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Monitoring Nutrient Impact on Bacterial Community Composition during Bioremediation of Anoxic PAH-Contaminated Sediment

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Marine harbor sediments are frequently polluted with significant amount of polycyclic aromatic hydrocarbons (PAHs) some of which are naturally toxic, recalcitrant, mutagenic, and carcinogenic. To stimulate biodegradation of PAHs in PAH-contaminated sediments collected from near Gwangyang Bay, Korea, lactate was chosen as a supplementary carbonaceous substrate. Sediment packed into 600 ml air-tight jar was either under no treatment condition or lactate amended condition (1%, w/v). Microbial community composition was monitored by bacteria-specific and archaea-specific PCR-terminal restriction fragment length polymorphism (T-RFLP), in addition to measuring the residual PAH concentration. Results showed that lactate amendment enhanced biodegradation rate of PAHs in the sediment by 4 to 8 times, and caused a significant shift in archaebacterial community in terms of structure and diversity with time. Phylogenetic analysis of 23 archaeal clones with distinctive RFLP patterns among 288 archaeal clones indicated that majority of the archaeal members were closest to unculturable environmental rDNA clones from hydrocarbon-contaminated and/or methanogenesis-bearing sediments. Lactate amendment led to the enrichment of some clones that were most closely related to PAH-degrading *Methanosarcina* species. These results suggest a possible contribution of methanogenic community to PAH degradation and give us more insights on how to effectively remediate PAH-contaminated sediments.

***Keywords*:** anaerobic biodegradation, bacterial community, archaea, polycyclic aromatic hydrocarbon, anoxic sediment

Marine harbor sediments are frequently polluted with hydrocarbon contaminants derived from fuel spills, industrial wastes, shipping activities, runoff, soot, and creosote-treated pilings (Wild and Jones, 1995). Aliphatic and monoaromatic components present in the sediments are often readily degraded or volatilized while polycyclic aromatic hydrocarbons (PAHs) tend to accumulate in the sediment due to their low aqueous solubility, low volatility, and high affinity for particulate matter (Cuypers *et al*., 1998; Karthikeyan and Bhandari, 2001).

Several PAH treatment technologies such as thermal destruction, extraction, chemical treatment, and bioremediation, have already been developed and being used. Among these treatment technologies, bioremediation holds a great promise for dealing with intractable environmental problems such as PAH accumulation (Henner *et al*., 1997; Taylor and Jones, 2001; Haapea and Tuhkanen, 2006; O'Mahony *et al*., 2006; Ferrarese *et al*., 2008).

Though little information was available on anaerobic biodegradation of PAHs in the past, a number of researches began to report biodegradation of PAHs under various anaerobic conditions (Coates *et al*., 1997; Hayes *et al*., 1999; Rothermich *et al*., 2002). Studies have focused mainly on anaerobic biodegradation potential of sediments, isolation of PAH-degraders, and the effects of inorganic or organic amendments. Only a few studies have been performed on the bacterial community changes during PAH degradation. The studies addressed mainly the changes of bacterial communities during aerobic degradation of hydrocarbons, whereas Chang *et al*. (2005, 2006) investigated the community structure of PAH-degrading cultures in minimal medium under methanogenic conditions.

In this study, the effect of lactate amendment on the rate of PAH biodegradation was determined and the microbial communities that were supposed to be responsible for the PAHs degradation were monitored by terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial and archaeal 16S rRNA gene. In addition, cloning and sequencing of archaeal 16S rDNA gene were carried out to characterize the microbial community components. To our knowledge, this is the first study to assess changes in archaebacterial communities during anaerobic PAH degradation using culture-independent methods.

Results of this study will be of big help in enhancing the rate of biodegradation in PAH contaminated sediments, thus eliminating or minimizing the hazardous threats of these compounds to humans. In addition, identifying the microbial communities responsible for PAH degradation will give us more insights on how to effectively treat PAHs in sediments through bioremediation process.

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Materials and Methods

Sample collection and incubation

Sulfide-blackened anoxic sediments and seawater were collected from an estuary adjacent to a petrochemical factory in the industrial area of Gwangyang Bay, Korea. Sediment samples were packed into canning jars and immediately sealed then transported back to the laboratory. Aliquots of the sediment were packed into 600 ml air-tight jars after treatments in a glove box as described previously (Bach *et al*., 2005). Treatments consisted of a no-treatment control (NTC) and lactate (1%, w/v) treated sediment. The sediments were incubated at 20°C for 120 days without shaking, and 1 ml sediment aliquots in triplicate were sampled using 1 ml blunt-ended syringe corers with 0, 15, 30, 60, 90, and 120 day intervals. PAH concentrations in the sediments were analyzed using a GC chromatograph (HP 6890) after extraction using CH_2Cl_2 (Bach *et al*., 2005).

