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Microbial Community Structure in Hexadecane- and Naphthalene-Enriched Gas Station Soil

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Shifts in the activity and diversity of microbes involved in aliphatic and aromatic hydrocarbon degradation in contaminated soil were investigated. Subsurface soil was collected from a gas station that had been abandoned since 1995 owing to ground subsidence. The total petroleum hydrocarbon content of the sample was approximately 2,100 mg/kg, and that of the soil below a gas pump was over 23,000 mg/kg. Enrichment cultures were grown in mineral medium that contained hexadecane (H) or naphthalene (N) at a concentration of 200 mg/l. In the H-enrichment culture, a real-time PCR assay revealed that the 16S rRNA gene copy number increased from 1.2×10^5 to 8.6×10^6 with no lag phase, representing an approximately 70-fold increase. In the N-enrichment culture, the 16S rRNA copy number increased about 13-fold after 48 h, from 6.3×10^4 to 8.3×10^5. Microbial communities in the enrichment cultures were studied by denaturing gradient gel electrophoresis and by analysis of 16S rRNA gene libraries. Before the addition of hydrocarbons, the gas station soil contained primarily *Alpha-* and *Gammaproteobacteria*. During growth in the H-enrichment culture, the contribution of *Bacteriodetes* to the microbial community increased significantly. On the other hand, during N-enrichment, the *Betaproteobacteria* population increased conspicuously. These results suggest that specific phylotypes of bacteria were associated with the degradation of each hydrocarbon.

Keywords: Denaturing gradient gel electrophoresis, gas station, hexadecane, microbial community, naphthalene

Petroleum contamination results from the leakage of aboveground and underground storage tanks, spillage during the transport of petroleum products, abandoned manufactured gas plants, other unplanned releases, and industrial processes. Petroleum hydrocarbons, such as gasoline and diesel fuel, are composed of complex mixtures of alkanes and aromatics, as well as nitrogen-, oxygen-, and sulfur-containing compounds (NSO compounds). The degradation of petroleum hydrocarbons in soil is affected by several physicochemical and biological factors: the number and types of microbial species present; the prevailing environmental conditions for microbial degradation (*e.g.*, oxygen and moisture content, temperature, pH, and nutrient levels); the type, chemical structure, quantity, and bioavailability of contaminants; and soil characteristics (*e.g.*, pore size and clay and organic material content and distribution). Microbes are primarily responsible for the degradation of pollutants in soil. Unlike sudden tanker accidents or explosions, a pipeline or a storage tank may leak for many years and gradually contaminate the surrounding soil. After prolonged exposure, communities of indigenous microorganisms can develop and adapt to petroleum compounds by changing their species composition [8, 15, 18].

For any successful bioremediation process, it is important to clarify the behavior of the microbial populations that utilize the hydrocarbon contaminants and those that, in turn, utilize their metabolites. An understanding of the structure of the entire microbial community, including microbial populations that are not responsible for degradation, is essential because nondegrading bacteria can affect the behavior of degrading bacteria through microbial interactions. To address the impact of petroleum contamination on the microbiology of ecosystems, some studies have investigated changes in the structure of the indigenous microbial community [5, 7, 9, 13]. However, information on changes in microbial communities that occur during bioremediation is limited.

Recently, molecular approaches based on the analysis of 16S rDNA sequences have been applied to the assessment of microbial diversity in environmental samples. Denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), and amplified rDNA restriction analysis (ARDRA) are the most commonly used techniques for this purpose [1, 3, 5, 11, 22].

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The soil in this study was collected from a gas station in Gyeongbuk, South Korea, that was abandoned in 1995 because of ground subsidence. The site has become heavily contaminated with gasoline and diesel fuel over time. Using DGGE and 16S rRNA gene library analyses, we investigated how the composition of the microbial community shifted during the degradation of hexadecane and naphthalene.

