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Biodegradation of Phenanthrene in Polycyclic Aromatic Hydrocarbon-Contaminated Wastewater Revealed by Coupling Cultivation-Dependent and -Independent Approaches

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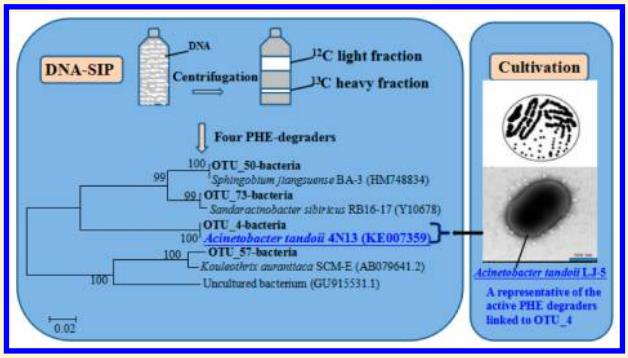
Supporting Information

ABSTRACT: The indigenous microorganisms responsible for degrading phenanthrene (PHE) in polycyclic aromatic hydrocarbons (PAHs)-contaminated wastewater were identified by DNA-based stable isotope probing (DNA-SIP). In addition to the well-known PHE degraders *Acinetobacter* and *Sphingobium*, *Kouleothrix* and *Sandaracinobacter* were found, for the first time, to be directly responsible for indigenous PHE biodegradation. Additionally, a novel PHE degrader, *Acinetobacter tandoii* sp. LJ-5, was identified by DNA-SIP and direct cultivation. This is the first report and reference to *A. tandoii* involved in the bioremediation of PAHs-contaminated water. A PAH-RHD$_\alpha$ gene involved in PHE metabolism was detected in the heavy fraction of ^{13}C treatment, but the amplification of PAH-RHD$_\alpha$ gene failed in *A. tandoii* LJ-5. Instead, the strain contained catechol 1,2-dioxygenase and the alpha/beta subunits of protocatechuate 3,4-dioxygenase, indicating use of the β-ketoadipate pathway to degrade PHE and related aromatic compounds. These findings add to our current knowledge on microorganisms degrading PHE by combining cultivation-dependent and cultivation-independent approaches and provide deeper insight into the diversity of indigenous PHE-degrading communities.



1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of hydrophobic organic compounds with fused aromatic rings that are generated from natural and anthropogenic processes and pose a serious threat to the health of all organisms.[1,2] Because of their high toxicity, mutagenicity and carcinogenicity, the United States Environmental Protection Agency has classified PAHs as priority pollutants since the 1970s.[3] Bioremediation has proven to be a cost-effective and environmentally friendly alternative to remove PAHs from contaminated sites.[4,5]

Considerable effort based on traditional cultivation-dependent approaches has focused on isolating and identifying cultivable PAHs degraders to explore the fate of PAHs. Hitherto, many microorganisms capable of degrading PAHs have been isolated and evaluated, most of which belong to the genera *Paenibacillus*,[6] *Burkholderia*,[7] *Stenotrophomonas*,[8] *Acinetobacter*,[9] *Alcaligenes*,[10] *Mycobacterium*,[11] *Arthrobacter*,[12] *Flavobacterium*,[12] *Aeromonas*,[12] *Vibrio*,[12] *Bacillus*,[13] *Micrococcus*,[13] *Nocardioides*,[13] *Marinobacter*,[13] *Pseudomonas*,[14] *Cyclotrophicus*,[15] *Sphingomonas*,[16] *Tistrella*,[16] and *Collimonas*.[17] Cultivation-based approaches provide clues about PAHs degraders and degradation pathways. Furthermore, functional genes associated with PAHs degradation, such as PAH-ring hydroxylating dioxygenases (PAH-RHDs)[18,19] and PAH-ring cleaving dioxygenases (PAH-RCDs, including catechol dioxygenase [CAT][20,21] and protocatechuate dioxygenase [PACH][22−24]), have been identified. However, it is difficult to obtain all the PAHs-degrading isolates in nature as the majority of microbes are uncultivable,[25] and this method greatly underestimates prokaryotic diversity.[26] In addition, cultivation-based method fails to explain the complex interactions among individuals within microbial communities in their native environment.[27]

Cultivation-independent methods, which can be used to effectively evaluate the prokaryotic diversity of complex systems,[28,29] have been used to evaluate microbial degradation of PAHs.[30−33] High-throughput methods have revolutionized the ability to investigate deeper into the microbial communities in environmental samples by providing higher resolution of microbial taxa compared with conventional cloning techniques.[34] However, these methods suffer from the inaccurate identification of the metabolic or functional features of the

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Table 1. Primers Used for the PCR of 16S rRNA, PAH-RHD and PAH-RCD Gene

target	primer	sequence (5′-3′)
16S rRNAs	515f	GTGCCAGCMGCCGCGGTAA
	806r	AACGCACGCTAGCCGGACTACVSGGGTATCTAAT
	27f	AGAGTTTGATCCTGGCTCAG
	1492r	GGTTACCTTGTTACGACTT
PAH-RHD	610f	GAGATGCATACCACGTKGGTTGGA
	911r	AGCTGTTGTTCGGGAAGAYWGTGCMGTT
	641f	CGGCGCCGACAAYTTYGTNGG
	933r	GGGGAACACGGTGCCRTGDATRAA
PAH-RCD	CAT1f	ATGTCGATACCGCACAAGGA
	CAT1r	TGCACGACGACGATCAACT
	CAT2f	CGCGACGACGATCTACTTCA
	CAT2r	CTGCAACTGGTCCTGTCGAT
	PACH1f	ACGCACAACGCAATACCGAT
	PACH1r	ACGACCACGCAAAGTGATGT
	PACH2f	TGAAACTCCATCTCAAACAGGTG
	PACH2r	ACTGTTTCGTCTTCGCCTTGT

targeted microorganisms.[34] Stable-isotope probing (SIP) is a cultivation-independent technique that circumvents the requirement of distinguishing microorganisms to assess metabolic responses and links identity to function.[35] It has been successfully used in environmental samples by feeding microbial communities stable isotope-labeled substrates (^{13}C or ^{15}N) to label the intracellular components (DNA, RNA, or proteins) and allowing the separation and characterization of the targeted but hidden functional microorganisms according to buoyant density, particularly those not amenable to cultivation.[17] To date, SIP has been used to identify a large number of PAHs-degrading bacteria.[27,32,33,36]

