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Screening and degrading characteristics and community structure of a high molecular weight polycyclic aromatic hydrocarbon-degrading bacterial consortium from contaminated soil

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Abstract

Inoculation with efficient microbes had been proved to be the most important way for the bioremediation of polluted environments. For the treatment of abandoned site of Beijing Coking Chemical Plant contaminated with high level of high-molecular-weight polycyclic aromatic hydrocarbons (HMW-PAHs), a bacterial consortium capable of degrading HMW-PAHs, designated 1-18-1, was enriched and screened from HMW-PAHs contaminated soil. Its degrading ability was analyzed by high performance liquid chromatography (HPLC), and the community structure was investigated by construction and analyses of the 16S rRNA gene clone libraries (A, B and F) at different transfers. The results indicated that 1-18-1 was able to utilize pyrene, fluoranthene and benzo[a]pyrene as sole carbon and energy source for growth. The degradation rate of pyrene and fluoranthene reached 82.8% and 96.2% after incubation for 8 days at 30°C, respectively; while the degradation rate of benzo[a]pyrene was only 65.1% after incubation for 28 days at 30°C. Totally, 108, 100 and 100 valid clones were randomly selected and sequenced from the libraries A, B, and F. Phylogenetic analyses showed that all the clones could be divided into 5 groups, Bacteroidetes, α-Proteobacteria, Actinobacteria, β-Proteobacteria and γ-Proteobacteria. Sequence similarity analyses showed total 39 operational taxonomic units (OTUs) in the libraries. The predominant bacterial groups were α-Proteobacteria (19 OTUs, 48.7%), γ-Proteobacteria (9 OTUs, 23.1%) and β-Proteobacteria (8 OTUs, 20.5%). During the transfer process, the proportions of α-Proteobacteria and β-Proteobacteria increased greatly (from 47% to 93%), while γ-Proteobacteria decreased from 32% (library A) to 6% (library F); and Bacteroidetes group disappeared in libraries B and F.

Key words: high-molecular-weight-PAHs; bacterial consortium; bacterial community structure; 16S rRNA gene library
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Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a large and diverse class of organic compounds. They are generally described as molecules which consist of three or more fused aromatic rings in various structural configurations (Kanaly and Harayama, 2000). Low-molecular-weight (LMW) PAHs (containing less than four rings) are acutely toxic, with some having effects on the reproduction and mortality rates of aquatic animals. High-molecular-weight (HMW) PAHs (containing four or more rings) such as benzanthracene and benzo[a]pyrene are mutagenic and carcinogenic (Dean-Ross et al., 2002; Samanta et al., 2002). Due to their hydrophobic nature, most PAHs in aquatic and terrestrial ecosystems bind to particulates in soil and sediments, rendering them less available for biological uptake, and they also bioaccumulate in food chains (Boonchan et al., 2000). Consequently, the US Environmental Protection Agency (EPA) identified 16 PAHs as priority pollutants; significant effort is being made to develop strategies for removing these pollutants from the environments (Vandermeer and Daugulis, 2007). Nevertheless, many physical, chemical and biological methods for decontamination have been applied and among which microbial biodegradation is the most promising method (Wilson and Jones, 1993) and the bioremediation with inoculation of efficient microbes has been proved to be successful, especially in the treatment of petroleum pollutions (Andreoni et al., 2004; Ringelberg et al., 2001; Siciliano et al., 2003; Zucchi et al., 2003).

Microorganisms play important roles in the degradation of PAHs in terrestrial and aquatic ecosystems. In recent years, many microbes capable of degrading LMW-PAHs have been reported, such as the members of genera *Pseudomonas*, *Mycobacterium*, *Bacillus* (Kanaly and Harayama, 2000; Luan et al., 2006; Kanaly et al., 2000; Warshawsky et al., 2007; Moody et al., 2004). The ability of bacteria to utilize PAHs as growth substrates has

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been documented extensively over the past few decades (Dean-Ross et al., 2002; Smith, 1990; Schneider et al., 1996). These studies share a common approach, to isolate bacteria from the environment and use the isolated strains as pure cultures to establish the metabolic pathways by which the bacterial strains can degrade PAHs. Such studies do not reflect the true complexity of PAHs degradation in natural environments where the bacteria are present in communities. Furthermore, contaminated environments commonly contain complex hydrocarbon mixtures, including LMW- and HMW-PAHs. It is not expected that a single isolate would degrade all these PAHs in a contaminated site. Actually, this can be better achieved by a consortium composed of different microorganisms, including some that can degrade a range of PAHs, and others capable of using partially degraded metabolites that could be toxic or inhibitory to some of the primary degraders (Sugiura et al., 1997; Casellas et al., 1998; Lafortune et al., 2009). In this case, to inoculate a consortium might be more efficient than just to inoculate a single pure culture in bioremediation of HMW-PAHs polluted sites. A few studies (Abalos et al., 2004; Boonchan et al., 2000; Luan et al., 2006) have focused on the degradation of HMW-PAHs by microbial consortium, and on the bacterial community structure associated with bioremediation of different environmental pollutants (Kaplan and Kitts, 2004; MacNaughton et al., 1999; Nogales et al., 2001). Some literatures described the specific phylotypes of bacteria relating both to inorganic nutrient added into the soil and to different phases of PAH degradation in nonspiked PAH-contaminated soil, by culture-independent analysis of 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE) and also by most probable number (MPN) method for determination of heterotrophs and PAH degraders in the microbial consortium (Viñas et al., 2005a; Lafortune et al., 2009). However, few were about the community structure and degrading properties of consortium from high level HMW-PAHs contaminated soil.

