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In situ biodegradation of phenanthrene in polycyclic aromatic hydrocarbon-contaminated sewage water revealed by coupling cultivation-dependent and -independent approaches

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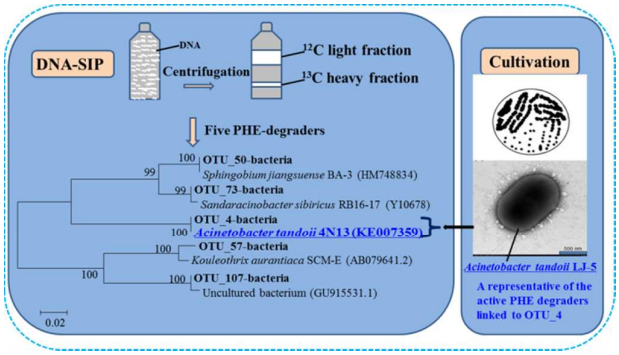
DNA-SIP
DNA
Centrifugation
^{12}C light fraction
^{13}C heavy fraction
Five PHE-degraders
OTU_50-bacteria
Sphingobium jiangsuense BA-3 (HM748834)
OTU_73-bacteria
Sandaracinobacter sibiricus RB16-17 (Y10678)
OTU_4-bacteria
Acinetobacter tandoii 4N13 (KE007359)
OTU_57-bacteria
Kouleothrix aurantiaca SCM-E (AB079641.2)
OTU_107-bacteria
Uncultured bacterium (GU915531.1)
0.02
Cultivation
Acinetobacter tandoii LJ-5
A representative of the active PHE degraders linked to OTU_4

17

18

ABSTRACT

The microorganisms responsible for degrading phenanthrene (PHE) in polycyclic aromatic hydrocarbon (PAH)-contaminated sewage water were identified by DNA-based stable isotope probing (DNA-SIP). In addition to the well-known PHE degraders *Acinetobacter* and *Sphingobium*, *Kouleothrix*, *Sandaracinobacter* and *Kouleothrixaceae* were found, for the first time, to be directly responsible for *in situ* 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 PAH-contaminated water. A PAH-RHD$_{\alpha}$ gene involved in PHE metabolism was detected in the DNA-SIP ^{13}C heavy fraction, but the amplification failed in *A. tandoii* LJ-5. Instead, the strain contained catechol 1,2-dioxygenase and the alpha and 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 that degrade PHE by combining cultivation-dependent and cultivation-independent approaches and provide deeper insight into the diversity of PHE-degrading communities *in situ*.

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 removing PAHs from contaminated sites.[4,5]

Considerable effort based on traditional cultivation-dependent approaches has focused on isolating and identifying cultivable PAH 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 *Agmenellum*, *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Berjerinckia*, *Burkholderia*, *Comamonas*, *Corynebacterium*, *Cyclotrophicus*, *Flavobacterium*, *Moraxella*, *Micrococcus*, *Mycobacterium*, *Marinobacter*, *Nocardioides*, *Pasteurella*, *Pseudomonas*, *Lutibacterium*, *Rhodococcus*, *Streptomyces*, *Stenotrophomonas*, *Sphingomonas*, *Vibrio* and *Paenibacillus*.[6-17] Cultivation-based approaches provide clues about PAH degraders and PAH degradation pathways. Furthermore, functional genes associated with PAH 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 PAH-degrading isolates in nature, as the majority of microbes are uncultivable,[25] and cultivation-based methods greatly underestimate 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 revolutionised the ability to investigate deeper into the microbial communities contained in environmental samples by providing higher resolution of microbial taxa compared with that of conventional cloning techniques.[34] However, these methods fail to accurately identify the metabolic or functional features of the targeted microorganisms.[34] Stable-isotope probing (SIP) is a cultivation-independent technique that circumvents the requirement of distinguishing organisms to assess metabolic responses and links identity to function.[35] It has been successfully used in environmental samples by feeding microbial communities stable isotope-labelled substrates (^{13}C or ^{15}N) to label the intracellular components (DNA, RNA, or proteins) and allowing the separation and characterisation of the targeted but hidden functional microorganisms according to buoyancy, particularly those not amenable to cultivation.[17] To date, SIP has been used to identify a large number of PAH-degrading bacteria.[27,32,33,36]