Phenanthrene (PHE) is a PAHs model compound used in biodegradation studies due to its ubiquity in nature and fused-ring angular structure.[13,17] A number of PAHs degraders have been identified in real-world habitats, such as soil or seawater, using DNA-SIP.[27,31,36−38] However, only a few investigators have successfully isolated the microbes belonging to the SIP-identified microorganisms,[31] leaving the gap of revealing their metabolic characteristics and exploring the functional populations actually responsible for pollutant degradation in situ. In the present study, DNA-SIP was applied to wastewater samples to link the indigenous bacterial taxa with their PHE biodegradation phenotypes. The PHE-degrading bacteria in wastewater microcosm were successfully characterized using DNA-SIP and the high-throughput sequencing was applied for more complete understanding of the bacterial communities contributing to PHE degradation. Additionally, a representative active PHE degrader (*Acinetobacter tandoii* LJ-5) was successfully isolated from the indigenous wastewater microbial community with a cultivation-based method in parallel. To explore the environmental significance of this strain, we focused on the functional genes encoding the alpha subunit of PAH-RHD (PAH-RHD$_\alpha$) and PAH-RCD (CAT and PACH) involved in PHE metabolism, by analyzing relevant sequences amplified from the ^{13}C-DNA-enriched fraction and *A. tandoii* LJ-5 DNA. We hope to provide novel information on the bioremediation of PAHs-contaminated wastewater, and potentially soils and sediments using a reliable theoretical basis.

2. MATERIALS AND METHODS

2.1. Sample Collection. Water sample (~10 L) was collected from untreated industrial wastewater at an oil refinery (37°49′N, 118°25′E; altitude, 37.49 m) located in Shandong Province, China, in September 2015. This wastewater was not yet handled with onsite wastewater treatment systems and directly discharged. After transport to the laboratory at 4 °C, a portion of the samples was stored at −20 °C for subsequent DNA extraction. The remaining samples were immediately stored at 4 °C within 24 h before PHE degradation and SIP experiments. The PAHs identified in the wastewater are listed in Supporting Information (SI) Table S1 (determined using gas chromatography−mass spectrometry as described below).

2.2. SIP Experiment. *2.2.1. SIP Microcosms.* The microcosm was constructed in a 150 mL serum bottle containing 50 mL of wastewater sample. Unlabeled PHE (99%; Cambridge Isotope Laboratories, Inc., Tewksbury, MA) or ^{13}C-labeled PHE ($^{13}C_{14}$−PHE, 99%; Cambridge Isotope Laboratories, Inc., Tewksbury, MA) at a final concentration of 10 mg/L was added to a bottle with a rubber stopper and an aluminum cap using a gastight syringe. Microcosms without PHE were used as the non-PHE control. The treatments with unlabeled PHE in filter-sterilized wastewater were used as the sterile control, which confirmed whether PHE degradation was attributed to biological activities in the biotic treatments. Each treatment was conducted in triplicates. All the microcosms were incubated in the dark with shaking at 120 rpm and room temperature (~25 °C). The serum bottles were opened each day for approximately 1 h in sterile hood to maintain the ambient oxygen level, as there was little loss of PAHs at the background concentrations in the sterile controls (data not shown). From the analysis of residual PHE on day 3 and day 6, all the PHE was completely degraded on day 6 (data not shown), and the samples were therefore taken on day 3 from each treatment for PHE analysis and DNA extraction.

2.2.2. Nucleic Acid Extraction and Ultracentrifugation. After centrifuging 50 mL of each water sample from the ^{12}C-PHE and ^{13}C-PHE treatments, total nucleic acids were extracted from the resulting cell pellets using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions.[39] DNA content was quantified

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using the ND-2,000 UV−vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Approximately 5 μg DNA was added to Quick-Seal polyallomer tubes (13 × 51 mm, 5.1 mL; Beckman Coulter, Pasadena, CA) and mixed with Tris-EDTA (pH 8.0)-CsCl solution at a final buoyant density (BD) of ∼1.77 g/mL. The BD was determined using a digital refractometer (model AR200; Leica Microsystems Inc., Buffalo Grove, IL). After balancing, the tubes were heat sealed and transferred to an ultracentrifuge (Optima L-100XP, Beckman Coulter) at 45 000*g* (20 °C) for 48 h. Subsequently, DNA in the tube was fractioned (400 μL each) and collected using a fraction recovery system (Beckman Coulter). After the BD measurements, the DNA fractions were purified using the method described by Sun et al.[40] The relationships between BD and the fraction number or DNA concentration are listed in SI Figure S1 and Figure S2, respectively.

2.2.3. High-Throughput Sequencing and Computational Analyses. Sequencing was conducted using an Illumina MiSeq sequencer with the standard pipeline and generated paired-end reads of 250 bp. The V4 hypervariable region of bacterial 16S rRNA in fractions derived from the ^{12}C-PHE and ^{13}C-PHE microcosms was amplified using the 515f/806r primer set (Table 1), with a sample-specific 12-bp barcode added to the reverse primer as described by Liu et al.[41] Reads were filtered if they contained primer mismatches, uncorrectable barcodes or ambiguous bases. Then, the qualified sequences were analyzed using the MOTHUR software package.[42,43] Sequences were assigned using an operational taxonomic unit (OTU)-based method to generate microbiome profiles.[44−46] OTU assignment based on 97% cutoff and the total number of 20503 sequences were used for subsampling in the present study.

