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The Genetic Diversity Analysis of the Bacterial Community in Groundwater by Denaturing Gradient Gel Electrophoresis (DGGE)

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This study employed two PCR-based 16S rDNA approaches, amplified rDNA restriction analysis (ARDRA) and denaturing gradient gel electrophoresis (DGGE), to characterize the bacterial community structure in groundwater. Samples were collected from groundwater for the use by private residences, as well as for industrial and agricultural purposes, in Ansan City. Each PCR product was obtained by PCR with eubacteria 16S rDNA and variable V3 region specific primer sets. After amplification, the 16S rDNA PCR products were digested with 4-base site specific restriction endonucleases, and the restriction pattern analyzed. The genetic diversity and similarity of the groundwater bacterial community was analyzed by eubacteria universal primer sets for the amplification of variable V3 regions of the bacterial 16S rDNA. The result of the bacterial community analysis, by ARDRA and DGGE, revealed the same pattern. The highest diversity was found in groundwater from site G1, which was used in residences. In the DGGE profile, a high intensity band was sequenced, and revealed to be *Pseudomonas* sp. strain P51.

Key words: ARDRA, bacterial community, DGGE, groundwater

Early studies on groundwater ecosystems have primarily identified and characterized microorganisms from a phenotypic perspective, such as colony morphotypes, Gram staining, and carbon substrate utilization patterns. However, the median percentages of viable and culturable cells in aquitards and deep vadose zones are usually ca. 1% and 0.01% of the total microscopic count (Brockman *et al.*, 1993). In other words, these investigations are hampered by an inability to culture microbes, since many microbiological properties of groundwater cannot be ascertained. The cultivability problem is particularly relevant to groundwater studies, where many microorganisms reside in a dormant or low-activity states, or a viable, but non-culturable, state. Therefore, numerous methods, such as culture-independent molecular techniques, have been developed and utilized to improve access to the diversity of microbial communities.

Of the various approaches for the understandings of microbial community structures in nature, comparative analysis of 16S rRNA sequence of microorganisms has been universally applied, due to the ubiquity of ribosomal RNA molecules in all microorganisms, to infer relationships among organisms (Pederson *et al.*, 1996; Wise *et al.*, 1999; Lee *et al.*, 2000). The rRNA molecules are comprised of highly conserved sequence domains, interspersed with more variable regions. In general, the essential rRNA domains are conserved across all the phylogenetic domains, thus universal tracts of sequences can be identified (Olsen *et al.*, 1986).

This study employed two PCR based-16S rDNA approaches, ARDRA (amplified rDNA restriction analysis) and DGGE (denaturing gradient gel electrophoresis), to characterize the bacterial community structures in groundwater. A very simple method for measuring the community diversity is ARDRA, which is based on the restriction digestion of a population of rDNA sequences directly amplified from an environmental sample. However, ARDRA has limited resolution for identifying a specific phylogenetic group within a complex community, as it can only utilize sequence information from restriction sites. Recently, a novel method, PCR followed by denaturing gradient gel electrophoresis (PCR-DGGE), was proposed for the study of the phylogenetic diversity of bacterial populations in environmental samples (Muyzer *et al.*, 1993). In this method, total microbial DNA is extracted from groundwater bacteria, and the bacterial 16S rRNA genes amplified by PCR with universal eubacterial primers. The PCR products of the same length, but with different internal sequences, can be separated by DGGE, according to their melting properties. The patterns obtained provide information on the underlying bacterial

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populations. This molecular method does not have the limitation of cultivation based methods, and hence can assess the diversity of the bacterial groups, including the non-culturable bacterial groups.

The aims of this study were to establish PCR-DGGE based technology for the analysis of microbial community structures in groundwater, without the need for isolation and identification of pure cultures.

Materials and Methods

Groundwater samples

Groundwater samples were collected from four sites in Ansan City during August 2000. Water for homes use was collected from a residential area (G1 site), industrial water from an industrial area (G2 site), agricultural water from an agricultural area (G3 site), and drinking water from a park area (G4 site) (Fig. 1). The samples were collected in sterilized 4 liter polyethylene bottles.