In this article, a HMW-PAHs-degrading bacterial consortium was enriched and screened from contaminated soil. Its degrading properties and community structure were investigated by HPLC and construction of 16S rRNA gene libraries at different transfers, in order to confirm the degrading stability and evaluate its application potential for *in situ* bioremediation of polluted soils.

1 Materials and methods

1.1 Chemicals

Pyrene (PYR), fluoranthene (FLU), chrysene (CHR), Indeno[1,2,3-cd]pyrene (IPY), benzo[b]fluoranthene (BbF), benzo[j]fluoranthene (BjF), 1,2-benzanthracene (BaT), dibenz[a,h]anthracene (DBA) and benzo[a]pyrene (BaP) were obtained from J&K Chemical (USA). Structures of some representative HMW-PAHs are shown in Fig. 1.

1.2 Soil sample

A soil sample was obtained from the abandoned site of Beijing Coking Chemical Plant, 10 cm beneath of the surface. HMW-PAHs levels in the soil sample were determined (mg/kg): pyrene (226 ± 21); fluoranthene (257 ± 26); chrysene (175 ± 16); benzo[a]anthracene (120 ± 9); benzo[b,k]fluoranthene (393 ± 31); benzo[a]pyrene (172 ± 8); dibenz[a,h]anthracene (45.7 ± 5).

1.3 Medium and culture conditions

BSM (basal salts medium) contained (g/L): $Na_2HPO_4{\cdot}12H_2O$, 2.0; KH_2PO_4, 1.0; $(NH_4)_2SO_4$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.1; and 5 mL trace elements solution; 1000 mL H_2O; pH 7.2–7.5. Trace elements solution (g/L): MoO_3, 4; $ZnSO_4{\cdot}5H_2O$, 28; $CuSO_4{\cdot}5H_2O$, 2; H_3BO_3, 4; $MnSO_4{\cdot}5H_2O$, 4; $CoCl_2{\cdot}6H_2O$, 4. HMW-PAH dichloromethane (DCM) (Beijing Chemical Works, China) stock solutions (10.0 g/L) were sterile by filtration (0.22 μm filtration membrane, Whatman, British). A portion of individual HMW-PAH DCM stock solution was added into an autoclaved bottle. After the DCM was evaporated, then autoclaved BSM medium was transferred into it to a final concentration of 100 mg/L for four-ring compounds, or 50 mg/L for five-ring compounds.

Unless otherwise stated, cultures were incubated on a

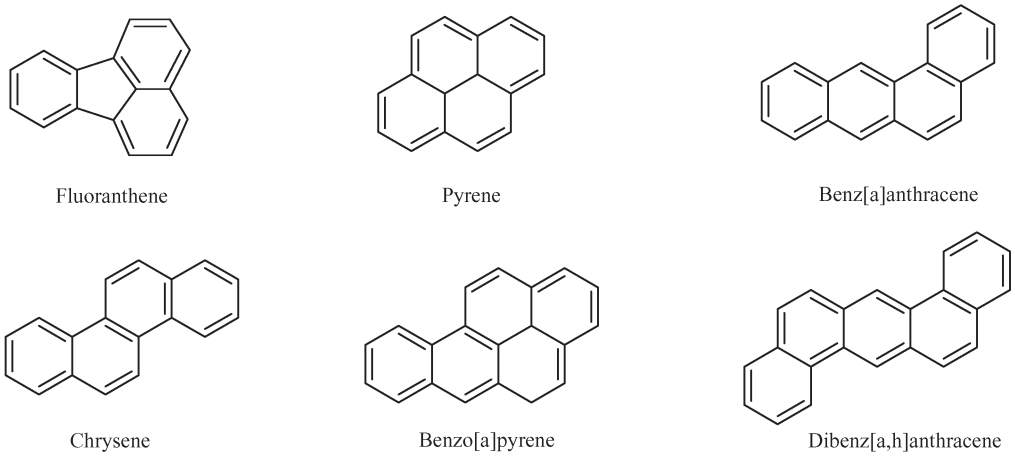

Fig. 1 Structures of some representative HMW-PAHs.

shaker at 160 r/min and 30°C, and all plates were incubated at 30°C.

1.4 Enrichment of the soil sample

Ten gram of soil sample was suspended in 100 mL of (BSM+PYR) on a shaker at 160 r/min overnight. Then this suspension was settled down for 30 min, and 10 mL supernatant was inoculated into a flask bottle with 90 mL BSM containing 100 mg/L PYR as sole carbon source. This inoculated bottle was incubated on a shaker at 160 r/min and 30°C for 7 days. Then, 10 mL enrichment was transferred to a fresh bottle and continued incubation for another 7 days. This procedure was repeated for five times in the PYR-containing medium.

1.5 Degradation of HMW-PAHs by bacterial consortium

The bacterial consortium was tested for its ability to grow on, and to degrade, a number of different HMW-PAHs as sole carbon source in triplicates, respectively. Non-inoculated bottles were set as abiotic controls. Bottles were incubated on a shaker at 160 r/min and 30°C and samples were taken for analysis after 0, 2, 4, 6, 8 days or 0, 7, 14, 21, 28 days. In addition, its degrading ability was also detected after several transfers (first, third and fifth) to confirm the function stability.

