J. Microbiol. Biotechnol. (2006), **16**(5), 704–715

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Analysis of Bacterial Diversity and Community Structure in Forest Soils Contaminated with Fuel Hydrocarbon

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Received: August 30, 2005
Accepted: September 24, 2005

Abstract Oil spill was found in 1999 from a diesel storage facility located near the top of Baekun Mountain in Uiwang City. Application of bioremediation techniques was very relevant in removing oil spills in this site, because the geological condition was not amenable for other onsite remediation techniques. For efficient bioremediation, bacterial communities of the contaminated site and the uncontaminated control site were compared using both molecular and cultivation techniques. Soil bacterial populations were observed to be stimulated to grow in the soils contaminated with diesel hydrocarbon, whereas fungal and actinomycetes populations were decreased by diesel contamination. Most of the diesel-degrading bacteria isolated from contaminated forest soils were strains of *Pseudomonas*, *Ralstonia*, and *Rhodococcus* species. Denaturing gradient gel electrophoresis (DGGE) analysis revealed that the profiles were different among the three contaminated sites, whereas those of the control sites were identical to each other. Analysis of 16S rDNA sequences of dominant isolates and clones showed that the bacterial community was less diverse in the oil-contaminated site than at the control site. Sequence analysis of the alkane hydroxylase genes cloned from soil microbial DNAs indicated that their diversity and distribution were different between the contaminated site and the control site. The results indicated that diesel contamination exerted a strong selection on the indigenous microbial community in the contaminated site, leading to predominance of well-adapted microorganisms in concurrence with decrease of microbial diversity.

Key words: Bioremediation, fuel, 16S rRNA, soil DNA, DGGE, bacterial community

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Oil spills and leaks from storage tanks occur frequently in land areas, causing great damage to the surrounding ecosystems, such as soil, groundwater, and surface water. Various regulations and agreements require the remediation of such contaminations. Massive oil pollution threatens public health, wildlife, and land development, and therefore, prompt to investigate cost-effective, safe, and less disruptive cleanup strategies [45].

Oil-contaminated sites are often located in regions that are difficult to access. It is not easy to treat contaminated soils at such remote sites with conventional remediation approaches, because of limited infrastructure and difficulty of transporting equipment. Bioremediation, the use of microorganisms to remove environmental contaminants, can be done onsite and is known to be an effective method for cleaning up oil-contaminated soil [41, 47]. Oil-degrading microorganisms are ubiquitous in the environment, and various bacteria, fungi, and algae able to degrade oil have been reported [3, 25]. Among these organisms, bacteria are considered to be mainly responsible for degradation of diesel fuel hydrocarbon contaminant in the environment [25, 31, 46].

Various methods have been used to characterize hydrocarbon-degrading microorganisms in soil. Traditionally, soil microbiological experiments have been focused on cultivation of individual microbial species that can be used for further phylogenetic and physiological characterizations [18]. However, cultivation has limited usefulness, since most of the soil microorganisms are known to be unculturable on laboratory media [5]. Molecular methods based on total soil microbial community DNA analysis can overcome the limitation of culture-based techniques. Analysis of 16S rDNA sequences cloned from environmental samples facilitated

detection and identification of unculturable microorganisms and confirmed the immense underestimation of naturally occurring bacteria in the environment [26]. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes have also been used to produce DNA banding patterns that represent the whole microbial community, allowing to detect significant changes of the microbial community structure in nature [17, 27, 30].

Hydrocarbon-degrading microbial populations were isolated and identified in various environments, including field soils, sediments, coast littoral zone, arctic soils, and Antarctic soils [3, 4, 9, 25, 29, 49], but little information is available about the diversity and changes of bacterial community in forest soils contaminated with diesel fuel hydrocarbon. In the temperate region, unlike field soil, forest soil is subjected to low temperature, large temperature fluctuations, and dry condition throughout the year. Thus, forest soil microbial communities may differ from those in polar soils, sediments, and field soils.

In this study, we analyzed the differences of bacterial communities between forest soils contaminated with diesel fuel hydrocarbon and uncontaminated control soils by using both molecular and cultivation techniques. The bacterial community structure of the contaminated site and its control site was analyzed by DGGE of specific DNA fragments of 16S rRNA genes. 16S rDNA sequences obtained by direct cloning from forest soil DNA and from isolated bacterial populations were analyzed and compared. Finally, the sequence diversity and distribution of the alkane hydoxylase (*alkB*) genes, which encode the key enzymes involved in the first step of alkane degradation, was analyzed between the contaminated soil and the control soil.

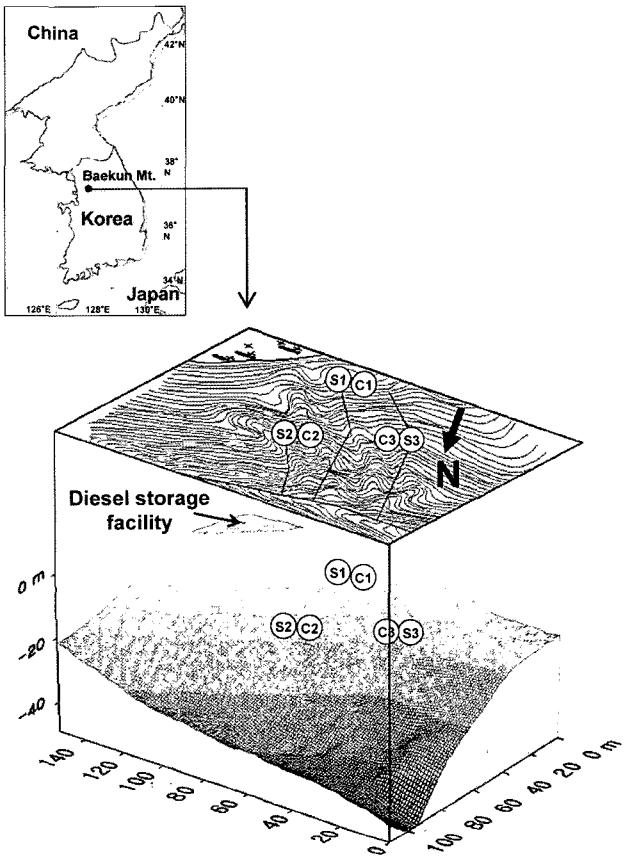

Fig. 1. Study sites contaminated with diesel hydrocarbon in Baekun Mountain.

