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Sphingomonas from petroleum-contaminated soils in Shenfu, China and their PAHs degradation abilitiesLisha Zhou^a, Hui Li^{a,b,*}, Ying Zhang^a, Siqin Han^a, Hui Xu^a^a Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, China^b State Key Laboratory of Forest and Soil Ecology, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, China

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ABSTRACT

Members of the *Sphingomonas* genus are often isolated from petroleum-contaminated soils due to their unique abilities to degrade polycyclic aromatic hydrocarbons (PAHs), which are important for in situ bioremediation. In this study, a combined phenotypic and genotypic approach using streptomycin-containing medium and *Sphingomonas*-specific PCR was developed to isolate and identify culturable *Sphingomonas* strains present in petroleum-contaminated soils in the Shenfu wastewater irrigation zone. Of the 15 soil samples examined, 12 soils yielded yellow streptomycin-resistant colonies. The largest number of yellow colony-forming units (CFUs) could reach 10^5 CFUs g^{-1} soil. The number of yellow CFUs had a significant positive correlation ($p < 0.05$) with the ratio of PAHs to total petroleum hydrocarbons (TPH), indicating that *Sphingomonas* may play a key role in degrading the PAH fraction of the petroleum contaminants at this site. Sixty yellow colonies were selected randomly and analyzed by colony PCR using *Sphingomonas*-specific primers, out of which 48 isolates had PCR-positive signals. The 48 positive amplicons generated 8 distinct restriction fragment length polymorphism (RFLP) patterns, and 7 out of 8 phylotypes were identified as *Sphingomonas* by 16S rRNA gene sequencing of the representative strains. Within these 7 *Sphingomonas* strains, 6 strains were capable of using fluorene as the sole carbon source, while 2 strains were phenanthrene-degrading *Sphingomonas*. To the best of our knowledge, this is the first report to evaluate the relationship between PAHs contamination levels and culturable *Sphingomonas* in environmental samples.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are considered hazardous due to their toxic, genotoxic, mutagenic and/or carcinogenic properties.¹ Microbial degradation is a major process in the successful removal and elimination of PAHs from contaminated soils.² The use of native or indigenous microbiota is of great interest for bioremediation of petroleum-polluted sites because they are often more useful and beneficial than commercial inocula that can be out-competed by indigenous microorganisms.³

Members of the genus *Sphingomonas*, which was proposed by Yabuuchi et al.,⁴ are frequently isolated from PAH-contaminated soils, suggesting that the *Sphingomonas* are probably key members of natural PAH-degrading microbial consortia.^{3,5,6} A number of studies demonstrated that *Sphingomonas* was one of the dominant populations in the microbial communities of PAH-contaminated soil and water samples.⁷⁻⁹

The Shenfu irrigation zone is located between Shenyang and Fushun city in Liaoning province and is the largest petroleum wastewater irrigation zone in China. The paddy fields in this region have been irrigated by oil-bearing wastewater discharged by the petroleum and coal-refining industry since the 1940s due to a lack of irrigation water. During the 1980-1990s, partial paddy fields were converted to upland in the up- and mid-stream. Mineral oil (especially PAHs) was the dominant contaminant in the Shenfu irrigation zone.¹⁰ Our previous study demonstrated that a large percentage of *Sphingomonas* species was present in the petroleum-contaminated soils of the Shenfu irrigation area using culture-independent methods,¹¹ indicating a potential link between *Sphingomonas* and the biodegradation of petroleum compounds in the Shenfu irrigation zone. However, due to the lack of a reliable system for the specific detection and isolation of culturable *Sphingomonas*, the functional strains in the environmental samples were only found at random.

Vanbroekhoven et al.¹² developed a streptomycin-based *Sphingomonas* growth medium that, together with the yellow pigmentation of *Sphingomonas*, facilitated the detection and isolation of culturable *Sphingomonas* from soils. However, this phenotypic detection method is not efficient or accurate because other bacterial taxa also produce yellow pigments and are resistant to streptomycin. A genotypic detection method that circumvents the major drawbacks inherent in the phenotypic detection method described above has been developed for the detection of specific groups. The 16S rRNA gene sequence offers ample possibilities to distinguish microorganisms of various genera and minimize false-positive reactions using genus-specific primers.^{13,14} PCR-based detection has proven to be useful for the identification of the genera *Clostridium*, *Pseudonocardia*, *Saccharopolyspora*, *Nocardioopsis* and *Saccharothrix* using genus-specific primers.¹⁴⁻¹⁶ In our previous study, a primer set targeting the *Sphingomonas* 16S rRNA gene was designed¹⁷; thus, we hypothesized that the use of genus-specific colony PCR combined with phenotypic identification would allow the specific detection of *Sphingomonas* isolates.

