Journal of Bioscience and Bioengineering
VOL. 107 No. 6, 646–651, 2009

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# Novel two-dimensional DNA gel electrophoresis mapping for characterizing complex bacterial communities in environmental samples

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Received 3 October 2008; accepted 2 February 2009

**Genomic DNA profiles such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE), and single-strand conformation polymorphism (SSCP) have been commonly used to characterize bacterial communities in environmental samples. We recently developed a two-dimensional gel electrophoresis (2-DGE) method with a combination of chain-length polymorphism analysis (CLPA) and DGGE analysis, in order to improve the DNA resolution and resolve complex environmental DNA fragments produced by polymerase chain reaction (PCR) amplification. The 2-DGE method can generate high-resolution DNA separation maps on the basis of the lengths and composition polymorphisms of DNA sequences. It can thus facilitate detailed analyses between bacterial communities in complex environmental systems such as soil or water. For the present paper, we further developed two novel 2-DGE methods using a combination of CLPA and TTGE (or CLPA and SSCP) and here describe their potential application to the characterization of bacterial communities in nature using clustering analyses. The results show that DNA amplicons can undergo more detailed separation by the two new mapping than by their corresponding 1-DGE fingerprints. Our findings also suggest that these two new 2-DGE mapping techniques are more easily carried out than previously described DGGE-based 2-DGE mapping because they do not require a chemical denaturing gradient gel.**



[**Key words:** Novel 2-DGE mapping; 16S rDNA; Bacterial community; Environmental sample; Cluster analysis]

In the natural environment, bacteria play vital roles in the biogeochemical and nutrient cycles of ecosystems, including contaminant removal and nitrogen fixation (1–3). Although our interest in bacterial communities is considerable, our knowledge of their diversity and the changes they undergo is relatively poor due to methodology limitations (4, 5). Bacterial characterization has been limited in the past because only 1% of bacteria observed under a microscope can be cultured in a laboratory (6–9). The development of molecular methods based on PCR amplification has given powerful support to traditional techniques; among these molecular methods, electrophoretic separation is an important approach to studying diversity of and changes in environmental bacterial communities. Amplified ribosomal DNA restriction analysis (ARDRA) (10) and terminal restriction fragment length polymorphism (T-RFLP) (11, 12) have been used to characterize bacterial communities on the basis of the size polymorphism of DNA fragments. Denaturing gradient gel electrophoresis (DGGE) (13), temperature gradient gel electrophoresis (TGGE) (14), temporal temperature gradient gel electrophoresis (TTGE) (15), and single-strand conformation polymorphism (SSCP) (16) were originally developed to detect point mutations in DNA sequences; however, they have since been expanded to the study of bacterial characterization in environmental samples, such as the molecular techniques associated with the gene encoding the 16S ribosomal RNA (rRNA) (17–21).

There is large variation in bacterial 16S ribosomal DNA (rDNA) sequences as a result of their evolution. Generally, the substitution of a base pair occurs more easily in DNA chains than an insertion or elimination; thus, bacterial communities that belong to the same taxonomical group tend to show changes in base composition, while those in different groups instead show differences in chain length (22). These differences or changes in DNA chains can be effectively analyzed using the above well-established electrophoresis techniques. For example, a change in size can be easily resolved on a polyacrylamide gel, while a change in guanine + cytosine (G+C) content can be monitored by DGGE, TGGE and TTGE based on the decreased mobility of a fork-like double-stranded DNA in polyacrylamide gels containing a linear chemical gradient or temperature gradient. Furthermore, conformational changes due to the substitution of one base pair can be detected by SSCP, which is based on the fact that single-stranded DNA has a sequence-specific secondary structure and that a single base difference in the DNA sequence can affect this secondary structure. Without a doubt, these aforementioned techniques are extremely powerful tools in describing and viewing DNA fragments produced by PCR amplification of genes from bacteria in environmental samples, using electrophoretic separation on a polyacrylamide gel. It is obvious, however, that it is difficult to achieve a high-resolution separation for DNA amplicons with characteristic of

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doi:10.1016/j.jbiosc.2009.02.002

**TABLE 1.** Universal primer sets used in the present study (30)

| [a]Primer name | [c]Sequence (5′–3′) | Target |
|---|---|---|
| Set I | | Most eubacteria |
| 27f | [b](GC clamp) AGAGTTTGATCMTGGCTCAG | |
| 109r | ACGYGTTACKCACCCGT | |
| Set II | | Most eubacteria |
| 357f | CCTACGGGAGGCAGCAG | |
| 518r | ATTACCGCGGCTGCTGG | |

[a] Primer name is from the 3′ end number of the 16S rRNA sequence of *Escherichia coli*.

