Two-Dimensional DNA Gel Electrophoresis Mapping: a Novel Approach to Diversity Analysis of Bacterial Communities in Environmental Soil

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The diversity analysis of bacteria is useful for the environmental assessment of soil. Traditional molecular-based methods such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) analysis achieve a low-resolution display of bacterial DNA fragments on a gel. To improve the resolution, a novel two-dimensional DNA gel electrophoresis (2-DGE) method was designed. This method can generate a high-resolution DNA map that facilitates the detailed analysis of soil bacteria. This map can be obtained by utilizing 2-DGE to separate genomic DNA fragments produced by polymerase chain reaction (PCR) amplification on the basis of chain length and G+C content. To develop this 2-DGE method further and to apply it to the assessment of bacterial diversity, we carried out a 2-DGE mapping of bacterial DNA fragments from different environmental soils and computed Shannon index as well as plotted rank-abundance curves on the basis of the relative intensity of each spot on the maps. DGGE mapping was also performed to compare the resolution of the two methods. 2-DGE mapping was capable of generating a higher resolution display by a factor of more than 2 using a DGGE fingerprint pattern on a piece of gel. Furthermore, the higher number of detected spots from the 2-DGE map enabled the assessment of differences in bacterial diversity in complex soil systems using a logarithmic normal rank-abundance plot.

[**Key words:** two-dimensional DNA gel electrophoresis, soil bacteria, 16S ribosomal DNA gene, diversity analysis, rank-abundance plot]

A gram of soil is estimated to contain more than 1×10^4 bacterial species (1), most of which play vital roles such as in toxin removal and nitrogen fixation in the biogeochemical and nutrient cycles of the ecosystem (2–4). Since some bacterial species become predominant, obscure, or extinct with a change in their soil environment, the diversity of bacteria in soil is considered to be an important environmental index. Previous studies of bacterial diversity in soil were mainly dependent on culture, with which only 1% of bacterial species can be isolated in the laboratory (5). In recent years, a number of culture-independent molecular methods have been developed and utilized for studying bacterial diversity in soil. These methods mainly include guanine plus cytosine (G+C) content determination (6), DNA reassociation and hybridization (7, 8), the use of DNA microarrays (9), and PCR-based approaches such as amplified ribosomal DNA restriction analysis (ARDRA) (10), terminal restriction fragment length polymorphism (T-RFLP) (11), and denaturing gradient gel electrophoresis (DGGE) (12). However, for screening DNA fragments amplified by poly-

merase chain reaction (PCR) analysis on a gel, these PCR-based molecular approaches are limited by their low resolution. Thus, it is difficult to resolve large bacterial diversity or detect differences between highly similar bacterial communities in environmental soil samples using these approaches. To resolve this issue, Isshi *et al.* (13) designed a novel two-dimensional DNA gel electrophoresis (2-DGE) combined with chain length polymorphism analysis and DGGE. 2-DGE is a genomic technique that resolves bacterial genomes into hundreds of fragments. Fisher and Lerman (14) used this technique to separate the genome of an *Escherichia coli* lambda lysogen to identify the insertion of a 48-kb lambda phage. Poddar and Maniloff (15) used 2-DGE to determine the genome sizes of three microorganisms: *Mycoplasma capricolum*, 724 kb; *Acholeplasma laidlawii*, 1646 kb; and *Haemophilus influenzae*, 1833 kb. Te Meerman *et al.* (16) demonstrated the usefulness of 2-DGE in an empirical linkage study of the red factor in cattle and suggested the application of this technique to mapping genetic traits in humans and animals. Malloff *et al.* (17) developed a bacterial comparative genomic hybridization method based on 2-DGE for comparison of more distantly related bacterial strains (of the same species or of different species

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within a genus). However, the resolution of these 2-DGE methods was insufficient in terms of the chain length of DNA fragments, and these techniques were mainly applied to the study of genomic changes and variation in the field of medicine. A novel 2-DGE method that can generate a high-resolution DNA map for resolving bacterial diversity in complex environmental soil systems has been suggested. The high-resolution map can be obtained by utilizing 2-DGE to perform a detailed separation of genomic DNA fragments. DNA fragments are first separated based on their size difference on a nondenaturing highly cross-linked polyacrylamide gel; parallel DGGE is then performed to resolve the difference in sequence composition (G+C content) between the fragments.

