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Isolation of polycyclic aromatic hydrocarbons (PAHs)-degrading *Mycobacterium* spp. and the degradation in soil

Jun Zeng[a,b,c], Xiangui Lin[a,b,*], Jing Zhang[a,b], Xuanzhen Li[a,b,c]

[a] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Beijing East Road, 71, Nanjing 210008, PR China
[b] Joint Open Laboratory of Soil and the Environment, Hongkong Baptist University & Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, PR China
[c] Graduate University of Chinese Academy of Sciences, Beijing 100049, PR China

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ABSTRACT

The goal of this study was to isolate PAHs degraders that can utilize PAHs associated with soil particulates and investigate the biodegradation of PAHs on agar plate, in liquid culture and soil. Two *Mycobacterium* strains (NJS-1 and NJS-P) were isolated from PAHs-contaminated farmland soil using enrichment based on soil slurry. The isolates could degrade five test PAHs including pyrene, phenanthrene, fluoranthene, anthracene and benzo[*a*]pyrene on plate, but showed different effects in liquid culture, especially for fluoranthene. Isolate NJS-1 was capable of utilizing benzo[*a*]pyrene as a sole carbon and energy source, and an enhanced degradation was observed when pyrene was supplied as cometabolic substrate. Reintroduction of the isolates into sterile contaminated soil resulted in a significant removal of aged pyrene and fluoranthene (over 40%) in 2-months incubation. In pyrene-spiked soil, the degradation of pyrene and fluoranthene increased to 90% and 50%, respectively. Comparing PAHs degradation on plate, in liquid culture and soil, we can conclude that there was corresponding degradation in different test systems. In addition, the degradation of aged PAHs in soil suggested the potential application of two isolates in further bioremediation.

1. Introduction

Pollution of polycyclic aromatic hydrocarbons (PAHs) in soil has recently become a matter of great concern due to their potential toxicity, mutagenicity and carcinogenicity [1]. These compounds enter into the environment via many ways including incomplete combustion of fossil fuels, waste incineration, as well as accidental spilling of hydrocarbons [2]. Because of their hydrophobic nature, most PAHs tend to sorb to the soil particulates, rendering them less available for biological uptake. High molecular weight (HMW) PAHs are considered to be more recalcitrant and may persist in the environment for a long time. For example, the half-life of five-ring PAH benzo[*a*]pyrene in soil was reported ranging from 229 to 1400 days [3]. Although the removal of PAHs is determined by many processes such as volatilization, photo oxidation, chemical oxidation and biological process, the microbial degradation and transformation are thought to be the principal one.

Pyrene, a four-ring PAH with structural similarity to several carcinogenic PAHs, was often used as a model HMW PAH for biodegradation [4]. The initial studies on the bacterial degradation of pyrene were carried out by Heitkamp and Cerniglia [5]. From then on numerous pyrene-degraders were isolated. Most of them belong to genus *Sphingomonas* [6,7], *Mycobacterium* and *Rhodococcus* [8,9]. These degraders could also utilize other PAHs, such as naphthalene, phenanthrene and fluoranthene, as a sole carbon source and energy [10,11]. Some of them were even capable of degrading benzo[*a*]pyrene when some cometabolic substrates were supplied [12], which suggested their important roles in the fate of PAHs in the environment [13]. The catabolic versatility of pyrene-degrader is partly due to the broad substrate specificity of the degradative enzyme they possessed, such as dioxygenases NidAB and NidA3B3 in *Mycobacterium vanbaalenii* PYR-1. These enzymes can convert many PAHs to corresponding *cis*-dihydrodiols [14,15]. It is of great interest to isolate and investigate such a versatile degrader.

However, PAHs degradation in soil is more complex than that in laboratory because the organic matter in soil may sorb hydrophobic PAHs strongly. Hence, the isolates that can degrade adherent PAHs binding to soil particulates will be useful in further application in soil [16,17]. In order to obtain bacteria that can degrade PAHs in soil, enrichment procedure based on soil slurry was used. In this study, two *Mycobacterium* species were isolated from PAHs-contaminated soil with pyrene as a sole carbon and energy source. The PAHs degradation was described both on plate and in liquid

* Corresponding author at: Department of Biology and Biochemistry, Institute of Soil Science, Chinese Academy of Sciences, Beijing East Road, 71, Nanjing 210008, PR China. Tel.: +86 025 86881312; fax: +86 025 86881000.
E-mail address: xglin@issas.ac.cn (X. Lin).