The relative abundance of each OTU was determined as described previously.[40] In total, 4186 OTUs were detected in all samples, and those with the top 100 relative abundances were selected for analysis. Bacteria represented by OTUs that were enriched in the heavy fractions from ^{13}C-PHE treatments compared with ^{12}C-PHE treatments were involved in PHE degradation. Finally, four OTUs (OTU_4, OTU_50, OTU_57, and OTU_73) were selected and aligned to *Acinetobacter* spp., *Sphingobium* spp., *Kouleothrix* spp. and *Sandaracinobacter* spp. (accession numbers: KX364043−KX364046), respectively, using the Greengenes database (version 13.8).[47,48] The phylogenetic information from the sequences was analyzed using the Basic Local Alignment Search Tool algorithm (National Center for Biotechnology Information, Bethesda, MD, USA) and MEGA ver. 4.0.[49]

2.3. Isolation of PHE Degraders by Enrichment and Cultivation. Raw wastewater (5 mL) was added to 50 mL minimal medium (MM) (SI Table S2, pH 7.0) with 1000 mg/L PHE (MM-P) as the carbon source. After a 25 °C incubation for 7 days, 5 mL of the culture medium were subcultured in 50 mL fresh MM-P medium and incubated under the same conditions for another 7 days. After three sequential rounds of enrichment, the enriched population was serially diluted and spread on MM-P agar. The plates were incubated at 25 °C for 4 days. In total, 10 colonies were isolated, purified by recultivation and identified. The growth curves were constructed, and the PHE degradation efficiencies of the isolated strain were evaluated in MM supplemented with different concentrations of PHE (100−1000 mg/L) in the dark for 7 days on a 180 rpm shaking plate at 30 °C. PHE degradation was determined using the method described in Section 2.5. After extraction of genomic DNA, the 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR) using the 27f and 1492r bacterial universal primers (Table 1).[50] The PCR products were gel-purified using a gel extraction kit (D2500-01; Omega Biotek, Norcross, GA), followed by cloning and sequencing as described by Li et al.[51] In addition, the morphological and physiological characteristics, GC content and DNA−DNA hybridization of the isolated strain were determined using previously described methods.[51] Growth conditions under different pH, temperature and salinity were set up following previously reported methods.[51] In order to obtain the optimal growth curve and PHE degrading efficiency of the isolated strain, cell counts were adjusted to approximately 1 × 10^7 colony forming units/mL at the beginning of the experiment using the dilution plate counting method.[52] Cell growth was evaluated by measuring the optical density of the culture at 600 nm (OD_{600}) after incubation for 18 h. Sterile controls without cells were also established. All tests were performed in triplicates, using the same standard and incubation conditions as those used for the microcosm experiment.

2.4. Detection of PAH-RHD and PAH-RCD Genes. The PAH-RHD$_\alpha$ gene in the heavy DNA fraction was investigated using two primer sets in Gram positive and Gram negative (GN) degraders, 641*f*/933r[53] and 610*f*/911r,[53] respectively (Table 1). Gradient PCR was performed at annealing temperatures of 52−62 °C.[54] However, only the PAH-RHD$_\alpha$ GN primer set produced a specific amplicon and was selected for this study. The amplification reactions were conducted according to previous methods.[54] The PAH-RCD genes (CAT and PACH) were amplified using the CAT1f/CAT1r, CAT2f/CAT2r, PACH 1f/PACH1r, and PACH 2f/PACH2r primer pairs listed in Table 1. All specific primer sets were designed based on published sequences of *A. tandoii* DSM 14970^T (GenBank assembly accession number: GCA_000400735.1) using Primer Premier 5.0 software. The CAT1f/CAT1r and CAT2f/CAT2r primer pairs were used to target two different types of CATA. The PCR program for these two primer sets was as follows: 3 min at 95 °C; 32 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 55 s; final extension at 72 °C for 5 min. The PACH 1f/PACH1r and PACH 2f/PACH2r primer pairs were used to target the alpha and beta subunits of PACH, respectively. The PCR program for PACH 1f/PACH1r and PACH 2f/PACH2r was as follows: 3 min at 95 °C; 32 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 40 s; final extension at 72 °C for 5 min. The PCR products were gel-purified using a gel extraction kit (D2500-01; Omega Biotek, Norcross, GA), followed by cloning and sequencing as described by Song et al.[55] The phylogenetic dendrograms were prepared using the method described above.

The partial PAH-RHD and PAH-RCD gene sequences obtained are available in GenBank with the following accession numbers: KX364042 for PAH-RHD, KX364048 and KX364049 for CATA and KX364050 and KX364051 for PACH. The GenBank accession number for the 16S rRNA gene obtained from isolated *A. tandoii* is KU168603.

2.5. PHE Analysis. The PHE concentrations in each microcosm treatment were analyzed on days 0 and 3 as follows. The water sample was spiked with 1000 ng deuterated PAHs as a surrogate standard and was extracted twice with dichloromethane (DCM). The extracted organic phase was concentrated to approximately 0.5 mL after solvent exchange with hexane and then purified using a silica gel/alumina column (8

