extension at 72°C for 2.0 min; 72°C for 20 min. Reaction mixtures (final volume, 100 μl) contained 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.1% Triton X-100, 200 μM each deoxynucleoside triphosphate, 0.1 μM each oligonucleotide primer, and 2.0 U of DynaZyme (Finnzymes Co.). DNA templates were added to a final concentration of 1 ng · μl^{-1}. The PCR products were electrophoresed in 2.0% agarose gel and stained with ethidium bromide.

SSCP-heteroduplex pattern analysis. The modified methods of SSCP-heteroduplex pattern analysis were used for the separations of amplified products (15). After being mixed 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 sample was heated for 3 min at 95°C and then cooled to room temperature before being loaded onto a gel. The samples were electrophoresed in a 6% acrylamide-bisacrylamide (49:1) gel (10% glycerol) with TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]) for 12 h at 550 v. In other experiments for the results without heteroduplex bands, alkali denaturing buffer (0.1 M NaOH, 20 mM EDTA) and a gel without glycerol were used to prevent the formation of heteroduplex DNAs. The loading dye (0.1% bromophenol blue plus 0.1% xylene cyanol FF in formamide) was added just before the sample was loaded. On terminating electrophoresis, the acrylamide gel was stained with 0.15% silver nitrate solution for 10 min, rinsed with distilled water, developed with a reagent (1.5% NaOH, 0.01% sodium borohydride, 0.15% formaldehyde) for 5 to 10 min, washed with distilled water, fixed with 0.75% sodium carbonate solution for 10 min, and rinsed again with distilled water. The silver-stained gel was covered with Saran wrap to prevent drying and photographed with GA-100 film (Fuji Co.) and an Autocompanica 690C camera (RBS Co.). The bands on the positive film were analyzed with a laser beam densitometer (UltroScan XL, Pharmacia Co.) for quantification.

Collection of natural bacterial population by filtration. Freshwater from Lake Soyang, Korea, was collected in a sterile bottle (2 liters) with a Niskin water sampler and stored in a cooler until it was received in the laboratory. In the laboratory, the water samples were prefiltered with a sterilized nitrocellulose membrane (pore size, 3.0 μm; Nuclepore Co.), and the filtrates were filtered again with a stirred ultrafiltration cell (Amicon Co.) and YM100 disk membranes (molecular weight cutoff, 100,000; Amicon Co.) under N_2 gas at constant pressure (20 lb/in^2). When the bacterial cells were observed by the acridine orange direct count method, most of them were smaller than 2.0 μm in diameter and cells greater than 3.0 μm were rare. Therefore, the bacterial cells collected on the 3.0-μm filter and ultrafilter were used for analyses of the particle-attached and free-living bacterial populations, respectively. To detach the bacteria from the membrane, the filter was soaked with a small volume of sterile STE buffer (0.1 M NaCl, 10 mM Tris, 1 mM EDTA [pH 7.6]) for 1 h at 4°C. The resuspended buffer was centrifuged (10,000 × *g*) for 15 min at 4°C, and supernatant was removed gently. This detaching step was repeated four times, and the collected cell pellet was stored at −70°C until used for DNA extraction.

Collection of a heterotrophic bacterial population by cultivation. Unfiltered samples (1 ml) for the analysis of heterotrophic bacterial population were inoculated on Zobell 2216e medium with distilled water and incubated for 2 weeks at 20°C. After the selection of plates containing 50 to 100 colonies, all bacterial colonies were put into microtubes with sterile toothpicks. Cultured bacterial samples were stored at −70°C until used for DNA extraction.

RESULTS

PCR. The nucleotide sequences of the primers used in this study are as follows: SRV3-1, 5′-CGGYCCAGACTCCTAC GGG-3′; SRV3-2, 5′-TTACCGCGGCTGCTGGCAC-3′ (Y is C or T). Amplified 16S rDNA fragments in the different bacterial species with these primers correspond to positions 330 to 533 in *E. coli*. Each nucleotide of the primers was conserved in more than 98.5% of 1,262 strains in the EMBL database, except nucleotides 4, 6, 7, and 8 in SRV3-1, which were conserved in 88.0 to 97.9% of the tested strains. To check our primers, the Check_Probe program served by the Ribosomal Database Project was used with the prokaryotic-small-subunit-rRNA database containing 2,709 eubacterial strains (17). When the number of permitted mismatches was 2, the results showed that 2,144 strains could be aligned with SRV3-1 and 2,259 strains could be aligned with SRV3-2.

Five gram-positive and seven gram-negative bacteria with different taxonomic positions were used to determine the optimal PCR condition for the diverse bacterial strains (Table 1). Ten strains whose 16S rRNA sequences were listed in the database had V3 regions of different sizes and contained a common recognition site (5′-CGCG-3′) for the restriction endonuclease (*Bst*UI). The expected sizes of PCR products from these strains could be divided into four groups: 205 bp in two strains, 204 bp in four strains, 184 bp in two strains, and 179 bp in two strains. To evaluate our universal primers, two strains whose 16S rRNA sequences were not known were also included in the experiments. After the optimization of PCR conditions, to confirm if the PCR products correspond to the V3 region of 16S rRNA gene, they were digested with *Bst*UI and analyzed by 2% agarose gel electrophoresis (Fig. 1). This result demonstrated that variable V3 regions of 16S rDNA were amplified successfully with our primers regardless of whether they were gram-positive or gram-negative bacteria. Therefore, the primer pair can be used to amplify the V3 region from the diverse bacterial populations in the environment.