Physico-chemical environmental factors

The temperature, pH, conductivity and salinity of samples were measured, with a Water Tester (Horiba U-10, Japan), immediately after sampling. The ammonia, nitrate, nitrite, and phosphate concentrations were measured according to standard methods (American Public Health Association, 1998). The analysis for trichloroethylene (TCE) and perchloroethylene (PCE) were accomplished by the methods of the National Institute of Environmental Research, according to the manufacturer's instructions.

Bacterial counting and isolation

The total bacterial count was determined by epifluorescence microscopy (Axioplan, Zeiss, Germany), after staining with 4, 6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980). Samples (100 μl) were spread on plates containing NA medium (8 g of beef extract, 5 g of peptone, and 15 g of agar per liter). The plates were incubated for 2 days at 37°C. From each of the four samples 8 isolates were selected, and subcultured several times on NA medium. The isolates were stored at -80°C in NA broth medium (8 g of beef extract, and 5 g of peptone per liter) containing 15% glycerol.

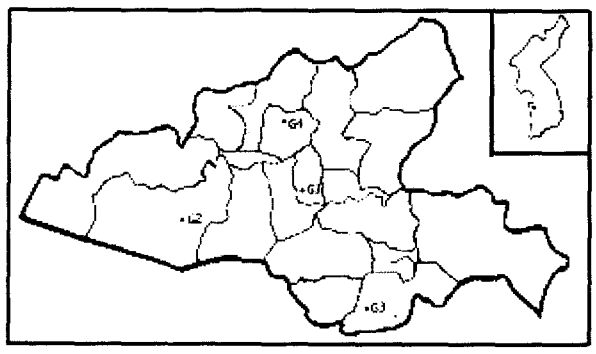

Fig. 1. Sites and Locations of the surveyed groundwater in Ansan City. G1, groundwater for residential use; G2, groundwater for industry; G3, groundwater for agriculture; G4, groundwater for drinking.

Genomic DNA extraction of cultured isolates

The genomic DNA from the bacterial cells was obtained using a modification of the method described by Sambrock *et al.* (1989). The bacterial cells from pure cultures were harvested by centrifugation (12,000 rpm) for 2 min, and the cell pellets mixed with 600 μl of lysis buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5], 0.5% SDS, and 100 μg/ml proteinase K) and incubated at 37°C for 1 h. After the addition of 100 μl 5 M NaCl, and 80 μl CTAB/NaCl, the samples were incubated at 65°C for 10 min. The samples were cooled to room temperature, followed by extraction of the aqueous phase with an equal volume of chloroform: isoamyl alcohol (24 : 1, v/v), and then with an equal volume of phenol: chloroform: isoamyl alcohol (25 : 24: 1, v/v), which was centrifuged at 12,000 rpm and 4°C for 10 min. Isopropanol (0.6×) was mixed with the aqueous phase, and centrifuged at 12,000 rpm and 4°C for 10 min. After removal of the aqueous phase, the DNA pellets were washed with 70% ethanol, and centrifuged at 12,000 rpm and 4°C for 10 min, The DNA pellets were dried under vacuum, and then dissolved in TE buffer (10 mM Tris-HCl, and 1 mM EDTA [pH 7.5]).