DNA extraction and purification

One gram of sediment (dry weight 0.5 g) was homogenized with 2× TENS solution (10 mM Tris-HCl, 40 mM EDTA, 200 mM NaCl, 2% SDS) in a 2 ml tube containing 0.5 g of zirconia glass beads (diameter 150~212 μm) using bead mill homogenizer (Bio-Spec Product, USA). The supernatant obtained by centrifugation (12,000×g, 4°C for 5 min) was extracted twice with 1 volume of phenol: chloroform: isoamylalcohol (25:24:1) mixture. The DNA was precipitated for 20 min in -20°C freezer after adding 1 volume of 0.3 M cold isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2). The pellet was washed with 70% (v/v) ethanol, dried, and then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to a final volume of 100 μl. After digestion of RNA with RNase A solution, DNA extracts were purified by polyvinylpolypyrrolidone (PVPP)-spin column chromatography according to the modified method of Cullen and Hirsch (1998).

PCR amplification of 16S rDNA

Two approaches were used for the amplification of 16S rDNA gene fragments which are suitable for analysis by T-RFLP (Ellis *et al*., 2003). The whole bacterial community genomic DNA was amplified using fluorescently labeled polymerase chain reaction (PCR) primer Hex-27F; 5'-Hexachlorfluorescein-AGA GTT TGA TCM TGG CTC AG-3' and the unlabeled reverse primer, 1522R; 5'-AAG GAG GTT ATC CAN CCR-3' as previously described (Giovannoi, 1991). For archaeal community Hex-21F; 5'-Hexachlorfluorescein-TTC CGG TTG ATC CYG CCG GA-3' and 958R 5'-YCC GGC GTT GAM TCC AAT T-3' were used (Delong, 1992). PCR was carried out in a 50 μl reaction mixture containing 1:10-diluted DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, dNTPs at 200 μM, 0.2 μM of each primer, 5% DMSO (Sigma, USA), 0.1% BSA (BMS, USA) and 5 unit of *Taq* polymerase (TaKaRa, Japan). Thermocycling was carried out on a thermocycler (Biometra, Netherlands) with an initial denaturation step of 94°C for 3 min; 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 4 min; cycling was completed by a final extension period of 72°C for 3 min. Positive and negative control reactions were also performed. Triplicate samples of different DNA extraction and separate PCR reactions for each sample were compared to assess the reproducibility. For each sample, the PCR products of three reactions were purified using the AccuPrep PCR Purification Kit (Bioneer, Korea), according to the manufacturer's instructions.

T-RFLP analysis

Approximately 50 ng of fluorescently labeled PCR products were fragmented by 2.5 U of restriction enzymes, *Hae*III and *Hha*I (Promega, Germany) with 1× reaction buffer and 0.01% of bovine serum albumin (BMS, USA). The fragmented DNAs were dried after ethanol precipitation, and rehydrated with 10 μl of formamide: ROX 2500 size standard (50:1, v/v). The mixture was denatured at 95°C for 5 min and cooled on ice for 5 min prior to running of the samples in 96-well optical plates on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA).

Cloning and colony PCR amplification

16S rDNA genes were amplified with nonfluorescent primers (27F, 1522R, 21F, and 958R) and the PCR products were purified using AccuPrep PCR Purification Kit (Bioneer, Korea). The purified 16S rDNA amplicons were then ligated into pGEM-T easy vector (Promega, Germany), and transformed into *E. coli* DH5α cells (Gray and Herwig. 1996). For colony PCR, white colonies were transferred to the PCR tube that contained 0.5 U EX *Taq* (TaKaRa, Japan), 1× buffer, 1.5 μM $MgCl_2$, dNTP at 200 μM, 0.2 μM of forward (T7) and reverse (SP6) primers, and sterilized water to 20 μl. After initial heating step of 10 min at 95°C, amplification reactions were performed with 30 cycles of denaturation (1 min at 95°C), primer annealing (1 min at 55°C), and extension (2 min at 72°C), with a final extension step at 72°C for 2 min.

Amplified 16S rDNA restriction analysis

To examine the RFLP patterns, colony PCR products were digested with restriction enzyme *Hha*I at 37°C for 4 h. The resultant fragments were analyzed by electrophoresis in 2% agarose MS gels (Roche, Germany).

Sequencing and phylogenetic analysis

The PCR products were purified using AccuPrep PCR Purification Kit (Bioneer, Korea), according to the manufacturer's instructions. A 16S rDNA clone, representing an RFLP family in a previously prepared 16S rDNA library, was bidirectionally sequenced at the 5' end by using primers T7 and SP6, and an ABI prism Big Dye terminators v3.1 cycle sequencing kit (Campbell and Cary, 2001). The reaction mixtures were cleaned using Wizard MagneSil sequencing reaction clean-up system (Promega, Germany) and sequenced with an ABI Prism 3100 Genetic Analyzer. 16S rDNA gene sequence data were edited with Chromas, version 1.45 (Technelysium, Australia). BLAST sequence similarity searches were performed on the National Center for Biotechnology Information (USA). The closest relatives were initially identified from 16S rDNA sequences aligned against known sequences in the GenBank database by using the gapped BLAST tool and the Ribosomal Database

Project databases. Sequences were assembled, aligned, and analyzed with the PHYDIT program (version 3.2). Then, the phylogenetic tree was constructed using the neighbor-joining method based on Jukes & Cantor distance (Jukes and Canter, 1969). The sequences determined in this study have been deposited in the GenBank database under the accession numbers EU616771 to EU616793.