SSCP-heteroduplex pattern analysis. To analyze the PCR products of 11 strains, the SSCP-heteroduplex patterns were observed by silver staining (Fig. 2). Bands of double-stranded DNAs of 179 to 205 bp were detected at the bottom of the acrylamide gel (Fig. 2b). The mobilities of single-stranded and heteroduplex DNAs were lower than those of double-stranded DNAs, and smaller products had higher mobilities (Fig. 2a). In addition, double-stranded and single-stranded DNAs were distinguished from each other by their stained colors. The double-stranded DNAs were dark brown, and the single-stranded DNAs were red-brown (data not shown). A PCR product from a single strain (*A. calcoaceticus*) showed two bands correspond-

TABLE 1. Standard strains used to evaluate the PCR and SSCP-heteroduplex analysis method

Species	KCTC strain no.	Group[a]	Size of PCR product (bp) (sizes of two fragments by *Bst*UI digestion)	Sequence similarity with *A. calcoaceticus* (%)
Bacillus megaterium	KCTC 3007	18	204 (73 + 131)	67.8
Bacillus subtilis	KCTC 1021	18	205 (73 + 132)	68.3
Micrococcus luteus	KCTC 1056	17	184 (73 + 111)	75.6
Nocardioides simplex	KCTC 9106	22	184 (73 + 111)	70.6
Staphylococcus aureus	KCTC 1916	17	204 (73 + 131)	70.9
Acinetobacter calcoaceticus	KCTC 2357	04	205 (73 + 132)	100.0
Enterobacter pyrinus	KCTC 2520	05	Unknown	Unknown
Escherichia coli	KCTC 1039	05	204 (73 + 131)	87.3
Helicobacter pylori	KCTC 2691	02	179 (73 + 106)	70.2
Pseudomonas cepacia	KCTC 2475	04	204 (73 + 131)	82.0
Zoogloea ramigera	KCTC 2531	04	179 (73 + 106)	77.6
Zymomonas mobilis	KCTC 1534	05	Unknown	Unknown

[a] Numbers within the group column represent the groups in *Bergey's Manual of Determinative Bacteriology* (11a). Group 02, aerobic/microaerophilic, motile, helical/vibrioid gram-negative bacteria; group 04, aerobic/microaerophilic gram-negative rods and cocci; group 05, facultatively anaerobic gram-negative rods; group 17, gram-positive cocci; group 18, endospore-forming gram-positive rods and cocci; group 22, nocardioform actinomycetes.

ing to two single-stranded DNAs derived from a double-stranded DNA (Fig. 2a, lane A). When the PCR products that were amplified from the mixtures containing genomic DNAs of *A. calcoaceticus* and 1 of the other 10 strains were loaded, different banding patterns caused by the combinations of strains were observed (Fig. 2a, lanes 1 to 10). Two bands of single-stranded DNAs produced from *A. calcoaceticus* were observed in all lanes, and additional single-stranded DNA bands from mixed strains were also detected in each lane. In addition, single-stranded DNAs of similar lengths were distinguished from each other by the different mobilities based on secondary structures.

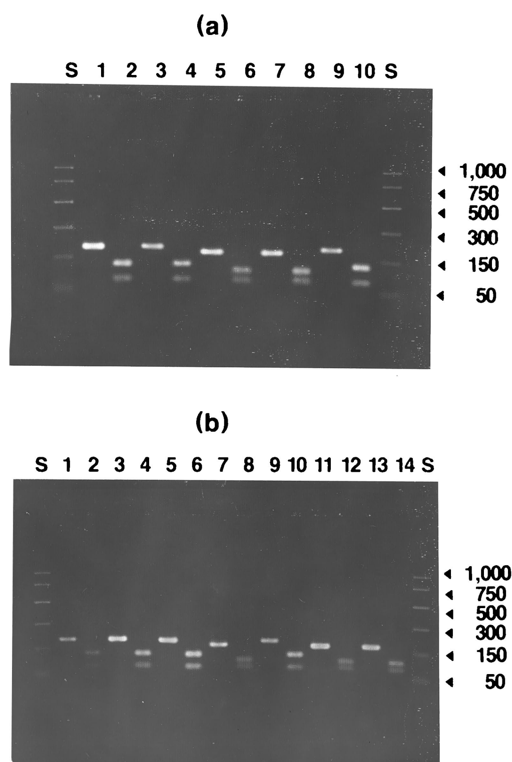

FIG. 1. *Bst*UI digestion pattern of the PCR amplification products for the bacterial genomic DNA samples. Odd-numbered lanes represent the PCR products, and even-numbered lanes represent the digested fragments. (a) *Bst*UI digestion pattern of the gram-positive strains. Lanes: S, size marker; 1 and 2, *Bacillus megaterium*; 3 and 4, *Bacillus subtilis*; 5 and 6, *Micrococcus luteus*; 7 and 8, *Nocardioides simplex*; 9 and 10, *Staphylococcus aureus*. (b) *Bst*UI digestion pattern of the gram-negative strains. Lanes: S, size marker; 1 and 2, *Acinetobacter calcoaceticus*; 3 and 4, *Enterobacter pyrinus*; 5 and 6, *Escherichia coli*; 7 and 8, *Helicobacter pylori*; 9 and 10, *Pseudomonas cepacia*; 11 and 12, *Zoogloea ramigera*; 13 and 14, *Zymomonas mobilis*.

