Phylogenetic Analysis of Phenanthrene-Degrading *Sphingomonas*

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Abstract Soil samples were obtained from 5 sites contaminated with polycyclic aromatic hydrocarbons (PAHs). These soil samples were cultured in using phenanthrene as a sole carbon and energy source, and 36 strains of phenanthrene-degrading bacteria were isolated from 3 sites. Most of them degraded 500 ppm of phenanthrene within 8 to 10 days, and these isolates could degrade a few other PAHs other than phenanthrene. Their genotypes were determined by restriction digests of the 16S rRNA genes [amplified ribosomal DNA restriction analysis (ARDRA)]. It was found that all the phenanthrene degrading isolates were included in 4 ARDRA types, and they showed a strict site endemism. 16S rDNAs of 12 strains selected from different sites were sequenced, and they were all confirmed as *Sphingomonas* strains. Their 16S rDNA sequences were compared for phylogenetic analysis; their sequence showed a similar result to ARDRA typing, thus indicating that these heterotrophic soil bacteria are not regionally mixed. In addition, it was found that the microbial diversity among sampling sites could be monitored by 16S rDNA PCR-RFLP pattern alone, which is simpler and easier to perform, without 16S rDNA sequence analysis.

Key words: PAH, phenanthrene, *Sphingomonas*

Interest in the biodegradation mechanisms and environmental fate of polycyclic aromatic hydrocarbons (PAHs) is prompted by their potentially nocuous effects on human health. PAHs are composed of fused aromatic rings in linear, angular, or cluster arrangements [11]. PAHs are divided into two classes: low-molecular weight PAHs (LMW PAHs), such as naphthalene, phenanthrene, anthracene, and fluorene that are composed of two or three fused aromatic rings; and high-molecular weight PAHs (HMW PAHs), such as pyrene, chrysene, and fluoranthene that are composed of four or more fused aromatic rings [1, 5].

The chemical properties of a PAH molecule are dependent partly on both molecular size, the number of aromatic rings, and the pattern of ring linkage. Moreover, an increase in the size and angularity of a PAH molecule generally results in increase in hydrophobicity and electrochemical stability. These hydrophobic compounds tend to increase affinity for organic matter and particles, and accumulate in organic compound-rich marine sediments and soils [2]. For example, half-lives in soil and sediment of the three-ring phenanthrene molecule have been reported to range from 16 to 126 days, while 299 to more than 1,400 days for the five-ring molecule benzo[a]pyrene (BaP) [18].

PAHs are present as natural constituents in fossil fuels, and are formed during the incomplete combustion of organic material, therefore present in relatively high concentrations in the products of fossil fuel refining. Petroleum refining and transport activities are major contributors to localized loadings of PAHs in the environment. In addition, PAHs are released into the environment by forest fires [29], gasoline and diesel fuel combustion [17], and smoking of tobacco [12]. PAH concentrations in the environment vary widely, depending on the proximity of the contaminated site to the production source, the level of industrial development, and the mode(s) of PAH transport. Hence, the need to develop practical bioremediation strategies for heavily impacted sites is evident [17].

Although PAHs are considered hazardous due to potential carcinogenic, mutagenic, and teratogenic effects, the discovery of PAH-degrading bacteria greatly helped to develop remediation processes of PAH-contaminated sites [5]. A variety of bacteria can degrade certain PAHs completely to CO_2 and metabolic intermediates, in order to obtain energy and carbon for their growth [11, 20, 26, 27].

Phenanthrene is considered as a prototypic PAH and serves as a signature compound to detect PAH contamination, since its chemical structure is found in carcinogenic PAHs, such as benz[a]anthracene. Phenanthrene has also been used as a model PAH to determine factors that affect the bioavailability, biodegradation potential, and rate of microbial degradation of PAHs in the environment [16, 19, 25].

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Recently, endemism studies of isolated microorganism in soil have been reported by Cho and Tiedje [4] who studied fluorescent *Pseudomonas*, and Fulthorpe *et al.* [7] who studied 3-chlorobenzoate-degrading bacteria. However, endemism of *Sphingomonas* that degrade phenanthrene is hardly known. Previously, 36 phenanthrene-degrading bacteria were isolated from PAH-contaminated soil, and they were characterized and identified. In this report, based on 16S rDNA sequence analysis their phylogenetic relationships and their endemicity according to sampling sites were studied based on RFLP analysis of amplified 16S rDNA.