1.6 Analytical procedures

Bacterial growth was determined by measuring the turbidity at 600 nm (OD_{600}) with a 7200 spectrophotometer (UNICO, Shanghai Optical Company, China), with the abiotic set containing HMW-PAH as control. The growth of consortium was calculated as Eq. (1):

$$OD_{600\,(1\text{-}18\text{-}1)} = OD_{600} - OD_{600(HMW\text{-}PAH)} \quad (1)$$

where, $OD_{600(1\text{-}18\text{-}1)}$ represents the growth of consortium 1-18-1, OD_{600} represents the turbidity value measured by spectrophotometer and $OD_{600(HMW\text{-}PAH)}$ represents the turbidity value of the remaining HMW-PAH in samples.

HMW-PAH was extracted with DCM. HPLC analysis of DCM extracts and HMW-PAH standards was performed using a liquid chromatograph system (1200 series, Agilent, USA), with an Agilent reverse C18 column (4.6 mm × 250 mm). The elute (0.7 mL/min) was a methanol-water gradient system as described by Boonchan et al. (2000). HMW-PAHs were detected at 254 nm. However, for the analysis of fluoranthene, the effluent gradient was change to: 0 min, 25:75; 0–30 min, ramp to 10:90; 30–50 min, isocratic at 10:90. The degradation of HMW-PAH was calculated as Eq. (2):

$$R_d = (C_0 - C_n)/C_0 \times 100\% \quad (2)$$

where, R_d (%) represents degradation rate, C_0 (mg/L) represents the initial concentration of HMW-PAH and C_n (mg/L) represents the remained concentration of HMW-PAH after incubation for n days.

1.7 Construction and analysis of 16S rRNA gene clone library

The bacterial diversity and the changes occurring in community structure were investigated by construction and analyses of the 16S rRNA gene clone libraries at different transfers (first, third and fifth transfer of liquid culture enrichments).

The genomic DNA of bacterial consortium 1-18-1 was extracted according to Martin-Laurent et al. (2001) using a bead-beater. DNA crude extract was purified by silica gel membrane Genomic DNA Extraction Kit (SBS Genetech, China). 16S rRNA gene was amplified by PCR using universal bacterial primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-ACGGCTACCTTGTTACGACT-3′). PCR amplification was performed in a 50 μL reaction volume with 2 μL template DNA, 1 μL of each primer, 5 μL 10× Taq Buffer, 4 μL dNTP mixture (2.5 mmol/L each), 0.5 μL Taq DNA polymerase (2.5 U/μL) and 36.5 μL ddH_2O. The amplification was performed at 95°C for 5 min, then 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, and finally an extension period of 10 min at 72°C. The PCR-amplified DNA products were purified by gel midi purification kit (TIAN, China) and then cloned into a PBS-T II vector (Takara, Japan) and transformed into *Escherichia coli* TOP10 according to standard procedures. Blue-white screening method was utilized for the selection of positive clones. The positive clones were then sequenced by TSINGKE (China), and the false-positive clones were ruled out. Sequences were compared with 16S rRNA gene sequences available in the NCBI database (http://www.ncbi.nlm.nih.gov/) by BLAST search. Multiple sequence alignments were performed using Clustal_X program. Unalignable and ambiguous bases were manually omitted. The phylogenetic tree was constructed from the evolutionary distance matrix calculated using the neighbour-joining method in the MEGA program (version 3.1, Kumar et al., 2004). Clones with more than 97% sequence similarity were considered as belonging to the same OUT according to Dłez et al. (2001). The library's coverage was calculated by Eq. (3):

$$C = (1–(n/N)) \times 100\% \quad (3)$$

where, C (%) represents coverage, N represents the total number of positive clones and n represents the number of OUT (Buckley et al., 1998).

2 Results

2.1 Enrichment of liquid cultures

Bacterial consortium 1-18-1 was obtained from soil from a contaminated site in Coking Plant when the liquid cultures were inoculated with the soil and enriched with PYR as sole source of carbon and energy in BSM after five serial transfers. HMW-PAHs utilization in the enriched cultures was evidenced by a visual decrease in the amount of PAH crystals, colour change in the medium and growth increase in bacterial biomass.

2.2 Degradation of HMW-PAHs by bacterial consortium 1-18-1

The PYR-enriched bacterial consortium 1-18-1 was able to utilize PYR, FLU and BaP as sole carbon and energy source for growth and was able to degrade these substrates efficiently as revealed by HPLC. For the first transfer of consortium 1-18-1, the degradation rate of PYR and FLU reached 82.8% and 96.2% after incubation for 8 days, respectively; while the degradation rate of BaP reached 65.1% after incubation for 28 days (Table 1). In addition, consortium 1-18-1 exhibited efficient degrading ability after several transfers, as shown in Table 1. More than 80% of PYR (100 mg/L) was degraded by the third and fifth generations after incubation for 8 and 28 days, respectively. However, consortium 1-18-1 was weak in degrading chrysene, indeno[1,2,3-cd]pyrene, benzo[b]fluoranthene, benzo[j]fluoranthene, 1,2-benzanthracene and dibenz[a,h]anthracene (data not shown).