The present study aimed to develop this 2-DGE method further and to apply it to the assessment of bacterial diversity in environmental soil by calculating Shannon index and plotting rank-abundance curves. For resolution comparison, DNA fragments were also analyzed by DGGE fingerprinting, which was primarily developed for detecting point mutations in DNA sequences. Both methods are based on the separation of PCR-amplified fragments coding for 16S ribosomal RNA (rRNA) with the characteristics of high conservation and variability in all prokaryotes. Total genomic DNA was extracted from four soil samples by the bead-beater method and purified using polyvinylpolypyrrolidone (PVPP) and Sepharose 4B spin columns; direct PCR amplification of the DNA fragments was then performed using universal primers in the V1 variable region of the 16S ribosomal DNA (rDNA) gene of bacteria. The PCR products were separated by 2-DGE and DGGE fingerprinting. The high-resolution DNA maps obtained by 2-DGE were used to analyze bacterial diversity by calculating the Shannon index and plotting rank-abundance curves.

MATERIALS AND METHODS

Soil samples   An unpolluted grass soil sample was collected inside the Yokohama National University campus and passed through a 2-mm-mesh sieve to remove all visible roots and other materials; three trichloroethylene (TCE)-contaminated moist soil samples from different polluted sites were also obtained from Adeka Corporation, Tokyo. All the samples were dried before genomic DNA was extracted. The environmental characteristics, including TCE concentration (measured using a GC-MS system), temperature, and pH, of the soil samples are listed in Table 1.

Genomic DNA extraction, purification, and quantitative determination   Total DNA was directly extracted from the soil samples using the following protocol based on the bead-beater method (18) and purified using two spin columns packed with PVPP and Sepharose 4B (19).

Bacterial cells in each dried soil sample weighing approximately 0.5 g were treated with 1.5 ml of a surfactant mixture solu-

tion containing 0.15 ml of sodium dodecyl sulfate (SDS, 10%, w/v) and 1.35 ml of sodium phosphate buffer (SPB, 0.2 M, pH 8). The bacterial cells were then disrupted with 0.5 g of glass beads of three sizes (0.1, 0.5, and 1 mm, each size forming one-third of the total weight of the glass beads) for 3–30 s/each at 252×g at 4°C with a Bend Smash (12BS-12R; Wakenyaku, Kyoto). After cooling to 4°C in ice water for 5 min, the tubes were centrifuged at 2820×g at 4°C for 15 min. Four-hundred-μl portions of clear aqueous phases were collected into 1.5-ml tubes, and 100 μl of EDTA (0.5 M, pH 8) and 40 μl of potassium acetate solution (5 M, pH 5.5) were added to each tube for protein precipitation. The mixture was incubated in a −20°C freezer for 30 min and then thawed. The mixture was then centrifuged at 9500×g at 4°C for 15 min. Five-hundred-μl portions of clear aqueous phases were collected into 1.5-ml new tubes, and DNA was precipitated by adding 300 μl of isopropanol and leaving the mixture to stand for 1 h at room temperature. Crude DNA pellets were collected by centrifugation at 9500×g at 4°C for 15 min and finally dissolved in 100 μl of TE buffer or sterile distilled water. The crude DNA solution was eluted through a prepared spin column packed with 0.15 g of PVPP by a 10 min centrifugation at 3120×g. The eluate was then eluted through another prepared spin column packed with 3 ml of Sepharose 4B by a 10 min centrifugation at 2540×g. The purified DNA was confirmed by 2% agarose gel electrophoresis and stored in −20°C freezer for subsequent analysis.

The yield of DNA extracted from 1 g of each soil sample was determined using the formula DNA yield (μg/g) = $500ABS_{DNA}$, where ABS_{DNA} is the DNA absorbance measured using a UV/VIS spectrometer (Ubest-55; Jasco, Tokyo) at 260 nm and 500 is the conversion coefficient in this study.