Materials and Methods

Soil

Soil was collected from the Y gas station (483.63 m^3) that was described in the Introduction. Soil samples were put in plastic bags, transported to the laboratory, and stored at 4°C. The soil, which was sieved to a particle size of <2 mm, consisted of 85% sand and 10% clay (*i.e.*, loamy sand). It contained 2.5% organic matter and had a pH of 6.49 in water. The total petroleum hydrocarbon (TPH) content of the soil near a gas pump was 23,367 mg/kg and that of the subsurface soil was approximately 2,100 mg/kg.

Enrichment Cultures

Triplicate cultures were initiated in 100-ml serum bottles containing 1 g diesel-contaminated soil and 20 ml of Bushnell-Haas medium (Difco, U.S.A.) spiked with 200 mg/l naphthalene or 200 mg/l hexadecane. Bottles were sealed with Teflon-coated butyl rubber stoppers and aluminum crimp seals. Bacteria were cultured at 150 rpm and 30°C for 7 days. At each time point, the replicate cultures of each hydrocarbon treatment were randomly sampled. A 2-ml aliquot of the solution in each sample was removed for plate counts and DNA extraction and the remaining solution was subjected to hydrocarbon analysis.

Cell Enumeration

For total bacterial 16S rRNA gene analysis, real-time PCR assays were performed with the DNA Engine Opticon continuous fluorescence detection system (MJ Research, U.S.A). The 20-μl reaction mixtures contained 0.2 μM of the 1055F and 1392R primer set [4], 1 μl of DNA (1 ng/μl), and 10 μl of 2× master mix from the DyNAmo HS SYBR Green qPCR kit (Finzymes, U.S.A.). The PCR program consisted of 5 min at 95°C and 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The assay was conducted three times, and negative controls without template were included in each PCR run. The standard curve for total bacterial 16S rRNA was made with genomic DNA extracted from *E. coli*.

Analysis of Hydrocarbons

Dichloromethane (50 ml) was added to the enrichment cultures, which were then thoroughly mixed for 10 min with a vortex mixer and sonicated for 1 h in a water bath (4°C). The supernatant was passed through a 0.45-μm filter. Extracts (1 ml) were analyzed on a gas chromatograph (Varian 3400CX; CA, U.S.A.) equipped with a flame ionization detector and a DB-1 column (30 m×0.32 mm; 0.25-μm film thickness; J&W Scientific, IL, U.S.A.). During analysis, the injector and detector temperatures were maintained at 250°C and 300°C, respectively. The column temperature was programmed to increase from 40°C to 170°C in 6°C/min increments, remain at 170°C for 3 min, rise from 170°C to 300°C in 8°C/min increments, and finally hold at 300°C for 10 min.

DNA Extraction and PCR–DGGE

Genomic DNA was extracted from the soil samples (dry wt, 0.5 g) and enrichment cultures (1 ml) with a FastDNA SPIN kit for soil (Bio101; CA, U.S.A.) according to the manufacturer's protocol. The quantity and purity of DNA were determined by measurement of the absorbance at 260 and 280 nm (NanoDrop ND-1000 spectrophotometer; U.S.A.). DNA extracts from the soil samples and enrichment cultures were then amplified with primers 341FGC and 536R for the 16S rRNA genes as described by Muyzer *et al.* [10]. Reactions were carried out in 50-μl volumes containing 1× PCR buffer, 2 mM dNTP mixture, 1 μM of each primer, template DNA, and 5 U *Taq* polymerase. The PCR conditions involved denaturation at 94°C for 5 min, followed by 30 cycles of 45 s of denaturation at 94°C, 45 s of annealing at 50°C, and 45 s of extension at 72°C.

DGGE was performed in a D-Code 16/16-cm gel system with a gel width of 0.7 mm (Bio-Rad, CA, U.S.A.). Samples were electrophoresed on 10% polyacrylamide gels with a 40–70% denaturing gradient. Gels were run at 60 V for 15 h, and then stained with ethidium bromide and destained twice in 1× TAE buffer for 15 min. To evaluate DGGE band patterns, images were converted, normalized, and analyzed with the Kodak 1.0 software package (Eastman Kodak Co., NY, U.S.A.). A hierarchical cluster analysis based on the Pearson correlation was conducted on the transformed data with SPSS 11.5 (SPSS Inc., IL, U.S.A.). The Shannon–Weaver (H') index of microbial diversity [16] was calculated according to the formula $H'=-\Sigma Pi\log Pi$, $Pi=ni/N$, where *ni is* the intensity of band *I* in the lane, and *N* is the total intensity of all bands in the lane. The Simpson index of dominance (D) [17] was calculated using the Pi value, $D=\Sigma Pi^2$. Richness (S) refers to the number of bands detected in a sample. The evenness (E_H), which is a measure of how evenly DGGE bands were distributed in a given sample, was calculated as $E=H/\ln(S)$.