The specific aim of this study was to investigate the relationship between indigenous culturable *Sphingomonas* in soils from the Shenfu petroleum-wastewater irrigation zone and

the contamination level by the direct and specific detection of culturable *Sphingomonas* from the soils. Additionally, the PAH-degrading capability of the isolated *Sphingomonas* was preliminarily characterized.

Materials and methods

Soil samples

Soil samples were collected from the Shenfu wastewater irrigation zone (41°50'46"-41°41'24"N, 123°44'43"-123°25'38"E), which is the biggest petroleum wastewater irrigation zone in China. The total length of the irrigation channel is approximately 70 km, and 15 sampling sites with different relative geographical positions, irrigation histories and soil management types were selected from up- to down-stream. The 15 sampling sites included 11 paddy soils (soils A-K) and 4 upland soils (soils L, M, N and O) that were referred to as soils A-O in the following text. The physicochemical soil characteristics and the PAH concentrations in the soil were reported in our previous study.¹¹ Typically, soil samples collected from the up- and mid-stream sites accumulated more petroleum contaminants compared with those from the down-stream sites.

Selective isolation and enumeration of putative *Sphingomonas*

The indigenous putative culturable *Sphingomonas* were isolated and enumerated using a previously described method.¹² The streptomycin-resistant yellow colonies that occurred on the plates were counted based on the formation of colony forming units (CFUs).

PCR and RFLP analysis of the *Sphingomonas* isolates

The identity of the yellow colonies grown on the streptomycin plates was confirmed by genus-specific colony PCR. The PCR protocol was used with the SA429f/933r primer set developed in our previous study.¹⁷ The amplicons with the expected size were further analyzed by RFLP analysis using three endonucleases [*Hae*III (GG/CC), *Hin*fI (G/ANTC) and *Msp*I (C/CGG)] at 37 °C for 2 h. The RFLP patterns were compared manually, and distinguishable RFLP patterns were considered to represent one phylotype.

Phylogenetic identification of *Sphingomonas* isolates

Two representatives of each phylotype were selected for nearly full-length 16S rRNA gene sequencing for phylogenetic identification. PCR amplification of the 16S rRNA genes was performed with the forward primer 27F and the reverse primer 1492R.¹⁸ The obtained sequences were used for phylogenetic analysis as described previously.¹¹

Measurement of PAH degradation

To determine the PAH-degrading capability of the isolated *Sphingomonas* strains, the pure strains were inoculated

into mineral salts medium (MSM) containing a single PAH compound as the sole carbon and energy source. The MSM at pH 7.0–7.2 contained 2 g K₂HPO₄, 0.5 g KH₂PO₄, 0.5 g NaCl, 0.5 g NH₄Cl, 0.2 g MgSO₄, 10 mg CaCl₂, and 1 ml of trace element solution per liter of water. The composition of the trace element solution was 2 g L⁻¹ FeSO₄·7H₂O, 2 g L⁻¹ MnSO₄·H₂O, 0.5 g L⁻¹ Na₂MoSO₄·2H₂O, 0.2 g L⁻¹ H₃BO₄, 0.4 g L⁻¹ CuSO₄·5H₂O, 0.5 g L⁻¹ ZnSO₄, 0.2 g L⁻¹ NH₄VO₃, 0.5 g L⁻¹ CoCl₂·6H₂O, and 0.2 g L⁻¹ NiCl₂·6H₂O. Eight PAHs, including naphthalene (NA), acenaphthene (ACY), fluorene (FLO), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLU), pyrene (PYR) and benzo[a]pyrene (BaP), were prepared at a concentration of 5 g L⁻¹ in acetone and filtered prior to analysis with a 0.22- μ m Teflon syringe filter.