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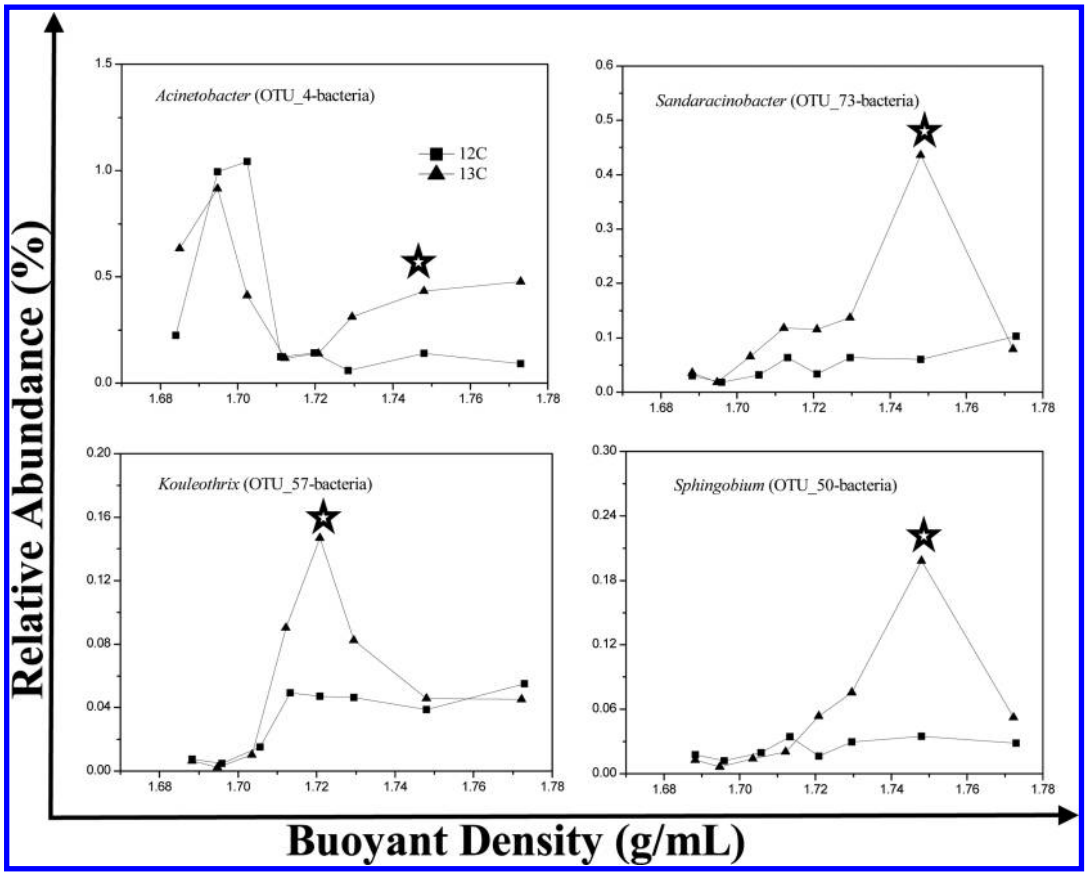

Figure 1. Shift tendency of OTU_4, OTU_50, OTU_73, and OTU_57 fragments. The relative abundance of the OTU_4, OTU_50, OTU_73, and OTU_57 fragments is in the fractions of different buoyant density (BD) of DNA extracted from the wastewater amended with either ^{12}C- or ^{13}C-labeled PHE.

mm i.d.) filled with anhydrous Na_2SO_4 (1 cm), neutral silica gel (3 cm, 3% deactivated) and neutral alumina (3 cm, 3% deactivated) from top to bottom, using 15 mL hexane/DCM (1:1, v/v) as the eluent. After concentrating the eluent to approximately 50 μL using a gentle stream of N_2, 1000 ng hexamethylbenzene were added as an internal standard to all samples before the instrumental analysis. The components of deuterated PAHs, standards and internal standard are listed in SI Table S3.

PHE was analyzed by gas chromatography (model 7890; Agilent Technologies, Santa Clara, CA), using a capillary column (DB-5MS; 30 m × 0.25 mm, 0.25 μm) and a mass spectrometric detector (model 5975; Agilent) as described by Jiang et al.[17]

3. RESULTS

3.1. PHE Biodegradation in Wastewater. PHE biodegradation in the ^{12}C-PHE and ^{13}C-PHE microcosms is shown in SI Table S4. The recovery rates of PHE during the extraction procedure were 70−85% in this study. The PHE concentration in the sterile treatment exhibited less decreases than those in the biotic treatments. Residual PHE was 11−13% and 12−13% in the ^{12}C-PHE and ^{13}C-PHE microcosms, respectively, suggesting that PHE biodegradation occurred in the biotic treatments. No significant difference ($p > 0.05$) was observed between the ^{12}C-PHE and ^{13}C-PHE treatments, consistent with our previous study.[55]

3.2. Bacteria Involved in PHE Degradation As Revealed by DNA-SIP. DNA extracted from the ^{12}C-PHE and ^{13}C-PHE microcosms was subjected to ultracentrifugation and fractionation, followed by high-throughput sequencing of each fraction. The relative abundance of the total 16S rRNA defined by the phylum, family and genus showed slight difference in the indigenous microbial communities between the samples from the ^{12}C-PHE and ^{13}C-PHE treatments (SI Figure S3).

The indigenous microorganisms responsible for ^{13}C assimilation were detected by assessing the relative abundances of specific OTUs in the ^{12}C-PHE and ^{13}C-PHE samples from each fraction. The results indicated that OTU_4 at a higher BD (>1.7209 g/mL) was enriched only in the ^{13}C-PHE sample, but not in the ^{12}C-PHE sample (Figure 1). Additionally, the relative abundances of OTU_50, OTU_73 and OTU_57 at higher BDs (>1.7209, > 1.7296 and 1.7122−1.7481 g/mL, respectively) were also higher in the ^{13}C-PHE samples than those in the ^{12}C-PHE samples. Comparing to the relative abundances of OTU_4, OTU_50, OTU_57, and OTU_73 in the same fractions of ^{12}C-PHE sample (0.13%, 0.03%, 0.04%, and 0.06%, respectively), the higher abundance in the heavy fractions from ^{13}C-PHE sample (0.43%, 0.20%, 0.15%, and 0.44%, respectively) indicated that microorganisms represented by OTU_4, OTU_50, OTU_73, and OTU_57 played a primary role in PHE degradation.

