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Bioremediation of polycyclic aromatic hydrocarbon-contaminated soil by a bacterial consortium and associated microbial community changes

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ABSTRACT

Bioremediation of a PAH-contaminated soil was carried out with a bacterial consortium enriched from the soil. The soil contained 9362.1 μg kg^{-1} of USEPA priority PAHs, 90.6% of which were 4- and 5-ring PAHs. After incubation for 56 days, 20.2% and 35.8% of total PAHs were removed from the soil with the addition of 10% and 20% of a bacterial consortium suspension. The soil microbial population increased in the early days but decreased by the end of the experiment. Denaturing gradient gel electrophoresis (DGGE) analysis of polymerase chain reaction (PCR)-amplified bacterial 16S rRNA gene fragments revealed that DGGE profiles of the soil with the addition of the consortium were clustered together and distinct from those of control soil. Sphingobacteria and Proteobacteria were found to be the dominant bacterial groups in the soil according to the sequence analysis of DGGE bands. The results indicate that incubation with a bacterial consortium may be a promising method for bioremediation of PAH-contaminated soils.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in the environment with potential mutagenicity and carcinogenicity. They are generated from natural combustion processes as well as from human activities (Luan et al., 2006). Low-molecular-weight PAHs are acutely toxic, with some having effects on the reproduction and mortality rates of aquatic animals, and most high-molecular-weight (HMW) PAHs (containing four or more benzene rings) are mutagenic and carcinogenic (Boonchan et al., 2000). Most of the pollutant hydrocarbons in the environment are often composed of mixtures of numerous homologous compounds and bound to particulates in soil and sediments, restricting their availability for biodegradation.

Bioremediation is one of the promising technologies to reclaim PAH-contaminated sites due to its relatively low cost and limited impact on the environment (Liebeg and Cutright, 1999). Microbial consortia have been widely used in cleanup of a number of pollutants in laboratory and field bioremediation studies. It is generally thought that microbial consortia are more effective than pure cultures in biodegradation of PAHs (Mueller et al., 1989; Tam et al., 2003). This is possibly because broader enzymatic capacity is achieved (Casellas et al., 1998), and the formation of toxic intermediate metabolites is counteracted by the selection of these dead-end products formed mainly by co-metabolism processes (Vinas et al., 2005a). Bouchez et al. (1999) found that a culture enriched from soil could readily mineralize a mixture of five PAHs but mixed cultures of two or three pure strains possessing the capacity to mineralize each of five PAHs achieved limited degradation of the mixture of five PAHs.

Although there have been some studies on the bioremediation of PAH-contaminated soils with microbial consortia, little is known about changes in microbial community structure during the bioremediation process. A few studies that have been described to date have been performed with soils with single contaminants, spiked soils or petroleum-contaminated soils (Vinas et al., 2005a). The objectives of the present study were to enrich an efficient PAH-degrading bacterial consortium, to investigate the bioremediation

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capability of the consortium in PAH-contaminated soil, and to analyze the changes in the microbial community during the bioremediation process using denaturing gradient gel electrophoresis (DGGE).

2. Materials and methods

2.1. Soil sampling and soil PAH extracts

Soil contaminated by PAHs was collected from the surface layer (0–15 cm) of an industrial area in WuXi, Jiangsu Province, China. The soil with pH 6.4 contains organic matter 19.2 g kg^{-1}, total nitrogen 1.0 g kg^{-1}, total phosphorus 0.5 g kg^{-1}, total potassium 14.2 g kg^{-1}, and CEC 21.5 cmol kg^{-1}. Soil was air-dried, homogenized, passed through a 2-mm sieve, and stored at 4 °C in the dark prior to use.

Ten grams of soil were Soxhlet-extracted with dichloromethane for 24 h, rotary evaporated to about 2 ml, and purified by a silica gel column (8 mm × 220 mm). After evaporation to dryness under N_2 streams, the extract was dissolved with 2 ml of N, N-dimethylformamide (DMF). About 200 ml of soil PAH extracts was obtained in this way.

