Isolations and consortia of PAH-degrading bacteria from the rhizosphere of four crops in PAH-contaminated field

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Abstract
Polycyclic aromatic hydrocarbon (PAH)-degrading bacterial consortia were enriched from the rhizosphere of four crops in PAH-contaminated field using phenanthrene, pyrene and benzo[a]pyrene as the sources of carbon and energy. The PAHs concentration in rhizosphere was lower than that in bulk soils, whereas, the extracted DNA amount was greater. Thirteen isolations belonged to five genera of *Firmicutes, Betaproteobacteria*, and *Gammaproteobacteria*. Eleven bands cut from DGGE gel profiles belonged to nine genera of *Firmicutes, Bacteroidetes*, *Alpha proteobacteria, Betaproteobacteria*, and *Gammaproteobacteria*. The bands of DGGE showed that the diversity of PAH-degrading bacteria was greater in rhizosphere. *Stenotrophomonas maltophilia* was a major population in either rhizosphere or bulk soil under maize, soybean and rape, while *Acidovorax avenae* was promoted exclusively in rhizosphere. *Lysinibacillus sphaericus* as a PAH-degrading bacterium was reported for the first time. This study indicated that the rhizosphere of vegetation in contaminated field contains higher diversity of population of PAH-degrading bacteria, and some exhibit the potential for biodegradation or bioremediation in rhizosphere.

Key Words
Bacterial communities, DGGE, smelting factory, PAH-degrading strains, rDNA

Introduction
Soil is an important pool for polycyclic aromatic hydrocarbons (PAHs) as its high partition coefficient between air and soil (Wilcke *et al.,* 1996). PAHs in surrounding posed considerable health risk to human beings because of its persistence and carcinogenicity (Ma *et al*., 2009a). Rhizoremediation is proposed as the most potential approach for PAH remediation in soil (Ma *et al*., 2009b). Soil microflora play vitally important role during rhizoremediation of xenobiotics (Johnsen *et al.,* 2005; Semple *et al.,* 2007). The interaction among microbial degrader, plant and PAHs in soil might be regulated through rhizosphere processes (de Carcer *et al.,* 2007). For investigating the effect of rhizosphere processes on PAH-degrading bacterial community, great efforts have been done in recent years. But many previous studies were implemented using spiked soils (Joner *et al.,* 2001), in which the effect of rhizosphere on PAH-degrading bacterial community is largely different from that in natural contaminated field. In this study, rhizosphere soils were sampled from a PAH contaminated field near a small smelting factory. We attempted to detect bacteria related to PAH degradation and evaluate the role of rhizosphere in PAHs remediation in soil.

Methods
Soil sampling
The soil samples were collected at a crop garden in Wuxi of Jiangsu province, China (31.6041N, 120.4759E), where suffered from the pollution of PAHs due to the coal combusion from a surrounding smelting factory. Various plots with rape, soybean, maize and oat were chosen separately. Rhizosphere samples were collected by removing the soil adhering to the plant roots after gently shaking. The falling soils collected by shaking were treated as bulk soils. Soils were lyophilized and passed through a 2-mm sieve. The extraction and cleanup scheme was based on US EPA 3550C and 3630C, respectively. The quantification method was according to the US EPA 8270D.

Enrichment of PAHs-degrading consortia and isolation of PAHs-degraders
About 5 g soils were added to 50 ml of mineral medium, and supplied with 0.5 ml of PAH mixture solution. The PAH mixture of phenanthrene, pyrene and benzo[a]pyrene was dissolved in acetone and filtered through a 0.22 μm pore film and added at final concentrations of 0.2, 0.1 and 0.02 g L^{-1} respectively. The solvent was allowed to evaporate on a rotary shaker before adding the sample or inoculation. Enrichment was conducted at 25°C and 120 rpm and kept from light for about 1 month. The enrichment cultures were transferred with 1 ml inoculums to 50 ml fresh medium with PAH spiked, and repeated two times every 2 weeks. About 10^{-4}

dilutions were spread on M8 agar plates and incubated at 25°C. Colonies that were different in morphology from each consortium were streaked onto fresh LB plates to obtain pure culture. Finally, 13 PAHs degrading consortia were obtained.

PCR amplification of the 16S rDNA genes and sequencing
Genomic DNA of isolated bacteria was prepared with the bacteria genome DNA extraction kit (Generay, Shanghai). The 16S rDNA genes were amplified from genomic DNA using the universal primer set 27f and 1502r. The thermal cycling parameters were a 5 min hot start at 94°C, followed 32 cycles of denaturation for 30s at 94°C, annealing at 53.5°C for 30s, and extension for 2 min at 72°C, with a final extension of 20 min at 72°C. The PCR products were cloned into pEASY-T1 simple clone vector (TransGen Biotech, Beijng) and sequenced using M13 prime by Shanghai Invitrogen.