PCR amplification   The purified template DNA (1 μl) was directly amplified by the touchdown PCR method in the V1 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 u/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. A pair of universal primers of the 16S rRNA gene, 27f (5′-AGAGTTTGATCMTGGCTCAG-3′, M=C:A=1:1, from positions 27–46 in the 16S rRNA of *E. coli*) and 109r (5′-ACGYGT TACKCACCCGT-3′, Y=C:T=1:1, K=G:T=1:1, from positions 109–126 in the 16S rRNA of *E. coli*), were used, and a GC clamp of 40 bases was added to the 5′-end of the forward primer. DNA was amplified using the following thermal cycle: 94°C for 5 min, followed by 10 cycles of 94°C for 1 min, 59°C (decrease of 0.4°C per cycle) for 30 s, 72°C for 1 min; 20 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 7 min.

Two-dimensional DNA gel electrophoresis (2-DGE)   The DNA fragments obtained in the touchdown PCR amplification of the V1 variable region of the 16S rDNA gene 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 on a 1-mm-thick, 10% (w/v) nondenaturing highly cross-linked polyacrylamide gel (bisacrylamide gel stock solution, 37.5:1; Spreadex Polymer NAB solution [10×:

TABLE 1.   Environmental characteristics of four soil samples

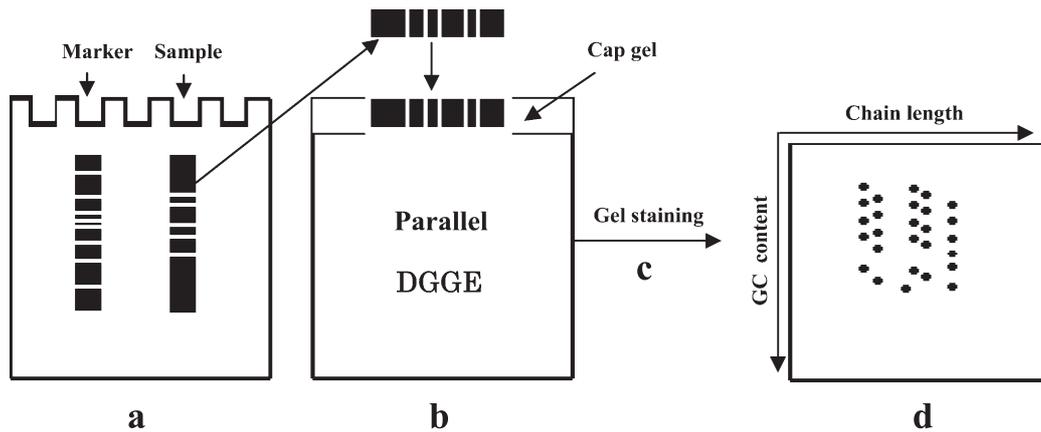| Characteristic	Sample			
	S1	S2	S3	S4
TCE concentration (mg/kg)	0	15.6	25.4	28.3
Soil temperature (°C)	16.8	14.1	13.2	13.6
pH	6.3	8.2	7.6	7.7

FIG. 1.   Graphical representation of 2-DGE mapping. (a) The DNA sample for analysis is separated by size on a nondenaturing polyacrylamide gel. (b) The gel strip with DNA is excised from the first-dimension gel and transferred to the top of a compound denaturing polyacrylamide gel. Parallel DGGE is performed on the basis of sequence composition difference. (c) The DGGE gel is stained with SYBR Green I in $1 \times$ TAE buffer. (d) Photograph of gel on UV Transillumination device for generating spot distribution map.