Figure 2 shows phylogenetic information for the PHE degraders represented by the above OTUs. OTU_4 belonging to the genus *Acinetobacter* (phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Pseudomonadales*, family *Moraxellaceae*) shared 100% similarity to partial 16S rRNA gene

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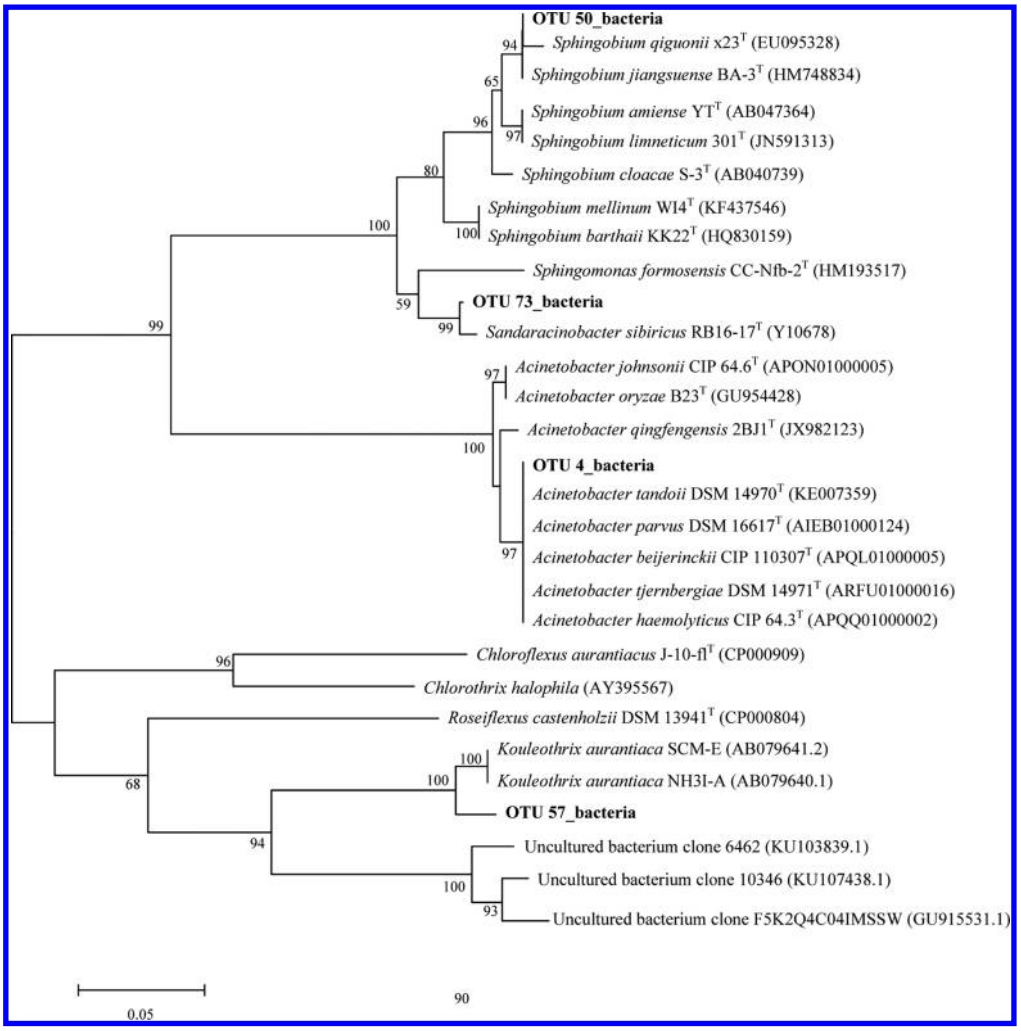

Figure 2. Phylogenetic tree of identified OTUs responsible for PHE degradation. Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of the bacteria corresponding OTU_4, OTU_50, OTU_57, OTU_73 and their representatives of some other related taxa. Bootstrap values (expressed as percentages of 1200 replications) > 50% are shown at the branch points. Bar 0.05 substitutions per nucleotide position.

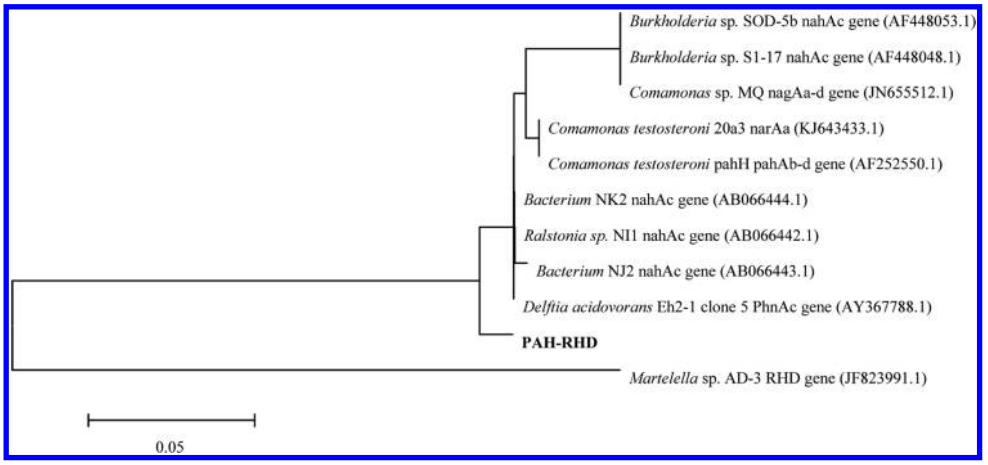

Figure 3. Phylogenetic analysis of ofamplified PAH-RHD$_\alpha$ GN gene based on the amino acid sequences from heavy fraction in ^{13}C-PHE microcosm. PAH-RHD$_\alpha$ gene shows 97% similarity with *Delftia acidovorans* Eh2-1 clone 5 *phnAc* gene.

sequence with strains *A. tandoii* DSM 14970^T (KE007359), *Acinetobacter parvus* DSM 16617^T (AIEB01000124), *Acinetobacter beijerinckii* CIP 110307^T (APQL01000005), *Acinetobacter tjernbergiae* DSM 14971^T (ARFU01000016), and *Acinetobacter hemolyticus* CIP 64.3^T (APQQ01000002) and formed a subclade with a high bootstrap value of 97. OTU_50 and OTU_73 were assigned to the genera *Sphingobium* and *Sandaracinobacter* within the same family *Sphingomonadaceae*