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Table 1
Concentrations of PAHs in autoclaved soils.

PAHs	Naturally contaminated soil ($\mu g\,kg^{-1}$ dry soil)	Pyrene-spiked soil ($\mu g\,kg^{-1}$ dry soil)
Naphthalene	0	0
Acenaphthylene	1603.24 ± 381.33	1374.22 ± 346.45
Fluorene	0	0
Phenanthrene	300.86 ± 71.36	290.60 ± 26.87
Anthracene	25.84 ± 4.18	22.57 ± 0.57
Fluoranthene	1170.54 ± 78.60	1122.36 ± 18.95
Pyrene	2489.59 ± 518.56	48490.07 ± 342.91
Benz[*a*]anthracene	729.96 ± 21.21	715.44 ± 19.46
Chrysene	628.71 ± 6.13	626.38 ± 19.49
Benzo[*b*]fluoranthene	1219.09 ± 3.35	1137.20 ± 4.63
Benzo[*k*]fluoranthene	441.88 ± 1.30	414.46 ± 0.31
Benzo[*a*]pyrene	875.14 ± 0.88	778.89 ± 4.03
Dibenzo[*a*,*h*]anthracene	191.22 ± 6.88	174.05 ± 5.47
Benzo[*g*,*h*,*i*]perylene	1577.44 ± 22.52	1518.81 ± 62.92
Indenol[*1*,*2*,*3-cd*]pyrene	350.12 ± 7.63	298.38 ± 1.84
Total	11603.62 ± 1051.13	56963.44 ± 15.15

Date are presented as mean ± standard deviations for the replicates.

culture, and the remediation in soil was further investigated. The result of degradation of aged PAHs in soil suggested a potential application of the isolates in practical remediation.

2. Materials and methods

2.1. Chemicals and media

Phenanthrene, fluoranthene, pyrene, anthracene and benzo[*a*]pyrene were purchased from Sigma–Aldrich (Shanghai, China), and the purity of the PAH was 97–99%. PAH stock solutions were 10 mg ml^{-1} in acetone. All other solvents and chemicals used were of reagent grade or better. Minimal medium (MM) contained (per liter) 0.2 g of $MgSO_4{\cdot}2H_2O$, 20 mg $CaCl_2{\cdot}2H_2O$, 10 mg $FeSO_4{\cdot}7H_2O$, 0.4 g KH_2PO_4, 0.6 g Na_2HPO_4, 20 mg $MnSO_4{\cdot}H_2O$, 1 g NH_4NO_3. Luria-Bertani (LB) medium contained 1% peptone, 0.5% yeast extract and 0.5% NaCl (pH 7.0).

2.2. Soil

Soil sample was taken from the 5 to 10 cm layer of a PAHs-contaminated farmland in Wuxi, Jiangsu Province, China. The soil pH was 6.4, organic matter 19.2 g kg^{-1}, total nitrogen 1.29 g kg^{-1}, total phosphorus 0.48 g kg^{-1}, total potassium 14.2 g kg^{-1}, water-holding capacity 35% and the total concentration of the 15 US Environmental Protection Agency (EPA) priority PAHs was 13.3 mg kg^{-1}. The soil was sieved to 5 mm and stored at 4 °C in darkness prior to use.

2.3. Isolation of pyrene-degrading bacteria

Enrichment was carried out by adding pyrene (100 mg l^{-1}) into soil slurry prepared in MM medium at 1:1 (w/v) ratio. The soil slurry was incubated with shaking at 200 rpm at 30 °C for 4 weeks in the dark. Pyrene was added again at the end of 2-week incubation to complement the degraded PAH. At the end of enrichment, aliquot (100 μl) of the sample dilution was spread on double-layer plates and incubated for 2–3 weeks at 30 °C.

The double-layer plates were prepared by a modification of Bogardt and Hemmingsen [18]. Briefly, overlayer was mixed 0.2 ml of an acetone solution of pyrene (8 mg ml^{-1}) with 4 ml MM medium solidified with 1% agar to give a final concentration of 400 mg l^{-1} of pyrene, and after the contents were mixed, the overlayer was immediately poured onto an underlayer of MM medium solidified with 1.5% agar. Colonies surrounded by a clearing zone were selected as pyrene-degrader and picked up from the plate and purified by repetitive streaking on another double-layer plate. Pure cultures were routinely cultured in the presence of pyrene for retaining its degrading capability.