[b] Sequence of GC clamp (17) is: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG-GCACGGGGGG-3′.

[c] M = C: A, Y = C: T, K = G: T, all at 1:1.

polymorphisms on a gel using one-dimensional DNA gel electrophoresis (1-DGE) alone. Thus, when evaluating large bacterial diversity or detecting differences among very similar bacterial communities in complex environmental samples, these techniques cannot provide a detailed bacterial DNA distribution display due to their low resolution caused by insufficient separation.

Two-dimensional gel electrophoresis (2-DGE) mapping on the basis of any two of the three DNA polymorphism parameters (e.g., chain length, G+C content, and conformation) is a constructive concept for improving the resolution of DNA fragments on a gel. 2-DGE mapping is a new genomic technique that resolves bacterial genomes into hundreds of fragments and may be a useful tool for bacterial community analysis (23). For instance, the large number of separated bacterial units derived from 2-DGE maps enables a detailed diversity comparison of bacterial communities in different environmental samples using logarithmic normal rank–abundance plots (24). Although previous researchers (25–29) have developed this technique, it has only been applied to studies of genomic changes and mutations in the field of medicine. We recently developed a 2-DGE mapping combining chain-length polymorphism analysis (CLPA) and DGGE fingerprinting and applied it to the assessment of bacterial diversity in soil (23, 24). In the DGGE-based 2-DGE mapping, however, the complex preparation of a chemical denaturing gradient is required, resulting in labor-intensive protocols. To further develop this technique, we describe here two novel 2-DGE mapping performed by using a combination of CLPA and TTGE (or CLPA and SSCP) and evaluate their potential application to the characterization of bacterial communities in complex environmental samples using clustering analyses.

## MATERIALS AND METHODS

**Environmental samples** Sediment samples were collected from the upstream and midstream areas of the Sagami River in Japan, and soil samples were obtained from the grass and tree-grown sites in the campus of Yokohama National University, Japan. After removing all visible debris such as plant matter and sand, samples were transferred to a laboratory under conditions of 4 °C ice-water and were immediately subjected to subsequent analysis.

**DNA extraction and purification** As previously described (24), total DNA was extracted from the environmental samples using the bead-beater method; it was then purified using two spin columns packed with polyvinylpolypyrrolidone (PVPP) and Sepharose 4B. In brief, bacterial cells were disrupted with three different sizes of glass beads after lysis treatment with a surfactant solution containing sodium dodecyl sulfate (SDS, 10%, w/v) and sodium phosphate buffer (SPB, 0.2 M, pH 8). Proteins were then precipitated with EDTA (0.5 M, pH 8) and potassium acetate solution (5 M, pH 5.5). Crude DNA pellets were collected by centrifugation after isopropanol precipitation. The crude DNA was purified by eluting through PVPP and Sepharose 4B spin columns. The purified DNA was confirmed by 2% agarose gel electrophoresis and stored in a freezer at −20 °C.

**PCR amplification** As previously described (24), the purified template DNA was directly amplified by the touchdown PCR method in the V1 and V3 variable region of the 16S rDNA gene, using a PCR Thermal Cycler (Dice mini; Takara, Otsu). The general reaction mixture used in this study contained 0.25 μl of Ex Taq (5 μ/μl), 5 μl of 10× Ex Taq buffer (20 mM $Mg^{2+}$ (10×)), 4 μl of dNTP mixture (2.5 mM of each dNTP), 1 μl of template DNA, 2 μl of each primer (final concentration of 0.5 μM), and sterile distilled water in a final volume of 50 μl. The universal primer sets used are listed in Table 1. PCR conditions in the present study were: 94 °C for 5 min, followed by 10 cycles of 94 °C for 1 min, 65 °C (decrease of 1 °C per cycle) for 30 s, and 72 °C for 1 min; 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 7 min.