DGGE analysis of the structure of the bacterial communities
Total DNA in soil and enrichment consortia were extracted with FastDNA SPIN Kit for soil (Qbiogene) and genome DNA extraction kit (Generay, Shanghai), respectively. PCR was performed with the total DNA of the consortia or the isolates as templates. Primers F338GC and R518 were used to amplify the variable V3 region of bacterial 16S rDNA genes for DGGE analysis (Muyzer *et al.,* 1993). The PCR procedure was as follows: an initial cycle of 5 min at 95°C, followed by 20 cycles of 45 s at 94°C, 1 min at 63.5°C, with a touchdown of 0.5°C per cycle and 45 s at 72°C, then followed by 10 cycles of 45 s at 94°C, 45 s min at 53.5°C and 45 s at 72°C, with a final extension of 10 min at 72°C. Electrophoresis was performed at 60°C in an 8% (w/v) polyacrylamide gel with a denaturant gradient ranging from 40% to 60% for 15 min at 30 V, then 280 min 165V in 1× Tris-acetate-EDTA buffer with Dcode Universal Mutation Detection system (Bio-Rad Labo-ratories, Hercules, CA). After electrophoresis, the gel was stained by SYBR Green I and the pictures were captured by an Tanon Imaging System and analyzed by ImageJ 1.42q (http://rsb.info.nih.gov/ij). The cluster analysis was carried out with R. Bands of interest on the DGGE gel were excised and cloned into the pEASY-T1 simple clone vector (TransGen Biotech, Beijng) and sequenced using M13 prime by Shanghai Invitrogen.

Phylogenetic analysis
The 16S rDNA and 16S rDNA V3 region sequences were aligned with published sequences from the GenBank database using the ClustialX program (Thompson *et al.,* 1997). Phylogenetic trees were constructed by the neighbor-joining method using the ape package of R software. Nearly full length 16S rDNA sequences of most phylogenetically related strains were selected from GeneBank database as reference strains.

Results
PAHs and DNA concentrations in rhizosphere
The sum of PAH concentrations in detected bulk soils ranged from 16 μg g^{-1} to 33 μg g^{-1}. Under all four crops, total PAHs concentration in rhizosphere was significantly lower than in bulk soils ($p<0.05$). Particularly under soybean, total PAHs concentration in rhizosphere was 40% less than in bulk soils (Figure 1. a). The extracted DNA amount from rhizosphere was greater than that from bulk soils (Figure 1. b).

Isolation and identification of PAH-degrading bacteria
Bacteria in enrichment consortia were isolated with M8 plate medium, purified with LB plate medium, and identified by sequencing of 16S rDNA (about 1500bp). Finally 13 strains of different 16S rDNA sequences were obtained and were identified belonging to five genera of the *Firmicutes* and *Proteobacteria* (including *Beta-*, and *Gammaproteobacteria*). All seven strains closely related to *Bacillus* genera (99%) have been proven to be PAH-degraders. Three *Proteobacteria* strains, which were most closely related to *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans,* and *Serratia marcescens* respectively, have all been reported for PAHs degradation. However, two *Lysinibacillus* strains were the first time being testified as PAH degrader in the present study. The Genbank accession numbers of isolations S1 to S11 were from GQ889238 to GQ889248.

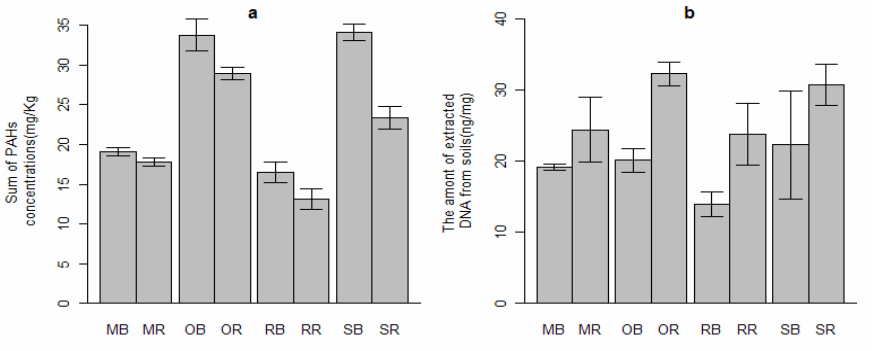

Figure 1. The sum of PAHs concentrations (a) and the extracted DNA amounts (b) in the tested rhizosphere and bulks soils. MB, OB, RB and SB represent the bulk soil in gardens of maize, oat, rape and soybean respectively; MR, OR, RR and SR represent the rhizosphere of maize, oat, rape and soybean respectively.

Table 1. Sequence analysis of bands retrieved from the 16S rDNA DGGE profiles.