Results and Discussion

Sample characteristics

Physicochemical characteristics of the sediments were analyzed in a previous study (Bach *et al*., 2005). The sediment contained a total PAH concentration (sum of the 16 US EPA priority pollutants) of 7,056.2 ng/g dry sediment, which was close to those reported in urbanized and industrialized area such as Pearl River (1,434~10,811 ng/g) (Mai *et al*., 2002). The measured concentrations of PAHs were compared with the ERL (effects range low) and ERM (effects range median) values that were used for assessment of aquatic sediment with a ranking of low to high adverse biological impact values (Long *et al*., 1995). Acenaphthylene, fluoranthene, pyrene, benzo[a] anthracine, chrysene, and the total PAH concentrations exceeded ERL, which indicates a potential biological impact. No PAH concentrations exceeded ERM.

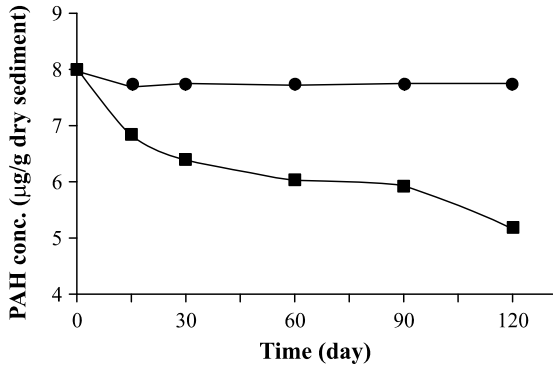

Fig. 1. Degradation of PAHs in anoxic sediments under no treatment condition and lactate amended condition during 120-day incubation. (●) no treatment control, (■) lactate amended sediment.