**2-DGE mapping with combined CLPA-TTGE analysis** The principle of this mapping is similar to that of the 2-DGE mapping previously described (24); the difference is that a temporal temperature gradient gel substitutes for a chemical denaturing gradient gel in the second-dimensional separation. The 4 μl 16S rDNA fragments obtained from PCR amplification of target DNA from the river sediment samples using primer set I were separated by 2-DGE using a DCode universal mutation system (Bio-Rad, Hercules, CA, USA), according to the following procedure. The amplified DNA fragments were first loaded onto a 1 mm thick, 10% (w/v) non-denaturing highly cross-linked polyacrylamide gel (bisacrylamide gel stock solution, 37.5:1; Spreadex Polymer NAB solution (10×: Elchrom Scientific, Cham, Switzerland)) with a final concentration of 10% (v/v) and run in 1× TAE electrophoresis buffer at a constant voltage of 100 V for 10 h (Fig. 1a). The gel strip with DNA bands was excised from the gel that was electrophoresed in the first dimension and transferred to the top of a compound polyacrylamide gel, prepared by attaching a 4% (w/v) non-denaturing polyacrylamide cap gel to the top of a 10% (w/v) polyacrylamide denaturing gel containing 7 M urea. TTGE was then performed on the basis of the melting characteristics of the DNA sequences in the 1× TAE electrophoresis buffer at 110 V for 8.5 h (Fig. 1b). The gel temperature in the electrophoresis process was set to increase gradually by 1 °C per hour, from a starting temperature of 60.5 °C to a final temperature of 69 °C. The electrophoresed gel was then washed and stained for 30 min with SYBR Green I (1:10,000 dilution; Molecular Probes, Eugene, OR, USA) in 1× TAE buffer (Fig. 1c). Finally, the stained gel was photographed with a UV transillumination device used to obtain a spot distribution map (Fig. 1d).

**2-DGE mapping with combined CLPA-SSCP analysis** This mapping is based on length polymorphism analysis and SSCP analysis of single-strand DNA (ssDNA) fragments produced by PCR amplification. Separation via this approach using a DCode universal mutation system can be done in the following steps. A total of 8 μl denaturing loading solution (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to 2 μl double-stranded DNA (dsDNA) fragments, which had been produced by amplifying template DNA from the soil samples with primer set II. The dsDNAs were denatured into ssDNA by incubating at 95 °C for 5 min and immediately cooling on ice. After 3 min, they were loaded onto a 1 mm thick, 10% (w/v) denaturing polyacrylamide gel (bisacrylamide gel stock solution, 37.5:1; final concentration of 7 M urea) and electrophoresed in 1× TBE buffer at a constant voltage of 150 V for 15 h at room temperature (Fig. 2a). The lane loading ssDNA bands was cut into a strip and transferred to a compound gel prepared by attaching a 4% (w/v) denaturing

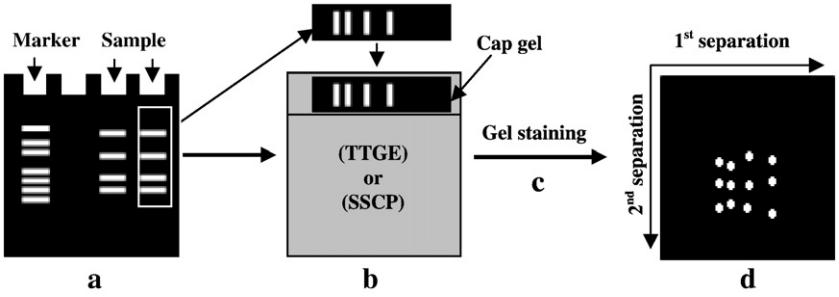


FIG. 1. Graphical representation of TTGE or SSCP-based 2-DGE mapping system. (a) The dsDNA or ssDNA samples to be analyzed are separated into band patterns on the basis of size differences on a polyacrylamide gel. (b) The gel strip loading DNA bands is excised from the first-dimension electrophoresed gel and transferred to the top of a TTGE or SSCP gel (or a second dimensional electrophoresed gel). The dsDNA or ssDNA fragments with the same size are then separated on the basis of their different GC content or sequence-specific secondary structure. (c) The TTGE or SSCP gel is stained, washed, and dried. (d) Photograph of gel under UV light, for generating 2-DGE spot distribution map.