2.4. Phylogenetic analysis

16S rDNA was amplified from genomic DNA of isolates using the set of primers 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ (8–27, *Escherichia coli* numbering) and 1492R: 5′-GGTTACCTTGTTACGACTT-3′ (1492–1510, *E. coli* numbering). The PCR products were purified and sent to be sequenced. The sequences were submitted to the GenBank database to align with published sequences using NCBI BLASTN. The accession numbers are AB548662 for NJS-1 and AB548663 for NJS-P. The software MEGA (version 4.1) was used to construct phylogenetic trees.

2.5. Growth on and degradation of pyrene

Utilization of pyrene as a sole source of carbon and energy was performed in liquid culture prepared by adding pyrene in acetone solution to empty sterile 250 ml flasks, followed by evaporation of acetone with gentle shaking. Each flask was added with 25 ml of MM medium to give a concentration of 100 mg l^{-1} of pyrene. Cultures of two isolates grown in LB medium for 3 days were harvested, washed, and resuspended in MM medium (A_{600}, 0.6). The resuspension was used as an inoculum (2.5 ml). Uninoculated flasks and flasks without PAH served as controls. All treatments including control were in triplicate. Cultures were incubated at 200 rpm at 30 °C in the dark and removed for analysis after 3, 6, 8, 10, 12 and 14 days. The growth was monitored by $O.D._{600\,nm}$. The rest of PAH in the culture was extracted with the same volume of dichloromethane. Aliquot (1 ml) of the extract was dried with gentle stream of nitrogen, and then dissolved in acetonitrile for ultra fast liquid chromatograph (UFLC) analysis.

2.6. Degradation of other PAHs and cometabolism of benzo[a]pyrene and pyrene

Degradation of other PAHs including phenanthrene, fluoranthene, anthracene and benzo[*a*]pyrene were assessed under two conditions, namely on solid agar (double-layer plates) and in liquid culture (MM). For the degradation on solid agar plate, single colony of the isolates was picked up and streaked on the double-layer plates consisting of single PAH. The concentration of PAH was 400 mg l^{-1} for phenanthrene and 100 mg l^{-1} for fluoranthene, anthracene and benzo[*a*]pyrene. The plates were incubated at 30 °C

in the dark for 10 days (except 1 month for benzo[*a*]pyrene) and a positive result was recorded if a clearing zone was observed around the colony. Degradation in liquid culture with single PAH (100 mg l^{-1} of phenanthrene, fluoranthene and anthracene and 50 mg l^{-1} of benzo[*a*]pyrene) as a sole carbon source and energy was performed for 2 weeks as described in Section 2.5.

Cometabolism of the two high molecular weight (HMW) PAHs was conducted in MM liquid medium with a mixture of benzo[*a*]pyrene (10 mg l^{-1}) and pyrene (100 mg l^{-1}). The treatments were in triplicate.

2.7. Reintroduction of bacteria into soil

PAHs degradation in soil was investigated in artificially and naturally contaminated soils. The naturally contaminated soil was used directly after the soil was autoclaved, while the artificially polluted soil was treated with PAH by spiking pyrene in acetone solution into autoclaved soil (50 mg kg^{-1}), followed by evaporation of acetone for 24 h at room temperature. The concentrations of the 15 EPA-PAH in soils are listed in Table 1.

Cells of NJS-1 and NJS-P, grown in LB medium for 3 days, harvested and resuspended in MM medium (A_{600}, 0.6) were inoculated into soils (50 g each) individually or together to give the initial bacterial population of 10^6 cells g^{-1} of soil. $HgCl_2$-sterilized (1 g kg^{-1}) and autoclaved controls without inoculation were set up similarly. All soil cultures were brought up to 60% water-holding capacity and incubated at 30 °C in the dark for 2 months. At the end of incubation, PAHs of the soils (1 g, dry weight) were extracted with 60 ml of dichloromethane in a Soxhlet apparatus for 24 h. The extracts were dried using a rotary evaporator and dissolved in acetonitrile. Purification with a chromatography column filled with activated silica gel was required before UFLC analysis.

2.8. Ultra fast liquid chromatograph (UFLC) and statistical analysis

PAHs were analyzed by shimadzu UFLC-20 system. A reversed phase column C_{18} (3 mm × 150 mm, particle size 2.2 μm) using mobile phase with acetonitrile/water (gradient elution 20 min at a constant flow rate 0.8 ml min^{-1}, 50 °C) was used to separate PAHs.

SPSS software (version 11.5) was used for statistical analysis and One-way ANOVA was used to determine the difference between treatments at a significance level of 0.05.