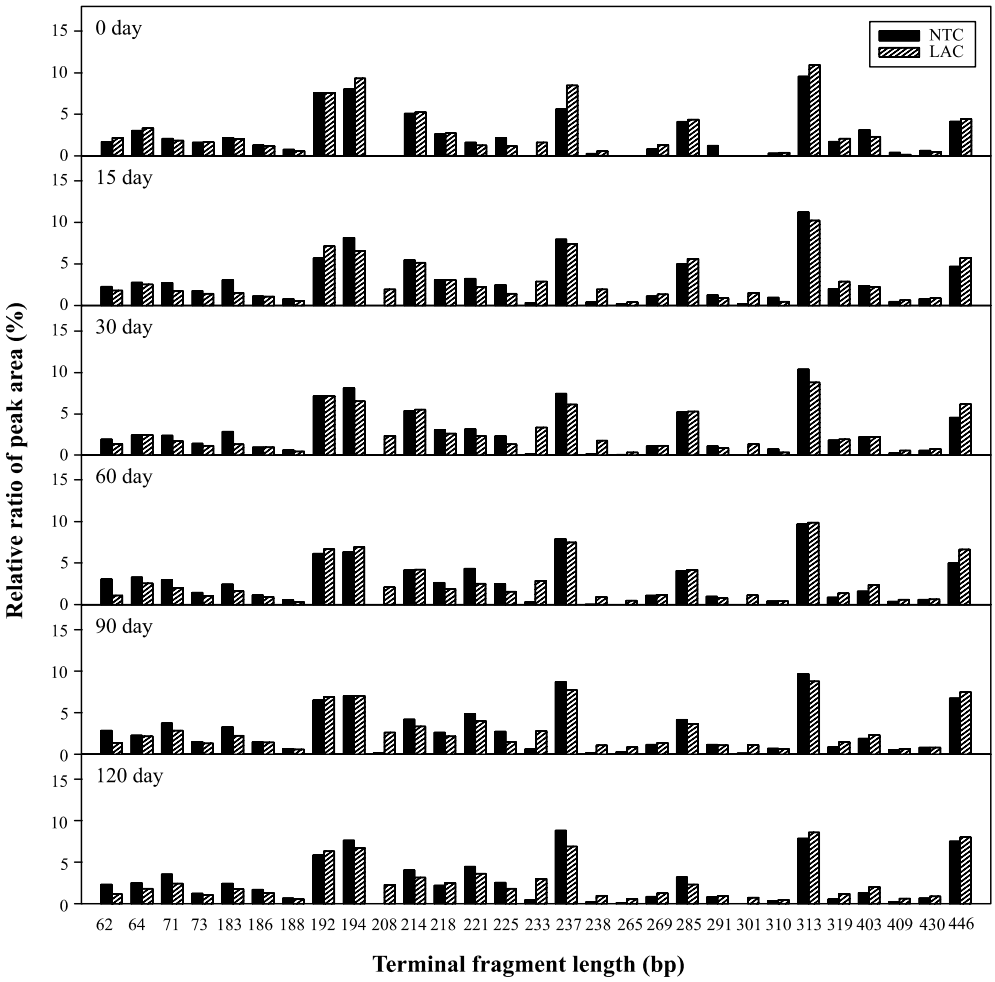

Fig. 2. T-RFLP analysis of bacterial community in anoxic sediment during PAH degradation. NTC, no treatment control; LAC, lactate amended sediment.

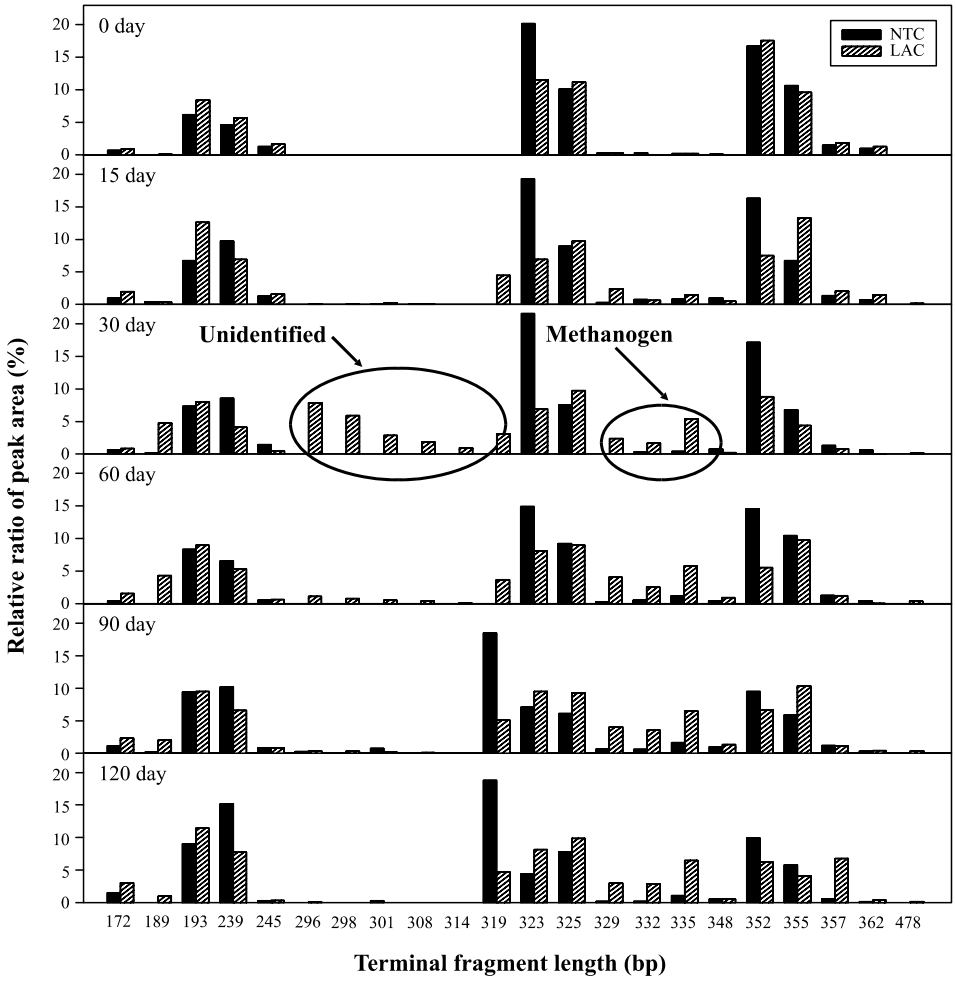

Fig. 3. T-RFLP analysis of archaeal community in anoxic sediment during PAH degradation. The shift in the community is shown over time. NTC, no treatment control; LAC, lactate amended sediment.

Effect of lactate on PAH degradation

Marine sediments contain lots of macronutrients as well as trace elements for microorganisms. However, organic compounds that are considered as co-substrates for PAH degradation processes are insufficient, since the microorganisms present in the sediment consumed these compounds rapidly. As denitrifying, sulfate-reducing, and methanogenic bacteria were found to be involved in PAH degradation in Gwangyang Bay sediment (unpublished data), lactate was used as co-substrate that can enhance the activities of these anaerobic heterotrophs in sediment environment (Chang *et al*., 2002; Yuan and Chang, 2007). Addition of lactate into the sediment enriched with anaerobic heterotrophs may hasten the possible role of these bacteria in PAH degradation processes. As shown in Fig. 1, PAHs were much more effectively degraded under lactate treated condition and lactate enhanced the degradation rate of most of individual PAH present in the sediment (Bach *et al*., 2005). These results indicate that addition of lactate enriched or activated anaerobic bacterial populations that were involved in PAH degradation processes and it was worthy to study changes in bacterial community structure.