MATERIALS AND METHODS

Isolation and Characterization of Phenanthrene-Degrading Bacteria

Soil samples were collected from 5 sites contaminated with PAH in November 2000. Surface soil samples were collected from an automobile industry site at a depth of 15 cm by using sterile conical tubes and were kept at 4°C until processing.

Minimal medium (MM) was used in enrichment cultures, isolation of phenanthrene-degrading bacteria, and the phenanthrene-degradation test. The composition of MM was as follows (per liter): $(NH_4)_2SO_4, 0.03$ g; NaCl, 2 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCl_2 \cdot H_2O$, 0.02 g; $KNO_3, 0.01$ g; and trace metal solution, 1 ml. The composition of trace metal solution was as follows (per liter): Fe-EDTA, 2 g; $ZnSO_4 \cdot 7H_2O$, 2 g; $FeSO_4 \cdot 7H_2O$, 1.5 g; $CuSO_4 \cdot 5H_2O$, 0.3 g; $CoCl_2 \cdot 6H_2O$, 0.2 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.1 g; $MnCl_2 \cdot 4H_2O$, 0.1 g. Solid MM contained 1.5% Bacto agar (Difco). PAH-contaminated soil samples were suspended in MM. After the addition of 500 ppm phenanthrene, the mixture was incubated at 28°C with shaking (180 rpm). When turbidity increased, a part of the culture was transferred into fresh medium and further incubated (3 times). Dilutions of soil slurry were inoculated on MM plates with phenanthrene as a sole carbon source (spraying). Colonies were chosen for their ability to form clear zones and were transferred to tryptic soy agar (TSA) plates.

PAH-degrading bacteria were characterized based on colony morphology and pigmentation, Gram staining, API (Analytical Profile Index) 20NE test strips (bioMerieux, Marcy-l'Etoile, France), and several physiological and biological tests.

Whole-cell fatty acid analyses were performed on all of the phenanthrene-degrading isolates by growing the cells at 28°C for 24 h on TSA plates. Cellular fatty acids were saponified, methylated, extracted, and analyzed by microbial identification system (MIDI, ChemStation Version 4.02).

PAH-Degradation Assay

To test whether the isolates could use phenanthrene as a sole carbon and energy source, the isolates were inoculated

into 100 ml of MM containing 500 ppm of phenanthrene to give an optical density of 0.15–0.2 at 660 nm. Culture medium was sampled during growth, and mixed with four volumes of a solvent mixture [acetone:methanol:hydrochloric acid=10:10:1 (v/v)] to dissolve solid phenanthrene, and its optical density at 660 nm was measured to estimate the concentration of bacterial cells using a spectrophotometer (Biomate 5, U.S.A.) [14]. Phenanthrene-degrading ability was monitored at indicated time intervals by sampling 5 ml of the culture media, and the remaining amount of phenanthrene was measured by using high-performance liquid chromatography (HPLC; Waters, U.S.A.). One thousand ppm of pyrene were added to each sample as an internal standard, the mixture was extracted three times with equal volumes of ethyl acetate, the remaining water was removed by anhydrous sodium sulfate, and ethyl acetate was evaporated by using a vacuum evaporator to extract the remaining phenanthrene. The dried residues were dissolved in 2.0 ml of acetonitrile and analyzed by HPLC (Waters, U.S.A.). Aliquots (20 μl) of this solution were injected onto an LC-PAH column (Supelco, U.S.A.) by using a 717 Autosampler (Waters, U.S.A.). The solvent system consisted of 100% acetonitrile, the flow rate was 0.8 ml min^{-1}, and PAHs were detected spectrophotometrically (254 nm). For integration of the chromatograms and quantification of the PAH amount, the software packet BREEZE was used.

To test whether the isolates could degrade PAHs other than phenanthrene, indole, naphthalene, fluoranthene, and pyrene were used. After inoculating each isolate on MM plate, 0.1 g of naphthalene crystal was added to the lid and colony production was observed. Two g each of fluoranthrene or pyrene were dissolved in 100 ml of acetone, sprayed onto inoculated MM plates, and clear zone production and color change around each colony were observed [22]. To test indole degradation, 0.01 g of indole was added on the lid of each petri dish after 24 h of incubation on TSA plates at 28°C, and production of indigo (blue color) was observed.