Cloning and Sequencing

Bacterial 16S rDNA fragments from soil and enrichment cultures were amplified using the universal primer set 9F and 1392R [19] and purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Each purified product was ligated into a pGEM-T easy vector (Promega Co., WI, U.S.A.) and transformed into competent *E. coli* DH5α cells as described by the manufacturer. Clones containing inserts of the correct size were sequenced with an ABI Prism 377 automated sequencer (Applied Biosystems, CA, U.S.A.). Sequences were then compared with those in the GenBank database by using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov).

Results

Cell Growth and Hydrocarbon Degradation

In the hexadecane (hereafter designated as H)-enrichment culture, the real-time PCR assay showed that the 16S rRNA gene copy number increased about 70 times without a lag phase for the first 3 days, from 1.2×10^5 to 8.6×10^6, and then slowly decreased (Fig. 1). On the other hand, in the

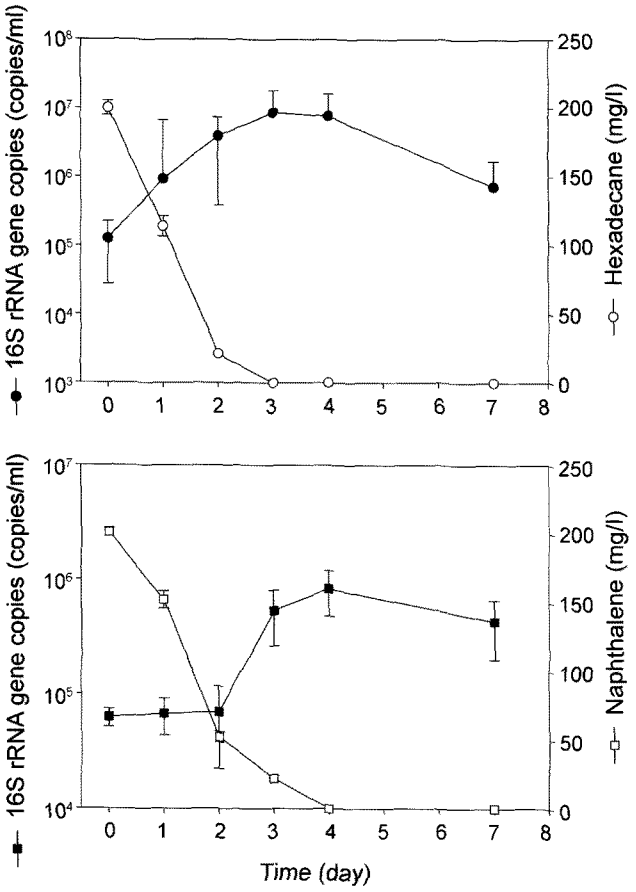

Fig. 1. Biodegradation of hydrocarbons.
Triplicate cultures were initiated in 100-ml serum bottles containing 1 g of diesel-contaminated soil and 20 ml of mineral medium spiked with 200 mg/l naphthalene or 200 mg/l hexadecane. Cells were cultured at 150 rpm and 30°C for 7 days. Data points represent the average of triplicate batch cultures randomly sampled on each day. Error bars represent standard deviation.

naphthalene (hereafter designated as N)-enrichment culture, the 16S rRNA gene copy number increased about 13 times, from 6.3×10^4 to 8.3×10^5, after 48 h (Fig. 1). Hexadecane was degraded by >80% within 48 h and was fully degraded after 3 days of incubation (Fig. 1). Naphthalene was degraded by ~75% within 72 h and was fully degraded after 4 days of incubation (Fig. 1). Hydrocarbons in both enrichments were degraded without a lag time.