MATERIALS AND METHODS

Forest Soil Samples and Culture Media

Diesel-contaminated soil samples were taken from three separate seepage sites (designated as S1, S2, and S3) located on Baekun Mountain (37° 21' N, 127° 0' E) in Korea, and their respective control soil samples (designated as C1, C2, and C3, respectively) were also taken from the nearby sites uncontaminated with diesel (Fig. 1). The forest soil sites under study, characterized as a sandy loam soil, have been contaminated with diesel continuously seeped from underground since 1998. Among the three sites, the site S3 has received especially more attention, because its overall total petroleum hydrocarbon (TPH) values were the highest of the three sites and its contamination resulting from continuous underground seepage was directly associated with contamination of a valley below this site. In the Spring of 2004, soil samples were taken from three randomly chosen locations in each site to a depth of 15 cm and were combined into one sample. All soil samples were sifted through a 2-mm sieve, placed in a plastic bag, and stored at 4°C until used. A 10-g soil sample from each site was homogenized with 95 ml of 0.85% sterilized saline solution by shaking at 200 rpm on a rotary shaker (Vision Scientific Co., Bucheon, Korea). Total counts of bacteria, actinomycetes, and fungi were measured with tryptic soy agar, sodium caseinate agar, and malt extract agar medium, respectively [47]. Dominant bacterial strains were isolated by plating appropriate dilutions of soil suspensions onto peptone-tryptone-yeast extract-glucose (PTYG) medium [6]. All cultures were incubated at 28°C for 4–7 days.

Most Probable Number Estimation and Isolation of Diesel-Degrading Microorganisms

A 10-g soil sample from each site was homogenized with 95 ml of a sterile 0·85% saline solution by shaking the preparation at 200 rpm on a rotary shaker. Samples (0.1 ml) of appropriate 10-fold dilutions were inoculated into five replicate MPN (most-probable-number) tubes containing 3 ml of diesel mineral medium (MMO mineral medium containing 1,000 ppm of diesel) [22]. The tubes were incubated at 28°C for 4 weeks, and degradation of diesel was analyzed by gas chromatography, with positive tubes

being scored as those with substantial cell growth and less than 20% of the diesel remaining. The population density of diesel degraders was estimated from MPN tables [1]. The culture of the terminal positive tube showing diesel degradation was diluted with sterile saline solution (0.85% NaCl) and spread onto PTYG (Peptone-Tryptone-Yeast extract-Glucose) agar medium, and then the plates were incubated at 28°C for 4 days. Single colonies were tested for diesel degradation in fresh diesel mineral medium before strain purification.

Identification of Dominant Bacterial Isolates and Diesel-Degraders

Dominant bacterial isolates obtained from the contaminated site S3 and its uncontaminated control site C3 (designated SV and CV, respectively) were subjected to repetitive extragenic palindromic (REP)-PCR to distinguish different strains by comparison of their genomic DNA fingerprints [11]. For taxonomic identification of the dominant bacterial isolates and diesel-degrading bacteria, total genomic DNA was extracted from each strain with a Wizard Genomic DNA Purification Kit (Promega, MA, U.S.A.). PCR amplification of nearly full-length 16S rRNA genes was performed with 27f and 1492r (*E. coli* 16S rRNA gene sequence numbering) as previously described [10, 24]. PCR products were purified by using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Sequencing was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, U.S.A.) according to the manufacturer's instructions with the sequencing primers 27f and 519r. Approximately 500 unambiguous nucleotide positions were used for comparison with the data in GenBank using the Basic Local Alignment Search Tool (BLAST) [2]. Sequences from nearest relatives were identified from the Ribosome Database Project (RDP) using the SIMILARITY-RANK program of the RDP [28]. The sequences were aligned by using ClustalX [42] and a phylogenetic tree was constructed by using the neighbor-joining method included in the PHYLIP software [13].

16S rDNA Clone Library Construction and Sequencing

Soil microbial community DNA of the contaminated site S3 and its control site C3 was extracted using a FastDNA Spin Kit (Qbiogene, Carlsbad, U.S.A.). PCR amplification and purification of nearly full-length 16S rRNA genes were performed as described above. The 16S rDNA PCR products from the contaminated site S3 and the control site C3 (designated SC and CC, respectively) were cloned into the pGEM-T Easy Vector as recommended by the manufacturer (Promega, Madison, U.S.A.). From each clone library, about 30 white colonies were randomly picked. Plasmid DNA was extracted by using the AccuPrep Plasmid Extraction Kit (Bioneer, Daejon, Korea). Sequencing and phylogenetic cluster analysis were performed as described above.

MPN-PCR Assays and Cloning of the *alkB* Gene

For PCR assays, DNA was extracted from soil samples of the contaminated site and uncontaminated control site using a FastDNA Spin Kit (Qbiogene, Carlsbad, U.S.A.). To quantify the number of *alkB*-coding DNA in the forest soils, 5-fold serial dilutions of soil DNA were made to 5^{-7} by adding 5.0 μl of DNA solution to 20.0 μl of DES solution provided by the FastDNA Spin Kit. The diluted DNA was then PCR amplified in three replicate tubes using the degenerate primer set TS2S and deg1RE [37]. PCR amplification was performed in 40 μl of reaction mixture containing 10× PCR buffer [160 mM $(HN_4)_2SO_4$, 670 mM Tris/HCl, pH 8.8, 25 mM $MgCl_2$, 0.1% Tween 20], 1.6 μl of the diluted DNA, 80 pmol each of primer, 200 μM each of dNTP (GeneCraft, Munster, Germany), and 1.6 U of *Taq* polymerase. PCR cycles consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 5 min. A PCR was scored positive if 40 μl of the PCR products yielded a visible band of the expected size (about 540 bp) after electrophoresis on horizontal 1.2% agarose gels. The MPN values were estimated from MPN tables [48].

For cloning of the *alkB* gene, the PCR products were pooled and fractionated on a 1.2% agarose gel. The amplified DNA band was removed and extracted with the QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany). The DNA was cloned into the pGEM-T easy vector as recommended by the manufactures (Promega, Madison, U.S.A.). From each clone library, about 30 white colonies were randomly picked. Plasmid DNA was extracted by using the AccuPrep Plasmid Extraction Kit (BIONEER). Sequencing was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, U.S.A.) by using primer deg1RE. Only the sequences that have the motif EHXXGHH at position 30 in the deduced amino acid sequence were accepted as alkane hydroxylase genes [37]. For the diesel-degrading bacteria isolated in this study, total genomic DNA of each strain was used for the *alkB* gene PCR. Approximately 500 unambiguous nucleotide positions were used for comparison with the data in GenBank using the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed as described previously [13].