The PAH degradation experiment was performed by monitoring the disappearance of PAH in live versus killed controls. The experiment was conducted in triplicate in sterile dry 250 ml Teflon-capped flasks. After all of the acetone in the PAH stocks was allowed to evaporate, 27 ml of MSM broth and 3 ml of inoculum (OD₆₀₀ = 0.8–1.0) were added to each flask at a final concentration of 200 μ g ml⁻¹ of NA, ANT, FLU, PHE, ANT, and FLU, 100 μ g ml⁻¹ of PYR, and 50 μ g ml⁻¹ of BaP. Triplicate flasks were inoculated with live bacteria and 4% formalin-killed controls. The cultures were incubated in the dark on a rotary shaker (150 rpm) at 30 °C for 7 days, and then the flask contents were extracted for 30 min with hexane (1:1, vol/vol). Flasks with PAHs but without the pure bacterial cultures were also run as a control. The extract was analyzed by high performance liquid chromatography (HPLC) using a Waters 2695 apparatus (Waters Inc., USA) equipped with an ultraviolet detector and a 250 \times 4.6 mm² Inertsil ODS-P (Dikma Inc., USA) reverse phase C18 column. The HPLC analysis was performed using methanol:water (90:10, v/v) as the mobile phase at a flow rate of 0.7 ml min⁻¹. PAH peaks were identified and integrated using the Masslynx software (Waters Corporation, Milford, MA).

Nucleotide sequence accession numbers

The nucleotide sequences have been deposited in the GenBank database under the following accession numbers: JF716058 to JF716065.

Statistical analysis

Analysis of all data was performed by ANOVA using SPSS version 16.0. Linear correlation coefficients were determined between different biological and biochemical parameters. *P* values below 0.05 were considered statistically significant.

Results and discussion

Enumeration of putative *Sphingomonas* by plating on streptomycin agar

Most *Sphingomonas* strains are characterized as yellow-pigmented, straight, rod-shaped bacteria. Additionally, resistance to streptomycin (Sm) is considered a common feature of *Sphingomonas*.¹² Hence, yellow colonies on plates containing Sm were counted as putative culturable *Sphingomonas*.

Previous real-time PCR analysis indicated that all 15 petroleum-contaminated soil samples gave positive signals for *Sphingomonas*.¹¹ However, in this study only 12 soils yielded yellow colonies on the selective LB plates with Sm. The number of yellow CFUs ranged from 0.6 \times 10³ to 1.6 \times 10⁵ CFUs g⁻¹ soil (Table 1). Provided that all of the yellow colonies belonged to the *Sphingomonas* genus, the counts of the *Sphingomonas* strains could only reach 10⁵ CFUs g⁻¹ soil, which was much less than the abundance (10⁷–10⁸ copies g⁻¹ soil) obtained by our previous real-time PCR assay in the same tested soils.¹¹ This discrepancy suggested that uncultured *Sphingomonas* accounted for a rather large proportion in the petroleum-contaminated soils, which was consistent with the results of Vanbroekhoven et al.¹² and Leys et al.¹⁹ Although culturable *Sphingomonas* represent only a small percentage in the polluted soils, it is nevertheless important to obtain pure *Sphingomonas* cultures for functional and mechanical characterization due to their potential roles in bioremediation.

The lowest amount of yellow CFUs was detected in soil K with light contamination, while the upland soil L with the highest ratio of PAHs to total petroleum hydrocarbons (TPH) had the highest number of yellow CFUs. A significant correlation (*P* < 0.05) was observed between the number of yellow CFUs and the ratio of PAHs to TPH (Table 1). It may be argued that the heavily PAH-polluted soils are too toxic for bacteria to survive, but this is certainly not the case for *Sphingomonas*. *Sphingomonas* are well-adapted to contaminated environments, especially those with high PAH to TPH ratios, indicating that members of the genus *Sphingomonas* are probably key members in natural PAH degradation. Indeed, many *Sphingomonas* strains have been isolated based on their ability to degrade a variety of PAHs, such as *Sphingomonas* sp. LB126 and *S. paucimobilis* EPA505.^{5,20}

We also found that *Sphingomonas* seemed to preferentially associate with the sand fraction in the soil because the number of yellow CFUs had a significant (*P* < 0.05) positive correlation with the proportion of the sand fraction. In contrast, a significant (*P* < 0.05) negative correlation was found between the number of yellow CFUs and the clay fraction (Table 1). The association of the bacteria with polluted soil particles was shown previously. Uyttebroek et al.²¹ reported that *Mycobacterium* mainly accumulated in the PAH-enriched clay fraction, indicating that *Mycobacterium* might experience advantages connected to substrate source attachment. However, to the best of our knowledge no research has been performed on the number of culturable *Sphingomonas* in different soil fractions in contaminated soils. Bastiaens et al.²⁰ compared two different procedures to isolate PAH-utilizing bacteria from PAH-contaminated samples and revealed that liquid enrichment mainly led to the isolation of *Sphingomonas*, while the membrane method exclusively led to the selection of *Mycobacterium*. *Sphingomonas* was postulated to prefer the sand habitat, in which the *Sphingomonas* were loosely associated with soil particles and thus obtained more oxygen to perform degradation.