Bacterial Community Structure

Band 1 in the DGGE gel (Fig. 2) was 97% similar to the 16S rRNA gene sequence of uncultured *Acinetobacter* sp. (DQ130041) (Table 1). This band appeared in both enrichment cultures but was not observed in the gas station soil. Bands 2, 3, and 4 were observed in both treatments and their 16S rRNA gene sequences were similar to those of *Acinetobacter* and *Burkholderia*, which are commonly detected in petroleum hydrocarbon-contaminated soil [14]. Bands 5, 6, 14, 15, and 17 were observed in the H-enrichment

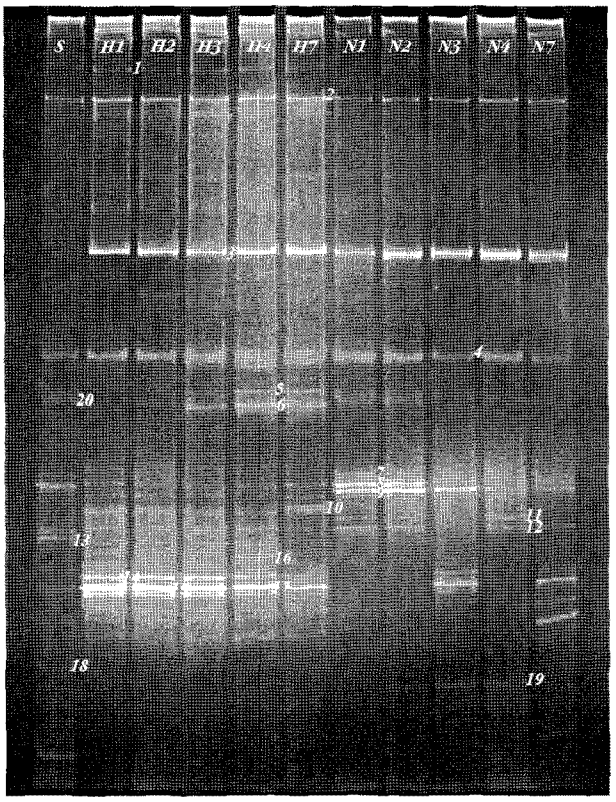

Fig. 2. Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments from hexadecane- and naphthalene-*amended enrichment cultures over time.*
The PCR fragments were run on 10% polyacrylamide gels with a 40–70% denaturing gradient. S, original Y gas station soil; H, hexadecane-enrichment culture; N, naphthalene-enrichment culture. The numbers in H1, H2, N1, and N2 *etc.* refer to days in each culture. Numbered DNA bands are discussed in the text.

culture. The intensity of three prominent bands (6, 14, and 15) increased during the culture period. Bands 5 and 6, which were observed after hexadecane was completely degraded, were apparent from 3 to 7 days of incubation. Bands 7, 8, and 9 were detected solely in the N-enrichment culture. These three bands were found to be associated with *Pseudomonas* spp.

Significant differences in the diversity of H-enrichment and N-enrichment DGGE band patterns were observed from day 1 of the incubation. The Shannon–Weaver diversity index (H') and Simpson dominance index (D) calculated from the PCR–DGGE results showed that the diversity was slightly higher in the H-enrichment cultures than in the N-enrichment cultures (Table 2). The diversity of the H-enrichment cultures gradually decreased, whereas the H' of the N-enrichment cultures decreased on day 1 and then recovered to almost the same level as that of the original gas station soil. The richness (S) of the H-enrichment cultures also increased, whereas that of the N-enrichment cultures decreased on day 1 (Table 2). The results of the clustering analysis based on the Pearson correlation showed

Table 1. Comparison of sequences derived from DGGE bands with BLAST database.