Elchrom Scientific, Cham, Switzerland] with a final concentration of 10% v/v) and run in $1 \times$ TAE electrophoresis buffer at a constant voltage of 110 V for 10 h (Fig. 1a). The gel strip with DNA was excised from the gel electrophoresed in the first dimension, and transferred to the top of a compound polyacrylamide gel prepared by attaching a 4% (w/v) nondenaturing polyacrylamide cap gel to the top of a 10% (w/v) polyacrylamide gel with denaturing gradient ranging from 35% to 65% (7 M urea and 40% formamide, v/v). Parallel DGGE was then performed on the basis of melting characteristics of DNA sequences in $1 \times$ TAE electrophoresis buffer at 60°C at 130 V for 9 h (Fig. 1b). The DGGE gel was then washed and stained for 30 min with SYBR Green I (1:10,000 dilution; Molecular Probes, Eugene, OR, USA) in $1 \times$ TAE buffer (Fig. 1c). Finally, the stained gel was photographed on a UV Transillumination device to obtain a spot distribution map (Fig. 1d).

Denaturing gradient gel electrophoresis (DGGE)   In order to compare resolution between 2-DGE and DGGE methods, we carried out a DGGE analysis of PCR-amplified 16S rDNA fragments, which were previously analyzed by 2-DGE. 16S rDNA samples were loaded onto the well of a 10% (w/v) polyacrylamide gel with denaturing gradient from 35% to 65%, and the electrophoresis was run at 60°C at 130 V for 9 h using the DCode universal mutation system. After electrophoresis, the gels were stained for 30 min with SYBR Green I (1:10,000) and photographed under UV light.

Diversity analysis of bacterial communities based on 2-DGE maps   We used Shannon index and rank-abundance plots to assess the diversity of bacterial communities derived from different environmental soil samples. Digitized gel images from 2-DGE were normalized and imported into the Lane and Spot Analyzer (version 6.0; Atto, Tokyo) software package. The number of spots and the relative intensity of each spot (the area integrated value of each spot) were measured and Shannon index (H′) was calculated using

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

where S is the number of spots, which is also considered to reflect bacterial abundance, and p_i was calculated as the ratio of a given spot intensity to the total intensity of spots ($p_i = n_i/N, N = -\sum_{i=1}^{S} n_i$), which is also called bacterial evenness.

Rank-abundance curves were obtained by plotting the relative intensity of each spot on the y-axis, which was represented on the logarithmic scale, and the rank of spot relative intensity on the x-axis (the most intense spot was assigned rank 1, the second intense spot was assigned rank 2, and so on).

RESULTS AND DISCUSSION

DNA extraction and PCR amplification   DNA extraction from an environmental sample is important in almost all molecular-based methods. Thus, the yield and purification of DNA are always recommended for capturing the entire repertoire of environmental bacteria. In this study, we used mechanical lysis based on the bead-beater method to extract DNA from four soil samples, and the DNA yields were 39.9, 9.8, 17.3, and 12.9 μg/g. This extraction method was used because it results in high recovery and high target DNA copy number, and is not labor-intensive (20).

The DNA fragments obtained by the PCR amplification of the V1 variable region of the 16S rDNA gene are suitable for 2-DGE analysis. The reason being that DNA fragments from this variable region show a high variation in size, as determined from the chain length and GC content of 200 bacterial 16S rDNAs derived from the ribosomal database project (RDP) (13). However, because of the small size (70–130 bp) of DNA fragments from the V1 variable region of the 16S rDNA gene, nonspecific fragments were easily generated when DNAs were amplified by PCR. Therefore, we employed direct amplification with one set of universal primers (27f and 109r) instead of the nested PCR method. This direct amplification was achieved using a relatively high primer concentration (0.5 μM) by controlling annealing temperature, which was reduced from 59°C to 55°C in 10 cycles, followed by 20 cycles at 55°C.