Genomic DNA extraction by filtering method

The procedure was performed as a modification of that described by Watanbe *et al.* (2000). The microorganisms from 4 liters of groundwater were collected by filtration with a 0.22 μm pore size membrane filter (type GV, Millipore) within 5 h of sampling. The membrane filter was soaked in 0.5 ml of a cell suspension buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.35 M sucrose, and 20 mg/ml lysozyme). After incubation for 10 min at 37°C, 0.75 ml of a lysing solution (100 mM Tris-HCl [pH 8.0], 0.3 M NaCl, 20 mM EDTA, 2% SDS, and 2% 2-mercaptoethanol) was added, and the suspension incubated at 70°C for 30 min. The membrane filter was then removed, and the solution extracted twice with phenol: chloroform (1:1, v/v). Ethanol, twice the volume, was added, to the sample, and incubated at -20°C for 2 h. Nucleic acids were precipitated by centrifugation at 12,000 rpm for 10 min, washed with 1 ml of 70% ethanol, and then dissolved in 0.5 ml of TE buffer containing 100 mg of RNase A. The solution was incubated at 37°C for 1 h, and the DNA precipitated by the addition of 2 volumes of ethanol, followed by washing with 1 ml of 70% ethanol. Finally, the DNA was dissolved in 0.2 ml of TE buffer. The concentration and purity of the DNA preparation were determined by absorption spectrophotometry (UV-160A, SHIMADZU, Japan).

PCR-amplified rDNA restriction analysis (ARDRA)

The procedure was performed as described by Jawad *et al.* (1998). Briefly, a ca. 1,500 bp fragment of the 16S rDNA gene was amplified using the universal primers 5'-GAGTTGGATCCTGGCTCAG and 5'-AAGGAGGGGATCCAGCC. The reaction mixture (50 ml final volume) consisted of 300 mM Tris-HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 20 pM of each primer, 20 mM of each dNTP, 2 U Taq polymerase (Super-Therm, England), and a 20 ng template. Amplification reactions were performed using an AMPLITRON®II THERMOLYNE (BARNSTEAD/THERMOLYNE, USA). After an initial denaturation at 95°C for 3 min, 32 cycles of 1 min at 95°C, 1 min at 50°C and 2 min at 72°C were performed. This was followed by a final extension at 72°C for 10 min. The restriction endonucleases, *Hae*III, *Msp*I, *Rsa*I and *Taq*I (Boehringer Mannheim, Germany), were used in separate reactions to digest the PCR products, according to manufacturer's instructions. The restricted DNA fragment was analyzed by horizontal electrophoresis in 3% agarose (FMC, USA). The electrophoresis was performed at 50 V for 2 h, and the gels stained with ethidium bromide (1 μg/ml), after which they were inspected under UV light and photographed with the SL-5GD-Photographic system. Differences in the restriction fragment patterns indicated differences in the DNA sequence. The restriction fragment lengths were calculated, and compared using an Image analyzer (Arcus II, Vilber Bio ID Loumat, France). A dendrogram was constructed from the distance matrix, using the unweighted pair group method, with the arithmetic means (UPGMA).

PCR-DGGE

The V3 region of the 16S rRNA genes (corresponding to positions 341-534 in *E. coli*) from the community DNA were amplified by PCR, using eubacteria specific primers, as described by Muyzer *et al.* (1993). The GC clamps described by Muyzer *et al.* (1993) were included on the 5' end of the forward primer (Table 2). The reaction mixture (50 μl final volume) consisted of 300 mM Tris-HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 20 pM of each primer, 10 mM of each dNTP, 2 U Taq polymerase (Super-Therm, England), and a 20 ng template. The thermocycling program for the touchdown PCR was as follows: initial denaturation was performed at 95°C for 5 min and then at 95°C for 1 min, followed by touchdown primer annealing from 55°C to 45°C (the annealing temperature was decreased 1°C every second cycle for the first 22 cycles, to touchdown at 45°C), followed by extension at 72°C for 1 min (for each of the 22 cycles), 18 more cycles were then performed at 95°C for 1 min, 45°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. The DGGE was performed using a V20-HCDC (SCIE-PLAS, England). The PCR samples were loaded onto 8% (wt/vol) polyacrylamide gels in 0.5×TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na-EDTA, pH 7.4). The 8% (w/v) polyacrylamide gels (bisacrylamide gel stock solution, 37.5:1) were prepared with a 20 to 80% gradient of denaturant (7 M urea and 40% deionized formamide). The electrophoresis was run at 60°C, for 3 h at 200 V. After the electrophoresis, the gels were stained for 45 min, with SYBR Green I nucleic acid gel stain (Molecular Probe, Netherlands), inspected under UV light and photographed.