Band	Closest match	Accession No.	Similarity (%)
1	Uncultured *Acinetobacter* sp.	DQ130041	97
2	Alkane-degrading soil bacterium	AF513979	99
3	*Acinetobacter calcoaceticus*	EU116047	96
4	Uncultured *Burkholderiaceae* bacterium	EF562274	98
5	Uncultured *Bacteriodetes* bacterium	AJ619082	87
6	Uncultured *Bacteriodetes*	EF179856	96
7	*Pseudomonas* sp.	AM410617	97
8	*P. fluorescens*	EF408245	97
9	Uncultured *Pseudomonas* sp.	EU097355	98
10	Uncultured division TM6	AY043962	93
11	*Pseudomonas* sp.	AY689052	96
12	*P. putida*	EF204245	93
13	Uncultured *Sinorhizobium* sp.	EF555026	99
14	Uncultured *Pseudomonas* sp.	EU341270	98
15	Uncultured *Pseudomonas* sp.	EF626822	99
16	*Flavobacterium*	AB264126	96
17	Uncultured *Acinetobacter* sp.	DQ130041	98
18	*Sphingomonas* sp.	AB299584	90
19	*Ralstonia* sp.	EU155874	97
20	Uncultured *α-Proteobacteria*	AY960235	96

that there was high similarity between the two treatments (data not shown).

16S rRNA Gene Clone Libraries

Before the addition of hydrocarbons, the microbial community in the gas station soil was composed mainly of *Alpha-* and *Gammaproteobacteria* (56% and 40% of total clones, respectively; Fig. 3 and Table 3). Most of the clones that were found to be related to *Alphaproteobacteria* were similar to *Sphingomonas* sp., whereas the dominant group among the *Gammaproteobacteria* consisted of species of *Pseudomonas*. *Deltaproteobacteria* and *Bacteroidetes* were not detected in this soil. In the H-enrichment culture, *Alphaproteobacteria* had decreased considerably (to 4% of total clones) after 7 days, whereas *Bacteroidetes* consisting of unculturable bacteria had increased (to 46% of total clones). Members of *Gammaproteobacteria* were observed to be at similar levels in the gas station soil and the H-enrichment cultures. On the other hand, in the N-enrichment cultures, *Alphaproteobacteria* declined to 16% of total clones, but *Betaproteobacteria* conspicuously increased to 26% of total clones. The dominant group among the *Betaproteobacteria* consisted of *Burkholderia* species. Furthermore, *Deltaproteobacteria* and *Bacteriodetes* were observed at lower levels. Results from the DGGE profiles and 16S rRNA gene clone analysis therefore suggest that specific phylotypes of bacteria were associated with the degradation of each of the two hydrocarbons.

Table 2. Diversity statistics for hexadecane- and naphthalene-amended enrichment cultures over time.

Time (h)	H'		D		S		E_H	
	H	N	H	N	H	N	H	N
0	1.157	1.157	0.083	0.083	19	19	0.905	0.905
24	1.132	0.919	0.099	0.147	22	14	0.843	0.802
48	1.077	0.947	0.114	0.141	22	14	0.802	0.862
72	1.094	0.988	0.116	0.124	25	17	0.782	0.803
96	1.091	0.965	0.107	0.135	24	19	0.790	0.754
168	0.999	1.015	0.136	0.124	19	17	0.781	0.825

H': Shannon–Weaver diversity index; D: Simpson diversity index; S: richness; E_H: evenness index.

Discussion

In the environment, a wide range of bacteria participate in the degradation of petroleum hydrocarbons. Because diverse bacteria are associated with different phases of pollutant degradation, a better interpretation of the microbial community dynamics during decontamination is important for the development of more efficient remediation techniques [13, 21].

Microbial communities in contaminated ecosystems tend to be dominated by organisms that are capable of utilizing or surviving in the presence of the contaminants. Our DGGE results indicate that obvious microbial shifts occurred during the degradation of each hydrocarbon. Indigenous *Pseudomonas* sp. and *Acinetobacter* sp. increased transiently in the early phase of the biodegradation of hexadecane and naphthalene, respectively. After 3 days of incubation, at which time the hydrocarbons were fully degraded, other

Table 3. GenBank database sequences having the highest-identity matches to clone libraries of gas station soil and enrichment cultures.