Two-dimensional DNA gel electrophoresis mapping The DNA fragments from the V1 variable region of the 16S rDNA gene were used for 2-DGE mapping. Band patterns from the first-dimensional electrophoresis are shown in Fig. 2. The sizes of the bands ranged from approximately 131 to 161 bp (GC clamp: 40 bp). In this procedure, Spreadex Polymer NAB solution was responsible for the high resolution of the gel due to its highly cross-linked nature (13, 21).
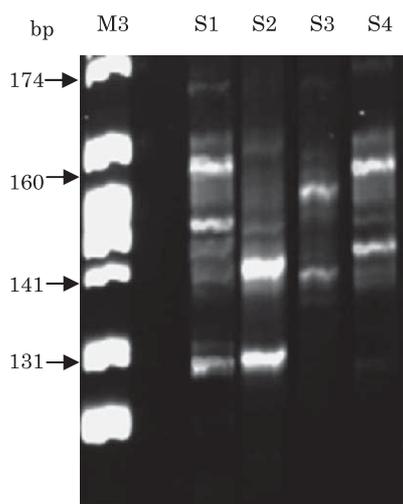
FIG. 2.   Chain length separation pattern based on size difference of 16S rDNA fragments of bacteria from four soil samples. Lanes: M3, size marker containing more than 50 DNA fragments in the size range from 50–622 bp; S1, unpolluted soil sample; S2, soil sample with 15.6 mg/kg TCE; S3, soil sample with 25.4 mg/kg TCE; and S4, soil sample with 28.3 mg/kg TCE.

Each band lane on the first-dimensional gel was cut into strips and separated into spot patterns by parallel DGGE on a polyacrylamide gel with denaturing gradient from 35% to 65% (Fig. 3a–d). In this protocol, the experimental results suggest that the adjustment of denaturing gradient produces a clear spot display. Figure 3a shows more than 50 spots on a strip of gel; this shows that the unpolluted grass soil sample had the highest bacterial diversity among the four samples. The 2-DGE maps from the TCE-contaminated samples showed spot differences in terms of number and distribution. It is interesting to note that some spots on these maps appeared to be sensitive to pollution. The number of spots from TCE-contaminated samples was small, but the relative intensity of some spots was high. This is because some bacteria can obtain more energy and nutrition due to the death of other bacterial species that cannot adapt to TCE-contaminated environment. Furthermore, a number of spots were observed at the position around 131 bp in slightly contaminated or unpolluted samples (S2 and S1), while a few or no spots were observed at this position in highly polluted samples (S3 and S4). Thus, these results indicate that the difference in spot distribution between 2-DGE maps can be used to assess the changes in the diversity of soil bacteria; moreover, because of the sensitivity of the spots to pollution, the maps can also monitor the quality of soil environments.

We consider that each spot on the 2-DGE map corresponds to one bacterial genus level or bacterial genus group level because only a small part (V1 variable region) of the entire 16S rDNA sequences of all bacteria was amplified by PCR. The position of each spot on these 2-DGE maps can be determined using a two-dimensional coordinate, in which the x-axis represents the size of DNA fragments and the y-axis represents the fragment constituent. The relative intensity of each spot, which was measured by calculating its area (integrated value) using the Lane and Spot Analyzer software package, was considered to reflect bacterial abundance. Thus, resolving the relationship between the relative intensity of each spot and the DNA sequences contained in these spots is also an important subject for an in-depth understanding of 2-DGE maps.

This technique is not only useful for revealing bacterial diversity and monitoring the quality of soil environment by modeling or simulation but also for identifying novel bacteria inhabiting environmental soil. Identification is achieved by extracting DNA from a spot on the high-resolution 2-DGE gel and performing cloning and sequencing analysis. The
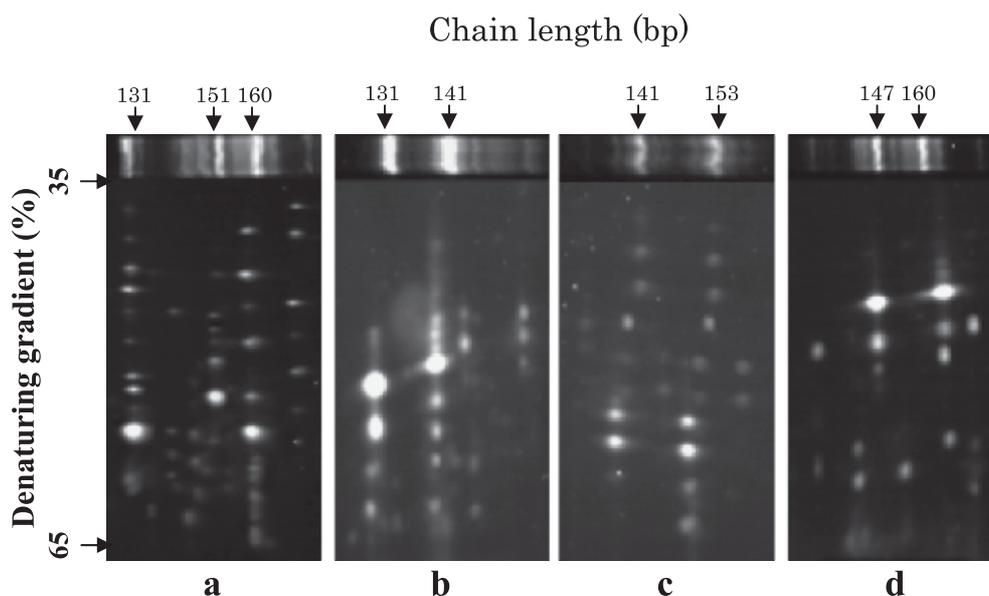