Sequencing of DGGE fragment

The DNA fragments to be sequenced were punched from the gel with sterile pipette tips, placed in sterilized vials, and 20 μl of sterilized water added. The DNA was allowed to passively diffuse into the water overnight at 4°C. Ten microliters of the eluate was used as the template DNA in the PCR, using the primers (no GC clamp 314f and 518r) and conditions described above. Following amplification, the PCR products were directly sequenced with an ABI Prism 3100 Genetic Analyzer. The sequences were aligned to the 16S rRNA sequences obtained from the database of the National Center for Biotechnology Information using a BLAST search.

Results

Analysis of physical and chemical environmental factors

Table 1 shows the physical and chemical characteristics of

Table 1. Physico-chemical environmental factors from groundwater in Ansan City

	G1	G2	G3	G4	drinking water criteria
Temperature(°C)	22.9	22.7	19.1	17.1	
pH	6.65	6.89	10.27	7.51	5.8~8.5
conductivity (ms/cm)	0.298	0.312	0.298	0.126	
salinity (‰)	0.01	0.01	0.01	0	
PO_4^{3-} (mg/l)	0.1417	0.0072	0.0094	0.009	
NH_4^+ (mg/l)	0.0039	0.0095	0.0240	0.0019	<0.5
NO_2^- (mg/l)	0.004	0.0039	0.0028	0.002	
NO_3^- (mg/l)	0.3112	0.1031	0.4128	0.0629	<10
TCE (mg/l)	ND	0.004	ND	ND	<0.03
PCE (mg/l)	ND	ND	ND	ND	<0.01

Table 2. Primer sequence and position

Primer (a)	Position (b)	Target	Sequence
GC341(f)	341-358	eubacteria, V3 region	5'-CCTACGGGAGGCAGCAG-3'
PRUN518(r)	518-534	universal, V3 region	5'-ATTACCGCGGCTGCTGG-3'
GC clamp(c)			5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3'

(a) f, forward primer; r, reverse primer; c, The GC clamp was attached to the 5' end of the GC341f primer.
(b) The numbering of position is based on *E.coli* 16S rRNA.

the groundwater samples. The results were found not to exceed the drinking water criteria. However, the presence of each pollutant was found to differ according to the groundwater location (high concentrations of phosphate in G1, TCE in G2, ammonia and pH in G3).

Measurement of total bacteria counting

The total bacteria counting results, by the DAPI method, were in the range 1.6~35.4×10^5 CFU. The total bacterial numbers were in the order 8.00×10^5, 1.54×10^6, 3.54×10^6, and 1.60×10^5 CFU at sites G1 to G4, respectively.

Extraction and quantitation of genomic DNA

In situ samples were concentrated using a Sterivex-GV (Millipore, USA) filter unit, and the genomic DNA extracted. For each site, the quantities of extracted genomic DNA were in the range of 0.021~0.230 μg/ml. The highest concentration was 0.230 μg/ml at the G3 site. These results were equal to those from the total bacteria counting. The quantities of the extracted genomic DNA were 0.073, 0.170, 0.230, and 0.021 μg/ml at sites G1 to G4, respectively.

Analysis of the microbial community in groundwater using ARDRA

The PCR amplification using the universal primer with the genomic DNA extracted from each groundwater site resulted in a PCR product of approximately 1500 bp. The PCR product (data not shown) of the 16S rDNA was compared with the fragment pattern by treatment with four restriction enzymes, which recognize four bases, and the genetic diversity and similarity in the groundwater microbial communities. Restriction enzymes were used with *Hae*III, *Msp*I, *Rsa*I, and *Taq*I, which proved the excellence of the restriction enzyme used in previous studies (Laguerre *et al.*, 1994; Brunel *et al.*, 1997; Pukall *et al.*, 1998; Ian *et al.*, 2000). The restriction enzyme fragment patterns of the 16S rDNA PCR products obtained are shown in Figs. 2 and 3.