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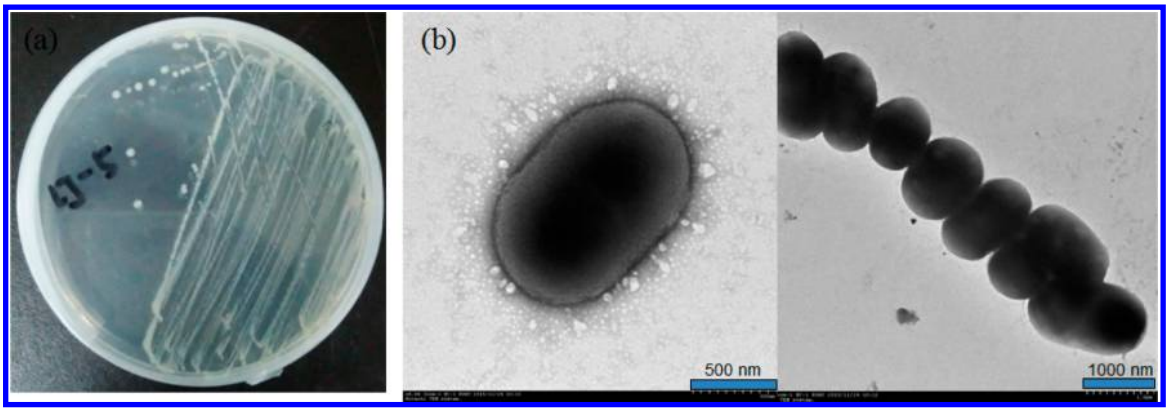

Figure 4. (a) Isolated *A. tandoii* LJ-5 colonies on MM-P agar plate; (b) Transmission electron micrograph of *A. tandoii* LJ-5 cells. Bar, 500 nm (left) and 1000 nm (right).

(phylum *Proteobacteria*, class *Alphaproteobacteria*, order *Sphingomonadales*), and they shared 100% similarity with *Sphingobium jiangsuense* BA-3T (HM748834) and 99% similarity with *Sandaracinobacter sibiricus* RB16-17^T (Y10678), respectively. OTU_57 was classified in the genus *Kouleothrix* (phylum *Chloroflexi*, class *Chloroflexi*, order *Roseiflexales*, family *Kouleothrixaceae*) and shared 97% similarity with *Kouleothrix aurantiaca* SCM-E (AB079641.2) and formed a subclade with a high bootstrap value of 100.

3.3. Presence of the PAH-RHDα Genes in the SIP Fractions. The PAH-RHD$_\alpha$ genes from GN bacteria were analyzed in the heavy fractions of ^{13}C-PHE treatment (marked with a star in Figure 1). In the present study, only one type of the PAH-RHD$_\alpha$ gene was detected, and showed 97% similarity with the PAH-RHD$_\alpha$ (PhnAc) gene of *Delftia acidovorans* Eh2-1 clone 5 (AY367788.1) (Figure 3).

3.4. Isolation and Characterization of PHE-Degrading Bacteria. We isolated PHE degraders from wastewater to characterize the bacteria corresponding to the four SIP-identified OTUs. Of all PHE-degrading isolates that belonged to the genus *Agromyces*, *Stenotrophomonas*, *Acinetobacter*, and *Pseudomonas*, only one bacterial strain, named *Acinetobacter tandoii* LJ-5, was identified in the contaminated wastewater samples after PHE enrichment. The partial 16S rRNA gene of this strain shared 100% similarity with the OTU_4 sequence and therefore belonged to the representative of the active PHE degraders linked to OTU_4. However, no strains belonging to the other three SIP-identified OTUs was successfully isolated from wastewater.

A. tandoii LJ-5 is a GN, rod-shaped, obligate aerobe lacking flagella, with a size of (0.7−1.0) × (1.0−1.5) μm (Figure 4). The *A. tandoii* LJ-5 colonies were circular, smooth, convex, and white pigmented with a colony diameter of 0.5−2.0 mm after growth on MM-P agar plates at 30 °C for 48 h. *A. tandoii* LJ-5 grew under different conditions, including 0−3% (w/v) salinity (optimum 0%), pH 5.0−9.0 (optimum pH 7.0) and temperatures of 25−40 °C (optimum 30 °C) (SI Figure S4). The metabolic characteristics of *A. tandoii* LJ-5 are listed in SI Table S5. *A. tandoii* LJ-5 has the highest nearly full 16S rRNA gene sequence similarity to that of *A. tandoii* DSM 14970^T (KE007359) (98.7%), whereas its similarity levels to other *Acinetobacter* strains are <97.0%. *A. tandoii* LJ-5 belongs to the genus *Acinetobacter* according to the neighbor-joining (SI Figure S5) phylogenetic dendrograms based on 16S rRNA gene sequences, and it forms a subclade with *A. tandoii* DSM 14970^T. The GC content of *A. tandoii* LJ-5 is 41.0 mol %, within the range of other *Acinetobacter* spp. (38.1−54.7 mol %). The DNA−DNA hybridization value for *A. tandoii* LJ-5 with *A. tandoii* DSM 14970^T is 90.11 ± 0.8%, which is significantly above the threshold value of 70% in the phylogenetic definition of a species.[56] Taken together, these results indicate that *A. tandoii* LJ-5 belongs to the species *A. tandoii*.

As shown in SI Figure S6, *A. tandoii* LJ-5 grew well in MM-P with PHE concentrations of 100−1000 mg/L under optimal growing conditions (pH 7.0 and 30 °C), suggesting strong tolerance of *A. tandoii* LJ-5 to high PHE concentrations. More than 60% of the PHE was biodegraded within 7 days at all PHE concentrations. Enrichment of *Acinetobacter* (OTU_4) was detected in the ^{13}C-PHE treatment, indicating that *A. tandoii* LJ-5 is a major indigenous PHE degrader.

3.5. Presence of PHE Metabolism-Related Genes in *A. tandoii* LJ-5. To further explore the environmental significance of *A. tandoii* LJ-5, we evaluated its functional genes involved in PHE metabolism. Although one PAH-RHD$_\alpha$ GN gene was detected in the heavy fraction of the ^{13}C-PHE sample, no PAH-RHD$_\alpha$ gene was successfully amplified from *A. tandoii* LJ-5 using the same primer set. However, the genes encoding CATA-1 and CATA-2 and the alpha and beta subunits of PACH-1 and PACH-2 were identified in *A. tandoii* LJ-5 in this study.