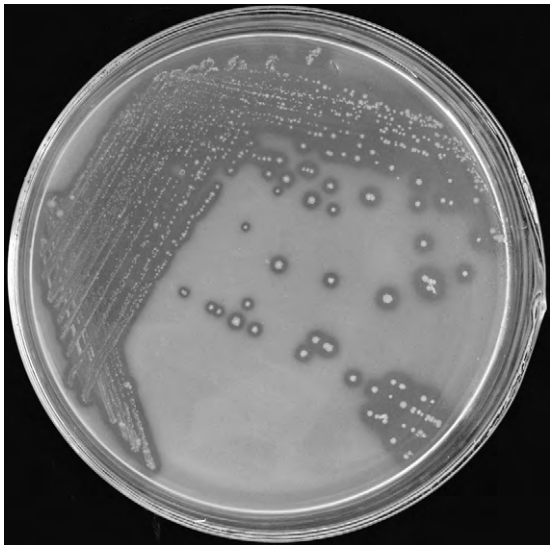

Fig. 1. Utilization of pyrene by isolated bacteria on minimal medium agar plate covered with an overlayer that was composed of pyrene with acetone as a carrier solvent. The plate was incubated for 3 weeks.

3. Results

3.1. Isolation of pyrene-degrading bacteria

Two morphologically distinct bacteria designated NJS-1 and NJS-P were isolated in pure culture by the formation of clearing zone on the double-layer plates (Fig. 1). Both of them were Gram positive and strongly acid-fast. The isolate NJS-1 was yellow, circular, and convex in colony morphology and coccus in cell morphology; NJS-P was pale-white, circular, convex and short-rod.

Phylogenetic analysis (Fig. 2) showed that both isolates were closely related to *Mycobacterium* and clustered together with some reported PAHs-degrading bacteria. The 16S rDNA sequence of NJS-1 had 99% identity to *Mycobacterium crocinum* strain czh-3 [9], a PAHs-degrading strain isolated from non-contaminated soil. NJS-P best matched to *Mycobacterium rhodesiae* strain JS60 (99% identity) [19] which was capable of degrading vinyl chloride.

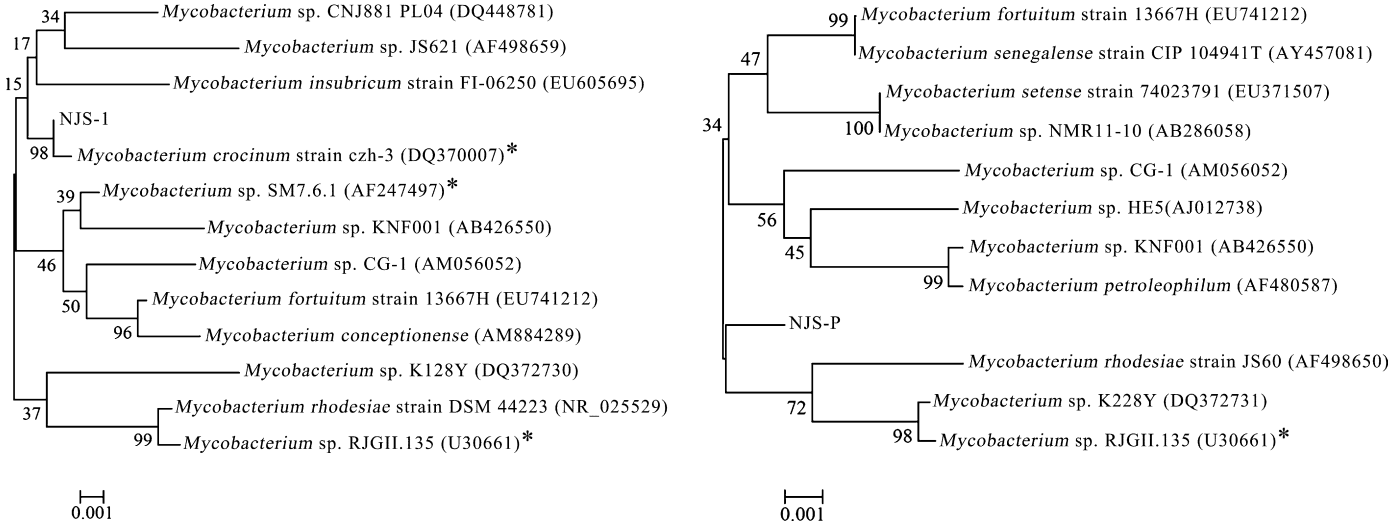

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rDNA sequences of the isolates NJS-1 and NJS-P. The trees were generated with 1000 replicates and the numbers at the nodes represent values for bootstrap probabilities. *Mycobacterium* isolates known to degrade PAHs are indicated with an asterisk (*).