Amplified 16S rDNA Restriction Analysis (ARDRA)

Sequence analyses of 16S ribosomal DNA (rDNA) were performed on the several isolates, by amplifying the 16 rRNA genes by PCR. The PCR primers were 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (GGYTA-CCTTGTTACGACTT) as indicated by Lane [24]. The PCR mixture (50 μl) contained 10x reaction buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, 1 mM DDT, 50% glycerol, 1% Triton X-100), 1.5 mM $MgCl_2$, 250 μM dNTPs, 20 pmol each primer, 2 units of *Taq* DNA polymerase (Promega, U.S.A.), and about 10 ng of DNA template. PCR amplification was performed using a PE480 (Perkin-Elmer, U.S.A.) programmed as follows: 5 min of denaturation at 95°C, followed by 30 cycles at 95°C for

1 min, 55°C for 1 min, and 72°C for 1.5 min, with a final extension at 72°C for 10 min. The amplified 1.5 kb PCR products were electrophoresed in a 1% agarose gel and purified using the DNA PrepMate II (Bioneer, Korea).

The purified 16S rDNA PCR products were digested with tetrameric restriction endonucleases AluI, HhaI, or HpaII, as recommended by the manufacturer (Promega, U.S.A.). The digestion products were resolved by electrophoresis with 2% MetaPhore agarose gels (FMC, U.S.A.). A 1 kb plus DNA ladder size marker (Gibco BRL, Germany) was run on both sides and in the central lane of each gel.

Gel images were photographed and stored as TIFF files. These digitized images were converted, normalized with the above-mentioned DNA size markers, and analyzed with a GelCompar program (Ver. 4.2; Applied Maths, Belgium). The rolling-disk background subtraction method was applied. For ARDRA patterns, a band-matching algorithm was used to calculate paired-wise similarity matrices with the Dice coefficient [13]. Cluster analyses of similarity matrices were performed by the unweighted pair group method using arithmetic averages (UPGMA) [28].

Sequencing and Phylogenetic Analysis of 16S rDNA

The sequences of purified 16S rDNA were determined using a Taq DyeDeoxy terminator cycle sequencing kit and a 310A DNA sequencer (Perkin-Elmer Applied Biosystems). 16S rDNA sequences were compared to sequences in gene banks using the BLAST (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) and FASTA3 (EMBL outstation European Bioinformatics Institute; http://www2.ebi.ac.uk/) programs to find the most similar sequences. The nucleotide sequences were aligned by using a Clustal X program (Ver. 1.8), and a phylogenetic analysis was performed by the TreeconW program (Bootstrap of 100 replicates, Ver. 1.3b, University of Antwerp, Belgium) [30].

RESULTS

Isolation and Characterization of Phenanthrene-Degrading Bacteria

Soil samples from 5 sites contaminated with PAHs were enriched to isolate phenanthrene-degrading bacteria. Thirty six colonies from 3 sites produced clear zones around them on minimal media sprayed with phenanthrene: 5 colonies were from Site 1, 16 colonies were from Site 3, and 15 colonies were from Site 4. All the phenanthrene-degrading isolates were Gram-negative rods, and produced colonies on TSA plates after 24 h of inoculation. Biochemical test by API 20 NE identified most of them as Sphingomonas paucimobilis, a few as Pseudomonas putida, but a few still could not be identified. Identification based on whole-cell

fatty acid composition showed slightly different results. Nevertheless, most of them were identified as Sphingomonas or Flavobacterium (data not shown). Recently, few PAH-degrading bacteria such as Pseudomonas and Flavobacterium were reclassified as Sphingomonas [18].

The isolates were tested for the ability to degrade a few PAHs other than phenanthrene, and most of them were found to be able to use naphthalene or indole as an energy and carbon source. They could convert indole into indigo and could produce colonies using naphthalene as vapor phase, 6 of them could degrade fluoranthene, but pyrene was not degraded by any of them (Table 1).

Table 1. Degradation of PAHs by the isolates.