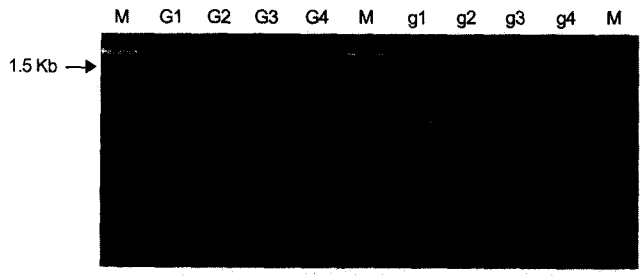

Fig. 2. Restriction pattern of PCR-amplified fragment of 16S rDNA genes digested with *Hae*III and *Msp*I. M, 1Kb DNA Ladder; G1 and g1, groundwater for residential use; G2 and g2, groundwater for industry; G3 and g3, groundwater for agriculture; and G4 and g4, groundwater for drinking. G was digested with *Hae*III and g with *Msp*I.

According to the type of restriction enzyme, the 16S rDNA fragment patterns were different, and showed notable genetic differences within the microbial communities. Restriction enzyme fragments of the 16S rDNA were represented in bands 3 to 14. Most fragments were found at the G1 site, and the fewest at sites G3 and G4.

The similarity between the patterns was calculated by matching the band positions, which were then clustered

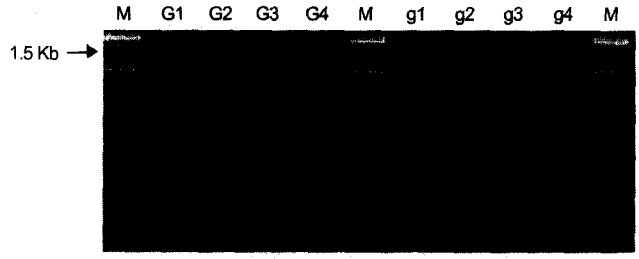

Fig. 3. Restriction pattern of PCR-amplified fragment of 16S rDNA genes digested with *Taq*I and *Rsa*I. M, 1Kb DNA Ladder; G1 and g1, groundwater for residential use; G2 and g2, groundwater for industry; G3 and g3, groundwater for agriculture; and G4 and g4, groundwater for drinking. G was digested with *Taq*I and g with *Rsa*I.

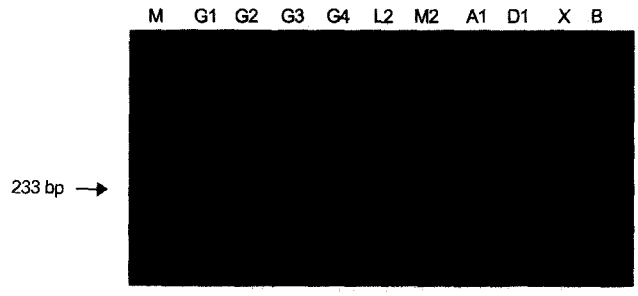

Fig. 4. PCR products of extracted DNA by a 16S rDNA primer. M, 100bp DNA Ladder; G1, groundwater for residential use; G2, groundwater for industry; G3, groundwater for agriculture; G4, groundwater for drinking; L2, isolate in groundwater for residential use; M2, isolate in industrial groundwater; A1, isolate in agricultural groundwater; D1, isolate in drinking groundwater; X, a sample obtained after PCR amplification of the mixed bacteria genomic DNAs (L2, M2, A1, and D1); B, negative control (D.W.).

by UPGMA, and a dendrogram constructed. The highest similarity coefficient was 76.5%, between the G3 and G4 site (Fig. 7).

Application of DGGE

The touchdown PCR technique, suggested by Muyzer *et al.* (1993), was used to amplify the 16S rDNA V3 region of the microbial community in the groundwater. This touchdown PCR technique was performed to minimize the nonspecific annealing primers to the non-target DNA, and the PCR product was approximately 233 bp (Fig. 4).