Phenanthrene (PHE) is a common PAH model compound used in biodegradation studies due to its ubiquity in nature and its fused-ring angular structure.[13,17] A number of *in situ* PAH degraders have been identified in real-world habitats, such as soil or seawater, using the DNA-SIP method.[27,31,36-38] However, only a few investigators have successfully isolated the microbes using traditional cultivation methods,[31] which help determine their metabolic characteristics and explore the functional populations actually responsible for pollutant degradation in the field. In the present study, DNA-SIP was applied to sewage water samples to link the bacterial taxa with their PHE

biodegradation phenotypes *in situ*. In addition, cultivation-based and high-throughput techniques were used to achieve a more thorough understanding of the bacterial communities contributing to PHE degradation. Here, a representative active PHE degrader (*Acinetobacter tandoii* LJ-5) was successfully isolated from the microbial sewage community. We focused on the functional genes involved in PHE metabolism to explore the environmental significance of this strain. The functional genes encoding PAH-RHD and PAH-RCD (CAT and PACH) were investigated by analysing relevant sequences amplified from the ^{13}C-DNA-enriched fraction and *A. tandoii* LJ-5 DNA. In this study, the PHE-degrading bacteria in sewage water was successfully characterised using DNA-SIP and cultivation-based methods. We hope to provide novel information on the bioremediation of PAH-contaminated sites using a reliable theoretical basis.

2. MATERIALS AND METHODS

2.1. Sample collection. Water samples were collected from sewage at an oil refinery (37°49′N, 118°25′E; altitude, 37.49 m) located in Shandong Province, China. After transport to the laboratory, a portion of the samples was stored at −20°C for subsequent DNA extraction. The remaining samples were immediately stored at 4°C for PHE degradation and SIP experiments. The PAHs identified in the sewage water are listed in Table S1 (determined using gas chromatography-mass spectrometry as described below).

2.2. SIP experiment

2.2.1. SIP microcosms. A 50 mL water sample was placed in a 150 mL serum bottle. Unlabelled PHE (99%; Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) or ^{13}C-labeled PHE ($^{13}C_6$-PHE, 99%; Cambridge Isotope Laboratories, Inc.) at a final concentration of 10 mg/L was added to a bottle with a rubber stopper and an aluminium cap using a gas-tight syringe. Microcosms without PHE were used as the non-PHE control, and those with unlabelled PHE in filter-sterilised sewage water were used as the sterile control. Each treatment was conducted in triplicate. All 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 to maintain the ambient oxygen level. On day 3 of incubation, samples from each treatment were removed for PHE analysis and DNA extraction.

2.2.2. Nucleic acid extraction and ultracentrifugation. After centrifuging 100 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, USA) according to the manufacturer's instructions.[39] DNA content was quantified using the ND-2,000

UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Approximately 5 μg DNA were added to Quick-Seal polyallomer tubes (13 × 51 mm, 5.1 mL; Beckman Coulter, Pasadena, CA, USA) 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, USA). After balancing and sealing, the tubes were 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 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. The V4 hypervariable region of bacterial 16S rRNA in fractions from samples derived from the ^{12}C-PHE and ^{13}C-PHE microcosms was amplified using the F515/R806 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 analysed using the MOTHUR software package.[42,43] Sequences were assigned using an operational taxonomic unit (OTU)-based method to generate microbiome profiles.[44-46]