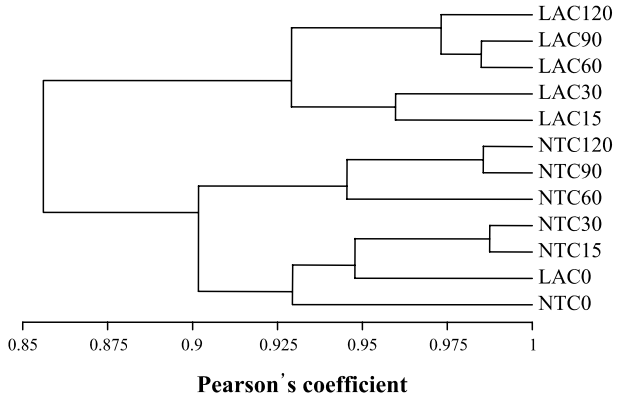

Fig. 4. Cluster analysis of bacterial community based on T-RFLP patterns. The dendrogram was produced by unweighted pair group method (UPGMA) clustering using a Pearson correlation based distance measure. NTC, no treatment control; LAC, lactate amended sediment.

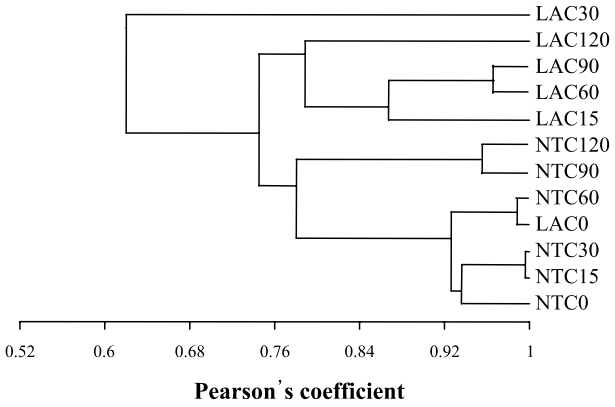

Fig. 5. Cluster analysis of archaeal community based on T-RFLP patterns. The dendrogram was produced by unweighted pair group method (UPGMA) clustering using a Pearson correlation based distance measure. NTC, no treatment control; LAC, lactate amended sediment.

Effect of lactate amendment on bacterial and archaebacterial community

T-RFLP patterns show that there were no substantial changes in bacterial community during 120 days of PAH degradation either in no-treatment control or lactate-treated sediment (Fig. 2 and 4). Only some of the minor peaks such as T-RFs 208, 233, 238, and 301 bp showed small variations (Fig. 2). In the archaebacterial community, however, T-RFLP patterns showed more significant changes in terms of community structure. The differences between the two treatment conditions are clearly shown by the number of T-RFs and the differences in the relative abundance of some diagnostic peaks (Fig. 3). T-RFs 189, 329, 332, and 335 bp started to be detected in the lactate treated sediment on 15th day and the relative ratios of peak area increased during 15∼60 day period when PAH degradation occurred actively. The other group of T-RFs 296, 298, 301, and 308 bp were also detected only in the lactate treated sediment on 30th day but rapidly disappeared thereafter. While very high

Table 1. Sequenced archaeal clones belonging to the phylum Euryarchaeota and their 16S rDNA gene sequence similarities to nearest cultured neighbor or nearest clone

Phylogenetic group	Representative clone (Accession number)	TRF size (bp)	Nearest neighbor (Accession number)	Similarity (%)
Methanosarcinales	ALAS 16 (EU616771)	332	EHB97 (AF 374285)	94.26
	ALAS 51 (EU616772)	329	*Methanosarcina semesiae* (AJ 012742)	97.89
	ALAS 79 (EU616775)	332	*M. lacustris* (AY 260431)	98.87
	ALAS 189 (EU616777)	329	*M. mazei* strain (AY 260432)	99.66
UAG1[a]	ALAS 95 (EU616776)	67	ZAR100 (AY 341269)	99.70
Unknown	ALAS 65 (EU616774)	332	NT305-CAT-A02 (AB 111491)	78.00
	ALAS 60 (EU616773)	332	SH05-CAT-A06 (AB 111023)	79.00

[a] UAG1, Unaffiliated Archaeal Group I

Table 2. Sequenced archaeal clones belonging to the phylum Crenarchaeota and their 16S rDNA gene sequence similarities to nearest cultured neighbor or nearest clone