Two additional dark bands, which were not identified from the individual experiments on a single strain, were also observed in mixed samples (Fig. 2a, lanes 1 to 10). From the evidence of stained colors, these new bands were double-stranded DNA. To identify new bands, these bands were cut from the gel and the DNAs were eluted in a small volume of TE buffer. When the DNAs of these bands were used as a template for PCR reamplification, their SSCP-heteroduplex pattern was identical to that of the mixed strains (Fig. 3). Therefore, the new bands were determined to be heteroduplex DNAs produced from single-stranded DNAs of each strain. The positions of heteroduplex DNA bands were related to the size of the DNA and the sequence homology of the two strains (Fig. 2c). As listed in Table 1, the strains had different sequence similarities to *A. calcoaceticus*. The heteroduplex DNAs of *A. calcoaceticus* and *E. coli* that showed the highest similarity value were observed at the bottom of the gel (Fig. 2c, lane 7). However, the bands produced from *A. calcoaceticus* and strains belonging to the low-G+C gram-positive group were located in the uppermost region (Fig. 2c, lanes 1, 2, and 5), and this group showed low sequence similarities to *A. calcoaceticus.*

The heteroduplex bands do not represent a single strain but a combination of two strains. Although the heteroduplex bands can be discerned by their stained color, they may form complex banding patterns in the analysis of natural populations. Because of these problems, there was a need for an improved method to obtain the results without heteroduplex bands. This was done by use of an alkali denaturing buffer (0.1 M NaOH, 20 mM EDTA) and a gel without glycerol. With these novel reagents, only single-stranded DNAs were observed in all lanes that were loaded with PCR products amplified from the mixed samples (Fig. 4). This approach can result in simple banding patterns in which a single band corresponds to a single species, with somewhat reduced resolution of the SSCP band patterns.

Sensitivity. The sensitivity of our methods was evaluated by analyzing PCR products from samples with different proportions of two strains, *A. calcoaceticus* and *Z. ramigera*. The genomic DNA of *A. calcoaceticus* was serially twofold diluted

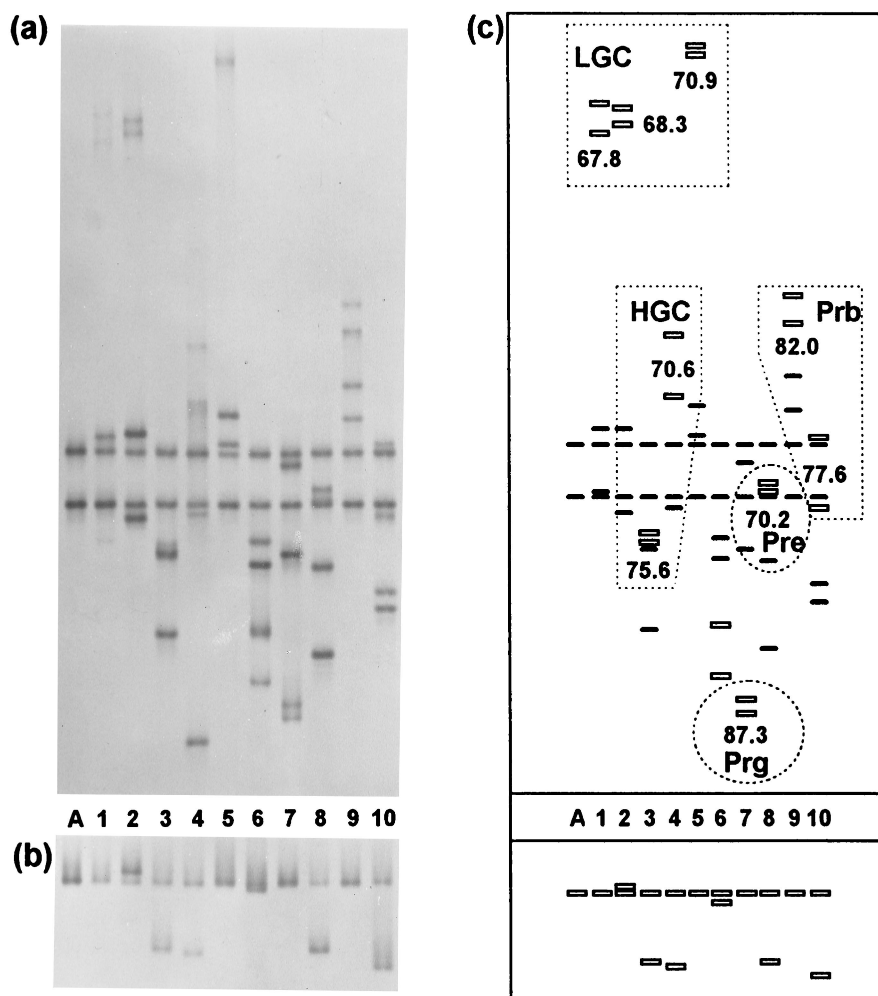

FIG. 2. SSCP-heteroduplex pattern analysis of the PCR amplification products of the mixtures of *A. calcoaceticus* and one of the other standard strains. (a) Single-stranded and heteroduplex DNA; (b) double-stranded DNA; (c) schematic diagram. Lanes: A, *A. calcoaceticus* alone; 1, mixed with *B. megaterium*; 2, mixed with *B. subtilis*; 3, mixed with *M. luteus*; 4, mixed with *N. simplex*; 5, mixed with *S. aureus*; 6, mixed with *E. pyrinus*; 7, mixed with *E. coli*; 8, mixed with *H. pylori*; 9, mixed with *P. cepacia*; 10, mixed with *Z. ramigera*. Numbers in panel c represent the sequence homology. HGC, gram-positive (high G+C) and relatives; LGC, gram-positive (low G+C) and relatives; Prb, beta *Proteobacteria*; Pre, epsilon *Proteobacteria*; Prg, gamma *Proteobacteria*. Symbols: ▬, single-stranded DNA; ▭, double-stranded and heteroduplex DNA.