The relative abundance of each OTU was determined as described previously.[40] In total, 4,186 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 samples compared with ^{12}C-PHE samples were identified as PHE degraders. Finally, five OTUs (OTU_4, OTU_50, OTU_57, OTU_73 and OTU_107) were selected and aligned to *Acinetobacter* spp., *Sphingobium* spp., *Kouleothrix* spp., *Sandaracinobacter* spp. and *Kouleothrixaceae* spp. (accession numbers: KX364043–KX364047), respectively, using the Greengenes database.[47,48] The phylogenetic information from the sequences was analysed 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 sewage water (5 mL) was added to 50 mL minimal medium (MM) (Table S3, pH = 7.0) with 1,000 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 and identified. The growth curve was calculated, and the PHE degradation efficiency of the isolated strain was evaluated in MM supplemented with different concentrations of PHE (100–1,000 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. Genomic DNA was extracted, and the 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR) using the 27f and 1492r bacterial universal primers (Table 1).[50] In addition, the morphological and physiological characteristics, GC content and DNA-DNA hybridisation of the isolated strain were determined using previously described methods.[51] Cell counts were adjusted to approximately 1×10^7 colony forming units/mL at the beginning of the experiment

154 using the dilution plate counting method.[52] Cell growth was evaluated by measuring the optical
155 density of the culture at 600 nm. Controls without cells were also established. All tests were
156 performed in triplicate, using the same standard and incubation conditions as those used for the
157 microcosm experiment.

158 **2.4. Detection of PAH-RHD and PAH-RCD genes.** The PAH-RHD$_\alpha$ gene in the heavy
159 DNA fraction was investigated in Gram positive and Gram negative (GN) degraders using two
160 primer sets, 642f/933r[53] and 610f/911r[53], respectively (Table 1). Gradient PCR was performed at
161 annealing temperatures of 52–62°C.[54] However, only the PAH-RHD$_\alpha$ GN primer set produced a
162 specific amplicon and was selected for this study. The amplification reactions were conducted
163 according to previous methods.[54] The PAH-RCD genes (CAT and PACH) were amplified using
164 the CAT1f/CAT1r, CAT2f/CAT2r, PACH1f/PACH1r and PACH2f/PACH2r primer pairs listed in
165 Table 1. All specific primer sets were designed based on published sequences of *A. tandoii* DSM
166 14970^T (GenBank assembly accession number: GCA_000400735.1) using Primer Premier 5.0
167 software. The CAT1f/CAT1r and CAT2f/CAT2r primer pairs were used to target two different
168 types of CATA. The PCR program for these two primer sets was as follows: 3 min at 95°C; 32
169 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
170 PACH1f/PACH1r and PACH2f/PACH2r primer pairs were used to target the alpha and beta
171 subunits of PACH, respectively. The PCR program for PACH1f/PACH1r and PACH2f/PACH2r
172 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
173 extension at 72°C for 5 min. The PCR products were gel-purified using a gel extraction kit
174 (D2500-01; Omega Bio-tek, Norcross, GA, USA), followed by cloning and sequencing as
175 described by Song et al.[55] The phylogenetic dendrograms were prepared using the method

176 described above.

177 The partial PAH-RHD and PAH-RCD gene sequences obtained are available in GenBank
178 under the following accession numbers: KX364042 for PAH-RHD, KX364048 and KX364049
179 for CATA and KX364050 and KX364051 for PACH. The GenBank accession number for the
180 16S rRNA gene obtained from isolated *A. tandoii* is KU168603.

181 **2.5. PHE analysis.** The PHE concentrations in each microcosm treatment were analysed on
182 days 0 and 3 as follows. The water sample was spiked with 1,000 ng deuterated PAHs as a
183 surrogate standard and was extracted twice with dichloromethane (DCM). The extracted organic
184 phase was concentrated to approximately 0.5 mL after solvent exchange with hexane and then
185 purified using a silica gel/alumina column (8 mm i.d.) filled with anhydrous Na_2SO_4 (1 cm),
186 neutral silica gel (3 cm, 3% deactivated) and neutral alumina (3 cm, 3% deactivated) from top to
187 bottom, using 15 mL hexane/DCM (1:1, v/v) as the eluent. After concentrating the eluent to
188 approximately 50 μL using a gentle stream of N_2, 1,000 ng hexamethylbenzene were added as an
189 internal standard to all samples before the instrumental analysis.