Phylogenetic group	Representative clone (Accession number)	TRF size (bp)	Nearest neighbor (Accession number)	Similarity (%)
MCG[a]	ALAS 26 (EU616779)	218	MA-A1-1 (AY 093446)	94.87
	ALAS 27 (EU616780)	63	Kazan-2A-38/BC19-2A-38 (AY 592014)	98.34
	ALAS 35 (EU616781)	355	MA-C1-3 (AY 093450)	96.57
	ALAS 41 (EU616783)	173	MA-B1-3 (AY 093447)	97.12
	ALAS 44 (EU616784)	203	Arc.118 (AF 005761)	93.25
	ALAS 53 (EU616785)	63	OHKA15.20 (AB 094560)	96.85
	ALAS 58 (EU616786)	358	MA-C1-3 (AY 093450)	89.93
	ALAS 63 (EU616788)	63	HTA-B10 (AF 418925)	98.55
	ALAS 68 (EU616789)	353	pGrfC26 (U 59986)	97.78
	ALAS 78 (EU616791)	240	MA-B1-3 (AY 093447)	95.10
	ALAS 127(EU616792)	173	MA-C1-3 (AY 093450)	99.34
	ALAS 140(EU616793)	85	Kazan-2A-33/BC19-2A-33 (AF 005765)	98.33
DSAG[b]	ALAS 36 (EU616782)	352	CRA8-27cm (AF 119128)	97.26
	ALAS 76 (EU616790)	199	VIM2-31 (AY 380641)	96.76
MHVG[c]	ALAS 25 (EU616778)	529	GoM GC234 621R (AY 211709)	97.30
SCG[d]	ALAS 61 (EU616787)	325	SCA1154 (U 62814)	98.97

[a] MCG, Miscellaneous Crenarchaeotic Group
[b] DSAG, Deep-Sea Archaeal Group
[c] MHVG, Marine Hydrothermal Vent Group
[d] SCG, Soil Crenarchaeotic Group

similarities were found among T-RFLP patterns of no treatment control sediments during 0~60 day period (Pearson's correlation coefficient, r>0.92), poor correlations were obtained between no treatment control and lactated treated sediment during same time period (Fig. 5).

Although it was found that sulfidogenic and denitrifying bacteria (bacterial community) were involved in PAH degradation in our preliminary study (unpublished data), a significant corresponding shift in bacterial community structure was not observed during lactate-enhanced PAH degradation. However, a strong correlation between lactate-induced PAH degradation and the shift in the archaebacterial community was observed and this result suggests a possible involvement of archaeal populations in metabolism of PAH degradation in the sediment.

Analysis of archaebacterial clone libraries

Based on T-RFLP results, archaebacterial community was chosen as a target for identification and phylogenetic analysis. A total of 288 archaeal clones were made from lactate amended sediment on day 30, which showed the most distinct T-RFLP pattern compared with that of zero-time sediment (Fig. 3 and 5). Twenty-three out of the 288 clones representing distinct RFLP patterns were selected for sequencing and the clones had similarity ranged from 78 to 99.66% to 16S rRNA gene sequences available in GenBank (Table 1 and 2).

Six major phylogenetic archaeal lineages were recovered from the PAH contaminated sediment. Seven representative clones were included in Euryarchaeota, four of these clones in *Methanosarcinalse*, one in Unaffiliated Archaeal Group I (UAG1), and two clones showing no clear involvement to any known archaea division. All of the other clones were included in Crenarchaeota, 12 representative clones in Miscellaneous Crenarchaeotic Group (MCG), 2 clones in Deep-Sea Archaeal Group (DSAG), and one clone each in Marine Hydrothermal Vent Group (MHVG) and Soil Crenarchaeotic Group (SCG). The phylogenetic distance of the clones to their closest relatives is shown in Fig. 6 and 7.