2.2. The PAH-degrading consortium

A PAH-degrading bacterial consortium was obtained from the PAH-contaminated soil. Enrichment cultures were established in 500-mL screw-cap Erlenmeyer flasks containing 100 mL sterile mineral salts medium (MSM) (Wang et al., 2005) and 5 mL soil PAH extracts. The consortium was maintained by fortnightly transfers into fresh medium (20-fold dilution) for 6 months incubated at 28 °C on a rotary shaker (200 rpm) in the dark. It was revealed by PCR–DGGE analysis that the most abundant populations of the consortium were related closely to *Mesorhizobium* sp., *Alcaligenes* sp. and *Bacillus* sp. The bacterial consortium have the capability of degrading most of the 16 USEPA listed PAHs in sterile mineral salts medium, especially 4- and 5-rings PAHs.

2.3. Soil microcosms

Microcosms were set up to investigate the capability of the consortium to bioremediate the PAH-contaminated soil. The consortium cultures were centrifuged at 4000 rpm for 20 min, washed twice with 100 mM of potassium phosphate buffer (pH 7.0), and suspended in MSM (2.1×10^7 cells ml^{-1}). 1 kg of non-sterile soil was mixed thoroughly with 10% (v/m) (Treatment A) and 20% (v/m) (Treatment B) of the consortium suspension. Sterile medium without inoculation with the consortium suspension served as control. Three replicates of each treatment were set up. Soil water content was adjusted to 60% of water holding capacity. All treatments were incubated at 28 °C in dark. After incubation for 0, 14, 28, and 56 days, soil was sampled for chemical and biological analysis.

2.4. Analysis of PAHs

Extraction and determination of 16 EPA priority PAHs was carried out according to the method of Ping et al. (2007). Analysis was conducted on a Shimadzu Class-VP HPLC system (Shimadzu, Japan), with a fluorescence detector (RF-10AXL). A reversed phase column C18 (VP-ODS 150 × 4.6 mm I. D., particle size 5 μm), using a mobile phase of water and acetonitrile mixture (4:6, v/v) at a constant solvent flow rate of 0.5 ml min^{-1}, was used to separate PAHs. The excitation and emission wavelengths for individual PAHs were set separately (Wu et al., 2008b).

2.5. Microbial analysis

Heterotrophic microbial population counts in soil were determined aerobically on days 0, 14, 28, and 56 using the most probable number (MPN) method as described previously (Taylor, 1962).

Total community DNA was extracted from 0.5 g soil using the FastDNA Spin Kit for Soil (Q-BIOgen, Irvine, CA) according to the manufacturer's instructions. The universal bacterial primers targeting the variable V3 region of the bacterial 16S rRNA gene, F338gc and R518 were used to amplify fragments. The GC-clamp was added to the F338gc primer (Maarit Niemi et al., 2001). The PCR conditions have been described previously (Wu et al., 2008a).

DGGE was performed on a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). PCR products were all separated in a 6% acrylamide gels with a denaturing gradient from 30% to 60% (100% denaturant corresponds to 7 M urea and 40% formamide). Gels were running in 1x TAE buffer at 60 °C and 80 V for 13 h, and stained for 30 min in 1x TAE containing SYBR Green I (Roche, Mannheim, Germany), documented by Gel Doc EQ gel documentation system (Bio-Rad Laboratories, Hercules, CA).

DGGE images were digitized and processed by Gelcompar II package (Applied Maths, Inc., Austin, TX) with default values. After normalization, bands with relative peak area intensities were included in further analysis. The similarity in the profiles of bands was calculated with Gelcompar II to construct a dendrogram using the unweighted pair group method (UPGMA) based on Pearson's similarity coefficient.

Predominant DGGE bands were excised and reamplified as described by Wilms et al. (2006). For purification, a second DGGE was run and bands with identical position to parent bands were excised, amplified again with primer pairs without GC-clamp. Sequencing of PCR products was carried out by Invitrogen (Shanghai, China). DNA sequences were compared to those in the GenBank. DGGE band sequences from this study have been deposited in the NCBI GenBank database under Accession Number EU401879–EU401892. The 16S rDNA sequences were aligned using Clustal X software, and the phylogenetic tree was generated using the neighbor-joining algorithms in Mega 4 software.