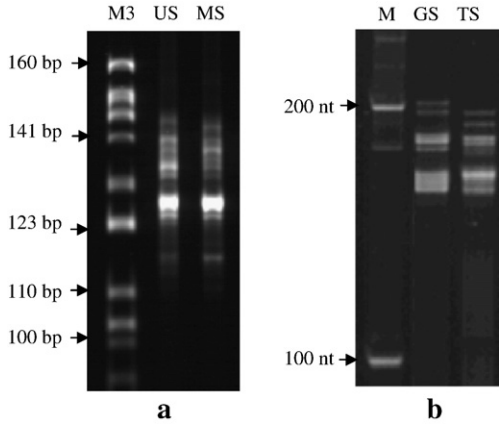


FIG. 2. CLPA banding patterns based on size differences for double-stranded 16S rDNA fragments from river sediment communities (a) and for single-stranded 16S rDNA fragments from soil communities (b). (a) Lanes: M3, size marker containing more than 50 DNA fragments in the size range from 50 to 622 bp; US, upstream sediment sample from the Sagami River; MS, midstream sediment sample from the Sagami River. (b) Lanes: M, 100 nt ladder size marker obtained from denaturing 100 bp ladder size marker composed of ten bands from 100 bp to 1000 bp; GS, grass-grown soil sample; TS, tree-grown soil sample.

polyacrylamide cap gel with 7 M urea to the top of a 0.75× MDE gel (FMC, Rockland, ME, USA) containing 10% glycerol. The ssDNA loaded in the strip gel then underwent an SSCP separation in Tris–glycine buffer (25 mM Tris–HCl, 200 mM glycine, pH 8.3) at 200 V for 19 h at 16 °C (Fig. 2b). The electrophoresed gel was stained with a 1:10,000 dilution of SYBR Gold (Molecular Probes, Eugene, OR, USA), washed and dried at room temperature (Fig. 2c), and photographed to obtain a spot image (Fig. 2d).

**TTGE and SSCP fingerprinting analyses** To check the position of spots on 2-DGE maps and compare resolution, TTGE analysis and SSCP analysis were carried out using the DCode universal mutation system. The double-stranded 16S rDNA fragments from river sediments were loaded onto the wells of a 10% (w/v) polyacrylamide denaturing gel containing 7 M urea, and the electrophoresis was run at 110 V for 8.5 h, increasing temperature gradually by 1 °C per hour from a starting temperature of 60.5 °C to a final temperature of 69 °C. The gel was washed and stained with SYBR Green I (1:10,000) and photographed under UV light after the electrophoresis. The single-stranded 16S rDNA fragments from soil samples were loaded on a 0.75× MDE gel (FMC, Rockland, ME, USA) containing 10% glycerol and then separated in Tris–glycine buffer (pH 8.3) at 200 V for 19 h at 16 °C. The gel was washed and stained with SYBR gold (1:10,000) and photographed under UV light after the electrophoresis.

**Clustering analyses based on 2-DGE maps and 1-DGE profiles** The digitization of 2-DGE maps, TTGE profiles and SSCP profiles was used for clustering analysis of bacterial communities in river sediment samples and soil samples. A "BioNumerics" software package (Version 5.10; Applied Maths; Sint-Martens-Latem; Belgium) was used to perform the analyses of 1-DGE and 2-DGE gels according to the instructions. In brief, the pairwise band on each 1-DGE gel was compared after treatments such as strip definition, calculation of densitometric curves, and normalization. The processing steps of 2-DGE gel images mainly include spot detection, calibration, normalization (gel alignment) and matching of 2-DGE gel spots. The normalization of 2-DGE gels makes use of a reference system to locate homologous spots on different gels. When a spot on one gel is linked to the same reference spot as a spot on another gel, these spots are considered the same bacterial species or bacterial species group. Pearson correlation coefficients were used to calculate similarities of the pairwise band on each 1-DGE gel and similarities between different 2-DGE gels since it is suited for identification of fingerprints (31). Phenograms were constructed using the unweighted pair group method with arithmetic average (UPGAMA) (32).