Isolate	Phenanthrene	Fluoranthene	Naphthalene	Pyrene	Indole
HS421	+++	–	–	–	++
HS430	+++	–	+++	–	+
HS442	+++	–	+++	–	+
HS451	+++	–	++	–	+
HS460	+++	–	++	–	+
HS411	+++	–	+++	–	+
HS402	+++	–	+++	–	+
HS413	+++	–	++	–	+
HS414	+++	–	+++	–	+
HS415	+++	–	–	–	+
HS416	+++	–	–	–	++
HS419	+++	–	+++	–	++
HS420	+++	–	–	–	+
HS412	+++	–	+++	–	++
HS440	+++	–	–	–	+
HS311	++	–	+++	–	+++
HS331	+++	–	+++	–	+++
HS351	+++	+	+++	–	+++
HS371	+++	–	+++	–	+++
HS391	++	–	++	–	+++
HS310	+++	–	+++	–	+++
HS301	++	–	+++	–	+++
HS330	++	–	+++	–	+++
HS380	++	–	+++	–	+++
HS111	++	–	–	–	++
HS122	+++	+	+++	–	+
HS152	+++	–	–	–	+
HS163	++	+	+++	–	+
HS182	+++	–	–	–	+
HS312	+++	–	+++	–	++
HS342	+++	–	+++	–	++
HS362	+++	–	+++	–	++
HS372	+++	–	+++	–	++
HS322	++	+	++	–	–
HS352	++	+	++	–	+
HS370	++	+	++	–	+

+++: excellent degradation.
++: good degradation.
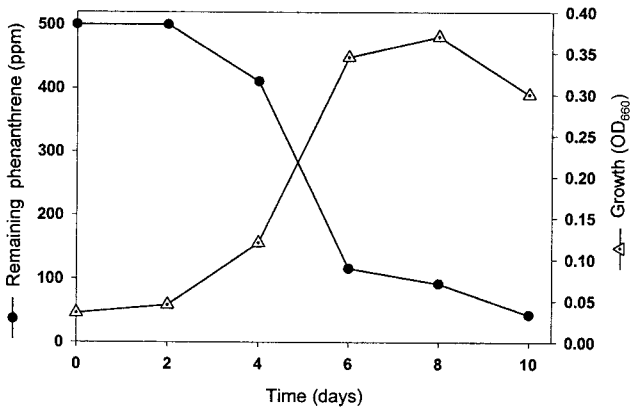+: moderate degradation.
–: no degradation.