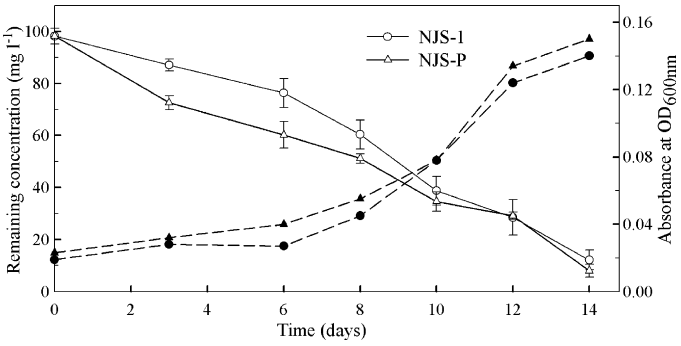

Fig. 3. Time course for pyrene degradation by isolates NJS-1 and NJS-P. The cultures were grown in minimal medium containing 100 mg l^{-1} of pyrene as a sole source of carbon and energy. Open symbols and closed symbols represent the degradation and the growth of strains, respectively.

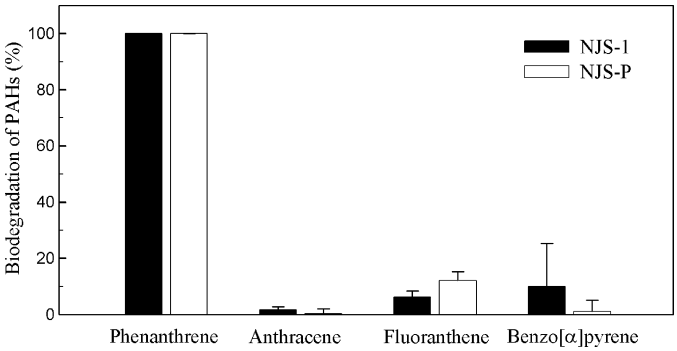

Fig. 4. Biodegradation of individual PAH in the liquid minimal medium for 2 weeks. Concentration of PAHs was 100 mg l^{-1} except benzo[*a*]pyrene (50 mg l^{-1}).

3.2. Growth on and degradation of pyrene

The time-courses of microbial growth and pyrene degradation with an initial concentration of 100 mg l^{-1} were determined at 200 rpm at 30 °C in the dark. As shown in Fig. 3, high amounts of pyrene (87.9% and 92% for NJS-1 and NJS-P, respectively) were removed after 2 weeks incubation, and an increase of the biomass corresponding to the decrease of remaining pyrene was observed.

3.3. Degradation of other PAHs and cometabolism of benzo[a]pyrene and pyrene

The isolates could clear plate overlayed with the test PAHs in the incubation time (1 month for benzo[*a*]pyrene and 10 days for the other PAHs). Phenanthrene and fluoranthene were utilized readily while anthracene and benzo[*a*]pyrene were more difficult to be degraded. Two isolates showed different degradation to fluoranthene and benzo[*a*]pyrene on plate according to the radius of the clearing zone and the incubation time (Table 2).

Differing from the degradation on solid agar plate, fluoranthene (100 mg l^{-1}) was difficult to be degraded in liquid culture. Only a small amount of fluoranthene (6.2% and 12.1% for NJS-1 and NJS-P, respectively) was degraded after 2-week incubation. In contrast, phenanthrene (100 mg l^{-1}) was degraded easily. High amounts of phenanthrene (both of 100%) were removed from the cultures after incubation. Isolate NJS-1 degraded 10.1% of benzo[*a*]pyrene (50 mg l^{-1}) as a sole carbon source, while no degradation was observed by NJS-P in the same condition (Fig. 4).

Stimulated degradation (enhanced from 10.1% to 22.5%) of five-ring benzo[*a*]pyrene (10 mg l^{-1}) in the presence of pyrene was observed in the culture of NJS-1 (Fig. 5), while only 1.4% was removed by NJS-P. However, degradation of pyrene (66.4% and 72.8% for NJS-1 and NJS-P, respectively) lowered in the assay compared with that in single PAH assay.

Table 2
PAHs biodegradation on the double-layer plate.