The degradation of HMW-PAHs by consortium 1-18-1 was accompanied with the growth of the consortium as shown in Fig. 2. The degradation of the substrates and the growth of the consortium were synchronous. Results (Fig. 2a) showed that the growth was in lag phase from day 0–2, then log phase from day 2–4, steady phase from day 4–8. The growth could reach maximum ca. OD_{600} 0.14 after incubation for 8 days, in the degradation of PYR, and the degradation rate of PYR gradually increased from 16.8% on day 2, to 64.1% on day 4 and 82.8% on day 8. Similar growth and degradation curves were also observed in the degradation of FLU with a maximal growth ca. OD_{600} 0.25 and degradation rate could reach 96.2% after incubation for 8 days (Fig. 2b). However, the degradation of BaP was much slow, the growth was in lag phase from day 0–14, then coming to a log phase from day 15–28 with a maximal growth ca. OD_{600} 0.30. The degradation rate of BaP could reach 65.1% after incubation for 28 days (Fig. 2c).

The degradation rates by consortium 1-18-1 were 10.4, 12.0 and 2.3 mg/(L·day) for PYR, FLU and BaP, respectively; higher than those of the bacterial consortium reported by Lafortune et al. (2009) (4.7 and 0.3 mg/(L·day) for PYR and BaP, respectively), and the bacterial consortium reported by Mueller et al. (1989) (10.3 and 7.3 mg/(L·day) for PYR and FLU, respectively). These data were also much higher than those of pure cultures, such as *Porphyrobacter* sp. B51 (4.0 and 1.2 mg/(L·day) for PYR and BaP, respectively, Gauthier et al., 2003) and a strain of *Burkholderia cepacia* (0.88, 0.85 and 0.40 mg/(L·day) for PYR, FLU and BaP, respectively, Juhasz et al., 1997).

Table 1 Degradation of HMW-PAHs by bacterial consortium 1-18-1*

PAH	Transfers of the consortium	Degradation rate (%)	Time (day)
Pyrene	First	82.8	8
	Third	80.5	8
	Fifth	83.3	28
Fluoranthene	First	96.2	8
Benzo[a]pyrene	First	65.1	28

* Values are the average of triplicates.

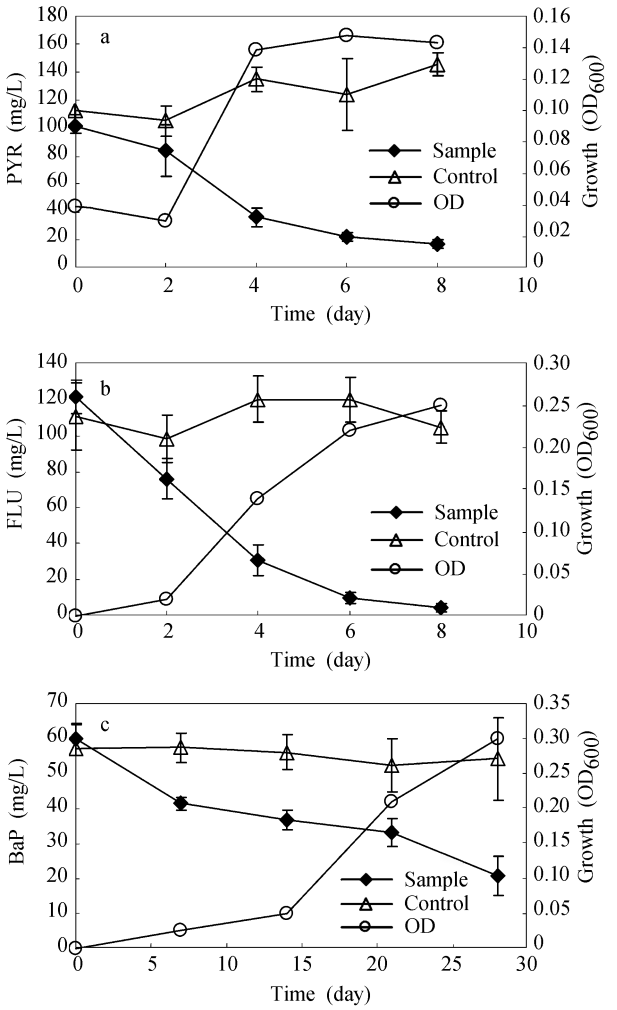

Fig. 2 Growth of consortium 1-18-1 on and the degradation of PYR (a), FLU (b), and BaP (c).

2.3 16S rRNA gene clone library

Three 16S rRNA gene clone libraries were constructed from different generations (first, third and fifth) of the consortium 1-18-1, and were designated libraries A, B and F, respectively. A total of 108, 100 and 100 valid clones were randomly selected from the libraries A, B, and F and sequenced; their coverage was 84.3%, 85% and 80.8%, respectively.

The results of the sequence similarity analysis of the clones from the 3 libraries are summarized in Table 2. A total of 39 OTUs were found in these libraries, indicating a very abundant bacterial diversity in the consortium 1-18-1. Among them, OTUs 5 and 20 presented in all libraries; OTUs 23 and 33 presented in both libraries A and B; OTUs 1, 2, 3, 21, 22 and 29 presented in libraries B and F; while other OTUs just presented in one library. 19 OTUs (48.7% of the total) belonged to α-Proteobacteria, 8 OTUs (20.5%) belonged to β-Proteobacteria, 9 OTUs (23.1%) belonged to γ-Proteobacteria, 2 OTUs belonged to Actinobacteria, and only one OTU belonged to Bacteroidetes.