Fig. 1. Typical phenanthrene degradation and growth of the isolates (HS312).

Minimal medium containing 500 ppm of phenanthrene was used to test the relationship between the growth rate and phenanthrene degradation. Usually, PAH exists as particles in liquid media and bacteria grow by adsorbing to these particles. Since these particles interfere with the measurement of bacterial growth, the method suggested by Iwabuchi *et al.* [14] was used. All isolates showed a similar pattern of relationship between the growth rate and degradation. After 2 days of lag period, cells entered the exponential period at 3 days followed by the death period after 8 days (Fig. 1). Phenanthrene degradation was well correlated with the growth. Almost no degradation was observed during the first 2 days, active degradation between 3 and 6 days, and then the degradation slowed down after 6 days, whereafter the growth decelerated until 10 days.
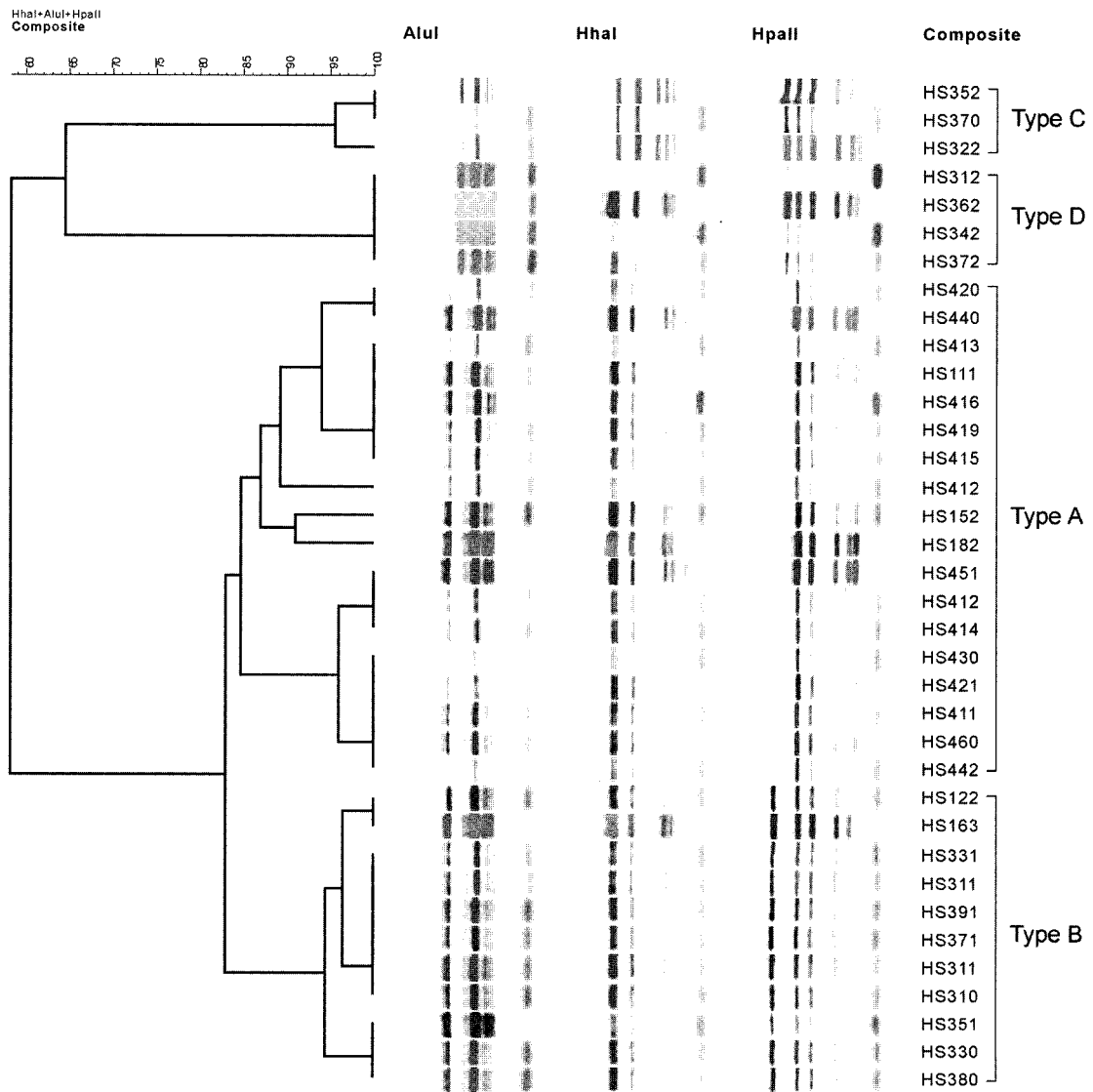