Strains	Degradation of PAHs on double-layer plate			
	PHE[a]	ANT[b]	FLT[b]	BaP[b]
NJS-1	+++[c]	+	++	++
NJS-P	+++	+	+++	+

Degradation was determined by the formation of a clearing zone surrounding the colony cultured for 10 days (except 1 month for BaP). Five PAHs investigated as a sole source of carbon and energy were PHE: phenanthrene; ANT: anthracene; FLT: fluoranthene; and BaP: benzo[*a*]pyrene.

[a] PAHs concentration was 400 mg l^{-1}.

[b] PAHs concentration was 100 mg l^{-1}.

[c] Abundance of the clear zone surrounding the colony. (+++) an obvious clear zone with large radius; (++) an obvious clear zone with small radius; (+) a clear zone formation but was not very obvious.

3.4. Reintroduction of bacteria into soil

$HgCl_2$-sterilized controls were set up to inhibit PAHs biodegradation, which was used to interpret that the PAHs disappearance of samples was caused by biological activity. The concentration of PAHs in $HgCl_2$-sterilized control did not change remarkably in the incubation period (date not shown).

Fluoranthene and pyrene were found to be most degradable when PAHs degraders were introduced (Table 3). In naturally contaminated soil, a large amount of fluoranthene (36.58 ± 0.87% to 49.23 ± 1.09%) and pyrene (48.55 ± 5.76% to 54.14 ± 7.43%) were removed after 2-month incubation, significantly higher ($P < 0.05$) than that of autoclaved control (10.06 ± 10.32% and 19.07 ± 0.20% for fluoranthene and pyrene, respectively). Stimulation of degradation was observed in pyrene-spiked soil, in which the degradation of fluoranthene and pyrene increased to over 50% and 90%, respectively. In addition, an increased degradation was also observed in autoclaved control (34.88 ± 1.56% and 58.80 ± 1.18% for fluoranthene and pyrene, respectively) (Table 3).

4. Discussion

It is well known that PAHs-degrading bacteria play an important role in the elimination of PAHs in soil. So far, many degraders have been discovered and the ordinary biochemical pathways of PAHs metabolism have been elucidated [4,20]. Therefore, the requirement to the degrader was promoted, which was capable of (1):

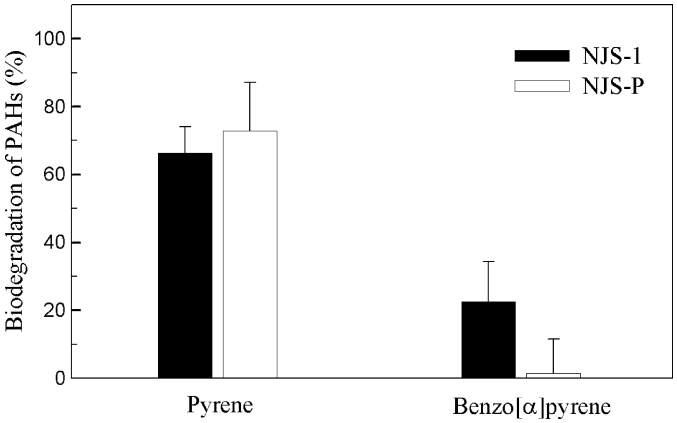

Fig. 5. Cometabolic biodegradation of benzo[*a*]pyrene (10 mg l^{-1}) and pyrene (100 mg l^{-1}) in the liquid minimal medium for 2 weeks.

Table 3
PAHs biodegradation in the soils for the 2-months incubation.