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

Soil bacterial community DNA was extracted using a FastDNA Spin Kit (Qbiogene). For pure culture, total genomic DNA was extracted from each strain with a Wizard Genomic DNA Purification Kit (Promega). PCR amplification of the 16S rRNA genes was performed with primers 1070f and 1392r (*E. coli* 16S rRNA gene sequence

numbering) as previously described [14]. The PCR product contained a GC clamp of 40 bases, added to the reverse primer, and had a total length of 392 bp, including the highly variable V9 region. PCR amplification and DGGE separation were performed as described previously [14, 22, 30]. After electrophoresis, the gels were stained with SYBR Green I for 15 min, rinsed for 20 min, and photographed with UV transillumination (302 nm).

Nucleotide Sequence Accession Numbers

The partial 16S rRNA gene sequences from the clone library have been deposited in the GenBank nucleotide sequence databases under accession numbers DQ136046 through DQ136137.

RESULTS AND DISCUSSION

Differences of Microbial Populations Between Oil-Contaminated Sites and Noncontaminated Sites

The total counts of bacteria, fungi, and actinomycetes in contaminated soils and control soils are shown in Fig. 2. The bacterial cell densities in three contaminated soils, which were in the range of 2.4×10^7–4.8×10^7 cells/g soil, were about 10-fold higher than those of three control soils, 2.3×10^6 to 2.7×10^6 cells/g soil. On the other hand, the fungal and actinomycetes population densities were lower in the contaminated soils than those in the control soils (Fig. 2). The population levels of diesel-degrading microorganisms in contaminated soils, which ranged from 1.8×10^4 to 1.5×10^5 cells/g soil, were about 400-fold higher than those in uncontaminated control soils (1.1×10^2 to 2.5×10^2 cells/g soil) (Fig. 2). The results suggest that soil bacterial populations are stimulated to grow on diesel hydrocarbon contaminated in the soils, whereas fungal and actinomycetes populations are more or less decreased by diesel contamination. This result was consistent with previous observations that bacterial communities are mainly responsible for degradation of diesel hydrocarbon contaminated in soils, leading to the increase of their populations [31, 46, 47].

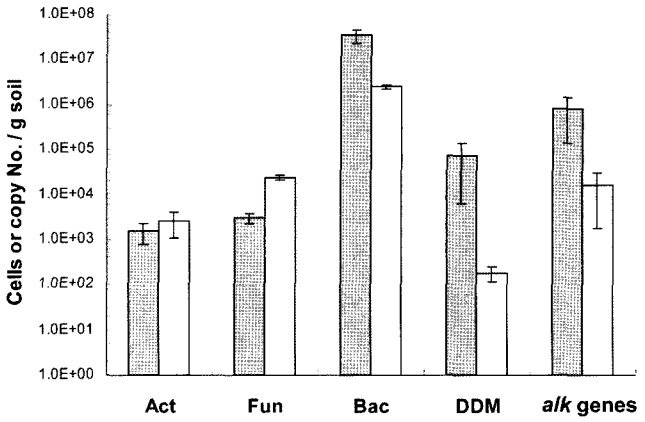

Fig. 2. Microbial populations and copy numbers of alkane hydroxylase gene in diesel-contaminated (filled) and control (blank) sites.
Act, actinomycetes; Fun, fungi; Bac, bacteria; DDM, diesel-degrading microorganisms; *alkB* genes, alkane hydroxylase genes.

Table 1. Nearest relatives of the diesel-degrading isolates based upon 16S rDNA sequence analysis.

Isolate	Accession number	Nearest relative	% Similarity*
PT01	DQ136046	*Luteibactor rhizovicina* LJ96T	98
PT02	DQ136047	*Caulobacter crescentus*	99
PT03	DQ136048	*Pseudomonas veronii*	99
PT04	DQ136049	*Dyella soli*	99
PT05	DQ136050	*Ralstonia eutropha* HAMBI2384	98
PT06	DQ136051	*Wautersia basilensis* strain C-10	99
PT07	DQ136052	*Ralstonia eutropha* HAMBI2384	99
PT08	DQ136053	*Ralstonia eutropha* HAMBI2384	98
PT09	DQ136054	*Pseudomonas stutzeri* strain SA1	98
PT10	DQ136055	*Pseudomonas stutzeri* strain SA1	97
PT11	DQ136056	*Ralstonia eutropha* HAMBI2384	99
PT12	DQ136057	*Alpha proteobacterium* TH-G33	98
PT13	DQ136058	*Serratia odorifera*	99
PT14	DQ136059	*Pseudomonas frederiksbergensis* strain JAJ28	98
PT15	DQ136060	*Burkholderia thailandensis* strain E264	99
PT16	DQ136061	*Pseudomonas stutzeri* strain S1	97
PT17	DQ136062	*Klebsiella milletis*	98
PT18	DQ136063	*Rhodococcus erythropolis* strain DSM43188T	99
PT19	DQ136064	*Rhodococcus erythropolis* strain DSM43188T	98

* Based upon partial 16S rDNA sequences.

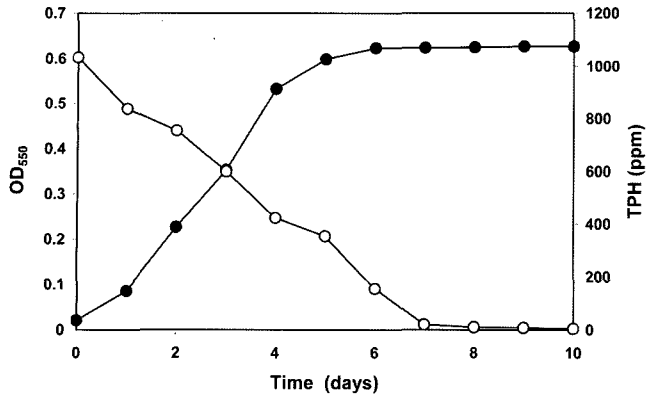

Fig. 3. Disappearance of diesel hydrocarbon (○) and growth of strain PT15 (●) during its growth in mineral medium.
Each point is the mean for two replicate liquid cultures.