## RESULTS AND DISCUSSION

**Chain-length polymorphism analysis (CLPA)** The CLPA analysis of 2-DGE mapping, as introduced in our studies, is very important to obtaining a clear 2-DGE map; i.e., whether a good-quality CLPA band image is generated or not will have a direct impact on obtaining a clear 2-DGE map. Our experimental results indicate that a poor-quality CLPA may cloud the 2-DGE display and lower the resolution. This will result in difficulties in carrying out detailed sequence analysis, by which we could otherwise excise spots of interest from the 2-DGE map. Generally, to improve DNA fragment resolution in terms of size, special chemical crosslink reagents such as Spreadex Polymer (NAB), polyvinylpyrrolidone (PVP) polymer, and glycerol are usually added to the gel solution. In our experiments, the Spreadex Polymer NAB solution (10×; Elchrom Scientific, Cham, Switzerland), which is highly cross-linked and allows full separation of DNA fragments that differ by as little as 1 bp on a 5-cm-long gel (33), was employed to achieve this purpose. Fig. 2 shows the results of CLPA analysis for river sediment samples (a) and soil samples (b). The results show that the CLPA gel achieves a detailed size separation from 110 bp to 160 bp (used in the TTGE-based 2-DGE mapping) and from 110 nt to 200 nt (used in the SSCP-based 2-DGE mapping).

In addition, subclass-level bacterial classification may be estimated by CLPA analysis of PCR-amplified DNA fragments in the V1 variation region of the 16S rRNA sequence (i.e., Proteobacteria α, β and γ can be subdivided and identified on the basis of the length of DNA fragments) (34). Although CLPA analysis can resolve a detailed separation on the basis of differences in DNA size, it cannot achieve high-resolution in sequence content because sequences with different GC-content but the same length contained in one CLPA band are difficult to separate on a CLPA gel.

**2-DGE mapping with combined CLPA-TTGE analysis and TTGE fingerprinting** TTGE-based 2-DGE maps of 16S rDNA fragments by amplifying template DNA extracted from the river sediments are shown in Fig. 3. For the identification of spot positions and a comparison of resolution, CLPA banding results ranging from 110 bp to 151 bp are shown in the top part of the figure; the banding patterns of TTGE separation ranging from 60.5 °C to 69 °C are shown in the right part of the figure. The resolution estimate, determined by measuring the number of bands and spots, indicates that in the TTGE-based 2-DGE mapping, 2-DGE provided higher resolution of amplicons produced by PCR amplification of DNA from the river sediment samples by a factor of more than 5, using 1-DGE with TTGE or CLPA.

The 2-DGE maps indicate that the new TTGE-based 2-DGE mapping can also achieve a detailed separation. This new method can be executed using the Dcode mutation detection system, in which

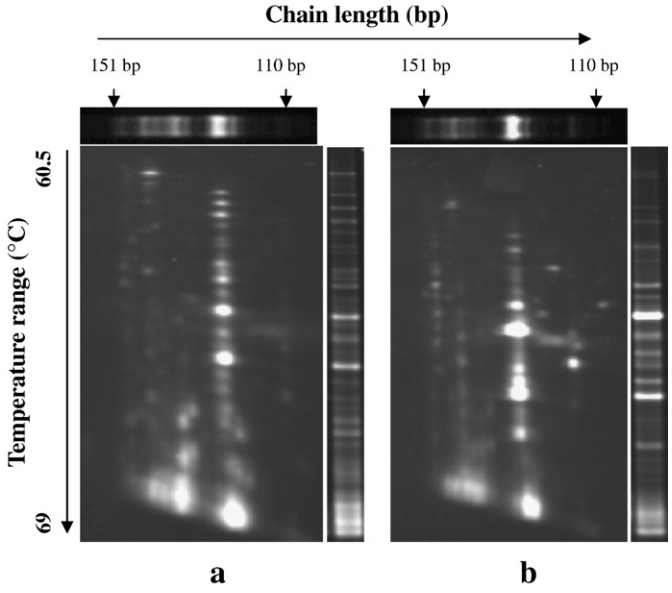


FIG. 3. Two 2-DGE maps of the 16S rDNA amplicons of bacteria from the upstream sediment sample (a) and the midstream sediment sample (b) from the Sagami River on the basis of the TTGE-based 2-DGE method. The top banding patterns of the maps show the results of CLPA, which was carried out at 100 V for 10 h at room temperature; the right banding patterns show the results of TTGE analysis, which was carried out at 110 V for 8.5 h with a temperature ramp rate of 1 °C/h and a temperature range between 60.5 °C and 69 °C.

the temperature range and ramp rate can be controlled and adjusted. Because DNA fragments loaded on the chain-length separation gel lane are separated depending on a temporal temperature gradient but not on a chemical gradient, the new mapping will eliminate the inconvenience of preparing a chemical denaturant gradient gel in the latter separation. If the samples to be analyzed are the same, two 2-DGE maps, one from each of the TTGE-based method and the DGGE-based method previously described, could theoretically generate the same 2-DGE maps if their running conditions are accurately adjusted.