PAHs	Degradation in the naturally contaminated soil (%)[a]				Degradation in the pyrene-spiked contaminated soil (%)			
	Autoclaved control	NJS-1	NJS-P	Cocultures[b]	Autoclaved control	NJS-1	NJS-P	Cocultures[b]
Naphthalene	NA	NA	NA	NA	NA	NA	NA	NA
Acenaphthylene	13.56 ± 7.84a	<0a	<0a	<0a	3.39 ± 31.08a	<0a	<0a	<0a
Fluorene	NA	NA	NA	NA	NA	NA	NA	NA
Phenanthrene	27.44 ± 7.29a	36.63 ± 10.98a	30.47 ± 1.9a	31.16 ± 15.60a	23.82 ± 0.14a	30.18 ± 20.20a	11.76 ± 15.75a	9.45 ± 2.63a
Anthracene	0.27 ± 8.11a	17.75 ± 19.23a	<0a	9.43 ± 5.22a	<0a	<0a	<0a	<0a
Fluoranthene	10.06 ± 10.32b	46.80 ± 2.60a	36.58 ± 0.87a	49.23 ± 1.09a	34.88 ± 1.56c	53.73 ± 0.62ab	49.80 ± 2.26b	56.05 ± 3.24a
Pyrene	19.07 ± 0.20b	52.47 ± 7.01a	48.55 ± 5.76a	54.14 ± 7.43a	58.80 ± 1.18c	90.12 ± 0.22a	87.53 ± 0.30b	90.58 ± 0.35a
Benzo[*a*]anthracene	2.92 ± 2.94a	<0a	<0b	1.58 ± 0.19a	0.35 ± 3.75a	2.86 ± 0.48a	2.10 ± 1.35a	2.76 ± 1.95a
Chrysene	<0a	<0a	<0a	2.47 ± 0.32a	2.47 ± 6.29a	2.83 ± 0.62a	2.51 ± 1.35a	5.65 ± 5.81a
Benzo[*b*]fluoranthene	4.21 ± 4.04a	<0ab	<0b	0.56 ± 1.43ab	<0a	<0a	1.09 ± 1.36a	1.30 ± 1.19a
Benzo[*k*]fluoranthene	4.42 ± 4.32a	0.33 ± 1.10a	<0a	1.62 ± 1.29a	<0a	<0a	0.86 ± 1.50a	0.72 ± 0.94a
Benzo[*a*]pyrene	<0a	<0a	<0a	<0a	<0a	<0a	<0a	<0a
Dibenzo[*a,h*]anthracene	4.98 ± 3.62a	4.29 ± 0.89a	<0a	0.76 ± 4.61a	2.66 ± 2.99a	<0a	0.31 ± 2.76a	2.93 ± 0.36a
Benzo[*g,h,i*]perylene	6.10 ± 3.39a	1.52 ± 2.54a	<0a	2.15 ± 4.22a	1.62 ± 6.38a	1.40 ± 0.39a	6.40 ± 0.87a	5.28 ± 0.15a
Indenol[*1,2,3-cd*]pyrene	10.43 ± 1.13a	8.11 ± 4.26a	1.62 ± 2.63a	7.29 ± 5.14a	<0a	<0a	<0a	<0a
Total	8.39 ± 3.72b	19.98 ± 3.06a	14.83 ± 2.32ab	21.27 ± 2.47a	51.36 ± 0.92c	78.98 ± 0.46a	77.24 ± 0.29b	79.62 ± 0.46a

Date are presented as mean ± standard deviations for the replicates. Different letters indicate significances between treatments ($P < 0.05$). NA: not applicable.
[a] Degradation was determined by using $HgCl_2$-sterilized (1 g kg^{-1}) soil as a control.
[b] Inoculated with NJS-1 and NJS-P.

utilizing HMW PAHs (five or more rings were better) and (2): degrading PAHs associated with soil particulates.

Enrichment based on soil slurry was different from the traditional procedure that transfers the enriched culture for many times. In this procedure, without any subculture, it just needs to incubate the soil slurry in 4 weeks and add pyrene once to supplement the consumed carbon source. Because there is no subculture, more species would retain in the culture, especially for the slow-growing bacteria. Traditional enrichment carried out in other studies showed the number of colonies forming clear zone was lower, even none in some tests, than that in soil slurry (data not shown). More important, soil particulates in slurry would act as a PAH-sorbing carrier for isolating the adherent PAHs-degrading strains [17]. As a result, two strains (NJS-1 and NJS-P) belonging to genus *Mycobacterium* were isolated. Bastiaens et al. [16] observed *Mycobacterium* was exclusively selected in culture enriched on hydrophobic membranes while *Sphingomonas* was mainly selected in the liquid enrichment. Actually, *Mycobacterium* species have been frequently reported as four-ring PAHs degraders, such as *M. vanbaalenii* PYR-1 [21], *Mycobacterium* sp. RJGII-135 [22], A1-PYR [23], LP1 [24] and SNP11 [25]. It has been established that the particular cell wall layer of mycobacteria, such as mycolic acids, is important for the degradation of hydrophobic compounds [26].