2.6. Statistical analysis

All the data obtained were subjected to one-way analysis of variance and post hoc Tukey test using the SPSS Version 13.0 statistical package. The results were tested for significance at the 5% level.

3. Results

3.1. PAH concentrations and composition in contaminated soil

The concentration and composition of PAHs in the contaminated soil and PAH extracts used in this study are shown in Table 1. The total concentration of 10 individual PAHs in the soil was 9362.1 μg kg^{-1} dry soil. Fluoranthene was present at the highest concentration among all the PAHs. The concentration of HMW-PAHs (4- and 5-rings) accounted for 90.6% of all the PAHs.

3.2. Changes in concentration of PAHs in the soil during bioremediation

Concentrations of individual and total PAHs in the soil under different treatments during the 56-day bioremediation are presented in Fig. 1. By the end of the experiment, 7.1% of total PAH removal was detected in the control soil. Significant differences ($P < 0.05$) were observed in treatment A (10% consortium) and

Table 1
Concentration and composition of PAHs in the contaminated soil and removal of individual PAHs under different treatments after 56 days of bioremediation.

PAH	Number of rings	Concentration (μg kg^{-1} soil)	PAH removal (%)		
			Control	Treatment A	Treatment B
Fluorene	3	36.4 ± 8.0	15.8 ± 5.4a	17.0 ± 1.5a	24.3 ± 4.6a
Phenanthrene	3	751.0 ± 108.9	17.4 ± 1.0a	19.7 ± 3.6a	37.3 ± 2.7b
Anthracene	3	88.8 ± 23.2	5.5 ± 1.3a	13.4 ± 2.9b	24.1 ± 4.5c
Fluoranthene	4	2024.6 ± 193.4	2.8 ± 0.7a	20.5 ± 3.2b	34.5 ± 2.4c
Pyrene	4	1785.4 ± 165.0	5.1 ± 0.5a	22.5 ± 2.5b	27.9 ± 3.1b
Benz[*a*]anthracene	4	791.0 ± 88.5	0.7 ± 0.7a	16.4 ± 2.0b	30.6 ± 3.2c
Chrysene	4	994.2 ± 136.7	15.3 ± 4.2a	27.6 ± 3.3b	41.2 ± 3.8c
Benzo[*b*]fluoranthene	5	1223.9 ± 155.4	1.7 ± 0.9a	11.8 ± 1.7b	44.2 ± 5.8c
Benzo[*k*]fluoranthene	5	476.2 ± 57.8	7.0 ± 1.9a	14.3 ± 3.5b	40.7 ± 2.7c
Benzo[*a*]pyrene	5	1190.8 ± 141.8	13.6 ± 1.9a	24.3 ± 2.7b	36.6 ± 3.2c
Total PAHs		9362.1 ± 1078.5	7.1 ± 1.5a	20.2 ± 2.1b	35.8 ± 3.2c

Data are presented as means ± SD. Means in the same row with a letter in common are not significantly different among treatments ($P < 0.05$).

treatment B (20% consortium), which lost 20.2% and 35.8% of total PAHs respectively.

The extents of degradation of individual PAHs are also shown in Table 1. In treatment A the highest PAH removal occurred in chrysene (27.6%), benzo[*a*]pyrene (24.3%) and pyrene (22.5%). In treatment B the highest degree of degradation was found in benzo[*b*]fluoranthene (44.2%), chrysene (41.2%) and benzo[*k*]fluoranthene (40.7%). In treatment B removal of benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and benzo[*a*]pyrene, fluoranthene, and benz[*a*]anthracene were significantly different ($P < 0.05$) from that of control soil and treatment A.

Over the 56 days the degree of degradation of 4-ring PAHs (21.8%) was higher than that of 3- and 5-ring PAHs (18.7% and 17.3%) in treatment A. In treatment B the removal of 5-ring PAHs was the highest (40.5%) and the losses of 3- and 4-ring PAHs were similar to one another (35.2% and 33.2%).