The amounts of yellow CFUs in 4 paddy soils (B, C, D and H) with low pH values (6.4–6.8) were much higher than the amounts in the other 5 paddy soils (A, F, I, J and K) with relatively high pH values (7.3–8.1). Correlation analysis showed a significant decrease (*P* < 0.01) in the amounts of

Table 1 – Total numbers of yellow streptomycin-resistant colonies in 12 soils from the Shenfu irrigation zone and the correlation between the number of yellow colonies and soil properties.

Soil No. ^a	Management type	No. of yellow colonies	Particle size (%)			pH	Total PAHs (mg kg ⁻¹)	TPH (mg kg ⁻¹)	Total PAHs/TPH (%)	HMW PAHs/TPH (%)
			Sand	Silt	Clay					
A ^U	Paddy	2.2 × 10 ³ ± 3.6	67	21	12	7.5	4.69 ± 417.3	1169.3 ± 78.8	0.40	0.28
B ^U	Paddy	6.0 × 10 ⁴ ± 1.1	86	12	2	6.5	2.56 ± 237.4	1266.3 ± 31.2	0.20	0.09
C ^U	Paddy	9.0 × 10 ⁴ ± 3.9	84	13	3	6.8	1.87 ± 154.4	1207.7 ± 75.9	0.15	0.12
D ^U	Paddy	7.2 × 10 ⁴ ± 1.1	84	13	3	6.8	1.35 ± 151.3	716.2 ± 31.1	0.19	0.15
E ^M	Paddy	N.D. ^b	42	24	34	8.1	4.04 ± 334.2	5243.6 ± 78.4	0.08	0.07
F ^M	Paddy	4.0 × 10 ³ ± 1.3	41	28	31	7.7	2.72 ± 241.9	1878.4 ± 77.9	0.14	0.07
G ^M	Paddy	N.D.	41	27	32	7.6	1.96 ± 210.4	661.4 ± 31.2	0.30	0.19
H ^D	Paddy	3.6 × 10 ⁴ ± 0.7	44	44	12	6.4	1.39 ± 201.9	666.2 ± 62.8	0.21	0.18
I ^D	Paddy	2.4 × 10 ³ ± 2.3	49	32	19	7.3	4.30 ± 188.0	937.1 ± 31.2	0.46	0.43
J ^D	Paddy	8.8 × 10 ³ ± 0.7	41	39	20	7.3	0.791 ± 68.1	497.0 ± 46.9	0.16	0.08
K ^D	Paddy	0.6 × 10 ³ ± 0.4	43	42	15	7.3	0.39 ± 31.7	277.2 ± 15.7	0.14	0.11
L ^U	Upland	1.6 × 10 ⁵ ± 5.4	59	27	14	7.3	2.99 ± 25.8	331.4 ± 15.6	0.90	0.74
M ^M	Upland	2.6 × 10 ³ ± 1.9	40	30	30	7.3	2.36 ± 79.3	604.4 ± 26.8	0.39	0.25
N ^D	Upland	N.D.	45	37	18	7.5	4.50 ± 35.4	2489.9 ± 72.8	0.18	0.13
O ^D	Upland	1.6 × 10 ⁴ ± 5.2	52	22	16	7.4	0.61 ± 102.3	220.8 ± 31.2	0.27	0.17
Correlation coefficient ^c			0.598 (P < 0.05)	-0.402 (P > 0.05)	-0.567 (P < 0.05)	-0.754 (P < 0.01) ^d	-0.112 (P > 0.05)	-0.259 (P > 0.05)	0.554 (P < 0.05)	0.541 (P > 0.05)

^a The locations of soil samples; U soils located up-stream; M soils located mid-stream; D soils located down-stream.

^b N.D.: not detected.

^c The correlation coefficient represented the linear correlation between the total number of yellow colonies and the soil parameter listed in that column.

^d The analysis was conducted with the 9 paddy soils that yielded yellow colonies.