Catabolic versatility of the isolates was verified from the degradation on double-layer plate, on which all of the test PAHs including benzo[*a*]pyrene could be degraded. *Mycobacterium* sp. HH1 isolated from mangrove sediments in Hong Kong [27] showed the similar degradation of phenanthrene, anthracene, fluoranthene and pyrene on agar plate, and Miller et al. [8] observed *Mycobacterium* sp. KMS and MCS, but not JLS, could degrade benzo[*a*]pyrene on plate. In contrast, the degradation in liquid culture showed different results, especially for fluoranthene. It showed that fluoranthene can readily be degraded on plate while it was hard to be utilized in the liquid culture. The same finding as Zhou et al. reported [27] suggested there were some differences between degradation on plate and in liquid culture. The strains of *Mycobacterium* may prefer to grow adhered with solid carrier rather than in shaken liquid culture due to the hydrophobic nature. The degradation in liquid culture also indicated phenanthrene was readily utilized while anthracene and benzo[*a*]pyrene was difficult to be degraded. Previous studies showed that the pyrene-degrader *Mycobacterium* sp. AP1 [13] and *M. pyrenivorans* 17A3^T [28] could utilize pyrene, phenanthrene and fluoranthene but not anthracene and benzo[*a*]pyrene, while *M. austroafricanum* GTI-23 [11] could degrade benzo[*a*]pyrene.

Until now, there has been no bacterial strain that was isolated to use benzo[*a*]pyrene as a sole source of carbon and energy [20,29]. Most of the studies on degradation of benzo[*a*]pyrene were carried out by the pyrene-degrader, and the degradation usually requires supplying cometabolic substrate [11,21,30]. *Mycobacterium* sp. NJS-1 was found capable of degrading benzo[*a*]pyrene as a sole carbon source. In the study by Juhasz et al. [31], pyrene-degraders *Pseudomonas cepacia* VUN 10,001, VUN 10,1002 and VUN 10,1003 were able to degrade 20–30% of benzo[*a*]pyrene as a sole carbon source after 63 days incubation at high cell density. Recent studies also showed *Ochrobactrum* sp. BL01 [32] and BAP5 [33], isolated from sea water and marine sediment, respectively, could degrade benzo[*a*]pyrene (over 20% in 2-week incubation) as a sole carbon source and energy. Improved degradation of benzo[*a*]pyrene was observed in the presence of pyrene. The stimulation could be interpreted as a positive analogue effect on enzyme induction [12,34,35]. The readily utilized substrate, such as pyrene, would stimulate PAHs-catabolic enzymes for the degradation of benzo[*a*]pyrene [36]. However, negative effect was also reported by McClellan et al. [37] that pyrene inhibited benzo[*a*]pyrene degradation by *Mycobacterium* sp. RJGII-135.

Reintroduction of *Mycobacterium* NJS-1 and NJS-P into contaminated soil led to the significant removal ($P < 0.05$) of indigenous fluoranthene and pyrene, which established their capability of degrading PAHs associated with soil particulates. Furthermore, the degradation of fluoranthene in soil indicated a solid phase benefited the PAHs degradation. Phenanthrene, another readily degraded PAH, was degraded to over 30%, but it showed no significant difference ($P > 0.05$) between the degradation in samples and autoclaved control (Table 3). It should be noted that the degradation which should be caused by unkilled indigenous degrader resulted from incomplete sterilization in general autoclaved process [22] was also observed in autoclaved control. Vinas et al. [38] and Li et al. [39] showed the incubation of soil at a proper water-holding capacity could lead to the degradation of indigenous PAHs. The enhanced degradation of indigenous fluoranthene (slightly increased to 50%) and that in autoclaved control (increased to 35%) in pyrene-spiked soil suggested the growth stimulation of degraders by exogenous PAH. It was reported by Miller et al. [8] and Johnsen et al. [40] that the number of culturable degrader and copy number of PAH dioxy-

genase gene increased after the incubation. Inoculation of NJS-1, NJS-P or cocultures showed no significant differences ($P > 0.05$) in naturally contaminated soil, while a relatively higher ($P < 0.05$) degradation with NJS-1 and cocultures was observed in pyrene-spiked soil (Table 3). Previous reports showed the cocultures could stimulate the degradation of PAH because one strain could utilize the intermediates generated by another strain [32,41–43], but such stimulation was not significant in our study.

Using enrichment based on soil slurry, two degraders belonging to genus *Mycobacterium* with catabolic versatility and capability of degrading adherent PAHs in soil were isolated. Investigation of PAHs biodegradation on agar plate, in liquid culture and soil demonstrated differences in degradation appeared in different test systems, and the capability of degrading aged PAHs in soil suggested the two isolates here might become a useful tool for remediation of PAHs in the environment.

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