3.3. Enumeration of the heterotrophic microbial population in soil

An MPN method was employed to evaluate the heterotrophic microbial population in the soil during the bioremediation process and the results are shown in Fig. 2. In control soil the size of the heterotrophic population remained constant until day 56. In contrast to the control soil, much higher microbial counts were observed when the consortium suspension was added in treatments A and B at day 0, and the highest microbial population (9.7×10^7 MPN g^{-1} soil) was detected in treatment B. However, the size of soil microbial population soon decreased. After 56 days the microbial population maintained in treatment B was only 1.3×10^7 MPN g^{-1} but this was still higher than that of other two treatments.

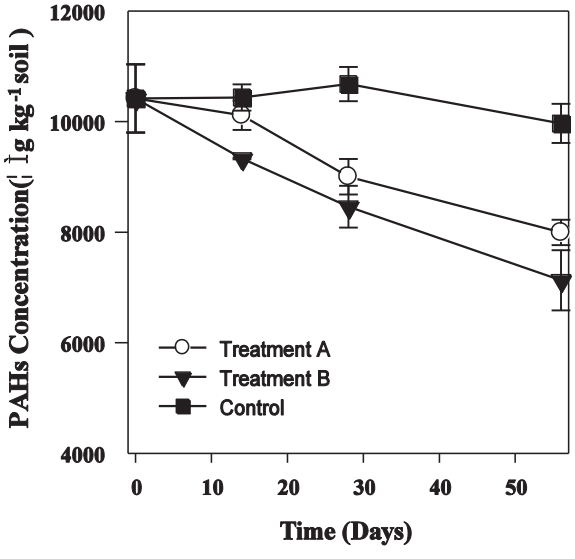

Fig. 1. Concentration of total PAHs in soil under different treatments over the 56-day bioremediation process (μg kg^{-1} soil). Each point represents the average value of triplicate samples.

3.4. Changes in soil bacterial community structure during the bioremediation process

A DGGE analysis of PCR-amplified 16S rRNA gene fragments was conducted to investigate differences in the bacterial populations present in soil with different treatments. Bacterial community profiles elucidated by DGGE of different soils collected at day 0 and day 56 are shown in Fig. 3. Three independent replicates per treatment and per sampling time were analyzed for DNA extraction, and there were no differences between them in DGGE profiles. At day 0, after the addition of the consortium suspension, the bacterial community profiles of the soils exhibited changes in contrast to the control soil. It also can be seen that there were more bands with deeper intensity in the soil sample of treatment B. After

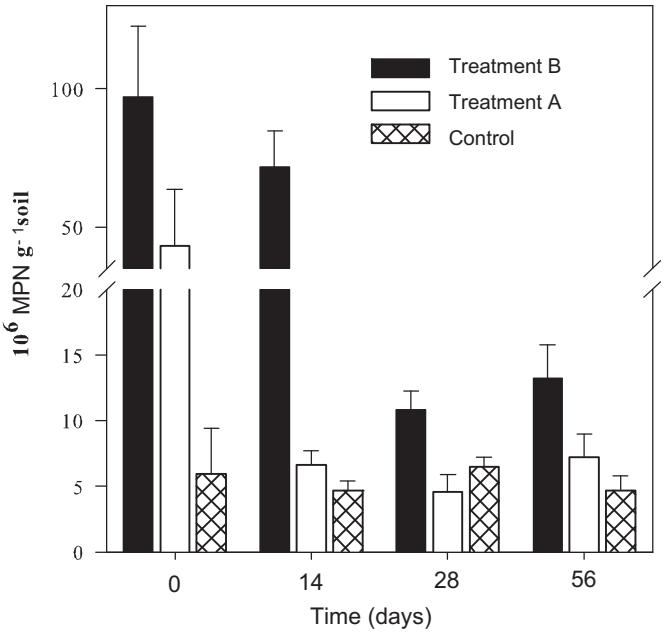

Fig. 2. Heterotrophic microbial populations in the soil of treatment A, treatment B, and control soil at days 0, 14, 28 and 56 over the course of the 56-day bioremediation.