Fig. 2. Restriction patterns of PCR-amplified 16S rDNA of the isolates. First digit of each isolate represents the site it is originated.

ARDRA Typing of Isolates

A more detailed analysis was performed with 36 strains isolated from 3 sites. To determine ARDRA type, 16S rRNA genes of 36 isolates were amplified by the PCR and the PCR products were digested with *Alu*I, *Hha*I, or *Hpa*II. The individual RFLP patterns are shown in Fig. 2. The combined *Alu*I, *Hha*I, and *Hpa*II restriction patterns of the amplified 16S rDNAs were used for cluster analysis by UPGMA. All the isolates of Site 4 produced ARDRA type A and the isolates of Site 3 produced ARDRA types B, C, and D (Fig. 3). The isolates of Site 1 which is located between Sites 3 and 4 were also separated into Types A

and B. These 4 ARDRA types were produced above genetic similarity level of 84%. *Alu*I differentiated ARDRA Types A and B from Types C and D, *Hha*I differentiated ARDRA Type C from Types A, B, and D, which means that Types A, B, and D are similar, and *Hpa*II differentiated ARDRA Types C and D from Types A and B.

Phylogenetic Analysis

16S rDNA of 12 representative isolates from 3 sites were partially sequenced and identified. In general, this 16S rDNA sequence analysis confirmed the result of the ARDRA typing. Strains HS122 and HS163 isolated from
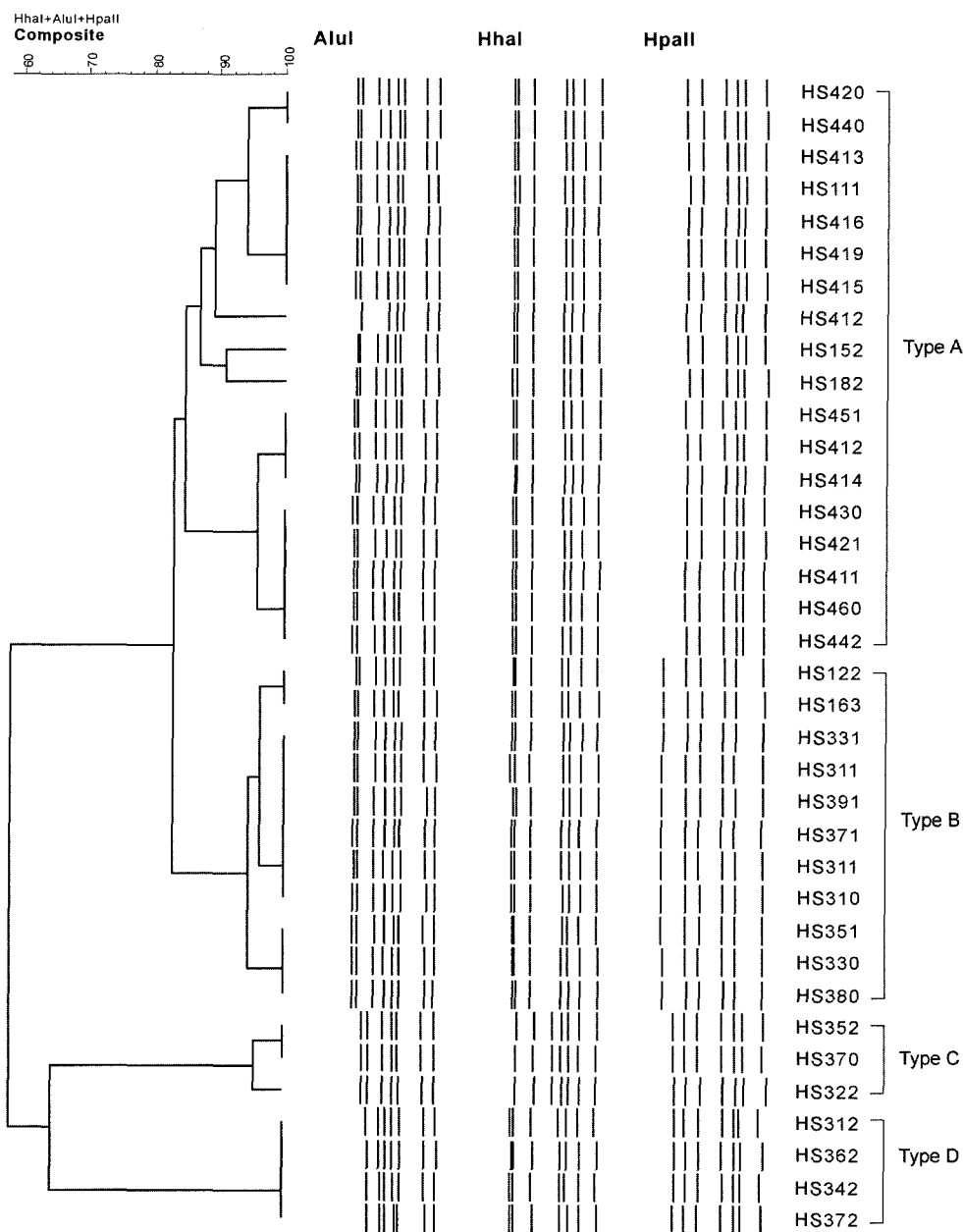

Fig. 3. Dice-UPGMA cluster analysis of combined *Alu*I, *Hha*I, and *Hpa*II restriction patterns of amplified 16S rDNA of the isolates.

Table 2. Identification of the selected isolates based on 16S rDNA sequence similarity.

Isolate	Best matches	Identities (%)	Partial sequence (bp)
HS122	*Sphingomonas* sp. G296-3	99.17	481
HS163	*Sphingomonas* sp. G296-3	99.20	497
HS311	*Sphingomonas chlorophenolica*	99.36	467
HS312	*Sphingomonas* CF06	99.14	464
HS351	*Sphingomonas chlorophenolica*	99.36	470
HS352	*Sphingomonas* CF06	99.40	501
HS362	*Sphingomonas* CF06	99.22	510
HS372	*Sphingomonas* CF06	99.06	532
HS380	*Sphingomonas chlorophenolica*	99.14	463
HS412	*Sphingomonas* sp. BRW2	98.65	520
HS416	*Sphingomonas* sp. BRW2	98.71	466
HS419	*Sphingomonas* sp. BRW2	98.38	495