in the mixture of target DNAs of the two strains, while that of *Z. ramigera* was kept constant. As shown in Fig. 5, the different intensities of DNA bands amplified from *A. calcoaceticus* were observed in the SSCP-heteroduplex profile. The double-stranded DNAs were not observed in the sample diluted to $1/2^4$. However, the bands of single-stranded and heteroduplex DNAs were detected in the samples serially diluted up to $1/2^5$ and $1/2^6$, respectively. This indicates that a bacterial population making up even 1.5% in the complex communities can be detected by heteroduplex bands.

Analysis of natural populations. The samples from surface water in Lake Soyang were analyzed to compare the culture isolation technique for heterotrophic bacterial populations with the direct filtration method in this study. In the SSCP-heteroduplex analysis, many distinctive bands in the separation pattern most probably derived from as many different bacterial species constituting microbial communities, were detected (Fig. 6). Although the same methods were used for DNA extraction, PCR, and SSCP-heteroduplex pattern analysis, the banding patterns of the cultivated bacterial sample (Fig. 6, lane 1) differed from those of the samples which were collected from the same sampling site by filtration (Fig. 6, lanes 2 and 3). It thus appears that the culture technique selects for bacteria which do not form predominant bands in the analysis of natural samples by the filtration method. In addition, more complex patterns were observed in directly filtered samples. The filtered sample showed more than 25 distinguishable bands, while the cultured sample showed 13 intensely stained bands. The result indicates that the existence of uncultivated bacteria in aquatic ecosystems and the direct filtration technique in our method is more useful for studying the diverse bacterial populations in environmental ecosystems.

The banding profiles of samples from an eutrophic pond and an oligotrophic lake, Lake Soyang, were also analyzed with a laser beam densitometer to observe the difference in the bacterial populations among trophic states (Fig. 7). The peak corresponding to the double-stranded DNAs of the large PCR products was greater than that of the small products in the eutrophic pond. In Lake Soyang, however, the peak corresponding to small products was higher and the peaks of het-

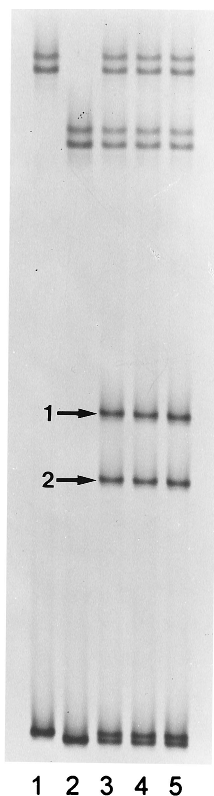

FIG. 3. SSCP-heteroduplex pattern analysis to confirm heteroduplex formation in mixed samples. DNAs loaded in lanes 1 to 3 are PCR products from the genomic DNA of *A. calcoaceticus* (lane 1), *Enterobacter pyrinus* (lane 2), and the genomic DNA mixture of the two strains (lane 3). For the last two lanes, the reamplified products of band 1 (lane 4) and band 2 (lane 5) were loaded.

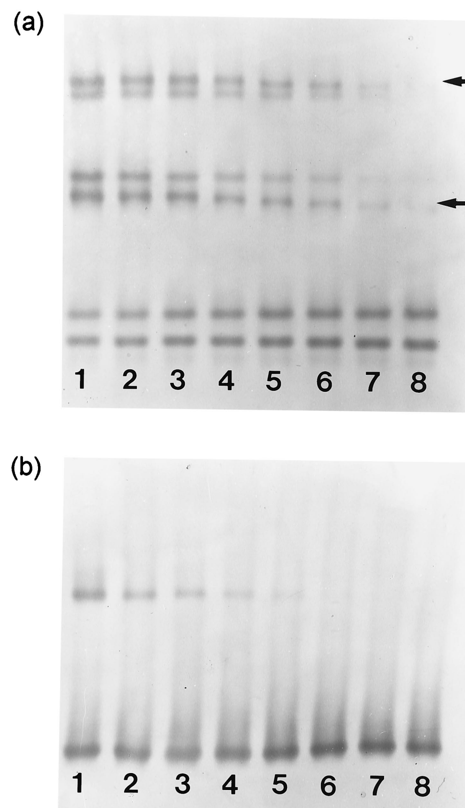

FIG. 5. SSCP-heteroduplex pattern analysis of the PCR amplification products for the genomic DNA mixtures of *A. calcoaceticus* and *Z. ramigera* in various ratios. The amount of genomic DNA of *A. calcoaceticus* was serially twofold diluted, while that of *Z. ramigera* was kept constant. The rows of heteroduplex bands are marked with arrows. (a) Single-stranded and heteroduplex DNAs; (b) double-stranded DNAs.

eroduplex and single-stranded DNAs differed from those in the eutrophic pond. We can infer that the structures of the bacterial communities in the two sampling sites differ from each other because of their different environments.