Four clones (ALAS16, ALAS51, ALAS79, ALAS189) out

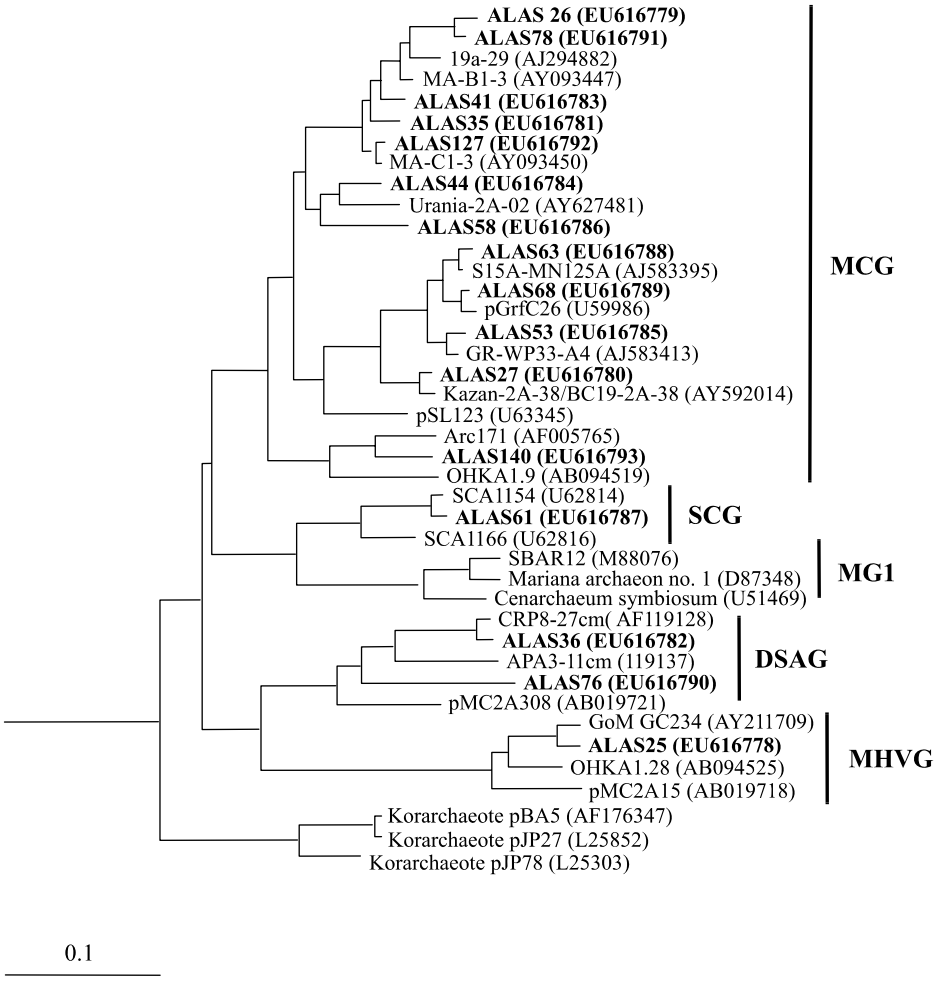

Fig. 6. Phylogenetic tree based on the 16S rDNA sequences of the Crenarchaeota clones obtained from PAH-contaminated sediment. The tree was constructed based on the neighbor joining method. The scale bar corresponds to 10 nucleotide substitutions per 100 nucleotide positions. MCG, Miscellaneous Crenarchaeotic Group; SCG, Soil Crenarchaeotic Group; MG 1, Marine Group 1; DSAG, Deep-Sea Archaeal Group; MHVG, Marine Hydrothermal Vent Group.