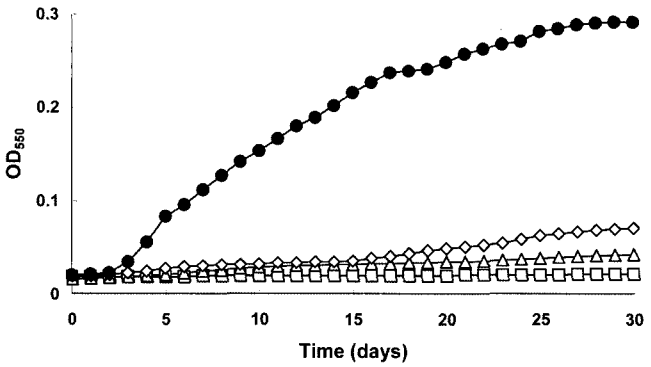

Fig. 5. Growth patterns of representative diesel-degrading isolates at 4°C.
Symbols: ●, strain PT15; □, strain PT02; △, strain PT07; ◇, strain PT10. Each point is the mean for two replicate cultures. OD_{550}, optical density at 550 nm.

Isolation and Characterization of Diesel-Degrading Bacteria

Diverse diesel-degrading bacteria were isolated from the contaminated soils, and their phylogenetic and degradation properties were investigated for potential use in bioremediation practices. 16S rDNA sequence analysis revealed that the isolates were closely related to members of the genera *Pseudomonas*, *Ralstonia*, *Burkholderia*, *Luteibacter*, *Caulobacter*, *Dyella*, *Wautersia*, *Serratia*, *Klebsiella*, and *Rhodococcus* (Table 1). Some of the genera, such as *Luteibacter*, *Dyella*, and *Wautersia*, have not been included in the previous reports [7, 15, 25]. Of the 19 isolates, 17 strains belonged to the *Proteobacteria* group, and the other 2 strains (PT18 and PT19) were associated with the *Actinobacteria*.

Figure 3 shows typical growth and degradation curves by strain PT15 on diesel mineral medium. At room temperature, it took about 7 days for 1,000 ppm of diesel hydrocarbon to be completely degraded by this isolate. The growth patterns of some representative strains on diesel mineral medium are shown in Fig. 4. Among the 19 isolates, strains PT15, PT18, and PT19 degraded and grew most quickly on diesel hydrocarbon with a small lag period. The other strains exhibited relatively slow growth on diesel mineral medium with lag periods. After 30 days of incubation at room temperature, strains PT01 and PT05 degraded about 89% and 64% of 1,000 ppm of diesel hydrocarbon in mineral medium, respectively, whereas strains PT11 and PT15 completely degraded all of them (Fig. 4). Oil degradation in nature is retarded by low temperature because of its negative effect on microbial metabolism and community composition, as well as on physical and chemical properties of oil [4]. Among the diesel-degrading isolates, strains PT01 and PT15 were able to degrade diesel relatively quickly even at low temperatures such as 4°C and 10°C, whereas the other isolates hardly degraded diesel at these low temperatures (Fig. 5). After 30 days of incubation at 4°C, strains PT15 degraded about 43% of 1,000 ppm of diesel hydrocarbon in mineral medium, whereas strains PT02, PT07, and PT10 degraded less than 10% of the diesel hydrocarbon. The psychrotolerant degradation property of the two isolates may be useful for enhanced degradation of diesel hydrocarbon at low temperatures on the top of the mountain.

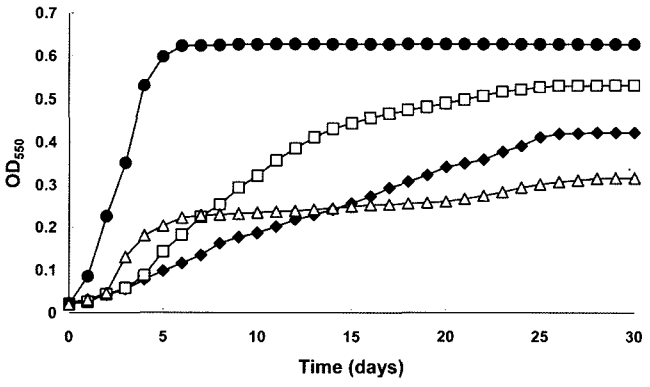

Fig. 4. Growth patterns of representative diesel-degrading isolates at room temperature.
Symbols: ●, strain PT15; □, strain PT11; ◆, strain PT01; △, strain PT05. Each point is the mean for two replicate cultures. OD_{550}, optical density at 550 nm.

DGGE Analysis

To investigate the impact of diesel contamination on the structure of the microbial community, total soil microbial DNAs extracted from forest soils were analyzed by DGGE after PCR amplification of the variable V9 region of the 16S rRNA gene. For the uncontaminated control soils, the DGGE profiles were quite similar to each other, suggesting that the indigenous soil microbial communities were spatially homogenous among the three control soils located distantly in the study site (Fig. 6). However, the DGGE profiles of the contaminated sites were very different from those of

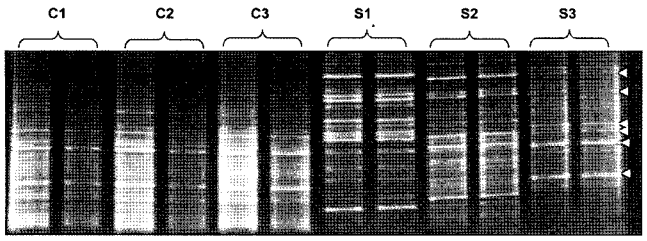

Fig. 6. DGGE fingerprint patterns of 16S rDNA fragments obtained from three contaminated sites (designated as S) and their control sites (designated as C).
Arrows indicate the 16S rDNA bands corresponding to *Sphingomonas*, *Kocuria*, *Pseudomonas*, *Ralstonia*, *Burkholderia*, and *Caulobacter* species, respectively.