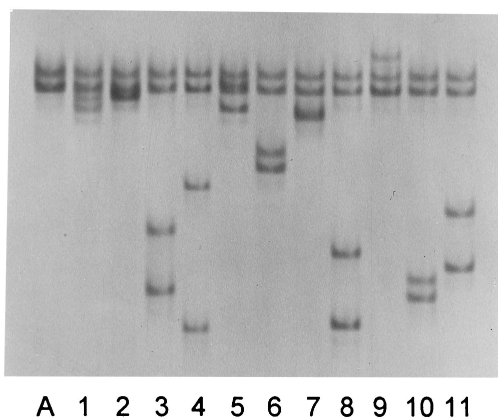

FIG. 4. SSCP pattern analysis of the PCR amplification products for the mixtures of *A. calcoaceticus* and one of the other standard strains. Lanes: *A. calcoaceticus* alone; 1, mixed with *B. megaterium*; 2, mixed with *B. subtilis*; 3, mixed with *M. luteus*; 4, mixed with *N. simplex*; 5, mixed with *S. aureus*; 6, mixed with *E. pyrinus*; 7, mixed with *E. coli*; 8, mixed with *H. pylori*; 9, mixed with *P. cepacia*; 10, mixed with *Z. ramigera*; 11, mixed with *Z. mobilis.*

DISCUSSION

The use of 16S rRNAs as an indicator for species diversity is providing a fresh approach for investigating the structure of microbial communities (35). However, any analytical procedure for the studies of the complex natural populations has the potential for some selectivity. The rRNA approaches may be affected by many factors such as biased sample collection, differential cell lysis and nucleic acid extraction, differential amplification, and selective cloning. In recent studies the effects of sample handling and of the number of 16S rRNA genes on PCR amplification and the relative efficiency of gene amplification were described (6, 29, 34). This makes it difficult to quantify the number of species in environmental samples by a molecular technique including PCR. Although biases in the molecular ecological methods are not yet well understood, they appear to be less limiting than those of the culture-based analyses. It thus appears that the rRNA approach, together with other molecular ecological techniques, has great potential for an analysis of microbial diversities. Therefore, we have tried to analyze the 16S rRNA fragments as a marker for microbial communities.

In this study, 16S rRNA nucleotide sequences of 1,262 bacterial strains were analyzed to amplify the most diverse fragments. Of nine variable regions (24), the sequences in the V3 region showed the greatest variance among the bacterial groups and conserved sequences in both ends of the fragment. Our universal primers (SRV3-1 and SRV3-2) have similar lo-

FIG. 6. SSCP-heteroduplex pattern analysis of the PCR amplification products for the genomic DNAs extracted from the bacterial colonies on the agar plate and bacterial cells collected by filtration. Lanes: 1, cultured on agar plate; 2, collected on ultrafilter; 3, collected on 3.0-μm-pore-size membrane filter.

cations and sequences to those (5′-CCTACGGGAGGCAGCAG-3′ and 5′-ATTACCGCGGCTGCTGG-3′) used by Muyzer et al. (23), but we can exclude the possibility of creation of 3′-end dimers. A comparison of the databases of rRNA sequences with our primers showed that mitochondrial and eukaryotic rRNAs had low homology (below 50%). It is unlikely that V3 fragments will be amplified from archaebacterial rDNAs because of low homology with one primer (SRV3-1). The chloroplasts phylogenetically related to cyanobacteria (38) can show amplified nucleotides of about 180 bp, but they are components of large eukaryotic cells and can be removed in the natural samples by prefiltration with a 3.0-μm-pore-size membrane.

The SSCP analysis is a simple and effective method for the detection of minor sequence changes in PCR-amplified DNA (33). The characteristic three-dimensional shape of nucleic acids alters the electrophoretic mobility of the fragments. The folded structure of single-stranded DNA is sensitive to a change of nucleotide sequences. It was reported that 97% of mutations in 100- to 300-base strands caused a mobility shift (11). However, many factors, such as gel matrix, temperature, fragment size, and sequence context, can influence the sensitivity of SSCP (16). It is known that complementary single strands are better separated in gels with low cross-linking (19). A gel with lower cross-linking is softer, has remarkably increased pore size, and seems to be more sensitive to conformation. It has been found empirically that the presence of low concentrations of glycerol (5 to 10%) in a gel frequently improves the separation of mutated sequences (26). The reason for this is unknown, but glycerol may affect the relative mobilities of single-stranded DNA because of its denaturing properties on the secondary conformation of macromolecules. Therefore, in this study, a low-cross-linking (49:1) polyacrylamide gel with glycerol (10%) was used for the SSCP-heteroduplex analysis. However, we could prevent the formation of heteroduplex bands only in the gel without glycerol. It was also found that the formamide loading dye interfered with denaturation if added at the same time as the alkali denaturant (39). This could be due to the chemical reaction between formamide and the alkali. Hence, the formamide dye was added to the sample just before it was loaded onto the gel, and a gel without glycerol was used to get results which are not affected by the heteroduplex DNAs. Although both SSCP and SSCP-heteroduplex analyses were done in this study, each method had advantages and disadvantages. In SSCP analysis, the heteroduplex bands could be removed by improved procedures but the resolution of the SSCP band patterns was reduced somewhat. In SSCP-heteroduplex analysis, the heteroduplex bands represented combinations of two strains but revealed a better resolution because of the inclusion of glycerol. Moreover, the heteroduplex bands were easy to detect because of their stained color. This property will be useful in analyzing the minor populations in complex communities.