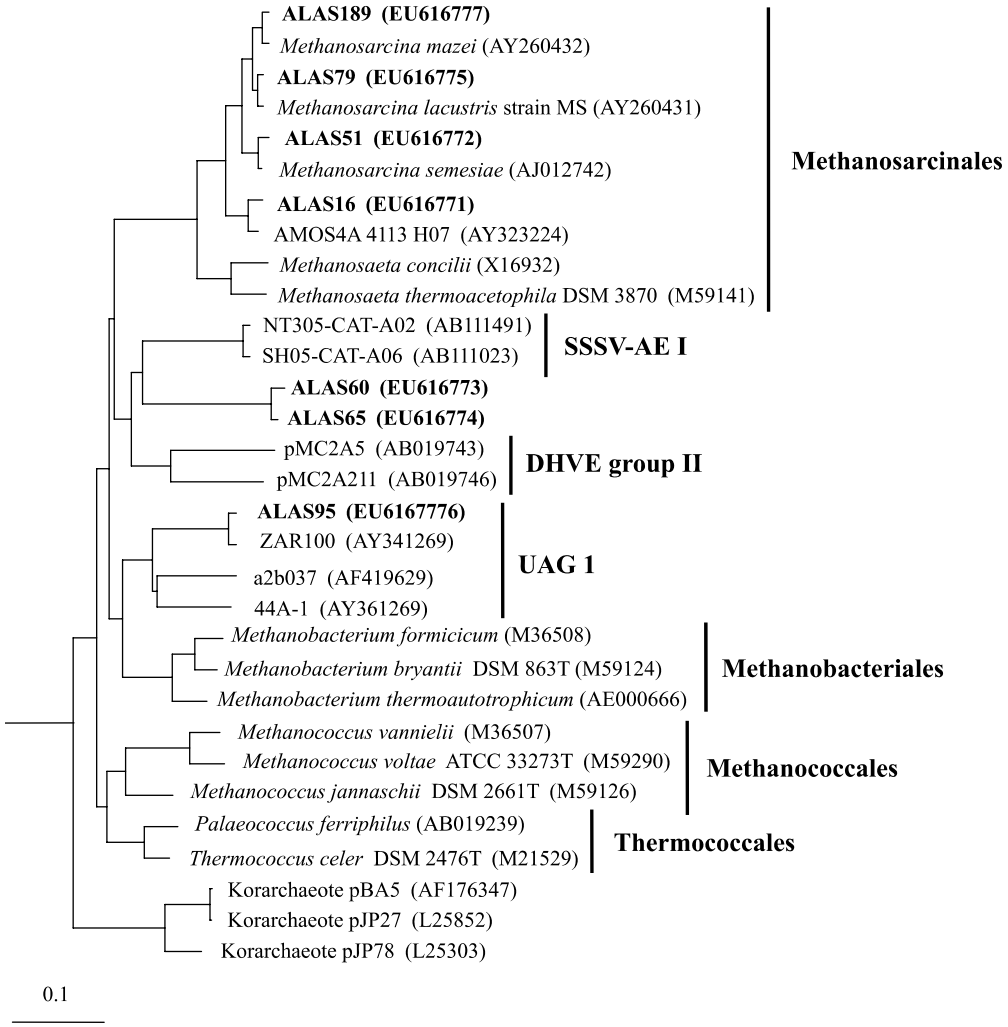

Fig. 7. Phylogenetic tree of the Euryarchaeota clones obtained from PAH-contaminated sediment. The scale bar corresponds to 10 nucleotide substitutions per 100 nucleotide positions. UAG 1, Unaffiliated Archaeal Group 1. SSSV-AE1, Suiyo Seamount Sub-Vent origin, Archaea domain, Euryarchaeota, GroupI; DHVE group II, Deep-Sea Hydrothermal Vent Euryarchaeotic group II.

of 23 clones were clustered into a culturable archaea group, *Methanosarcinales*, and their closest relatives were *Methanosarcina* sp. which were known to utilize CO_2-type, methyl type and acetotrophic substrates (Madigan *et al*., 2003). Among these closest relatives, *Methanosarcina semesiae* (AJ 012742) and *Methanosarcina lacustis* (AY 260431) were reported as predominant archaea in phenanthrene-degrading methanogenic cultures initiated with Baltimore Harbor sediments (Chang *et al*., 2005, 2006). Chang *et al*. (2005) also reported that archaeal 16S rDNA could be only successfully amplified with archaeal primers in the PAH-enriched methanogenic cultures, but not in PAH-free methanogenic cultures. Moreover, all of the four clones had sequence-determined T-RF sizes of 332 or 329 bp, which showed the most corresponding shift to the extent of PAH degradation (highlighted as Methanogen in Fig. 3). The other clones belonging to euryarchaeal group were also closely grouped within hydrocarbon-degrading archaeal communities. Clone ALAS95 belonged to the UAG1 group which has been commonly detected from naturally or anthropogenically hydrocarbon impacted sediments (Watanabe *et al*., 2002; Elshahed *et al*., 2004), anaerobic digester sludge (Sekiguchi *et al*., 1998), and hydrocarbon-contaminated aquifer (Dojka *et al*., 1998). The two ungrouped clones, ALAS65 and ALAS 60 were closest to SSSV-AE1 (Suiyo Seamount Sub-Vent origin, Archaea domain, Euryarchaeota, group 1) with 16S rDNA sequence similarities of 78∼79%. Members of SSSV-AE1 have been reported in deep-sea hydrothermal vent (Higashi *et al*., 2004). Among 16 clones belonging to Crenarchaeota, 75% (12 clones) belonged to the MCG group which has been detected in wide range of methane-related subsurface ecosystems as dominant members of archaeal community (Kim *et al*., 2005; Parkes *et al*., 2005; Biddle *et al*., 2006). Interestingly, 6 clones out of 12 MCG-group clones exhibited high sequence similarity to species which were identified in methane hydrate-bearing deep marine sediments (Reed *et al*., 2002) and these clones had sequence-determined T-RF sizes of 353 to 358 bp, which correspond to the dominant

fragments on day 0 (Fig. 3). The other 4 clones in Crenarchaeota belonged to DSAG, MHVG, and SCG groups, which have been reported from deep sediments in hydrate zones (Inagaki *et al*., 2006), black smoker (Takai *et al*., 2001a), and deep gold mines (Takai *et al.*, 2001b).

Response of the bacterial community to bioremediation treatment of contaminated environments has been observed in several studies (Zucchi *et al*., 2003; Connon *et al*., 2005; Saul *et al*., 2005), but the evaluation of the behavior of archaeal community is very limited. In this study, we observed a dynamic behavior of archaeal populations during PAH degradation in anoxic sediment. Though it was not successful to clone some of the diagnostic T-RFs in archaeal community (shown as unidentified in Fig. 3), the addition of lactate clearly shifted the structure of methanogenic communities and this was accompanied by enhanced PAH degradation. These results suggest that methanogenesis-bearing archaeal populations are likely involved in PAH degradation process and future studies focused on the linkage between the activity of methanogens and PAH degradation will be necessary.

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