the control sites and, moreover, exhibited distinct band patterns to each other even among the three contaminated sites (Fig. 6). The DGGE result is consistent with the previous observation that the variation of microbial communities in contaminated Antarctic sediments was greater than the variation in the control sediments [32]. The changes of DGGE patterns indicated that the indigenous microbial communities of the forest soils were significantly affected by diesel contamination and, subsequently, several microbial populations well-adapted to diesel contamination became dominant, as shown by several intense DNA bands in the contaminated soils (Fig. 6). When the dominant bacteria and diesel-degrading isolates obtained from the contaminated soils were analyzed together by DGGE, it was observed that the intense 16S rDNA bands in the DGGE profiles corresponded with those of *Sphingomonas*, *Kocuria*, *Pseudomonas*, *Ralstonia*, *Burkholderia*, and *Caulobacter* species (Fig. 6, indicated by arrows). In general, soil microbial communities were found to be quite complex and stable, as shown by high similarities between DGGE banding patterns throughout the year [12, 38]. However, environmental factors, such as flooding and treatment of contaminants, caused dramatic changes of microbial community structures in soils [21, 22, 34]. In our study, a diesel contamination from 1998 apparently acted as a strong selection pressure on the indigenous microbial community of each forest soil site, leading to distinct DGGE profiles in the diesel-contaminated soils. The *in situ* physicochemical factors, such as aeration, water-logging, and TPH values, were different among the three contaminated sites, which could have led to selection of different microbial populations in the three separate sites, as shown by their distinct DNA band patterns in DGGE analysis. Specifically, the site S3 exhibited the highest TPH values (427.2–11,056.3 mg/kg soil) among the three contaminated sites, and has received major public concern about valley contamination below this site. Hence, our study hereafter was focused on the site S3 and its control site C3 for a comparative understanding of their microbial community structures and diversities, possibly providing a specific insight into which bacterial groups were stimulated by the diesel contamination.

Table 2. A variety of diversity indices obtained, based on 16S rDNA sequence groups[a] of the isolates and clones.

Parameter	SV[b]	CV[b]	SC[b]	CC[b]
Total No. of isolates or clones	32	30	28	29
Total No. of sequence groups	9	16	22	26
Sequence group richness[c]	8.8	16.0	22.0	25.2
Shannon index (H)[d]	1.91	2.47	2.99	3.22
Evenness (e)[e]	0.87	0.89	0.97	0.99
Simpson's dominance (c)[f]	0.18	0.08	0.06	0.04

[a]A sequence group is defined as a group within which each isolate or clone has >97% 16S rDNA sequence similarity to each other.
[b]SV, isolates from the contaminated site; CV, isolates from the uncontaminated control site; SC, clones from the contaminated site; CC, clones from the uncontaminated control site.
[c]Calculated by rarefaction for a standard sample size of 30 (for isolates) and 28 (for clones).
[d]Calculated as $H=-\sum P_i \ln P_i$, where P_i is the proportion for each sequence group.
[e]Calculated as $e=H/\ln S$, where S is the total number of sequence groups.
[f]Calculated as $c=\sum(P_i)^2$.

Diversity Analysis of Dominant Isolates and Clones

Bacterial diversity indices obtained from 16S rDNA sequences of the dominant isolates and clones were compared between the diesel-contaminated site S3 and the uncontaminated control site C3 (Table 2). Richness, evenness, and diversity indices of the 16S rDNA sequence phylotype of both the isolates and the clones were lower in the contaminated site (SV and SC) than its control site (CV and CC). This result is consistent with other works that reported reduction of bacterial diversity in oil-contaminated environments, such as Antarctic sediments and beach soils [32, 34]. Moreover, larger diversity was obtained by the molecular-based method (CC and SC) than the culture-based method (CV and SV). Rarefaction analysis confirmed the relative phylotype

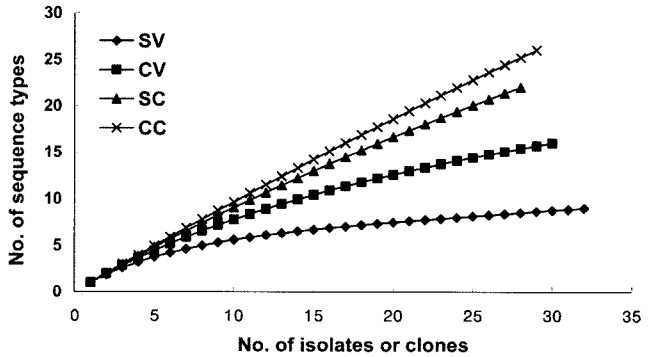

Fig. 7. Rarefaction curves generated for 16S rDNA sequence groups of the isolates and clones.
SV, isolates from the contaminated site; CV, isolates from the control site; SC, clones from the contaminated site; CC, clones from the control site.

richness of each library (Fig. 7). The steepness of the slopes of the phylotype richness curves increased in libraries obtained from SV to CC. The Simpson's dominance index for the SV library was much higher than the indices for the other libraries, suggesting that the SV library was dominated by several phylotypes. Overall, the calculated indices clearly revealed that the bacterial community of the uncontaminated control site was more diverse than that of the contaminated site, and that the DNA-based method was able to detect more diverse bacterial species than the cultivation-based method.

rDNA Sequence Analysis

16S rDNA sequences of the dominant bacterial isolates and clones obtained from the diesel-contaminated site S3 and its uncontaminated control site C3 were compared (Figs. 8 and 9). Among 119 sequences of isolates and clones, 96 sequences (80.7%) showed greater than 97.0% similarities to reference sequences of the RDP and the GenBank database. Twenty-two sequences (18.5%) had similarity values between 96.0% and 90.0%, and only one sequence (SC16) had less than 89.0%. Three of the determined sequences were not assigned to any recognized genus or division.