Table 2 also shows that of all 108 clones in library A could be grouped into 17 OTUs. Predominant OTUs were OTU 39 (clone A1), OUT 28 (clone A4), OUT 20 (clone A9), OUT 23 (clone A25) and OUT 5 (clone

Table 2 Sequence similarity analysis of the clones from 16S rRNA gene libraries A, B and F

OTU	Library	Presentative clone	Number/Percentage of clone	Similarity (%)	The nearest phylogenetic neighbor (accession number)	Phylogenetic group
1	A	–	0	–	Uncultured Caulobacteraceae	α-Proteobacteria
	B	B2	8 (8.0%)	97	bacterium clone GC1m-1-59	
	F	F96	10 (10.1%)	98	(EU641273)	
2	A	–	0	–	*Rhizobium* sp. SL-1	α-Proteobacteria
	B	B23	13 (13.0%)	100	(EU556969)	
	F	F29	7 (7.1%)	99		
3	A	–	0	–	*Sphingosinicella* sp.	α-Proteobacteria
	B	B25	13 (13.0%)	100	OC5S (AB429069)	
	F	F17	3 (3.0%)	99		
4	A	A28	1 (1.0%)	99	Uncultured	α-Proteobacteria
	B	–	0	–	*Brevundimonas* sp.	
	F	–	0	–	(FJ192619)	
5	A	A43	12 (11.1%)		*Mesorhizobium* sp. W5	α-Proteobacteria
	B	B80	3 (3.0%)	98	(AY429705)	
	F	F25	2 (2.0%)			
6	A	–	–	–	Uncultured bacterium	α-Proteobacteria
	B	B108	1 (1.0%)	100	clone nbw779f05c1	
	F	–	–	–	(GQ009805)	
7	A	A29	1 (1.0%)	93	Uncultured Sphingomonadaceae	α-Proteobacteria
	B	–	0	–	bacterium clone (EU642103)	
	F	–	0	–		
8	A	A30	1 (1.0%)	99	*Hyphomicrobium* sp. LAT3	α-Proteobacteria
	B	–	0	–	(AY934489)	
	F	–	0	–		
9	A	–	0	–	Uncultured *Sphingomonas* sp.	α-Proteobacteria
	B	–	0	–	clone Plot4-G09 (EU449628)	
	F	F61	1 (1.0%)	98		
10	A	–	0	–	Uncultured	α-Proteobacteria
	B	–	0	–	α-Proteobacterium	
	F	F100	2 (2.0%)	99	(AM936473)	
11	A	–	0	–	Uncultured soil bacterium	α-Proteobacteria
	B	–	0	–	clone MK41 (EF540431)	
	F	F16	1 (1.0%)	95		
12	A	–	0	–	*Nordella* sp. P-63	α-Proteobacteria
	B	–	0	–	(AM411927)	
	F	F15	2 (2.0%)	99		
13	A	–	0	–	Uncultured α-Proteobacteria	α-Proteobacteria
	B	–	0	–	bacterium	
	F	F57	1 (1.0%)	97	(CU924818)	
14	A	–	0	–	Uncultured bacterium	α-Proteobacteria
	B	–	0	–	clone nbt14a04 (FJ892825)	
	F	F41	1 (1.0%)	100		
15	A	–	0	–	*Ochrobactrum* sp. ST11	α-Proteobacteria
	B	–	0	–	(FJ982918)	
	F	F69	7 (7.1%)	99		
16	A	A64	1 (0.9%)	98	*Mesorhizobium* sp.	α-Proteobacteria
	B	–	0	–	(AB196496)	
	F	–	0	–		
17	A	–	–	–	Uncultured *Mesorhizobium* sp.	α-Proteobacteria
	B	B131	1 (1.0%)	99	(AM934992)	
	F	–	–	–		
18	A	A87	1 (1.0%)	96	Uncultured bacterium	α-Proteobacteria
	B	–	0	–	clone F1Y	
	F	–	0	–	(DQ860031)	
19	A	–	–	–	*Mesorhizobium*	α-Proteobacteria
	B	B139	1 (1.0%)	100	*metallidurans*	
	F	–	–	–	(AM930381)	
20	A	A9	19 (17.6%)	98	*Hydrogenophaga*	β-Proteobacteria
	B	B1	36 (36.0%)		*intermedia* strain C1	
	F	F3	33 (33.3%)		(FJ009392)	
21	A	–	0	–	Uncultured bacterium	β-Proteobacteria
	B	B6	2 (2.0%)	98	clone nbw621a07c1	
	F	F8	6 (6.1%)	98	(GQ111116)	
22	A	–	0	–	Uncultured	β-Proteobacteria
	B	B8	12 (12.0%)	99	bacterium	
	F	F9	19 (19.2%)	99	(AJ863383)	
23	A	A25	12 (11.1%)	98	*Janthinobacterium* sp.	β-Proteobacteria
	B	B21	2 (2.0%)	98	A1-13 (AB252072)	
	F	–	0	–		