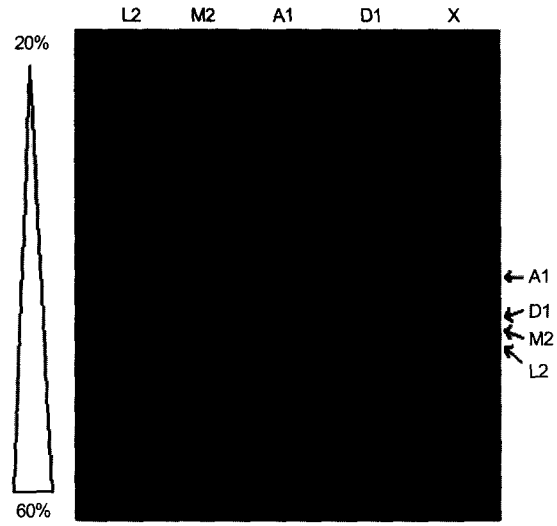

Fig. 5. DGGE analysis of 16S rDNA fragments of different isolates obtained after PCR amplification. L2, isolate in groundwater for residential use; M2, isolate in industrial groundwater; A1, isolate in agricultural groundwater; D1, isolate in groundwater drinking; X, a sample obtained after PCR amplification of the mixed bacteria genomic DNAs (L2, M2, A1, and D1). Percent values indicate the percentage of denaturants at each position.

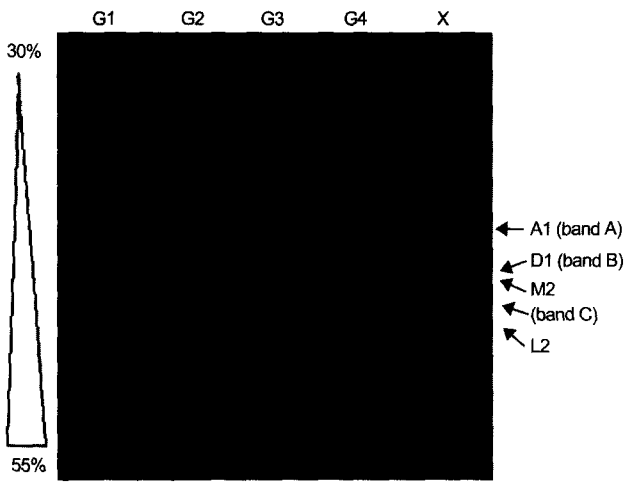

Fig. 6. DGGE analysis of 16S rDNA fragments obtained after PCR amplification of genomic DNA from uncharacterized bacterial community. G1, groundwater for residential use; G2, groundwater for industry; G3, groundwater for agriculture; G4, groundwater for drinking; X, a sample obtained after PCR amplification of the mixed bacteria genomic DNAs (L2, M2, A1, and D1). Percent values indicate the percentage of denaturants at each position.

Fig. 5 shows the DGGE analysis pattern of PCR products obtained after amplification of the genomic DNA of four purified bacterial strains isolated from the groundwater, and of the mixed bacterial genomic DNAs. Substantial separation of the 16S rDNA fragments derived from the four different bacterial isolates was observed when they underwent separate electrophoresis. When a mixture of the different isolates template DNAs was used, a similar separation pattern was observed, and the DGGE found to provide an immediate display of the constituents of the groundwater population.

Analysis of microbial community using DGGE

The 233 bp of PCR products obtained from the 16S rDNA amplification of the genomic DNA, directly extracted from each site, were applied to the DGGE, and 5~12 bands confirmed (Fig. 6). The bands in the DGGE profile corresponded to the 16S rRNA fragments, but differed in their nucleotide sequence, reflecting the distinct numerically dominant microbial populations in the community. Moreover, the intensity of the PCR product was believed to be proportional to the abundance of the template, and therefore, the abundance of each population (Muyzer *et al.*, 1993). Therefore, band A, which was common to all sites, suggests this is the major dominant population, and bands B and C, which had high intensities at the G3 and G4 sites, were abundant in each population.

That the diversity of the microbial population of each site was confirmed by the band profiling, with the highest appearing at the G1 site. Whereas, the total bacterial counting and DNA yield were high in G3, but the microbial community diversity was low. Therefore, the G3 site was characterized by the dominance of a few organisms (bands A, B, C), and consequently, a low diversity. The similarity coefficient was the highest between sites G3 and G4, at 73% (Fig. 8).