Site 1 showed best matches with *Sphingomonas* sp. G296-3 (99.17% and 99.20%, respectively); strains HS311 (99.36%), HS351 (99.36%), and HS380 (99.14%) from Site 3 matched best with *Sphingomonas chlorophenicola*; strains HS312 (99.14%), HS352 (99.40%), HS362 (99.22%), and HS372 (99.06%) also from Site 3 matched best with *Sphingomonas* CF06; and strains HS412 (98.65%), HS416 (98.91%), and HS419 (98.38%) from Site 4 matched best with *Sphingomonas* sp. BRW2 (Table 2). A phylogenetic tree of these 12 isolates was drawn based on 16S rDNA sequences (Fig. 4). This phylogenetic analysis showed
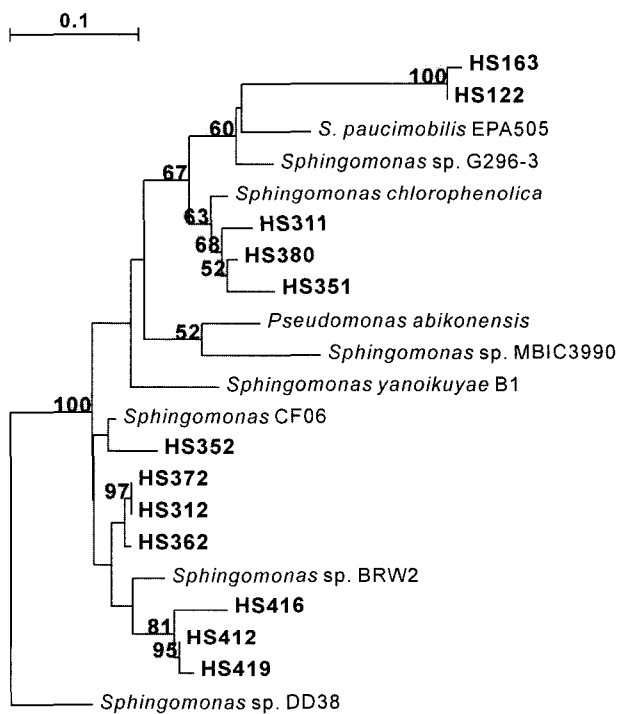
Fig. 4. Phylogenetic tree of the selected isolates.

endemism in Site 1, Site 3, and Site 4. In other words, all 3 sites that had phenanthrene-degrading bacteria showed endemism. It might be premature to make any conclusion on comparison of 16S rDNA sequence analysis to ARDRA typing in phylogenic analysis. However, in the present case, 16S rDNA sequence analysis appeared to be slightly more sensitive than ARDRA typing.

DISCUSSION

Phenathrene-degrading bacteria reported so far include *Pesudomonas* [6], *Aeromonas* [21], *Alcaligenes* [23], *Flavobacterium* [3], *Comamonas* [9], *Norcardiodies* [15], *Cycloclasticus* [8], and *Mycobacterium* [10]. Recently a few PAH-degrading bacteria such as *Peudomonas* and *Flavobacterium* have been reclassified as *Sphingomonas* [18]. Thirty-six phenanthrene-degrading bacteria have been isolated from PAH-contaminated soil, and tentative identification of the isolates by API test and MIDI test showed *Sphingomonas*, *Pesudomonas*, and *Flavobacterium*, however, 16S rDNA sequencing analysis identified all of them as *Sphingomonas*.

The isolates could use naphthalene, indole, fluoranthene, as well as phenanthrene as a sole carbon and energy source. They have excellent phenanthrene-degrading ability by degrading 500 ppm of phenanthrene in 8–10 days. ARDRA typing with three tetrameric restriction enzymes revealed 4 well-defined groups of *Sphingomonas* in the isolates. One of the 4 ARDRA types originated from Site 4 and the other 3 types were from Site 3. 16S rDNA sequence analysis identified the isolates from Site 4, which comprise ARDRA Type A, as *Sphingomonas* sp. BRW2, ARDRA Type B isolates from Site 3 as *Sphingomonas* CF06, and Types C and D as *Sphingomonas chlorophenolica* but Types C and D were in different branches in the phylogenetic tree (Fig. 4). The fact that the ARDRA typing and 16S rDNA sequence analysis gave identical results strongly suggest that the microbial diversity among sampling sites could be monitored by 16S rDNA PCR-RFLP pattern alone without 16S rDNA sequence analysis. Our results also show strong endemism in Sites 3 and 4, and *Sphingomonas* diversity in Site 3.

Acknowledgment

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