There is a lot of secondary-structure conservation in 16S rRNA. It may seem that only limited sequence changes can cause detectable structural changes of the molecule in the SSCP analysis. However, some variable regions of 16S rRNA show great variations among the genera and species. The example in Fig. 8 illustrates unique structural heterogeneities in the nucleotide 450 stem-loop region of the 16S rRNA molecule which characterize the major divisions of eubacterial strains (10). The stem and loop of *Agrobacterium tumefaciens*, which belongs to the alpha *Proteobacteria* group, are smaller than those of other groups, while *Bacillus subtilis*, which belongs to the group of low-G+C gram-positive bacteria, has a large stem-loop structure. The other strains show variable sizes

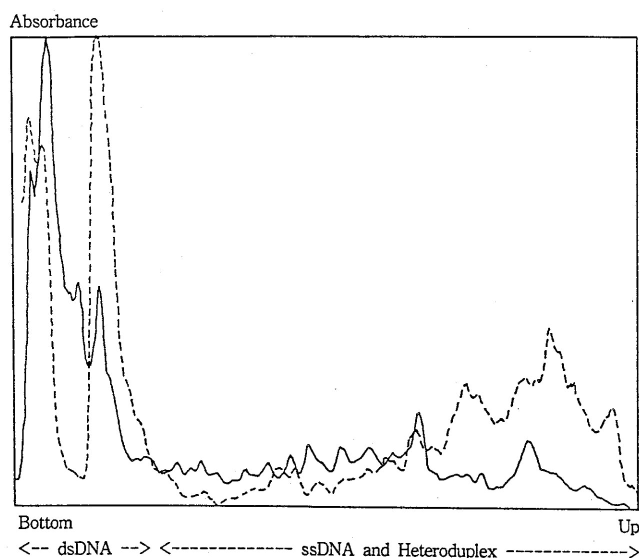

FIG. 7. Densitometric analysis of samples in Lake Soyang (solid line) and a eutrophic pond (dotted line). dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

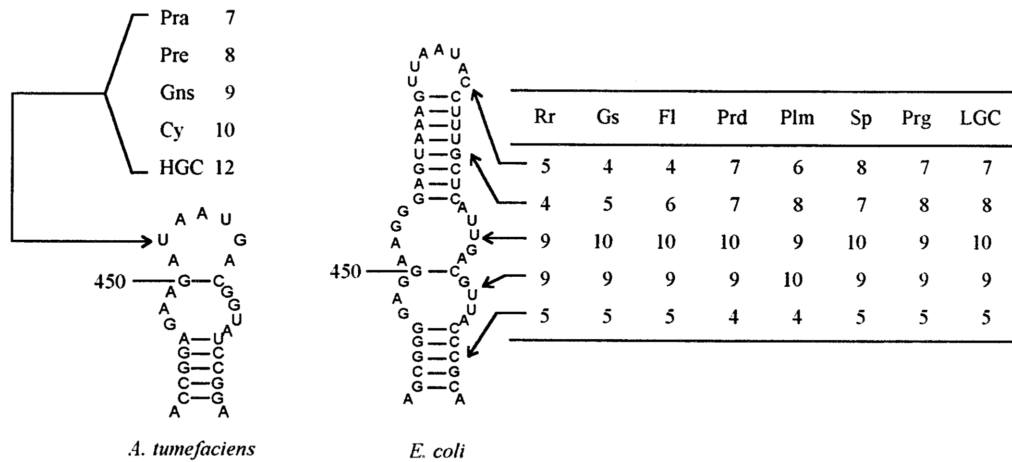

FIG. 8. Secondary-structure models for the nucleotide 450 stem-loop region of the 16S rRNA of 13 bacterial strains. The strains belong to different phylogenetic groups. Numbers represent the stem length or the number of bases forming each loop. The groups and strains are as follows: Pra, *Agrobacterium tumefaciens*; Pre, *Campylobacter sputorum*; Gns, *Thermomicrobium roseum*; Cy, *Synechococcus* sp. strain 6301; HGC, *Arthrobacter globiformis*; Rr, *Thermus thermophilus*; Gs, *Chlorobium vibrioforme*; Fl, *Bacteriodes fragilis*; Prd, *Myxococcus xanthus*; Plm, *Planctomyces staleyi*; Sp, *Spirochaeta aurantia*; Prg, *Escherichia coli*; LGC, *Bacillus subtilis*. For the full name of each group, see Table 2.

TABLE 2. Matrix of PCR product sizes and phylogenetic groups in the database

Size (bp) of PCR products	Group[a]																			Total
	Ch	Cy	Fi	Fl	Fu	Gns	Gs	HGC	LGC	Plm	Pra	Prb	Prd	Pre	Prg	Rr	Sp	Tt	Ua	
175																1				1
176										1	1									2
177								1								1				2
178									2		7									9
179				2		2			57		88	1		27	1		5		1	184
180		2						2	2		2			23			3			34
181		4				1		2	3									2	1	13
182		1			2			4	3				1							11
183					21			16											1	38
184					1			84	2											87
185								7												7
186								5	1											6
187								6												6
188								1	1	1										3
189								1	2								1			4
190								2		1						1				4
191								1								3	1			5
192								2								16				18
193								1								1				2
194																1				1
195								5												5
196			10				4	50	1										1	66
197			3	3				5												11
198				12				4	4											20
199			2	45	1			3	1											52
200				7					2											9
201				1				1		1			1		1					5
202								1	1	2	1	1			2				1	9
203									7			6	10		15				1	39
204	3			3	1				178			50	6		163		21	1	4	430
205									110				22		2		18	1	2	155
206									5				2		1		1			9
207									7								1			8
208											1				1		5			7
Total	3	7	15	73	26	3	4	204	389	6	100	58	42	50	186	24	56	4	12	1,262