The CATA-1 (KX364048) and CATA-2 (KX364049) translated amino acid sequences showed high homology with those of CATA from *Acinetobacter junii* (WP_004961950.1, 92%) and *Acinetobacter schindleri* (WP 004809441.1. 93%), respectively, as illustrated in SI Figure S7a. SI Figure S7b shows the high homologies of *A. tandoii* LJ-5 PACH-1 (KX364050) and PACH-2 (KX364051) at the amino acid level compared with the alpha subunit of PACH from *Acinetobacter bouvetii* DSM 14963 (WP_005011151.1, 99%) and the beta subunit of PACH from *Acinetobacter johnsonii* XBB1 (WP_058952216.1, 99%) and *Bacillus mycoides* (WP_044740784.1, 99%).

4. DISCUSSION

Some studies have successfully applied DNA-SIP in the detection of indigenous microorganisms involved in PHE biodegradation.[27,36−38] Our study employed DNA-SIP and identified four OTUs directly responsible for indigenous PHE biodegradation, such as the phylotypes affiliated with *Acinetobacter*, *Sphingobium*, *Kouleothrix*, and *Sandaracinobacter* from PAHs-contaminated wastewater. Here, only rare species were enriched in the heavy fraction of ^{13}C-PHE microcosms, showing the low abundance of functional PHE degraders in

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water samples. Nevertheless, the results from SIP experiment confirm their roles in metabolizing PHE and it is the strong evidence for their primary roles in PHE metabolism. It should be noted that the majority of the abundant bacteria, especially members of the genus *Pseudomonas*, might play the roles in degrading other organic pollutants but are not directly linked to PHE degradation, since many other organic carbons existed in the wastewater and PHE was only one of them promoting the rare PHE-degrading species.

The genus *Sphingobium* was first described by Takeuchi,[57] and 41 species in this genus have been isolated and reported (http://www.bacterio.cict.fr/s/sphingobium.html). *Sphingobium* is a well-known PAHs-degrading genus in the family *Sphingomonadaceae*.[57,58] Some strains in this genus metabolize a wide range of PAHs, such as naphthalene, PHE, anthracene, fluoranthene, pyrene, and benzo[a]pyrene.[59−63] However, no study has used SIP to demonstrate the PHE-degradation capacity of indigenous *Sphingobium*. The genus *Sandaracinobacter* also belongs to the family *Sphingomonadaceae*. Until now, only one species (*Sandaracinobacter sibiricus)* has been isolated and reported in this genus.[64] The phylogenetic analysis of SIP-identified OTU_73 suggests its close relationship to *S. sibiricus* RB16−17^T (Figure 2). *S. sibiricus* is an obligate aerobic phototrophic bacterium that contains bacteriochlorophyll *a*, which is light-harvesting complex II and the reaction center.[64] This bacterium tolerates and reduces high levels of tellurite.[64] The phototrophic cyanobacteria are reported as suitable candidates in bioremediation of crude oil due to their strong growth ability under various conditions. Narro et al. reported that the cyanobacteria *Oscillatoria* sp. strain JCM could degrade naphthalene.[65] The other cyanobacterial species *Oscillatoria salina*, *Aphanocapsu* sp., and *Plectonema terenbans* were found to degrade crude oil.[66] However, they have not been linked previously to PHE degradation; thus, our present results provide a strong evidence that some microbes in this genus are primarily responsible for PHE degradation in wastewater.

The phylogenetic analysis of the indigenous microorganisms represented by OTU_57 suggested their close relationship to *Kouleothrix aurantiaca* SCM-E (Figure 2). *K. aurantiaca* SCM-E was first isolated by Kohno from activated industrial waste sludge.[67] The genus *Kouleothrix* belongs to phylum *Chloroflexi* (family *Kouleothrixaceae*, class *Chloroflexi*), which is one of the earliest diverging lineages of bacteria and was first defined by Garrity and Holt in Bergey's Manual of Systematic Bacteriology.[68] Class *Chloroflexi* is one of at least five major *Kouleothrix* subgroups, and all known species in this class have a multicellular filamentous morphology.[69] A number of studies have indicated that microorganisms in the phylum *Chloroflexi* are closely related to PAHs degradation. Shahi et al. showed that *γ-Proteobacteria*, *Chloroflexi*, *Firmicutes*, and *δ-Proteobacteria* were the most dominant bacterial phyla in petroleum-contaminated soil from a coastal site at an old petroleum sludge storage pit in Turkey.[70] Bacterial species belonging to *γ-Proteobacteria*, *δ-Proteobacteria*, and *Chloroflexi* change dramatically after the treatment with PAHs, indicating that PAHs play key roles in bacterial community diversity.[71] Muangchinda et al. reported that indigenous microbes from the phylum *Chloroflexi* degrade PAHs and provided bioremediation information for Antarctic soils and sediments,[72] although PAHs contaminants such as PHE and pyrene decreases the abundance of *Chloroflexi* during PAHs remediation.[73,74] However, *Kouleothrix* has not been linked previously to PHE metabolism; thus, it is unclear whether these microbes are directly involved in PHE degradation. Our results provide unequivocal evidence that some microorganisms in this taxa are primarily responsible for PHE degradation in the complex indigenous microbial community of PAHs-contaminated wastewater.

Acinetobacter, belonging to *γ-Proteobacteria* and to the order *Pseudomonadales*, is a GN, nonmotile and strictly aerobic bacteria. These bacteria are widespread in natural environments, including hydrocarbon-contaminated sites.[75,76] Members of *Acinetobacter* possess versatile metabolic capabilities, such as pathways for degrading aromatic and hydroxylated aromatic compounds.[77] Since the early days of taxonomic research, the ability to degrade aromatic compounds has been a common characteristic used to identify microbes in the genus *Acinetobacter*.[9,77,78] Hereinto, some strains metabolize PAHs, such as PHE, acenaphthene and pyrene.[79−82] Degradation of PHE by *Acinetobacter* has not been identified using DNA-SIP prior to this study. Our results demonstrate that indigenous bacterium *A. tandoii* LJ-5 metabolizes PHE. *A. tandoii* was first described by Emma et al. in 2003 but was not previously associated with PAHs degradation.[83] Our results provide *A. tandoii* LJ-5 reference data for application in PAHs-contaminated wastewater treatment.