We can calculate the melting temperature range for a known sequence using a software package such as MacMelt (Bio-Rad); however, it is difficult to determine the melting temperature range of a DNA sequence from a bacterial community derived from an environmental sample, because we do not know what inhabits the samples. Despite the difficulty in performing exact calculations, the temperature range can be estimated on the basis of sequence size. Additionally, determining temperature ramp rate is essential for obtaining high-resolution DNA map; theoretically, reducing ramp rate can achieve high resolution, but running times for routine screening will need to be increased, requiring a long running gel. In the present study, the experimental results suggest an optimal temperature range from 60.5 °C to 69 °C and temperature ramp rate of 1 °C/h to achieve good separation.

The TTGE used in the second separation of the new system differs from TGGE, although they are both based on the principle of DGGE, in which the separation of dsDNA fragments is dependent on the melting behavior of the sequences on a denaturing polyacrylamide gel. TGGE has a fixed temperature gradient from the top to the bottom of the gel (35). Meanwhile, in TTGE temperature is gradually increased, and a linear temperature gradient over the length of the electrophoresis run is formed in a polyacrylamide gel with a constant concentration of urea. Compared to TGGE, TTGE allows for easier temperature modulation over time; if the temperature range remains the same, TTGE can provide a wider separation range, which in turn increases sensitivity (15, 36).

Despite the simplified protocol inherent in the new system, the experimental results suggest that a longer gel may give better separation. Compared to the previous DGGE-based 2-DGE mapping, longer running times are required for sufficient separation in the new mapping.

**2-DGE mapping with combined CLPA-SSCP analysis and SSCP fingerprinting** For the DGGE or TTGE-based 2-DGE mapping, a GC-rich sequence or so-called GC clamp is necessary, in order to increase the detected percentage of the sequence variants. In addition, a denaturing gradient environment is always required for separating dsDNA on the basis of the melting behaviors of different dsDNA sequences. Here, we developed another new 2-DGE mapping by using CLPA in combination with SSCP analysis. The SSCP analysis requires neither GC-rich sequence nor gradient gels; thus, it is a simple electrophoretic technique that has been developed for detecting mutations (37, 38), and it has been used widely to analyze bacterial communities in the natural environment.

Fig. 4 shows SSCP-based 2-DGE maps of the 16S rDNA fragments produced by PCR amplification of DNA extracted from the soil samples. For position check and a comparison of resolutions, the CLPA banding patterns ranging from 160 nt to 200 nt are shown at the top of the figure; the SSCP fingerprinting patterns are shown at the right of the figure. The resolution estimate, determined by measuring the number of bands and spots, shows that the resolution of 2-DGE mapping was more than twice that of 1-DGE fingerprinting.

In this 2-DGE mapping, the first-dimensional separation of the ssDNA is performed on the basis of length polymorphism on a polyacrylamide gel, in which urea (7 M) is added to preclude the formation of secondary and tertiary structures of ssDNA fragments and to ensure that separation depends only on size differences. The

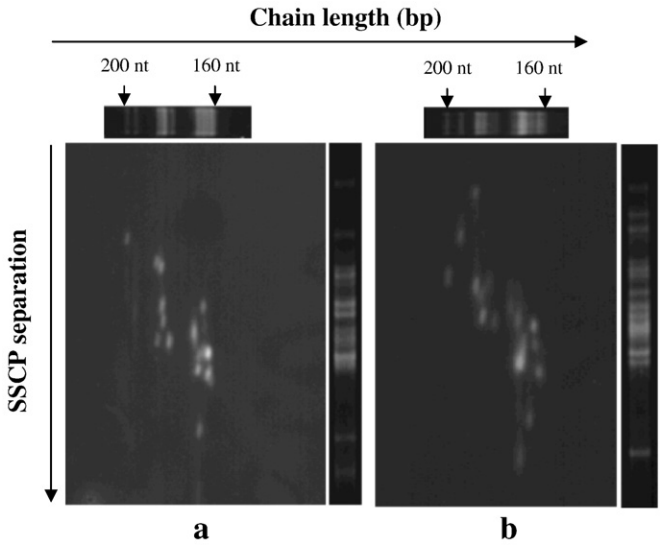


FIG. 4. Two 2-DGE maps of the single-stranded 16S rDNA amplicons of bacteria from the grass-grown soil sample (a) and the tree-grown soil sample (b) from the campus of Yokohama National University on the basis of the SSCP-based 2-DGE method. The top band figures of the maps show the results of CLPA, which was carried out at 150 V for 15 h at room temperature; the right band patterns show the results of SSCP analysis, which was carried out at 200 V for 19 h at 16 °C.