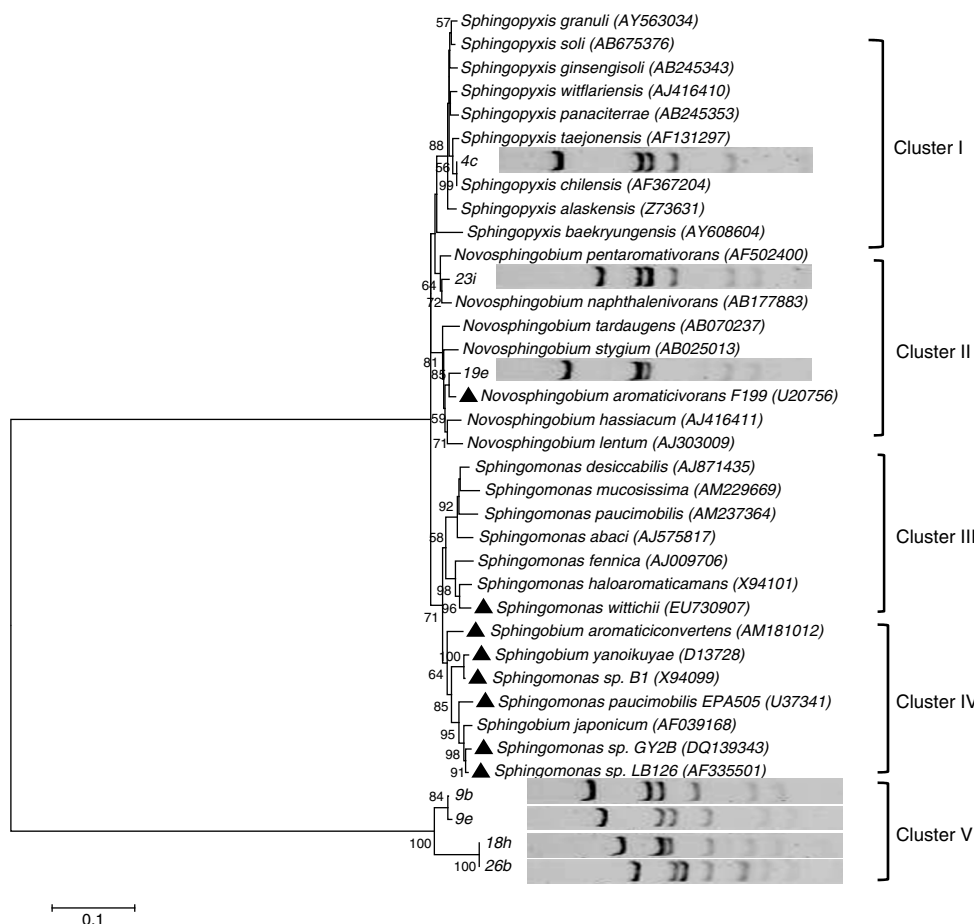


Fig. 1 – Phylogenetic tree based on the alignment of 16S rRNA sequences of seven isolates and related sequences of the genus *Sphingomonas* was constructed using the neighbor-joining algorithm. The scale bar represents 0.1 substitutions per base position. The numbers at the nodes indicate the percentages of occurrence in 500 bootstrapped trees. Species harboring PAH-degrading isolates are indicated by “▲”. The accession numbers for selected species, which were suggested by Yabuuchi et al.^{4,23} for genus *Sphingomonas* and include *Sphingomonas sensu stricto*, *Novosphingobium*, *Sphingobium*, and *Sphingopyxis*, at this time are given in parentheses.

yellow streptomycin-resistant CFUs with the increase in soil pH (the analysis was conducted with the 9 paddy soils that yielded yellow colonies) (Table 1). Thus, our data indicated that the culturable *Sphingomonas* preferred an acidic pH in petroleum-contaminated soils. Previous studies showed that *Sphingomonas* could survive in PAH-polluted soils with pH values ranging from 7.0 to 8.9,^{7,12,19,22} but the suitable soil pH for the removal of PAHs by *Sphingomonas* strains ranged between 6.0 and 7.0.⁶ This may explain to some extent why more culturable *Sphingomonas* were detected in soils with a relatively low pH value in this study. Nevertheless, the effect of soil pH on culturable *Sphingomonas* in petroleum-contaminated soils has not been previously reported. This finding raises further interest in the relationship between soil pH and the amounts of culturable *Sphingomonas* (especially the PAH-degrading isolates) in polluted soils.