Among the cultivated bacterial isolates, many isolates obtained from both the contaminated site S3 and its control site C3 were commonly associated with α-*Proteobacteria* and γ-*Proteobacteria* (Fig. 8). However, the low G+C Gram-positive group and the β-*Proteobacteria* group were found only in the isolates obtained from the control site, whereas the *Cytophaga-Flexibacter-Bacteroides* (CFB) group was found only in the isolates from the contaminated site (Fig. 8). In the study of Saul *et al.* [35] with Antarctic coastal soil, the low G+C Gram-positive group was also found only in control soils, but the CFB group comprised a minor group in hydrocarbon-contaminated soils. On the other hand, among the bacterial clones obtained from soil DNA, many clones from both the contaminated site and the control site were commonly associated with diverse phylogenetic groups such as *Proteobacteria*, low G+C Gram-positives, *Actinobacteria*, and *Acidobacteria* (Fig. 9). In addition, the clones obtained from the control site were also associated with other divisions, such as *Planctomycetes*, *Verrucomicrobia*, green nonsulfur (GNS) group, and Cyanobacteria (Fig. 9),

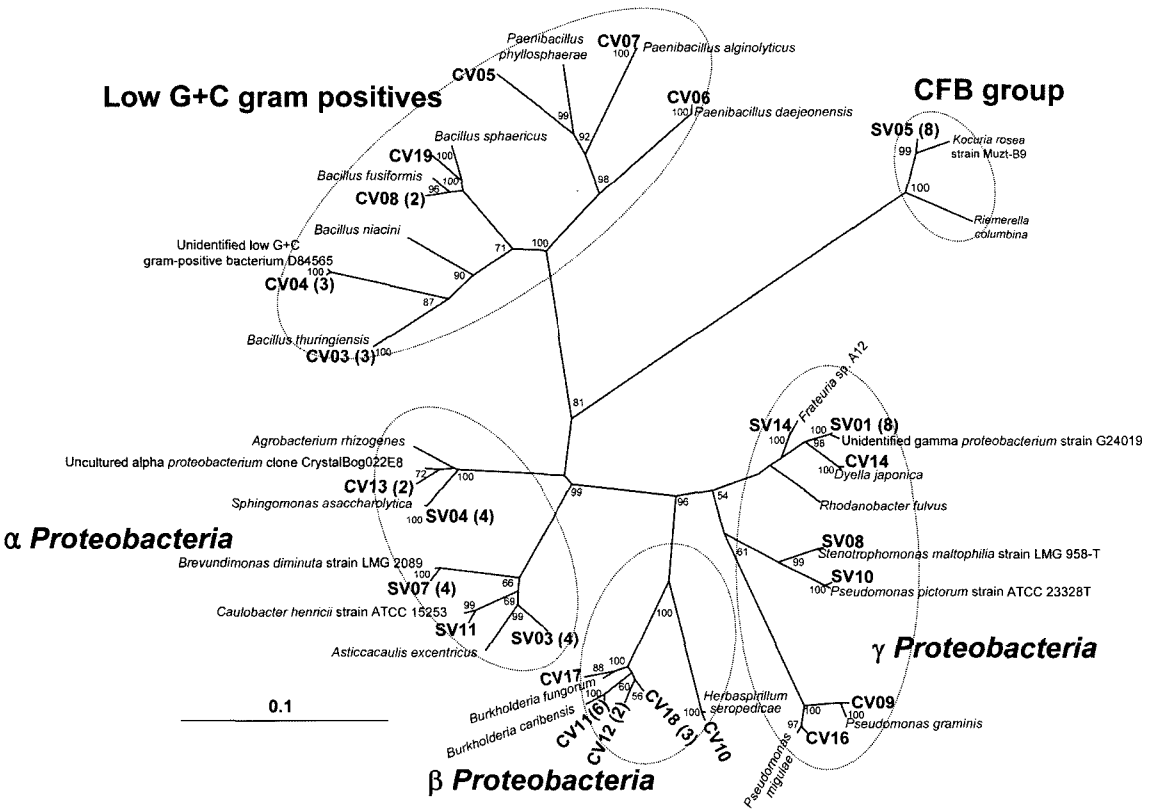

Fig. 8. Unrooted tree showing the phylogenetic relationship of bacterial isolate rDNA fragments (in bold) and reference strain (in italic) rDNA sequences.

Analyses are based on approximately 500-bp rDNA sequences. The scale bar represents 0.1 estimated change per nucleotide, and the numbers indicate boostrap values representing percent confidence of 1,000 replicate analyses. The number of isolates within a sequence type (>97% similarity) is indicated in parentheses if the type contains more than two isolates.

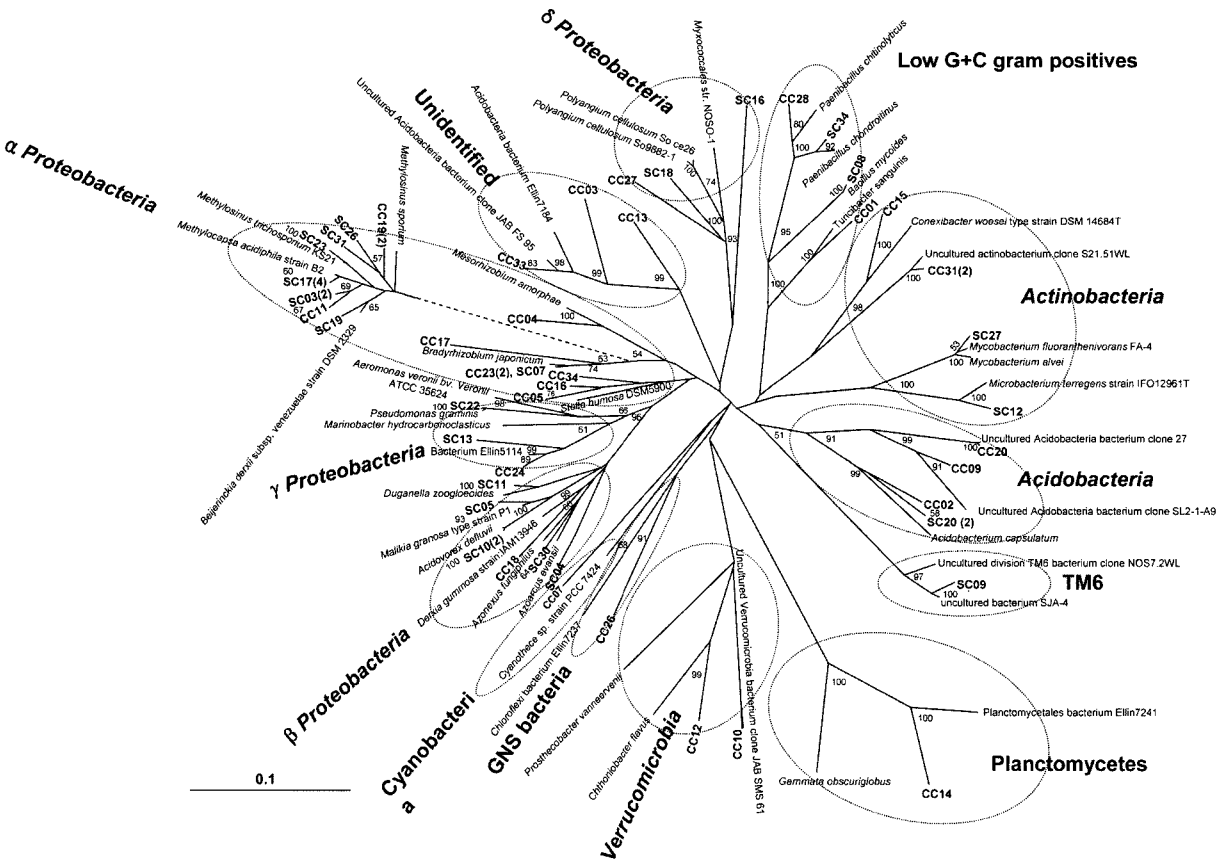

Fig. 9. Unrooted tree showing the phylogenetic relationship of clone rDNA fragments (in bold) and reference strain (in italic) rDNA sequences.