[a] Numbers within the group and total columns represent the number of strains. Ch, chlamydiae; Cy, cyanobacteria; Fi, fibrobacters; Fl, flavobacteria and relatives; Fu, fusobacteria and relatives; Gns, green nonsulfur bacteria; Gs, green sulfur bacteria; HGC, gram-positive (high G+C) and relatives; LGC, gram-positive (low G+C) and relatives; Plm, planctomycetes and relatives; Pra, alpha *Proteobacteria*; Prb, beta *Proteobacteria*; Prd, delta *Proteobacteria*; Pre, epsilon *Proteobacteria*; Prg, gamma *Proteobacteria*; Rr, radioresistant micrococci and relatives; Sp, spirochetes; Tt, *Thermotogales*; Ua, uncertain affiliation.

of the stem and loop, and PCR products amplified with our primers include this stem-loop region. If PCR products of the strains belonging to the same group have an identical shape, they will not produce distinguishable bands in the SSCP analysis. For example, *M. luteus* and *N. simplex* belong to the high-G+C gram-positive group, and *A. calcoaceticus* and *E. coli* belong to the gamma *Proteobacteria* group. However, the locations of bands differed within the same group (Fig. 2). Furthermore, it was found that because of its high resolving power, polyacrylamide gel electrophoresis can distinguish most conformational changes caused by subtle sequence differences such as one base substitution in a several-hundred-base fragment (33). Therefore, the SSCP technique can be applied to separate 16S rDNA fragments which are amplified from the samples of complex bacterial populations.

The results obtained with natural samples prepared from all the cultured bacterial colonies and cell pellets concentrated by nitrocellulose membrane filtration or ultrafiltration show that more complex patterns were obtained in directly filtered samples (Fig. 6). This is in agreement with the opinion that the cultured bacteria represent fewer than 10% of the microorganisms found in the natural environment (1) and with reports of viable but nonculturable organisms in many bacterial strains (20, 25). In addition, the samples filtered for the analyses of particle-attached and free-living bacterial populations showed different banding patterns. This implies that the community structures of particle-attached and free-living bacteria in freshwater differ because of different habitats; results similar to this were observed in the marine ecosystem. The rRNA genes of the <10-μm free-living and the aggregate-attached cells are fundamentally different (5). Whereas most rRNA genes recovered from the prefiltered sample were related to the alpha proteobacterial SAR11 cluster, most macroaggregate-attached rRNA clones were related to the *Cytophaga-Flavobacterium* cluster, the *Planctomycetales*, or the gamma *Proteobacteria.*

16S rRNAs isolated from natural bacterioplankton samples were diverse but fell predominantly into several distinct phylogenetic groups (8). Most of the clones constructed from Sargasso Sea bacterioplankton populations belonged to the cyanobacteria and the alpha or gamma subclass of proteobacteria (22). The analysis of a subsurface water sample from the Pacific Ocean yielded similar results (32). When the matrix of the expected sizes of PCR products was constructed from the database and our primers, the product sizes ranged from 175 to 208 bp, showed typical patterns for each bacterial group, and could be divided into several clusters on the basis of their phylogenetic categories (Table 2). If we assume that the above-mentioned gram-negative bacteria are also predominant in freshwater, we can infer that the peaks of double-stranded DNAs, which were measured at about 204 and 179 bp in the SSCP-heteroduplex analysis, represent beta and gamma *Proteobacteria* and alpha *Proteobacteria*, respectively. These peaks for eutrophic pond and oligotrophic lake water organisms were different from each other (Fig. 7). Therefore, our results can be interpreted to show that gamma and beta *Proteobacteria* groups are more abundant in an eutrophic environment, while the alpha group is predominant in an oligotrophic lake, as long as rDNA copy numbers do not vary greatly for bacteria in the aquatic ecosystem. This means that the methods described here are useful for the analysis of bacterial populations affected by the trophic state.

In conclusion, the novel method described here can provide an immediate display of all the members of a complex bacterial population. It is less time-consuming and laborious and offers several advantages with respect to previous approaches. The method does not require radioactive substrates and long PCR primers containing GC clamps. Rapid and detailed information can be obtained by the reamplification and sequencing of separated bands in the gel. By using fluorescence-labeled primers or other sets of primers for specific bacterial groups, resolving powers can be improved and overlapping of bands in gels can be decreased. Therefore, a newly developed method consisting of direct filtration, DNA extraction, amplification of the V3 region with PCR, and densitometric analysis of SSCP or SSCP-heteroduplex patterns is useful for the study of temporal and spatial changes in bacterial community structures in various aquatic ecosystems.