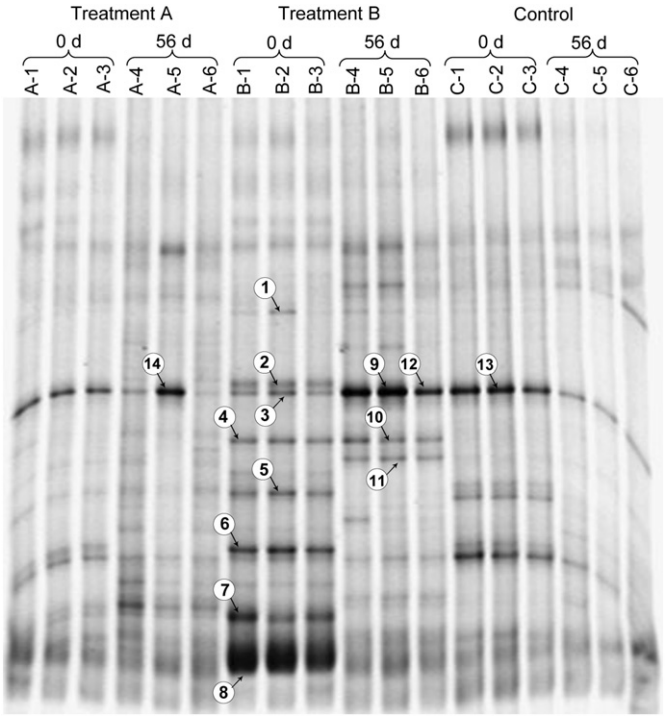

Fig. 3. DGGE profiles of bacterial community composition in soils of treatment A (lane A-1 to A-6), treatment B (lane B-1 to B-6), and control (lane C-1 to C-6) at day 0 and day 56. The bands marked with arrows were excised and sequenced (Table 2).

56 days of incubation, in the lanes of the different treatments, a significant shift in community structure was noted by the presence and absence of some bands.

The dendrogram of the UPGMA analysis of the DGGE patterns for the soil with different treatments is presented in Fig. 4. This shows that the soil samples at different days (days 0 and 56) are clustered into two main groups. Furthermore, the DGGE profiles of treatment A and treatment B were clearly different from the control soil at both day 0 and day 56.

Prominent bands were excised, PCR-amplified, and sequenced (indicated by arrows in Fig. 3). The closest relatives of DGGE bands are shown in Table 2 and most of the sequences exhibited levels of similarity greater than 95%. Bands 3, 9, 12, 13, and 14 appeared at the same positions in all treatments, which was the most abundant band in the soil, were detected as Sphingobacteria. Several of the dominant bands observed in the soil of day 0, such as band 2 (*Pedobacter* sp.), band 5 (uncultured Sphingobacteriales), band 6 (*Bacillus* sp.), band 7 (uncultured Sphingobacteriales), and band 8 (*Paenibacillus* sp.) were not present or present at relatively reduced levels on day 56. On the other hand, band 11 (Uncultured Proteobacterium) was a new dominant band which appeared at day 56 in treatment B.

A phylogenetic tree was constructed to show the relationship of all the partial 16S rDNA sequences representing the respective excised DGGE bands (Fig. 5). The neighbor-joining analysis revealed that most of 14 the bacterial sequences were affiliated with Sphingobacteria (9 sequences, 64.3%), especially uncultured Sphingobacteriales (7 sequences, 50%). The remaining sequences were primarily related to Proteobacteria (3 sequences, 21.4%) and Firmicutes (2 sequences, 14.2%).