Clones	BLAST results	Accession No.	Identity (%)
Y gas station soil	*Rhizobium* sp.	AY864736	99
	Rhizobium tropici	D12798	99
	Sphingomonas sp.	EF062503	99
	Sphingomonas mucosissima	DQ447783	98
	Novosphingomonas subarticum	AY167828	98
	Alcaligenes sp.	DQ459259	98
	Enterobacter aerogenes	AM184247	98
	Pseudomonas oryzibitans	AM262973	98
	P. stutzeri	AJ008106	98
	Pseudomonas sp.	AF229886	97
	Pseudomonas sp.	AJ551097	99
	P. fluva	AM411071	99
	P. abietaniphila	AJ011504	99
	P. putida	AE015451	98
	Acinetobacter calcoaceticus	AF159045	97
	Methylobacterium sp.	AY741717	96
	Strenotrophomonas maltophilia	AY445079	97
Hexadecane-enrichment	Uncultured *Bacteriodetes*	EF075333	94
	Uncultured soil bacterium	DQ123668	98
	Marinobacter flavium	AY517632	93
	Uncultured *Cytophagales* bacterium	AF361199	90
	Uncultured bacterium	DQ334627	93
	Uncultured bacterium	DQ001618	99
	Uncultured marine bacterium	DQ071113	90
	Flavobacterium sp.	DQ173003	96
	Alcaligenes hongkongensis	AY251391	93
	Epilithonimonas tenax	AF493696	97
	Acinetobacter radioresistens	AY568489	96
	A. xiamenensis	EF030545	95
	Geobacter metallireducens	CP000148	94
	Pseudomonas sp.	AM396910	99
	P. agarici	D84005	98
	P. fluorescens	AB046999	93
	Uncultured *Acinetobacter*	DQ130041	99
	Uncultured *Gammaproteobacterium*	AY622230	97
	Uncultured *Deltaproteobacterium*	AY395371	95
Naphthalene-enrichment	*Azospirillium* sp.	AF411852	99
	Alcaligenes hongkongensis	AY251391	99
	Ralstonia sp.	AB212234	94
	Ralstonia sp.	DQ847128	94
	Azohydromonas australica	AB188124	99
	Burkholderia sp.	DQ490284	99
	Chryseobacterium sp.	DQ521273	97
	Acinetobacter xiamenesis	EF030545	94
	A. calcoaceticus	DQ250143	96
	A. radioresistens	AY568493	96
	Acinetobacter sp.	DQ834356	97
	Trichloroacetic-acid-degrading bacterium	AF532186	99
	Xanthomonas sp.	AJ786786	98
	Pseudomonas cremoricoloranta	AB060137	91
	Bdellovibrio bacteriovorus	BX842648	99
	Caulobacter sp.	AY62223	97
	Uncultured *Alphaproteobacterium*	AF5230701	95
	Uncultured *Gammaproteobacterium*	AY622230	98

microorganisms became more prevalent [7]. In addition, analysis of 16S rRNA gene libraries revealed that the proportions of *Pseudomonas* sp. and *Acinetobacter* sp. decreased considerably during incubation compared with their proportions in the original gas station soil. Previous studies have demonstrated that a shift in microbial