Sequencing of DGGE fragment

For the determination of the more specific community structure traits, a sequencing analysis of the specific bands

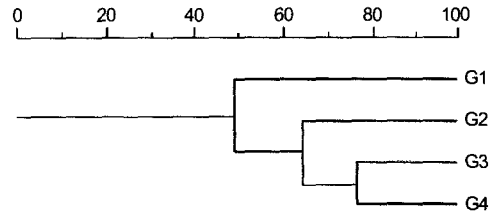

Fig. 7. Dendrogram of genetic similarity matrix value of 16S rDNA genotypes analyzed by PCR-ARDRA from uncharacterized bacterial community. G1, groundwater for residential use; G2, groundwater for industry; G3, groundwater for agriculture; and G4, groundwater for drinking.

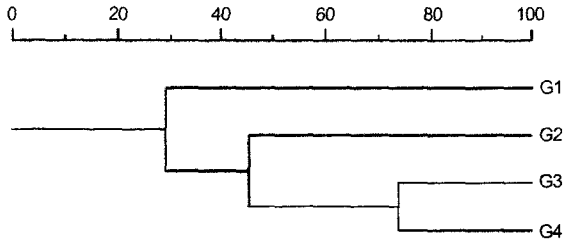

Fig. 8. Dendrogram of genetic similarity matrix value of 16S rDNA genotypes analyzed by PCR-DGGE fragments from uncharacterized bacterial community. G1, groundwater for residential use; G2, groundwater for industry; G3, groundwater for agriculture; and G4, groundwater for drinking.

DGGE band B

301

CCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATG

GGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGAT

GAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAA

CGAAAAGACTCTGGTTAATACCTGGGGTCCATGACG

GNACCGTAAGAATAAGCACCGGCTAACTACGT

476

Fig. 9. Sequence analysis of DGGE band fragment. N; ambiguous nucleotide. *Pseudomonas* sp. P51 positions (arrows) and region to which the annealing forward primer was the target (underline) are indicated.

was performed. Band B, which had high intensity in sites G3 and G4, was sequenced. The results of the alignment to the 16S rRNA sequence, using the BLAST network service, suggested a 99% identity with that of *Pseudomonas* sp. strain P51 (position 301~476). The sequencing results are shown in Fig 9.

Discussion

This study used ARDRA and DGGE to analyze the genetic diversity of the microbial populations in groundwater. These molecular techniques are based on the separation of gene PCR-amplified fragments coding for the 16S rRNA. A number of reports have suggested the limitations with PCR-mediated methods for quantitative analyses; possible biases are known to be associated with the DNA extraction (Wintzingerode *et al.*, 1997) and PCR amplification steps (Reysenbach *et al.*, 1992; Suzuki and Giovannoni, 1996; Wintzingerode *et al.*, 1997). Therefore, the samples were concentrated and extracted using a Sterivex-GV filter unit for the accumulation of the bacterial community to a high concentration. This nucleic acid extraction method is suitable for PCR amplification (Leff *et al.*, 1995), is able to minimize DNA destruction, and is sufficient for DNA extraction, as the DNA disruption is small, and many types of 16S rDNA can be amplified.

The ARDRA method has been applied to microbial communities from groundwater. It was first used to identify medically important bacteria (Vaneechoutte *et al.*, 1993), but these investigations only focused on a specific bacteria group. Recently, the ARDRA technique was used for the characterization of environmental communities, due to its ability for rapid comparison of the microbial populations present in different ecosystems (Pukall *et al.*, 1998). However, the ARDRA method lacks sensitivity in the detection of nondominant members, has limited resolving power of the band size on agarose gels, lacks quantitative information, and only shows relative diversity. These problems were also found in this study.