Continued

24	A	A10	1 (1.0%)	99	Uncultured bacterium	β-Proteobacteria
	B	–	0	–	clone R1B-10 (FJ167447)	
	F	–	0	–		
25	A	–	0	–	*Bordetella* sp. AU7049	β-Proteobacteria
	B	–	0	–	(EU082148)	
	F	F1	1 (1.01%)	99		
26	A	A11	1 (0.9%)	94	Uncultured *Delftia* sp.	β-Proteobacteria
	B	–	0	–	clone 1P-1-A23	
	F	–	0	–	(EU704715)	
27	A	–	0	–	*Acidovorax* sp. TS7	β-Proteobacteria
	B	B35	1 (1.0%)	99	(EU073073)	
	F	–	0	–		
28	A	A4	20 (18.5%)	98	Uncultured bacterium	γ-Proteobacteria
	B	–	0	–	clone (EU881323)	
	F	–	0	–		
29	A	–	0	–	Uncultured Xanthomonadaceae	γ-Proteobacteria
	B	B15	5 (5.0%)	99	bacterium clone LW9m-6-27	
	F	F58	3 (3.0%)	99	(EU640462)	
30	A	–	–	–	*Pseudomonas* sp. LS227	γ-Proteobacteria
	B	B129	1 (1.0%)	100	(FJ937929)	
	F	–	–	–		
31	A	–	0	–	Uncultured Xanthomonadales	γ-Proteobacteria
	B	–	0	–	bacterium clone 1099920970957	
	F	F68	2 (2.0%)	99	(EF434327)	
32	A	–	–	–	Uncultured soil bacterium	γ-Proteobacteria
	B	–	–	–	clone PAH-feed-52 (DQ123783)	
	F	F126	1 (1.0%)	100		
33	A	A66	6 (5.6%)	100	Uncultured γ-Proteobacterium	γ-Proteobacteria
	B	B81	1 (1.0%)	100	(AJ871073)	
	F	–	0	–		
34	A	A34	3 (2.8%)	99	*Pseudomonas* sp.	γ-Proteobacteria
	B	–	0	–	(AJ002813)	
	F	–	0	–		
35	A	A20	2 (1.9%)	99	*Acinetobacter haemolyticus*	γ-Proteobacteria
	B	–	0	–	strain BA56 (FJ263930)	
	F	–	0	–		
36	A	A81	4 (3.7%)	99	*Stenotrophomonas* sp.	γ-Proteobacteria
	B	–	0	–	(EU438980)	
	F	–	0	–		
37	A	–	0	–	*Mycobacterium gilvum*	*Actinobacteria*
	B	–	0	–	strain 23-45 (EU168036)	
	F	F40	1 (1.0%)	99		
38	A	A2	2 (1.9%)	100	*Actinobacterium* RG-38	*Actinobacteria*
	B	–	0	–	(AY561600)	
	F	–	0	–		
39	A	A1	21 (19.4%)	96	Uncultured Flavobacteria	Bacteroidetes
	B	–	0	–	bacterium clone (EF651134)	
	F	–	0	–		

"–" represents absence.

A43), counting 19.4%, 18.5%, 17.6%, 11.1% and 11.1% of the total clones, and representing uncultured Flavobacteria bacterium, uncultured γ-Proteobacterium, *Hydrogenophaga intermedia* strain C1, *Janthinobacterium* sp. A1-13 and *Mesorhizobium* sp. W5, respectively. Clones represented uncultured bacterium accounted for 47.18% in library A. Of all 100 clones in library B could be grouped into 15 OTUs. Predominant OTUs were OTU 20 (clone B1), OUT 2 (clone B23), OUT 3 (clone B25) and OUT 22 (clone B8), counting 36.0%, 13.0%, 13.0% and 12.0% of the total clones, and representing *Hydrogenophaga intermedia* strain C1, uncultured α-Proteobacterium, *Rhizobium* sp. SL-1 and *Sphingosinicella* sp. OC5S, respectively. Clones represented uncultured bacterium accounted for 25% in library B. Meanwhile, of all 100 clones in library F could be grouped into 19 OTUs. Predominant OTUs were OTU 20 (clone F3) , OUT 22 (clone F9) and OUT 1 (clone F96), counting 33.3%, 19.2% and 10.1% of the total clones, and representing *Hydrogenophaga intermedia* strain C1, uncultured β-Proteobacterium and uncultured Caulobacteraceae bacterium clone GC1m-1-59, respectively. Clones represented uncultured bacterium accounted for 36.4% in library F.

2.4 Phylogenetic relationship of the clones from 16S rRNA gene libraries

Phylogenetic analyses based on the sequences of the clones from all three libraries demonstrated that all the clones formed five main groups in the un-rooted tree (Fig. 3). Clone A1 along, together with an uncultured Flavobacteria bacterium clone, formed a distinct linkage of Bacteroidetes group. Clones A2 and F40, together with other two Actinobacteria strains, formed the distinct Actinobacteria group; clones A87, F41 and other 19 clones (Fig. 3) formed the largest α-Proteobacteria group; clones A10, F1 and other 11 clones (Fig. 3) formed the