Phylogenetic identification of isolates

Sixty streptomycin-resistant yellow colonies were randomly selected from the 12 petroleum-contaminated soils and

tested with *Sphingomonas* specific-PCR to exclude the non-*Sphingomonas* strains. A *Sphingomonas* species type strain *Sphingomonas paucimobilis* (1.3773, China General Microbiological Culture Collection Center) was used as the positive control. Simultaneously, 12 randomly chosen non-yellow pigmented colonies with different morphologies were tested together with the yellow colonies. No positive signals were displayed when *Sphingomonas* specific-PCR was performed on these non-yellow pigmented colonies (data not shown). A total of 48 out of the 60 yellow colonies produced amplicons of the expected size, indicating that the streptomycin medium together with the yellow pigment was only a semi-selective method for the identification of *Sphingomonas* species. The combination of this phenotypic detection method with *Sphingomonas* genus-specific PCR allowed genotypic confirmation of the yellow pigmented colonies, thereby facilitating the specific detection and isolation of culturable *Sphingomonas* from soil samples.

The 48 amplicons could be divided into 8 different phylotypes according to the RFLP analysis (Fig. 1). Sequence analysis showed that no sequence differences existed between the two representative strains from each phylotype; thus, only one

Table 2 – Degradation rate of eight PAHs by *Sphingomonas* strains.^a

PAH	% Degradation						
	4c	23i	9b	18c	10c	19e	9e
FLO	87.2 ± 3.8	85.4 ± 4.5	74.9 ± 4.7	83.0 ± 5.4	68.7 ± 3.1	63.2 ± 1.9	N.G.
PHE	64.8 ± 7.7	60.6 ± 6.3	N.G.	N.G.	N.G.	N.G.	N.G.
NA	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
ACE	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
ANT	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
FLU	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
PYR	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
BaP	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.

NA – naphthalene; ACE – acenaphthene; FLO – fluorene; PHE – phenanthrene; ANT – anthracene; FLU – fluoranthene; PYR – pyrene; BaP – benzo[a]pyrene; N.G. – no growth.

^a The presented degradation rate was calculated after 7 days of incubation on a rotary shaker at 30 °C, and the initial concentration of the eight PAHs was 200 µg ml⁻¹ of NA, ANT, FLU, PHE, ANT, and FLU, 100 µg ml⁻¹ of PYR, and 50 µg ml⁻¹ of BaP.

strain was selected for further analysis. The results showed that 7 phylotypes matched significantly (99–100% similarities) with the *Sphingomonas* 16S rRNA gene sequences in GenBank. Of these 7 *Sphingomonas* phylotypes, two representative strains (9b and 9e) both shared similarities of 99% with *Sphingomonas aromaticivorans* (currently nominated as *Novosphingobium aromaticivorans*); however, RFLP analyses indicated that they were different strains (Fig. 1). One phylotype (2 out of the 48 total streptomycin-resistant yellow colonies) was not affiliated with *Sphingomonas*, but with *Erythrobacter*. It is not unexpected that *Erythrobacter* was detected together with *Sphingomonas* using the phenotypic and genotypic methods developed in this study because streptomycin resistance and yellow pigmentation have been reported previously for *Erythrobacter* strains (i.e., *E. litoralis*).¹² Additionally, both *Sphingomonas* and *Erythrobacter* belong to the family *Sphingomonadaceae*, and the sequences of primer set SA429f/933r showed 100% similarity to the 16S rRNA gene sequences of *Erythrobacter longus* (data not shown). Thus, it can be inferred that the phenotypic and phylo-methods developed in the present study that aimed to specifically isolate *Sphingomonas* members could inevitably select a minor fraction of other groups from *Sphingomonadaceae*.

The phylogenetic tree (Fig. 1) constructed by aligning the isolated *Sphingomonas* sequences with sequences of typical species of genus *Sphingomonas* showed that only 3 representative strains (23i, 4c and 19e) clustered with known cultured *Sphingomonas* sequences. Strain 19e was closely related to *Novosphingobium aromaticivorans* F199, which could utilize fluorene and naphthalene as the sole carbon source²⁴; this result suggested that strain 19e might have the capability for PAH degradation. The other 4 isolated strains (9b, 9e, 18h and 26b) were not placed in groups with known *Sphingomonas* but were grouped in a single cluster, indicating that they might represent new members of the genus *Sphingomonas*. Further analyses are needed to determine their physiological and biochemical characteristics.