The analysis of the microbial community structure was followed by the DGGE technique, suggested by Muyzer *et al.* (1993), which is widely applied to microbial ecology studies as a molecular biological technique associated with 16S rDNA (Fries *et al.*, 1997; Brinkhoff *et al.*, 1998; Murry *et al.*, 1998; Rosado *et al.*, 1998; Bruns *et al.*, 1999; Sievert *et al.*, 1999). There has been concern regarding chimera formation during the PCR amplification process (Liesack *et al.*, 1991; Reysenbach *et al.*, 1992; Suzuki and Giovannoni, 1996), so to avoid this problem, the shorter and highly variable V3 region of 16S rRNA was chosen for the DGGE analysis. For optimal DGGE separation, a 40 bp GC clamp was incorporated into the 5' primer, which was necessary for the optimal resolution of the fragments in the denaturing gradient gel.

The denaturant gradient is also very important. In Fig. 5, the homoduplex molecules (see the M2, D1 band in mixture X) were confirmed, and were caused by ambiguity in the identical positions of the bands in the DGGE gel, with a 20~60% denaturant gradient. This problem can be addressed by exploiting a particular advantage of DGGE, i.e., using narrower gradients (30~55%), to provide a high resolution of the DGGE profiles of particular parts of the original profile (Fig. 6).

The microbial communities within contaminated ecosystems tend to be dominated by those organisms capable of utilizing or surviving the contaminants (Atlas *et al.*, 1991; Macnaughton *et al.*, 1999). Therefore, disturbances, such as the introduction of pollutants into the ecosystem, have been found to reduce the diversity of bacterial communities (Sayler *et al.*, 1982; Atlas and Bartha, 1998; Eichner *et al.*, 1999). In this study, pollutants that had been introduced into each groundwater, according to their utility, were confirmed. The G3 site showed an especially high concentration of ammonia, and therefore a high pH. This means that nitrogen pollutants, i.e., fertilizer, had been introduced into the groundwater. The G3 site, which had the highest bacterial level, was found to have reduced bacterial community diversity due to the introduction of pollutants into the ecosystem, and the introduction of these pollutants into the soil ecosystem caused direct con-

tamination of the groundwater. To determine the more specific traits in the community structure, a sequencing analysis of the specific bands was performed. As a result of the sequencing, band B was proved to be *Pseudomonas* sp. strain P51, which utilizes 1, 2, 4-trichlorobenzene as a substrate for growth (Ogawa and Miyashita, 1999). This may prove that the diversity of a microbial community is reduced by specific pollutants.

In this study, ARDRA and DGGE were applied to analyze the genetic diversity of the microbial populations in groundwaters. As described above, these methods show similar results because they are based on the PCR amplification of 16S rDNA. However, with the result of these methods, represented by a dendrogram, little difference is evident. This is because DGGE has a high sensitivity for the detection of nondominant organisms.

DGGE provides an immediate display of the constituents of a population in both qualitative and semiquantitative ways, and is less time-consuming and laborious. The primers used in this study were also specific to all eubacteria, but other primers can be designed to determine the genetic diversity among species from specific eubacterial groups or from other kingdoms, such as the eukaryotes and archaebacteria (Muyzer *et al.*, 1993). This technique has advantages that are ideally suited for monitoring the dynamics of microbial communities influenced by environmental changes (Henckel *et al.*, 1999), which detect specific microorganisms by hybridization after band separation (Muyzer *et al.*, 1993; ØvreÅs *et al.*, 1997; Santegoeds *et al.*, 1998). However, several different studies have shown that bacterial populations making up 1% or more of a total community can be detected by PCR-DGGE (Murry *et al.*, 1996). Despite these limitations, molecular methods can reveal the presence of microorganisms not detectable by classical cultivation techniques (Iwamoto *et al.*, 2000).

For the characterization of a complex microbial community structure, a multi-dimensional approach must be followed: an analysis of the phylogenetic relation by sequencing (Henckel *et al.*, 1999; Wise *et al.*, 1999), correlation between bacterial communities, with physicochemical environmental factors (Boon *et al.*, 2000), andsubstrate utilization patterns (Duineveld *et al.*, 1998).

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