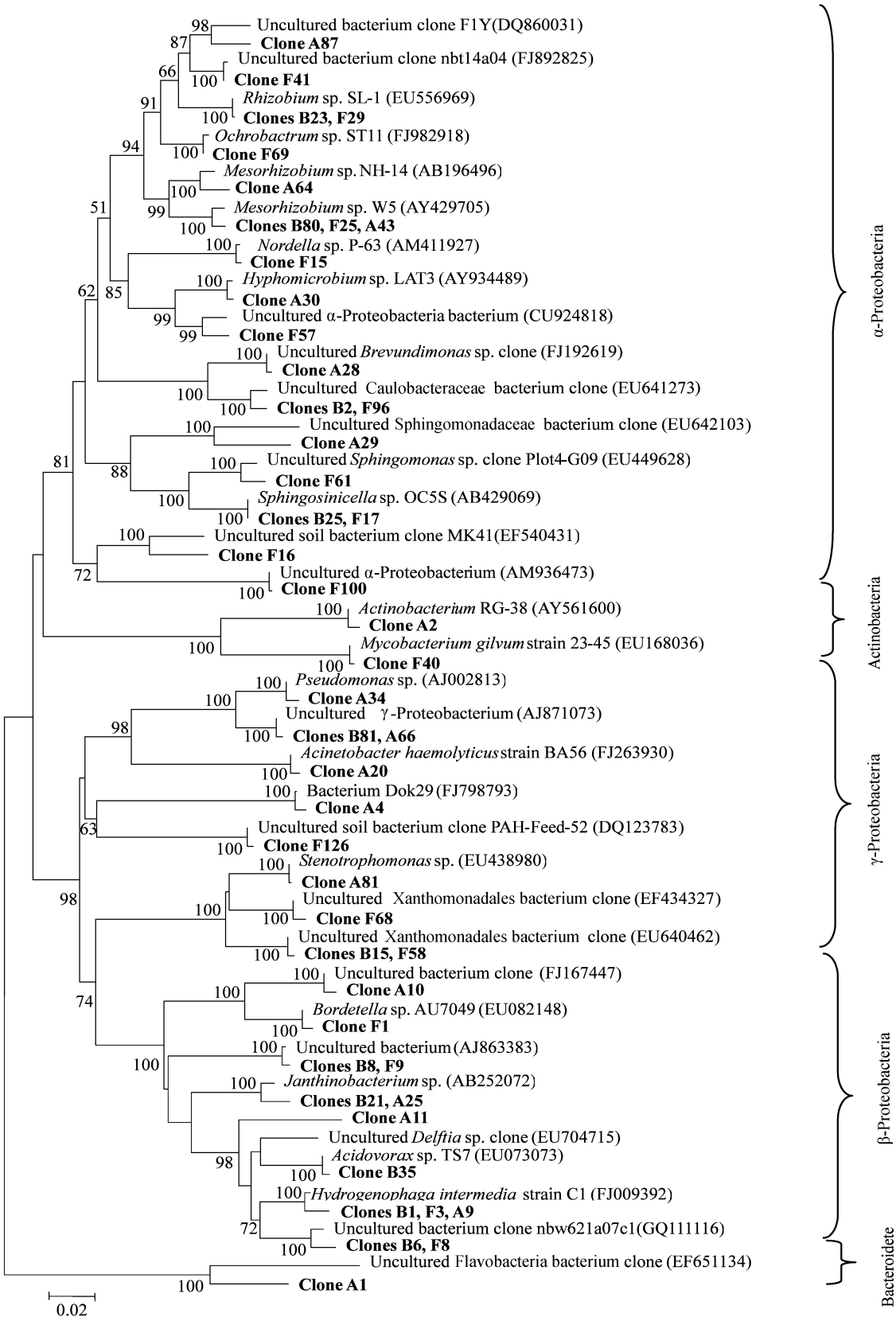

Fig. 3 A neighbour-joining tree based on the sequence of clones from libraries A, B and F, showing the phylogenetic position and relationship of the clones.

β-Proteobacteria group, the other 10 clones formed the γ-Proteobacteria group.

3 Discussion

Findings of several studies have been published on the response of the bacterial community to bioremediation treatment of soils polluted with crude oil or specific hydrocarbon classes (Zucchi et al., 2003; Viñas et al., 2005a; Lafortune et al., 2009), but most of them evaluated the behavior of the microflora during degradation process and lacked information about the bacterial community dynamics after serial transfer. In this study, we analyzed bacterial community dynamics after serial transfers for the degradation of HMW-PAHs and evaluated the changes occurring in the structure of the bacterial population detected by constructing 16S rRNA gene clone library.

A bacterial consortium 1-18-1 was obtained from soil during liquid culture enrichment of the BSM enriched by using PYR as sole carbon and energy source. Bacterial consortium 1-18-1 had a strong ability to degrade pyrene, fluoranthene and benzo[a]pyrene. In addition, consortium 1-18-1 exhibited efficient degrading ability after several enrichments, as shown in Table 1. More than 80% of PYR (100 mg/L) was degraded by the third and fifth generations after incubation for 8 and 28 days, respectively. These results might suggest that consortium 1-18-1 can be useful in the bioremediation of HMW-PAHs contaminated sites. In most cases, it had been proved that only the mixed microbial population which through complex interactions have an ability to degrade HMW-PAHs, and this is of great significance for the bioremediation of contaminated soils (Zucchi et al., 2003; Vandermeer and Daugulis, 2007; Luan et al., 2006).

The community structure of consortium 1-18-1 was also investigated by construction of 16S rRNA gene libraries at three different transfers (first, third and fifth transfers). Phylogenetic analysis demonstrated that consortium 1-18-1 comprises members of three main groups, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria. 16S rRNA gene clone library analysis and principal-component analysis confirmed a remarkable shift in the composition of the bacterial community due to serial transfers. In the three libraries (A, B and F), the proportions of α-Proteobacteria, β-Proteobacteria and γ-Proteobacteria are 16%, 31% and 32% (A), 40%, 53%, and 7% (B), and 33%, 60%, and 6% (F) (Fig. 4), respectively; and these changes might be the reason for the decrease in degrading-ability of 1-18-1 after many transfers.