FIG. 3.   High-resolution maps based on 2-DGE of 16S rDNA fragments of bacteria from four soil samples. (a) 2-DGE map of bacterial 16S rDNAs from sample S1. (b) 2-DGE map of bacterial 16S rDNAs from sample S2. (c) 2-DGE map of bacterial 16S rDNAs from sample S3. (d) 2-DGE map of bacterial 16S rDNAs from sample S4. The top band figures of the maps show the results of chain length analysis.

excision of the target spot from the 2-DGE gel can be simplified by the fact that high resolution enables the minimization of fragment contamination.

Some spots may contain more than one bacterial genus owing to the insufficient separation of some DNA fragments. Spot detection by staining may affect the assessment of bacterial diversity. Despite these limitations, because of the improvement in the resolution of DNA fragments on a gel, 2-DGE provides a better qualitative and semi qualitative approach to investigating the diversity of soil bacteria. In addition, although 2-DGE requires an additional electrophoresis run (compared with DGGE), its procedure is not very complicated. Therefore, it is a powerful technique for assessing bacterial diversity or identifying novel bacteria in environmental soil. The application of this technique may also be expanded to other environmental systems such as water and food.

Denaturing gradient gel electrophoresis   To compare the resolution of 2-DGE with that of DGGE for the separation of genomic DNA fragments, PCR products were subjected to single-dimensional separation by parallel DGGE. The DGGE profiles are shown in Fig. 4. A total of 7 to 20 bands were detected from lanes S1 to S4; their denaturing gradient ranged from 35% to 65%. DGGE was first developed for detecting gene mutation but is now widely applied as a molecular technique associated with the coding of the 16S rRNA gene to study microbial diversity (22–24). The phylogenetic information of bacterial species can be derived by the cloning and sequencing analysis of DNA extracted from DGGE bands (12, 25). Although DGGE enables an immediate qualitative and semi qualitative scanning of genetic information of bacterial species in soil samples, its low resolution makes it difficult to reveal large bacterial diversity or detect differences between highly similar bacterial communities in soil since no clear display of DNA separation is easily obtained.

Resolution comparison of two patterns   Figure 5 shows the comparison between band pattern and spot pattern. The band patterns a_1, b_1, c_1, and d_1 represent DGGE profiles corresponding to each DNA sample; the spot patterns a_2, b_2, c_2, and d_2 represent 2-DGE profiles. A total of 25 to 50 spots can be clearly observed on the four 2-DGE
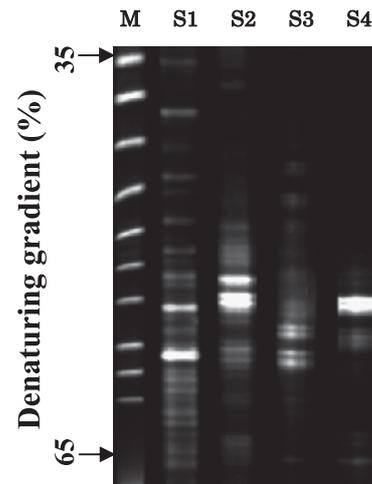