4. Discussion

As far as microbial biodegradation is concerned, the most important problem is the method for enrichment or isolation of the

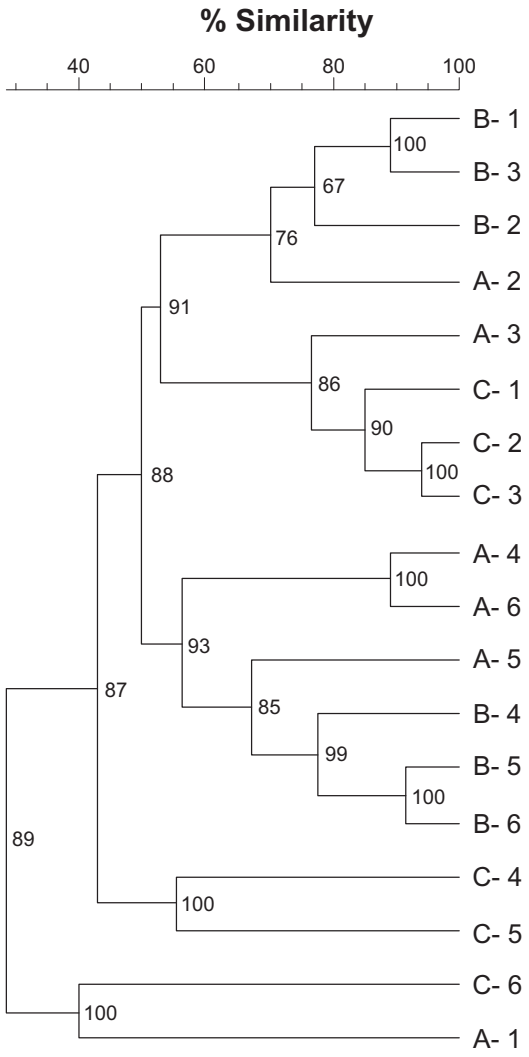

Fig. 4. Cluster analysis of community composition for different soil samples.

degraders. In previous studies pure PAH compounds (Boonchan et al., 2000; Jacques et al., 2007) or creosotes (Mueller et al., 1989; Vinas et al., 2005a) were most commonly used to enrich PAH degraders. In our study a bacterial consortium was enriched from the soil with the PAH extracts as an energy source, which contained 28779.2 μg l^{-1} of total PAHs. The PAH extracts might contain other organic matters besides PAHs, but their composition was more similar to the soil than simple addition of pure PAHs. The consortium cannot utilize DMF (data not shown), so this method is feasible for the enrichment of PAH-degraders.

Microbial consortia have been used in several studies to remediate PAH-contaminated soils. Ruberto et al. (2006) found that a combination of bioaugmentation with a consortium and biostimulation with organic nutrients caused a significant removal (46.6%) of phenanthrene after 56 days of incubation in Antarctic soil. Vinas et al. (2005b) found that in a bioremediation of heavily creosote-contaminated soil for 200 days, PAHs were significantly degraded (83–87%).

Compared to previous studies, our study showed that after bioremediation for 56 days, 20.2% and 35.8% of total PAH removal was detected in the soil with addition of 10% and 20% consortium. Observed losses of PAHs were probably the result of both primary utilization and co-metabolism (Mueller et al., 1989). This indicates that the consortium can survive in non-native soil and compete

Table 2
Sequence analysis of the major DGGE bands appearing in Fig. 3.

Band	Accession no.	Closest organisms in GenBank database (Accession no.)	Phylum or class	Similarity (%)
1	EU401890	*Pedobacter* sp. MSCB-14 (EF103211)	Sphingobacteria	100
2	EU401885	*Pedobacter* sp. hp16 (JN637320)	Sphingobacteria	100
3	EU401886	Uncultured Sphingobacteriales bacterium clone CCF88 (JN541184)	Sphingobacteria	100
4	EU401880	Uncultured proteobacterium clone GASP-WC2W2-B05 (EF075246)	Proteobacteria	96
5	EU401881	Uncultured Sphingobacteriales bacterium clone 366 (FJ645651)	Sphingobacteria	100
6	EU401882	*Bacillus* sp. JC46 (FN430771)	Firmicutes	96
7	EU401884	Uncultured Sphingobacteriales bacterium clone CG77 (JN541173)	Proteobacteria	99
8	EU401883	*Paenibacillus* sp. MP7 (AJ582394)	Firmicutes	93
9	EU401879	Uncultured Sphingobacteriales bacterium clone DMS07 (FJ536877)	Sphingobacteria	99
10	EU401887	Uncultured proteobacterium clone GASP-KC1W1_F10 (EU299390)	Proteobacteria	98
11	EU401888	Uncultured Proteobacterium clone GASP-MA1W2_A08 (EF662734)	Proteobacteria	100
12	EU401891	Uncultured Sphingobacteriales bacterium clone DMS05 (FJ536875)	Sphingobacteria	99
13	EU401889	Uncultured Sphingobacteria bacterium clone BF-48 (HM238164)	Sphingobacteria	100
14	EU401892	Uncultured Sphingobacteriales bacterium clone DMS11 (FJ536881)	Sphingobacteria	100