Strains (Genbank Accession No.)	Closest type strains in Genbank data base (Accession No.)	Length of fragment (bp)	Similarity (%)
B1(GQ 889238)	*Bacillus subtilis* FQ06 (GQ360038.1)	197	100
B2(GQ 889239)	*Caulobacter sp.* 3-3 (FJ605177.1)	172	100
B4(GQ 889240)	*Bacillus pumilus* NAPCC-1 (FJ458437.1)	195	98
B5(GQ 889241)	*Bacillus sp.* CAt2NG (GQ272359.1)	198	100
B6(GQ 889242)	*Stenotrophomonas maltophilia* (GQ287630.1)	197	100
B7(GQ 889243)	*Erythromicrobium ramosum* (GQ284449.1)	172	98
B8(GQ 889244)	*Acidovorax avenae* (AY512827.1)	197	98
B9(GQ 889245)	*Labrys sp.* LLQ-6 (FJ549002.1)	172	100
B10(GQ 889246)	*Rhizobium sp*. D255c (AB480418.1)	172	100
B11(GQ 889247)	*Burkholderia sp*. lxb-13 (GQ249223.1)	197	100
B12(GQ 889248)	*Cytophaga sp.* SSL03 (EU395843.1)	192	100

DGGE analysis of the bacterial structure

The diversity of enriched PAH-degrading bacterial consortia, presented as the bands of DGGE gel profile, was sharply lower than that of bacterial community diversity in bulk soil. There were dozens of bands in each lane of the DGGE gel profile for bacterial community both in rhizosphere and bulk soils. About ten bands were, however, observed in each lanes of the DGGE gel profile of PAH-degrading bacterial consortia. The bacterial community diversity was different between rhizosphere and bulk soils, except under rape. Among the bands, *Stenotrophomonas maltophilia* (B6) was stronger than other bands in all lanes. *Acidovorax avenae* (B8) tended to be stronger in rhizosphere under all crops (Table 1). According to the results of cluster analysis, the discrepancies of PAH-degrading bacterial community diversity among crop speices were obvious, and were much less than those between rhizosphere and bulk soils.

Bacterial identification by band sequencing

All strong bands in DGGE gel profile were subjected to DNA sequencing. Eleven sequenced bands covered nine genera of *Firmicutes, Bacteroidetes*, and *Proteobacteria* (including *Alpha-*, *Beta-*, and *Gammaproteobacteria*). The Genbank accession numbers of sequenced bands were from GQ889249 to GQ889261. The isolated strains did not match the strong bands in DGGE gel profile, except that both B1 and S1 were identified as *Bacillus pumilus* with similarity of 100% and 99%, respectively. The species in PAH-degrading enriched consortia were much broader than that of the isolated strains. Among the bands in consortia, B1, B4 and B5, belonging to *Firmicutes,* were closely related to *Bacillus subtilis* FQ06 (100%), *Bacillus pumilus* NAPCC-1 (98%), and *Bacillus cereus* HB59 (100%), respectively. B2, B7, B9 and B10, four genera of *Alphaproteobacteria,* were closely related to *Caulobacter sp.*3-3 (100%), *Erythromicrobium ramosum* THWCS10 (98%), *Labrys sp.* LLQ-6 (100%), and *Rhizobium sp*. D255c (100%), respectively. Two *Betaproteobacteria* bands*,* B8 and B11, were closely related to *Acidovorax avenae* and *Burkholderia sp. lxb-13* with similarities of 98% and 100% respectively. Band B6 was closely related to *Stenotrophomonas maltophilia* of *Gammaproteobacteria,* and band B13 was closely related to *Cytophaga sp.* SSL03 of *Bacteroidetes.* Most of corresponding species for each band have been widely reported to be PAH degrader or be isolated from PAHs polluted environment. With the sequences of 16S rDNA of all isolated strains and DGGE bands, a rooted phylogenetic tree was constructed. The biggest group was *Firmicutes,*

which was composed of ten strains and three bands, and included ten *Bacillus* and three *Lysinibacillus sphaericus*. The second biggest group was *Alphaproteobacteria,* which was composed of four bands as *Caulobacter sp., Erythromicrobium sp.*, *Labrys sp.*, and *Rhizobium sp*. respectively. *Betaproteobacteria* included two bands and one strain, *Gammaproteobacteria* group included one band and two strains, and *Bacteroidetes* group only had one band. Expect *Lysinibacillus* strains, all other bacteria species have been reported for PAH degrading or resisting capacity.

Conclusion

This study indicated that the rhizosphere of vegetation in contaminated field contains higher diversity of population of PAH-degrading bacteria. And some of the identified PAH-degrading bacteria have the potential for PAHs bioremediation in rhizosphere, among which two *Lysinibacillus* strains were reported as PAH degrader for the first time in the present study.

Acknowledgments

This work was jointly supported by the National High Technology Research and Development Program of China (863 Program, 2007AA061101), the National Natural Science Foundation of China (20977077, 40971136, 40701075), and the Key Scientific and Technological Program of Zhejiang Province (2008C13024-3).

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