FIG. 4. DGGE fingerprint pattern based on composition difference of 16S rDNA fragments of bacteria from four soil samples. The denaturing gradient ranged from 35% to 65%. Running conditions: 130 V, 60°C, 9 h. Lanes: M, DGGE marker; S1, unpolluted soil sample; S2, soil sample with 15.6 mg/kg TCE; S3, soil sample with 25.4 mg/kg TCE; and S4, soil sample with 28.3 mg/kg TCE.

maps, although running DNA samples twice may result in a small loss in DNA, and some weak intensity spots cannot be detected by the present methods. Only 7 to 20 bands were detected on the DGGE gel. Thus, the resolution of 2-DGE was more than twice that of DGGE. This high-resolution display was achieved by 2-DGE. In the horizontal direction, a band from DGGE was separated into several DNA spots with the same GC content but different chain lengths. In the vertical direction, a band used in chain length analysis was separated into a number of DNA spots with the same chain length but different GC contents. This high resolution was difficult to achieve using single DGGE or chain length polymorphism analysis. The high resolution can be attributed to a detailed separation in chain length (1 bp difference) using a highly cross-linked polyacrylamide gel, and the size of DNA fragments can easily be deciphered according to marker size. However, we could not obtain the size of DNA fragments from the DGGE band pattern. This is the advantage of the novel approach using 2-DGE.
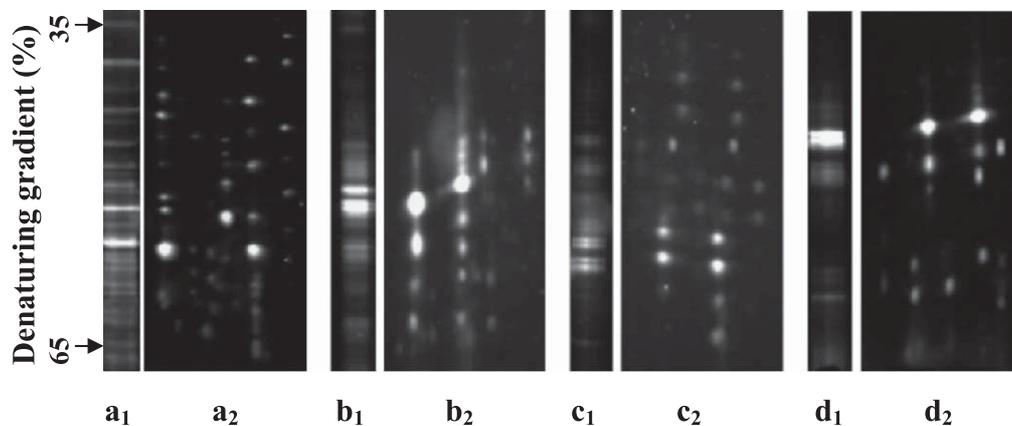

FIG. 5. Maps of resolution comparison between 2-DGE and DGGE. a_1, b_1, c_1, and d_1: DGGE fingerprint patterns; a_2, b_2, c_2, and d_2: 2-DGE spot patterns.
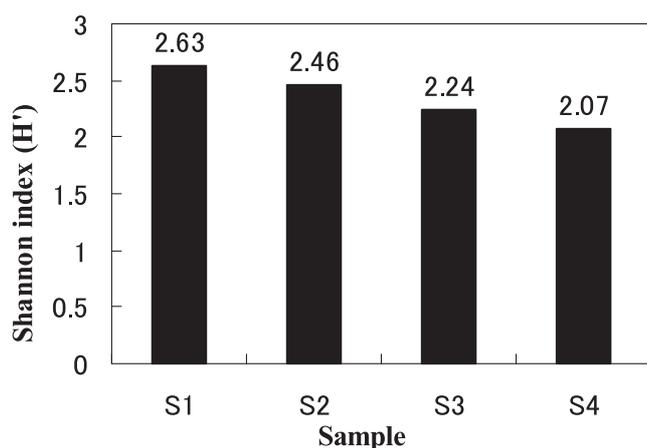
FIG. 6. Graph of the Shannon biodiversity index (H′) based on 2-DGE maps. S1, unpolluted soil sample; S2, soil sample with 15.6 mg/kg TCE; S3, soil sample with 25.4 mg/kg TCE; and S4, soil sample with 28.3 mg/kg TCE.