with indigenous microbial populations. It was also shown that the bioremediation efficiency was in proportion to the biomass of the consortium added to the soil. There was 7.1% of total PAH loss observed in the control soil, which may have been because the soil was non-sterile, and the addition of mineral salts medium stimulated the biodegradation capability of indigenous microorganisms in the soil (Li et al., 2008).

It is generally accepted that the biodegradation of low-molecular-weight PAHs occurs much more rapidly and extensively than that of HMW-PAHs (Nam et al., 2001). However, high degradation of the latter would have been obtained in some conditions. In the bioremediation of an aged PAH-contaminated soil by a microbial consortium, Li et al. (2009) observed that the biodegradation of 5–6 rings PAHs was significantly higher than that of 2–4 rings PAHs. In our study the degradation of 3-ring PAHs was less than that of 4- or 5-ring PAHs, perhaps due to the bacterial consortium being enriched from a soil containing more than 90% of HMW-PAHs, and it had a greater capability to degrade HMW-PAHs.

Bioremediation of PAH-contaminated soil with specific isolates of bacteria or fungi has been monitored in other studies showing opposite results. Some successful examples have shown significantly enhanced degradation of HMW-PAHs (Kotterman et al.,

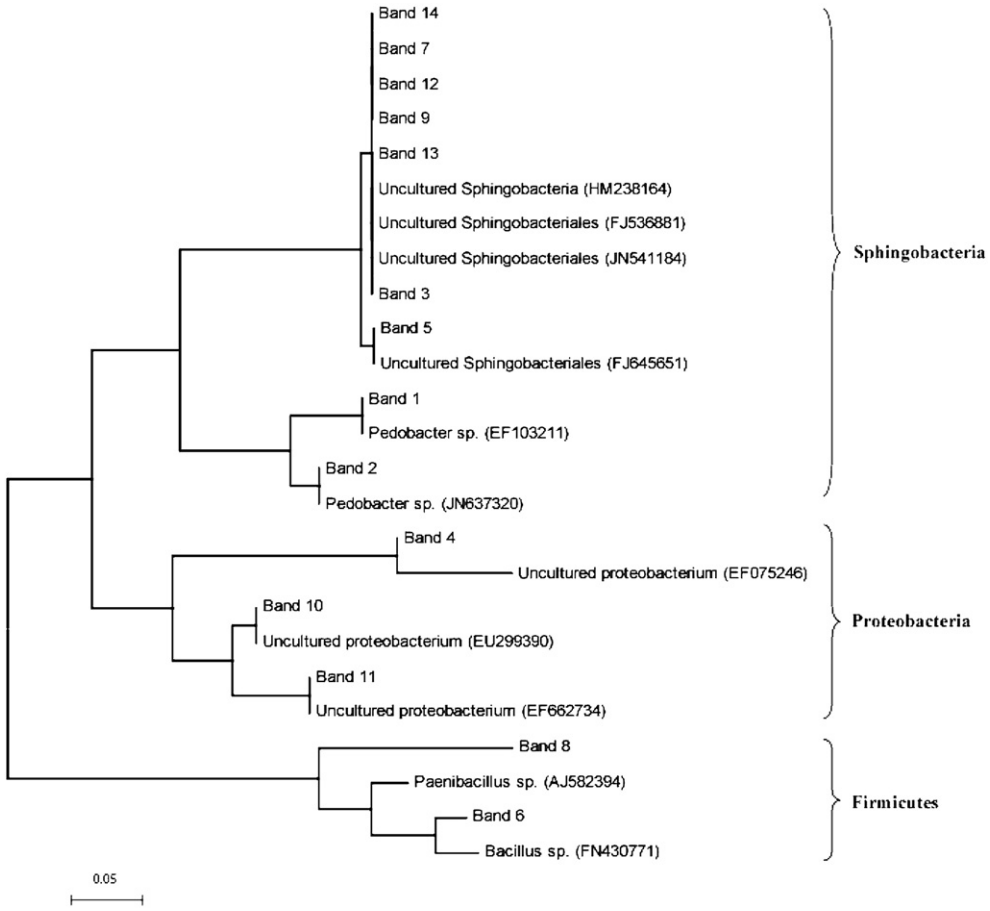

Fig. 5. Phylogenetic tree based on 16S rDNA-V3 sequences representing the respective DGGE bands in Fig. 3. Bootstrap analysis is based on 500 replicates. Scale indicates 5% sequence divergence.

1998; Boonchan et al., 2000), in a similar fashion to in our experiment. These results were especially important for HMW-PAHs and could be promising for bioremediation of PAH-contaminated soils.

Monitoring the microbial community by DGGE analysis provided an effective way of visualizing the microbial changes occurring during the bioremediation process. In the present study the DGGE analysis showed that the incubation of the consortium had an impact on the microbial community structure of the soil. However, at the end of the experiment the DGGE profiles of all treatments were significantly different from that of day 0. Disappearance of some dominant populations (*Bacillus* sp., *Caulobacter* sp., and *Paenibacillus* sp.) and appearance of a new population (*Proteobacterium*) at day 56 indicate that the microbial structure of microcosms had changed greatly throughout the entire experiment.