Analyses are based on approximately 500-bp rDNA sequences. The scale bar indicates 0.1 estimated change per nucleotide, and the numbers indicate boostrap values representing percent confidence of 1,000 replicate analyses. The number of clones within a sequence type (>97% similarity) is indicated in parentheses if the type contains more than two clones.

which are found in most soils [8]. The tendency of decreasing bacterial diversity with hydrocarbon contamination was also observed in coastal soils, where various taxonomic divisions such as *Acidobacterium*, *Actinobacteria*, CFB, *Deinococcus/Thermus*, and *Proteobacteria* were detected in a control clone library, whereas only two divisions such as *Actinobacteria* and *Proteobacteria* were obtained from the contaminated soil clone library [35]. In the present study, the greatest numbers of isolates and clones were associated with the *Proteobacteria*, which were found in both the contaminated site and the control site. It was reported that, among the *Proteobacteria*, the γ-subgroup was preferentially selected in oil-contaminated beach sediments [20, 34]. In our study, dominance of the γ-subgroup was observed in the diesel-degrading isolates and the cultivated isolate library obtained from the diesel-contaminated site, but no group appeared to dominate in the clone library obtained from soil DNA.

The isolates and clones from the control site were associated with four and eleven bacterial divisions, respectively, whereas those from the contaminated site were associated with three and eight divisions, respectively. The results suggest that the molecular-based method allows detection of much more diverse bacterial populations than the culture-based method, and that the bacterial diversity of the uncontaminated control site is higher than that of the diesel-contaminated site. It is of note that two dominant groups of isolates, represented by isolates SV01 and SV05, were found in the cultivated bacterial library from the diesel-contaminated site S3, but not detected in the control site (Fig. 8). The isolates SV01 and SV05 were closely affiliated with *Frateuria* species and *Kocuria rosea*, respectively, which were isolated from acid mine drainage and ice core, respectively [19, 50]. Since these isolates were not able to grow on diesel, they appeared to gain benefit from metabolic interactions with other indigenous diesel-degrading microbial populations in the contaminated soils. The results suggest that diesel contamination exerts a strong selection on the indigenous microbial community in the contaminated site, leading to predominance of well-adapted microorganisms in concurrence with decrease of microbial diversity.

Monitoring Functional Catabolic *alkB* Genes

The alkane hydroxylase gene (*alkB*) plays an important role in hydrocarbon degradation by microbial populations at sites contaminated with oil. PCR amplification with degenerate primers specific for the *alkB* genes was performed to investigate the population levels and the *alkB* gene sequence diversity of the hydrocarbon-degrading bacteria in the contaminated soil and the control soil.

MPN-PCR assays showed that the numbers of the *alkB*-coding DNA were in the range of 2.0×10^5 to 1.5×10^6 copies/g soil and 7.3×10^3 to 3.2×10^4 copies/g soil in the contaminated soils and the control soils, respectively