Diversity analysis of bacterial communities in environmental soil

Shannon index based on 2-DGE maps   Figure 6 shows the graph of the Shannon biodiversity index calculated from the intensity of spots on 2-DGE maps of the four soil samples. Shannon index (H′) has been widely used in measuring biodiversity in ecological systems. The advantage of using this index is that it considers both the number and evenness of species. Two properties of this index have been demonstrated: (i) H′=0, only when there is one species in the population and (ii) H′=maximum, only when each species in the population is evenly represented. In our study, the unpolluted soil sample (S1) showed the highest bacterial diversity (H′=2.63) and the smallest Shannon index (H′=2.07), that is, the lowest bacterial diversity was observed in the soil sample with 28.3 mg/kg TCE (S4).

Rank-abundance plot based on 2-DGE maps   The use of rank-abundance plots is a simple method of representing both bacterial abundance and evenness. It also overcomes the problems with the use of biodiversity indices such as the Simpson index and Shannon index, which cannot exhibit the relative roles played by different variables in their calculation.

The rank-abundance plot has been used to estimate bacterial abundance in recent studies (1, 26, 27). From the high-resolution map obtained from 2-DGE, we plotted rank-abundance curves in a two-dimensional chart by measuring the relative intensity of each spot. The results for the different samples are shown in Fig. 7. The rank of spot relative intensity was plotted on the x-axis and spot intensity was plotted on the y-axis, which was represented on the logarithmic scale. The relative intensity of each spot corresponds to bacterial abundance. Thus, bacterial diversity from different environmental soils can be compared using rank-abundance curves. Generally, bacterial abundance in each soil sample depends on spot number, and the slope of such curve determines bacterial evenness. A curve that extends farthest to the right denotes the highest bacterial abundance, and the smallest gradient of the curve indicates the greatest bacterial evenness because the abundances of different bacteria are
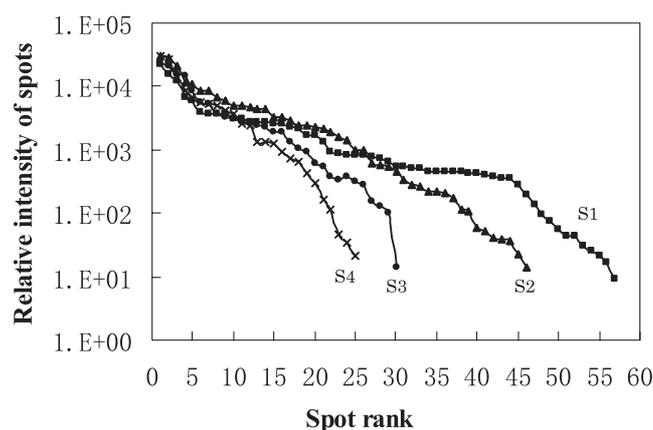

FIG. 7. Rank-abundance plots based on 2-DGE maps. The spot rank is plotted on the x-axis (the most intense spot is assigned rank 1, the second intense spot is assigned rank 2, and so on). The relative intensity of each spot is plotted on the y-axis, which is represented on the logarithmic scale. S1, unpolluted soil sample (squares); S2, soil sample with 15.6 mg/kg TCE (triangles); S3, soil sample with 25.4 mg/kg TCE (circles); and S4, soil sample with 28.3 mg/kg TCE (crosses).

similar. As shown in Fig. 7, the shape of the S1 curve represented the highest bacterial abundance and the greatest bacterial evenness. The spots decreased in number and the curves became steeper in gradient as TCE concentration increased in the other curves. These results indicate that TCE, an important pollutant, affects bacterial diversity in soils. The rank-abundance curves based on DGGE fingerprint patterns were also plotted (data not shown). However, these curves could not reveal a clear contrast in the diversity of bacteria from different environmental samples. Thus, the high-resolution map based on 2-DGE appears to have a potential advantage in plotting the rank-abundance curve used for the analysis of bacterial diversity in environmental soil.

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