second separation is carried out on an SSCP gel, based on differences in mobility in the ssDNA sequences. To obtain high-resolution ssDNA fragments in the SSCP step, we used a high-grade MDE polyacrylamide gel and a Tris–glycine buffer instead of TBE buffer. In addition, in the second separation of SSCP, the running temperature should be empirically determined. In our study, 16 °C was optimal for achieving good separation of PCR amplification products of DNA from soil communities.

Although SSCP provides a simple electrophoretic protocol for community analysis of natural bacteria, it also has a major limitation: a high rate of re-annealing of ssDNA after an initial denaturation during electrophoresis (39). This disadvantage is especially critical with highly diverse bacterial communities from environmental samples (40). For the new SSCP-based 2-DGE mapping, however, performing the first separation of ssDNA fragments on a gel containing a 7 M constant concentration of urea may preclude or reduce such re-annealing. In addition, because both CLPA and SSCP analysis do not need a GC clamp to increase the detection percentage of ssDNA in this system, a primer sequence can preclude the addition of GC-rich sequences. Thus, the SSCP-based 2-DGE mapping contributes not only to improving the separation of ssDNA fragments, but also to overcoming the limitations caused by SSCP separation alone. This may be useful for the analysis of high ssDNA concentrations with high bacterial diversity.

**Clustering analyses based on the novel 2-DGE mapping patterns** The novel 2-DGE methods provide higher-resolution mapping patterns of DNA fragments produced by PCR amplification of genes from natural samples than 1-DGE banding patterns; thus, 2-DGE methods may enable detailed bacterial community analyses such as diversity assessment using the Shannon diversity index and a logarithmic normal rank–abundance plot (24). To further develop these applications to the characterization of bacterial communities in environmental samples, we performed cluster analyses of bacterial communities from river sediment samples and soil samples, based on two novel 2-DGE maps, using the BioNumerics software package (the resulting phenograms are not shown). The clustering analysis on the basis of TTGE-based 2-DGE maps of bacterial communities from the

sediments collected from upstream and midstream in the Sagami River indicated a similarity of only 0.33. This may suggest environmental pressure on the bacterial communities in the Sagami River due to pollution. Similarly, the clustering analysis of SSCP-based 2-DGE maps of soil communities from the grass and tree-grown sites showed a similarity of 0.62. This indicates a difference in bacterial community structures among the two different soil samples. Moreover, we carried out clustering analyses based on TTGE banding profiles from river sediment samples (similarity: 0.81) and SSCP banding patterns from soil samples (similarity: 0.78). One interesting finding was that the similarity value based on 2-DGE maps was lower than that based on their corresponding 1-DGE profiles despite each technique using the same compared samples. This may be because 2-DGE mapping achieve such high resolution that some small different or close OTUs contained in one 1-DGE band are separated into several different spots on a 2-DGE map. This detailed separation results in detailed comparisons between bacterial communities from different environmental samples. Thus, such high-resolution maps may result not only in a better understanding of the basic microbial ecosystem, but also in the ability to monitor detailed environmental changes prompted by human pollution or climate change.

Undoubtedly, these two new methods are more easily performed because a chemical denaturing gradient is not required. Like the previously described DGGE-based 2-DGE mapping, they can also generate a detailed separation of PCR-amplified DNA fragments. Furthermore, a three-dimensional DNA electrophoresis mapping is possible on the basis of DNA sequence differences in size, composition, and conformation. Although it would be difficult to realize under the current experimental conditions, we could theoretically do so using computer simulations.

In spite of its merit, the detection and characterization of DNA amplicons from environmental samples are still restricted to the predominant species in the community. This is mainly because there are many problems inherent in using PCR and DNA-extraction techniques to recognize bacterial communities in environmental samples. With improvements in these techniques, the future of 2-DGE mapping *vis-à-vis* its use in microbial ecology will be promising.

## ACKNOWLEDGEMENTS

This study was supported by the Global Center of Excellence (Global COE) Program "Environmental Risk Management for Bio/Eco-Systems," of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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