Utilization of PAHs as a sole source of carbon and energy

Seven *Sphingomonas* isolates representing 7 phylotypes were assessed for their ability to use 8 different individual PAHs as

their sole carbon and energy source in liquid culture. Growth was considered positive when increases in turbidity were observed; positive growth responses were confirmed by analyzing the disappearance of the PAH with HPLC. Increases in turbidity and the disappearance of fluorene were observed in six of the seven tested strains (9b, 26b, 4c, 18h, 19e and 23i). Measurements of the degradation rates showed that *Sphingomonas* 4c could degrade 87.2% of the fluorene within 7 days at 30 °C when the initial concentration of fluorene was 100 mg L⁻¹. Significant variation ($P < 0.01$) in the fluorene degradation rate was observed among the 6 strains (Table 2). Additionally, strain 4c and strain 23i were capable of growing on phenanthrene (Table 2). Under the same incubation conditions, *Sphingomonas* 4c and *Sphingomonas* 23i could remove 64.8% and 60.6% of phenanthrene in the liquid culture, respectively. Overall, *Sphingomonas* strain 4c showed a capability to degrade fluorene and phenanthrene with a relatively high efficiency, indicating that it might serve as a promising functional strain for field studies of in situ bioremediation.

The selected *Sphingomonas* isolates all failed to grow on the media supplemented with the other six PAH compounds as the sole carbon sources (Table 2). Although only 6 PAH-degrading *Sphingomonas* strains were obtained in the study, based on the fact that most of the bacteria were uncultured and the limitations of the isolation method it is reasonable to deduce that the diversity of PAH-degrading *Sphingomonas* strains in petroleum-contaminated soils is higher than observed in the present data.

The 6 PAH-degrading strains originated from 8 soil samples (A, C, D, H, I, K, L and O) with different contamination levels. Despite the fact that all of these soils contained a relatively high ratio of high molecular weight (HMW) PAHs to TPH (Table 1), none of the strains isolated in this study could utilize HMW-PAHs. The probable reason for this result may be explained by three facts. First, several studies have indicated that *Sphingomonas* are more adapted to the degradation of PAHs with relatively high bioavailability, such as phenanthrene.^{5,25,26} Second, the co-existence of bacteria/bacteria or bacteria/fungi known catabolic cooperation is ubiquitous in soil. Thus, we may speculate that *Sphingomonas* may be involved in HMW-PAH degradation by living on the metabolites of other bacteria or fungi rather than

on the HMW-PAH itself. In the present study, degradation of HMW-PAHs was performed by pure strains but not by mixed cultures, which may provide a reduced chance of isolating HMW-PAH-degrading *Sphingomonas* strains. Last, our previous result suggested that the uncultivable *Sphingomonas* strains were the dominant ones in these contaminated soils¹¹; hence, the uncultured *Sphingomonas* strains might play important roles in the degradation of HMW-PAHs.

Genetic analysis of several PAH-degrading pathways in *Sphingomonas* strains revealed the presence of a unique group of genes for aromatic compound degradation that were distantly related to those in other genera.²⁴ Additionally, the catabolic genes from fluorene-utilizing *Sphingomonas* were unlike the other degradative genes involved in the degradation of other PAH components.²⁷ The protocatechuic acid catabolic gene clusters found in the fluorene-degrading *Sphingomonas* sp. strain LB126 displayed a strong similarity with genes found in the lignin-degrading *Sphingomonas paucimobilis* strain SYK-6.^{24,27} However, little information is available concerning the identification of the dioxygenase genes involved in the first step of PAH degradation in *Sphingomonas* sp. LB126 or other *Sphingomonas* strains.²⁸ Notably, the strains 9b and 9e were both identified as *Sphingomonas aromaticivorans*; however, only 9b (but not 9e) could degrade fluorene. It should be noted that not all *Sphingomonas* strains have the ability to degrade PAHs. This may reflect an ability of some *Sphingomonas* strains to more effectively recruit PAH-degrading genes as an adaptive response in PAH-contaminated soils.²⁹ Moreover, this finding could indicate that different strains, even if they belong to the same species, might represent different functional groups. With the development of comparative genomics, similarities and differences between the genomes of strains 9b and 9e could be compared to reveal how the degrading genes were obtained or lost.

Conflict of interest

The authors declare that there is no conflicts of interest.

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