It can be seen from the DGGE profiles and sequences alignment that most of the dominant bacterial species in the soil were previously reported PAH-degraders. Nine of the 14 sequenced DGGE bands belong to class Sphingobacteria, which are often isolated from contaminated soils as degraders of fluorene, naphthalene, anthracene, phenanthrene, fluoranthene, pyrene, and Benzo [*a*]pyrene (Daane et al., 2001; Shi et al., 2001). Another 3 sequenced bands were related to class Proteobacteria, whose members are known to degrade HMW-PAHs (Rentz et al., 2005; Vandermeer and Daugulis, 2007). In an analysis of the members of a consortium that mineralized HMW-PAHs by DGGE, Lafortune et al. (2009) screened 11 bands and 86 clones, of which 85% belonged to the Proteobacteria group. In a similar study, Vinas et al. (2005b) found that 85.7% of sequenced DGGE bands of a PAH-degrading consortium belonged to Proteobacteria.

We observed that addition of the bacterial consortium suspension significantly increased the microbial populations in soil, especially during the first two weeks, and then declined by the end of the experiment. This result indicated that the microorganisms which released into the environment often have difficulty in establishing themselves in their new ecological niche. This result is in agreement with previous reports (Vinas et al., 2005a; Ho and Banks, 2006). This phenomenon, together with the decrease in DGGE dominant bands, may suggest that although the addition of exogenous microorganisms can affect the microbial number and structure in soil, it cannot remain stable in the long term. Soil chemical, physical, and biological complexity may cause the decline in the inoculated population (Veen et al., 1997; Simarro et al., 2011). Therefore, it is necessary to provide sufficient nutrients and PAH-degrading microorganisms to the soils to improve the bioremediation efficiency of PAH-contaminated soils (Boopathy, 2000; Gogoi et al., 2003).

In conclusion, biodegradation of PAHs, especially HMW-PAHs, has been observed to occur during the bioremediation process, with an associated change in the relative abundances of the organisms constituting the microbial community. This research will be useful in developing an important technique that may lead to improved bioremediation rationales and approaches. This bacterial consortium might be a promising candidate for bioremediation of aged PAH-contaminated soils.

Acknowledgments

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