The presence of the distinctive PAH-RHD$_\alpha$ GN gene in the heavy DNA fraction from the ^{13}C-PHE microcosm suggests it may be the functional genes associated with the PHE degrading strains of either *Acinetobacter*, *Sphingobium*, *Kouleothrix*, or *Sandaracinobacter* identified by SIP. Failure to amplify this PAH-RHD$_\alpha$ gene from *A. tandoii* LJ-5 might be attributed to (1) incompatibility of the primers used in this study with the functional genes present in this PHE degrader or (2) a different PHE degradation mechanism present in *A. tandoii* LJ-5. *Acinetobacter* genes that encode enzymes catabolising aromatic compounds are enriched in five genomic loci within 25% of the genome,[84] whereas the metabolic genes of other aromatic compound degraders, such as microbes in the genus *Sphingomonas* or *Pseudomonas,* are scattered throughout their genome.[85,86] The mechanism is unclear, but some preliminary evidence suggests that syntenic localization of the genes associated with this metabolic pathway relieves the energy burden on the transcriptional and translational machinery.[87] Metabolism of many aromatic compounds produces the intermediate metabolites catechol and protocatechuate via the *β*-ketoadipate pathway. In the present study, we found that *A. tandoii* LJ-5 expresses genes involved in two parallel branches of the *β*-ketoadipate (ortho) pathway (CATA and PACH).[88] The enzyme protocatechol 3,4-dioxygenase cleaves the aromatic ring to form the intermediary protocatechol. It contains the Fe (III) as a prosthetic group and many subunits α and β form different quaternary structures $(\alpha\beta)_n$, where n is a number varying between 3 and 12.[89] Some microorganisms, such as *Acinetobacter lwoffii*[90] and *Pseudomonas aeruginosa*,[23] can produce PACH for the biodegradation of organic compounds. The presence of PACH suggests that *A. tandoii* LJ-5 degrades PAHs and related aromatic compounds via the ortho-cleavage pathway for compounds funnelled through protocatechuate (via PACH).[22] Catechol 1,2-dioxygenase involved in ortho-cleavage pathway is widespread in microorganisms.[91] The initial enzyme of the *β*-ketoadipic acid pathway is responsible for the microbial degradation of aromatic compounds, belonging to the class of enzymes that cleave the aromatic ring to form the intermediary catechol.[92] Successful amplification of CATA also indicates that *A. tandoii* LJ-5 metabolizes catechol through the catechol branch of the ortho-cleavage pathway.[93] Previous

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studies have suggested that the CATA route is preferred under low-contamination conditions.[94,95] The presence of ortho-cleavage for catechol probably helps *A. tandoii* LJ-5 in metabolizing PAHs in the present PAHs-contaminated wastewater.

It is interesting to point out that the beta subunit of PACH of *A. tandoii* LJ-5 also groups with those in *Bacillus mycoides*, which is a non-*Acinetobacter* strain. Horizontal gene transfer (HGT) explains how bacteria acquire DNA from foreign species beyond the host range of mobile genetic elements or bacteriophages.[96] Our results hinted the occurrence of HGT in the present PAHs-contaminated wastewater. HGT has been reported for many genera of bacteria, including *Acinetobacter* and *Bacillus*.[96] It is also identified recently as a vital component in the formation of biofilm.[97] However, the extent and role of HGT by bacteria in natural environment and nutrient-limited habitats remains to be fully understood.

This is the first study to apply a culture-independent DNA-SIP technique to identify the bacterial taxa responsible for PHE degradation in PAHs-contaminated wastewater. The results provide unequivocal evidence that *Acinetobacter*, *Sphingobium*, *Kouleothrix*, and *Sandaracinobacter* are involved in biodegradation of PHE in wastewater, none of which has been previously reported as indigenous PHE-degrading microorganisms using SIP. *Sandaracinobacter* and *Kouleothrix* have not been previously linked to PHE degradation. Moreover, given that few bacteria linked to PHE metabolism have been isolated from real-world habitats,[31] this study identified *A. tandoii* LJ-5 as a PHE degrader by DNA-SIP and revealed its functions by characterizing the functional PHE metabolic genes and pathways. This is the first report of the role of *A. tandoii* in bioremediation of PAHs-contaminated water. These results expand our current knowledge on indigenous microorganisms that degrade PHE by combining both cultivation-dependent and cultivation-independent approaches.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04366.

(Table S1) Concentrations of PAHs in wastewater; (Table S2) the components of minimal medium; (Table S3) The components of deuterated PAHs, standards and internal standard; (Table S4) residual PHE percentage in wastewater after 3 days of incubation; (Table S5) metabolic characteristics of *A. tandoii* LJ-5; (Figure S1) correlation between fraction number and buoyant density (g/mL) from DNA extracted from water samples in ^{12}C-PHE and ^{13}C-PHE treatments; (Figure S2) correlation between DNA concentration (ng/μL) and buoyant density (g/mL) from DNA extracted from water samples in ^{12}C-PHE and ^{13}C-PHE treatments; (Figure S3) relative abundance of 16S rRNA defined taxa by phylum (a), family (b) and genus (c) in ^{12}C-PHE and ^{13}C-PHE treatments; (Figure S4) growth curve of *A. tandoii* LJ-5 under different pH, temperature and salinity conditions; (Figure S5) neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of *A. tandoii* LJ-5 and representatives of other related taxa; (Figure S6) growth curve and PHE degrading efficiency of *A. tandoii* LJ-5 in mineral medium supplied with different concentrations of PHE as carbon source; (Figure S7) phylogenetic tree based of CATA and PACH sequences from strain LJ-5. There are 16 pages, 5 tables and 7 figures (PDF)

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The authors declare no competing financial interest.

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