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REFERENCES

1. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1994. Identification of uncultured bacteria: a challenging task for molecular taxonomists. ASM News **60:**360–365.
2. **Amann, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58:**614–623.
3. **Bond, P. L., P. Hugenholtz, J. Keller, and L. L. Blackall.** 1995. Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors. Appl. Environ. Microbiol. **61:**1910–1916.
4. **Britschgi, T. B., and S. J. Giovannoni.** 1991. Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. Appl. Environ. Microbiol. **57:**1707–1713.
5. **DeLong, E. F., D. G. Franks, and A. L. Alldredge.** 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. **38:**924–934.
6. **Farrelly, V., F. A. Rainey, and E. Stackebrandt.** 1995. Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl. Environ. Microbiol. **61:**2798–2801.
7. **Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1992. Novel major archaebacterial group from marine plankton. Nature (London) **356:**148–149.
8. **Giovannoni, S. J., and S. C. Cary.** 1993. Probing marine systems with ribosomal RNAs. Oceanography **6:**95–104.
9. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170:**3584–3592.
10. **Gutell, R. R., N. Larsen, and C. R. Woese.** 1994. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. Microbiol. Rev. **58:**10–26.
11. **Hayashi, K.** 1991. PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. PCR Methods Applic. **1:**34–38.

11a. **Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams.** 1994. Bergey's manual of determinative bacteriology, 9th ed. The Williams & Wilkins Co., Baltimore.

12. **Jensen, M. A., J. A. Webster, and N. Straus.** 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. **59:**945–952.
13. **Kenerly, M. E., E. A. Morgan, L. Post, L. Lindahl, and M. Nomura.** 1977. Characterization of hybrid plasmids carrying individual ribosomal ribonucleic acid transcription units of *Escherichia coli*. J. Bacteriol. **132:**931–949.
14. **Kiss, A., B. Sain, and P. Venetianer.** 1977. The number of rRNA genes in *Escherichia coli*. FEBS Lett. **79:**77–79.
15. **Lee, S.-T., S.-K. Park, K.-H. Lee, S. A. Holmes, and R. A. Spritz.** 1995. A non-radioactive method for simultaneous detection of single-strand conformation polymorphisms (SSCPs) and heteroduplexes. Mol. Cells **5:**668–672.
16. **Liu, Q., and S. S. Sommer.** 1994. Parameters affecting the sensitivities of dideoxy fingerprinting and SSCP. PCR Methods Applic. **4:**97–108.
17. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The Ribosomal Database project. Nucleic Acids Res. **22:**3485–3487.
18. **Martinez-Murcia, A. J., S. G. Acinas, and F. Rodriguez-Valera.** 1995. Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol. Ecol. **17:**247–256.
19. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
20. **Morgan, J. A., G. Rhodes, and R. W. Pickup.** 1993. Survival of nonculturable

Aeromonas salmonicida in lake water. Appl. Environ. Microbiol. **59:**874–880.

21. **Moyer, C. L., F. C. Dobbs, and D. M. Karl.** 1994. Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi seamount, Hawaii. Appl. Environ. Microbiol. **60:**871–879.
22. **Mullins, T. D., T. B. Britschgi, R. L. Krest, and S. J. Giovannoni.** 1995. Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. Limnol. Oceanogr. **40:**148–158.
23. **Muyzer, G., E. C. De Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
24. **Neefs, J. M., Y. Van de Peer, P. De Rijk, S. Chapelle, and R. De Wachter.** 1993. Compilation of small ribosomal subunit RNA structures. Nucleic Acids Res. **21:**3025–3049.
25. **Oliver, J. D., F. Hite, D. McDougald, N. L. Andon, and L. M. Simpson.** 1995. Entry into, and resuscitation from, the viable but nonculturable state by *Vibrio vulnificus* in an estuarine environment. Appl. Environ. Microbiol. **61:**2624–2630.
26. **Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi, and T. Sekiya.** 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc. Natl. Acad. Sci. USA **86:**2766–2770.
27. **Orita, M., Y. Suzuki, T. Sekiya, and K. Hayashi.** 1989. A rapid and sensitive detection of point mutations and genetic polymorphisms using polymerase chain reaction. Genomics **5:**874–879.
28. **Raskin, L., L. K. Poulsen, D. R. Noguera, B. E. Rittmann, and D. A. Stahl.** 1994. Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. Appl. Environ. Microbiol. **60:** 1241–1248.
29. **Rochelle, P. A., B. A. Cragg, J. C. Fry, R. J. Parkes, and A. J. Weightman.** 1994. Effect of sample handling on estimation of bacterial diversity in marine sediments by 16S rRNA gene sequence analysis. FEMS Microbiol. Ecol. **15:**215–226.
30. **Rochelle, P. A., J. C. Fry, R. J. Parkes, and A. J. Weightman.** 1992. DNA extraction for 16S rRNA gene analysis to determine genetic diversity in deep sediment communities. FEMS Microbiol. Lett. **100:**59–66.
31. **Ruano, G., and K. K. Kidd.** 1992. Modeling of heteroduplex formation during PCR from mixtures of DNA templates. PCR Methods Applic. **2:**112–116.
32. **Schmidt, T. M., E. F. DeLong, and N. R. Pace.** 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173:**4371–4378.
33. **Sheffield, V. C., J. S. Beck, A. E. Kwitek, D. W. Sandstrom, and E. M. Stone.** 1993. The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. Genomics **16:**325–332.
34. **Suzuki, M. T., and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:**625–630.
35. **Ward, D. M., M. M. Beteson, R. Weller, and A. L. Ruff-Roberts.** 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. Adv. Microb. Ecol. **12:**219–286.
36. **Ward, D. M., R. Weller, and M. M. Beteson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **344:**63–65.
37. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Truper.** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37:** 463–464.
38. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
39. **Yap, E. P. H., and J. O. McGee.** 1994. Non-isotopic single-strand conformation polymorphism (SSCP) analysis of PCR products, p. 165–177. *In* H. G. Griffin and A. M. Griffin (ed.), PCR technology: current innovations. CRC Press, Inc., Boca Raton, Fla.