The bacterial diversity in bacterial consortium 1-18-1 was abundant as revealed by the clone libraries (Table 2). The first generation (library A) contained 17 OTUs, and the predominant bacteria were the clones represented by uncultured Flavobacteria bacterium, uncultured α-Proteobacterium, *Hydrogenophaga intermedia* strain C1, *Janthinobacterium* sp. A1-13 and *Mesorhizobium* sp. W5. The third generation (library B) contained 15 OTUs, and the predominant bacteria were the clones represented *Hydrogenophaga intermedia* strain C1, uncultured

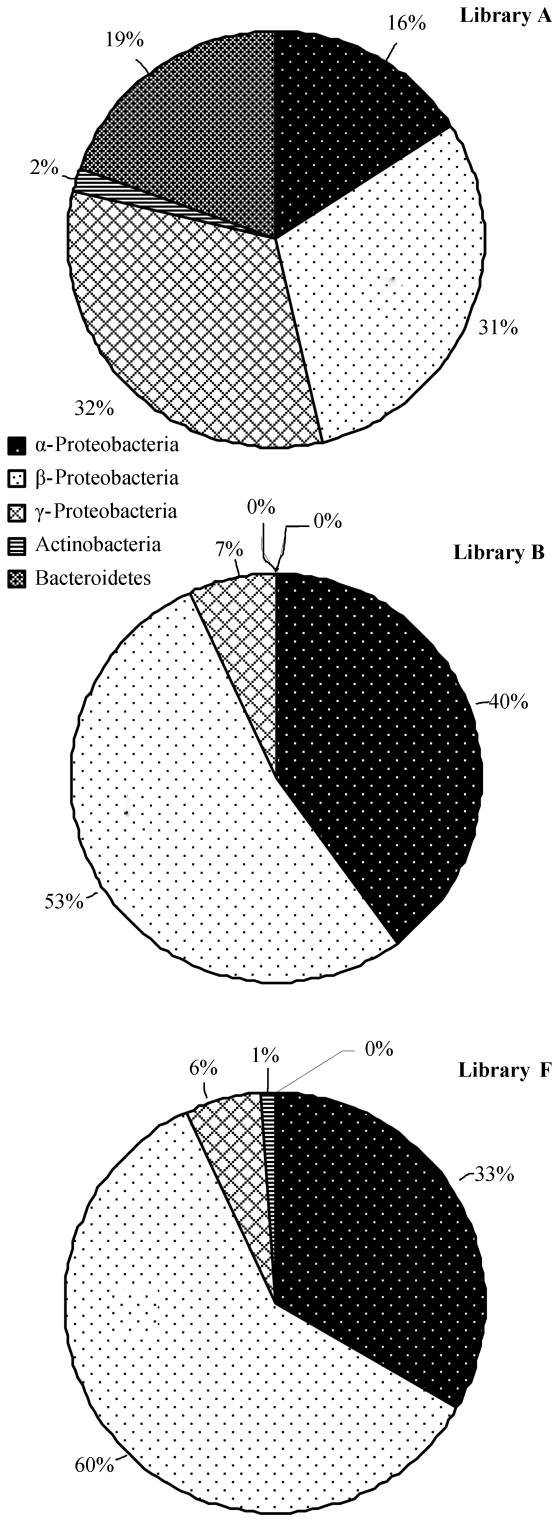

Fig. 4 Bacterial groups in clone libraries A, B and F.

α-Proteobacterium, *Rhizobium* sp. SL-1 and *Sphingosinicella* sp. OC5S. The fifth generation (library F) contained 19 OTUs, and the predominant bacteria were the clones represented *Hydrogenophaga intermedia* strain C1, uncultured α-Proteobacterium and uncultured Caulobacteraceae bacterium. Other studies also reported a large diversity within bacterial communities in PAH-degrading consortia. From a consortium enriched on a PAH mixture including

pyrene, Viñas et al. (2005b) identified 19 different microorganisms or phylotypes; Kanaly et al. (2000) detected 12 DGGE migrating bands in a BaP-degrading consortium and Lafortune et al. (2009) detected 20 DGGE migrating bands in HMW-PAHs-degrading consortium which was enriched from creosote-contaminated soil in a TLP system using silicone oil as the water-immiscible partitioning phase.

After serial transfers of pyrene-containing BSM, the structure of microbial community has changed (Fig. 4). The reason for the decline of 1-18-1 (fifth generation) in the ability to degrade pyrene may be due to the change in structure of microbial community. Through analyzing the bacterial community composition of three 16S rRNA gene clone library (A, B and F), members of Bacteroidetes were just observed in the library A (first generation), suggesting that this group was not the main degrading bacteria; the proportion of γ-Proteobacteria in the three library was gradually reduced, inferring that this group may be related to the degradation of HMW-PAHs.

4 Conclusions

A bacterial consortium was screened out from HMW-PAHs contaminated soil. This consortium could utilize and degrade several HMW-PAHs, including pyrene, fluoranthene and benzo[a]pyrene efficiently. Its community structures at different transfers were analyzed by construction of 16S rRNA gene libraries. All the clones could be divided into five groups, Bacteroidetes, α-Proteobacteria, Actinobacteria, β-Proteobacteria and γ-Proteobacteria, and the predominant groups were α-Proteobacteria, γ-Proteobacteria and β-Proteobacteria. The structure of microbial community had changed after serial transfers which influenced the degradation capability.

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