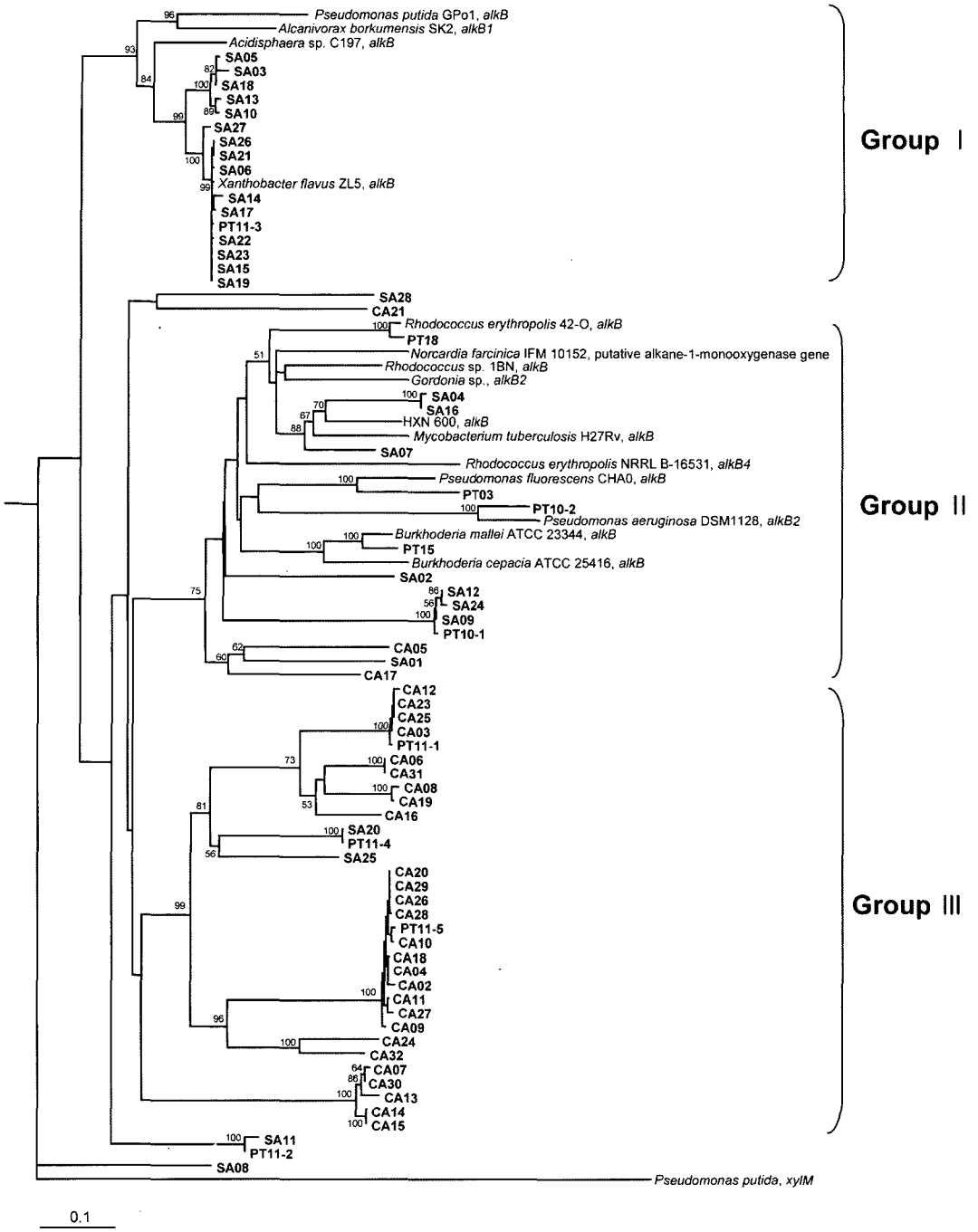

Fig. 10. Neighbor-joining dendrogram of alkane hydroxylase gene sequences from soil clones and isolates (in bold) and reference strains (in italic).

Clone sequences from the contaminated site (SA) and uncontaminated control site (CA), and sequences from diesel-degrading bacteria isolated in this study (PT) are indicated. Bootstrap values are indicated above each branch. The scale bar indicates 0.1 estimated change per nucleotide. The *xylM* (xylene monooxygenase gene) sequence of *P. putida* [37] was included as an out-group sequence.

(Fig. 2). The numbers of *alkB*-coding DNA were much higher in the diesel-contaminated sites than those in the control sites, which was similar to the distribution pattern of the diesel-degrading bacteria. However, the numbers of *alkB*-coding DNA were 1–2 orders of magnitude higher than the population levels of the diesel-degrading bacteria in the corresponding sites, possibly due to dead or dormant microorganisms, which was consistent with the observation of Sei *et al.* [36]. The result suggested that the contamination of diesel hydrocarbon increased the numbers of the *alkB*-coding DNA by stimulating the growth of bacterial populations able to degrade *n*-alkanes in the forest sites under study.

To investigate the sequence diversity and distribution of the alkane hydroxylase genes between the contaminated site and its control site, PCR-amplified *alkB* genes obtained from soil microbial community DNAs were cloned and sequenced. Most of the cloned *alkB* genes were of the same length (540 bp including primers) and showed substantial homology with database sequences. Overall, the levels of the *alkB* gene sequence similarity were in the range of 58.2% to 99.6%. Figure 10 shows a phylogenetic tree based on the *alkB* gene sequence alignments. Most of the genes were clustered in three large groups (I, II, and III). Group I consisted of the genes cloned only from the contaminated soil and was closely related with the *alkB* gene of *Xanthobacter flavus* ZL5, which were isolated from enrichment with cyclohexane [43]. Group II was mainly composed of the genes cloned from the contaminated soil, with only two out of ten sequences coming from the control soil, and was related with the *alkB* gene sequences of various bacteria, such as *Rhodococcus*, *Pseudomonas*, *Burkholderia*, *Nocardia*, and *Mycobacterium* species. Most of these reference strains were able to grow on *n*-alkanes [16, 37, 45]. In contrast, group III was mainly composed of the genes obtained from the control soil. The clones of group III were not closely related with any alkane hydroxylase gene from GenBank, and therefore, their specific functions are difficult to infer. Of the diesel-degrading bacteria isolated in this study, strains PT03, PT10, PT15, and PT18, which were identified as *Pseudomonas*, *Burkholderia*, and *Rhodococcus* species, respectively, were observed to have alkane hydroxylase genes very similar to the clones of group II (Fig. 10). On the other hand, three alkane hydroxylase genes of the diesel-degrading isolate PT11, which was identified as a *Ralstonia* species, showed high similarities (55.0 to 100.0%) to the cloned *alkB* genes of group III.

It is of note that the cloned *alkB* genes tend to cluster in different groups between the contaminated soil and its control soil. The alkane hydroxylase genes are known to be widespread among microorganisms in nature [33]. Previously, Sotsky *et al.* [39] demonstrated by Southern blotting that genes with high sequence identity to the *alkB* gene occurred in a large portion of the microbial population in oil-contaminated environments. A majority of bacteria able to grow on hydrocarbon possessed genes with high sequence similarity to the *alkB* gene, and some strains even had multiple alkane hydroxylase systems with different sequences in a single bacterium [23, 36]. The reference strains of group I were observed to be responsible for degradation of shorter alkanes, whereas those of group II were involved in degradation of alkanes with a wider range of chain lengths [23, 36]. In our study, since most of the *alkB* genes cloned from the contaminated soil belonged to groups I and II, the alkane hydroxylase genes of these groups appeared to play important roles in diesel hydrocarbon degradation in the contaminated site. The results indicated that contamination of diesel hydrocarbon exerted a strong pressure on the indigenous microbial community, leading to selection of well-adapted bacterial populations harboring specific alkane hydroxylase genes involved in degradation of hydrocarbon.

In conclusion, this work shows that diesel contamination gives a substantial impact on the indigenous microbial community of the forest soil, leading to dramatic changes of microbial populations, as shown by alterations of soil microbial population levels and DGGE profiles in the contaminated soils. The results of both the cultivation method and the molecular method revealed that the bacterial diversity in the contaminated site was much decreased, possibly owing to the selection pressure imposed by diesel contamination. The selection effect of diesel contamination led to different diversity and distribution of the alkane hydroxylase genes in microbial populations between the contaminated site and the control site. Since microorganisms are mainly responsible for the degradation of environmental contaminants in soil, information on the bacterial community, including the diesel degraders, would help in the removal of oil spills in geologically-unmanageable forest soils under study.

Acknowledgments

This study was supported by a grant from the National Instrumentation Center for Environmental Management (NICEM) of Seoul National University, the Research Institute of Technology (RIT) of Samsung Corporation, the RDA Genebank Management Program from Genetic Resources Division, National Institute of Agricultural Biotechnology, and the BioGreen 21 Project.

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