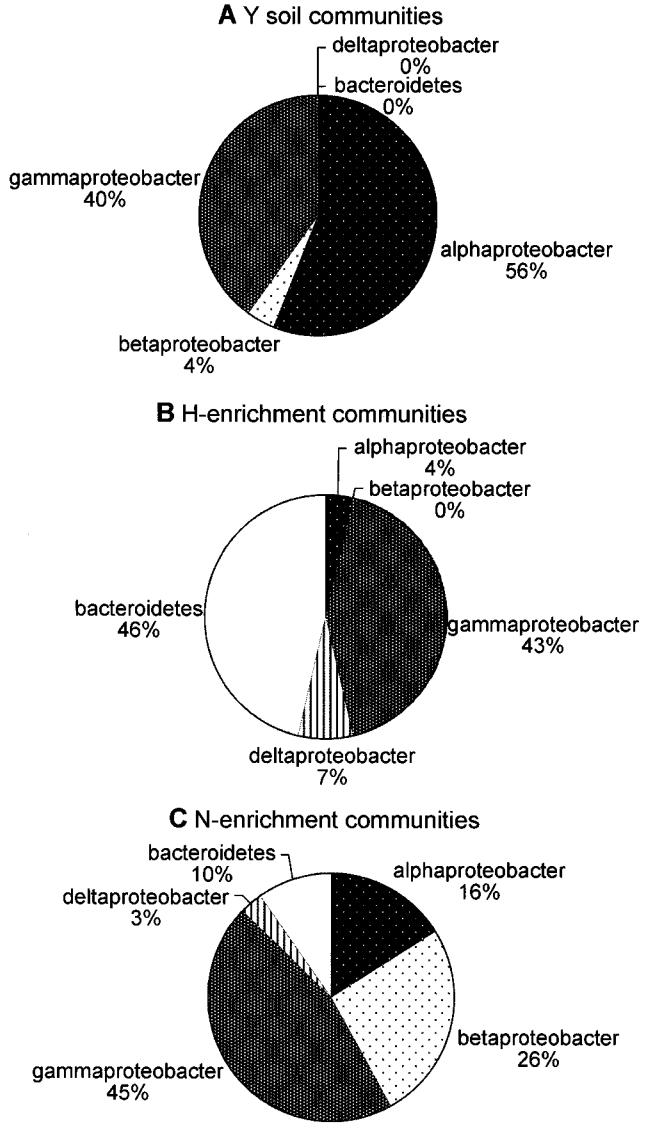

Fig. 3. Relative contributions of different phylogenetic groups to microbial communities.
A. Y gas station soil microbial community. **B.** Hexadecane-enrichment culture (200 mg/l). **C.** Naphthalene-enrichment culture (200 mg/l).

community structure from *Alpha-* and *Betaproteobacteria* to *Gammaproteobacteria* occurs when a microbial community is exposed to a mixture of aromatic hydrocarbons [2, 12, 20].

Bacteroidetes consist of three large classes of bacteria that are found in a wide variety of environments. *Actinobacter* such as *Nocardia* and *Rhodococcus* spp. have frequently been found in petroleum hydrocarbon-contaminated areas [14]. However, in this study, *Actinobacter*-like 16S rRNA gene sequences were not detected by molecular techniques in either the original soil or during hydrocarbon enrichment.

The microbial communities in the H- and N-enrichment cultures were notably different from each other after 1 day of incubation. Although H' was not significantly different between the two enrichments during the incubation periods, H' for the H-enrichment was slightly higher than that for the N-enrichment throughout the time course. From the beginning of the incubation, the number of 16S rRNA gene copies determined by real-time PCR assay as well as richness (S) was higher in the H-enrichment than in the N-enrichment. In addition, the number of 16S rRNA gene copies in the N-enrichment increased after 3 days of incubation while S values decreased compared with those of the original soil. Therefore, hexadecane might be more bioavailable as a substrate than naphthalene. Notably, it has been reported that *Pseudomonas* species, the predominant microorganisms found in this study, possess alkane hydroxylase and naphthalene dioxygenase activities [9, 23]. *Burkholderia* sp., *Ralstonia* sp. and *Sphingomonas* sp. also take part in hydrocarbon degradation [4, 6, 24]. In the present study, the proportion of total hydrocarbon-degrading bacteria increased with time during culturing, indicating another shift in microbial community structure.

Although metabolites produced by hydrocarbon-utilizers were not analyzed, this study demonstrates that specific phylotypes of bacteria were associated with the degradation of each hydrocarbon. The roles of other microorganisms that appeared following degradation of the mother compounds and that might assist the degrading bacteria through microbial interactions are still largely unknown. Monitoring bacterial populations responsible for the degradation of contaminants could facilitate an increase in the efficiency of the bioremediation process and contribute to our understanding of microbial community dynamics. Furthermore, to gain an understanding of the entire microbial community, it is important to study the spatial and temporal dynamics of microbial community shifts.

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