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

193 **3. RESULTS**

194 **3.1 PHE biodegradation in sewage water.** PHE biodegradation in the ^{12}C-PHE and
195 ^{13}C-PHE microcosms is shown in Table S2. The PHE concentration in the sterile treatment
196 exhibited fewer decreases than those in the biotic treatments. Residual PHE was 11–13% and
197 12–13% in the ^{12}C-PHE and ^{13}C-PHE microcosms, respectively, suggesting that significant PHE
198 biodegradation occurred in the biotic treatments. No difference ($p > 0.05$) was observed between
199 the ^{12}C-PHE and ^{13}C-PHE treatments, consistent with our previous study.[55]

200 **3.2. Bacteria involved in PHE degradation as revealed by DNA-SIP.** DNA extracted from
201 the ^{12}C-PHE and ^{13}C-PHE microcosms was subjected to ultracentrifugation and fractionation,
202 followed by high-throughput sequencing of each fraction. The relative abundance of 16S rRNA
203 defined by the family taxon showed no difference in the microbial communities between the
204 samples from the ^{12}C-PHE and ^{13}C-PHE treatments (Figure S3).

205 The organisms responsible for ^{13}C assimilation were detected during screening by comparing
206 the relative abundances of specific OTUs between the ^{12}C-PHE and ^{13}C-PHE samples from each
207 fraction. The results indicated that OTU_4 at a higher BD (>1.7209 g/mL) was enriched only in
208 the ^{13}C-PHE sample, but not in the ^{12}C-PHE sample (Figure 1). Additionally, the relative
209 abundances of OTU_50, OTU_73, OTU_57 and OTU_107 at higher BDs (>1.7209, >1.7296,
210 1.7122–1.7481 and 1.7209–1.7481 g/mL, respectively) were also higher in the ^{13}C-PHE samples
211 than in the ^{12}C-PHE samples. The higher abundance in the heavier fraction indicates that
212 organisms represented by OTU_4, OTU_50, OTU_73, OTU_57 and OTU_107 play a primary
213 role in PHE degradation.

214 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 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 haemolyticus* 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* (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 and OTU_107 were classified in the genus *Kouleothrix* (phylum *Chloroflexi*, class *Chloroflexi*, order *Roseiflexales*, family *Kouleothrixaceae*) and family *Kouleothrixaceae* (genus unclassified), respectively. OTU_57 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 analysed in the heavy fractions (^{13}C-labeled DNA, marked with a star in Figure 1). In the present study, only one PAH-RHD$_\alpha$ GN gene was detected, affiliated with the PAH-RHD$_\alpha$ (PhnAc) gene from *Delftia acidovorans* Eh2-1 clone 5 (AY367788.1) (Figure 3).

3.4. Isolation and characterisation of PHE-degrading bacteria. We isolated PHE degraders from sewage water to characterise the bacteria corresponding to the five SIP-identified OTUs. Of all PHE-degrading isolates, only one bacterial strain, named *A. tandoii* LJ-5, was identified in

237 contaminated sewage water samples after PHE enrichment. This strain shared 100% similarity
238 with the OTU_4 sequence and was therefore representative of the active PHE degraders linked to
239 OTU_4. However, no strains belonging to the other four SIP-identified OTUs were successfully
240 isolated from sewage water.

241 *A. tandoii* LJ-5 is a GN, rod-shaped, obligate aerobe lacking flagella, with a size of (0.7–1.0) ×
242 (1.0–1.5) μm (Figure 4). The *A. tandoii* LJ-5 colonies were circular, smooth, convex and white
243 pigmented with a colony diameter of 0.5–2.0 mm after growth on MM-P agar plates at 30°C for
244 48 h. *A. tandoii* LJ-5 grew under different conditions, including 0–3% (w/v) salinity (optimum
245 0%), pH 5.0–9.0 (optimum pH = 7.0) and temperatures of 25–40°C (optimum 30°C) (Figure S4).
246 The metabolic characteristics of *A. tandoii* LJ-5 are listed in Table S4. *A. tandoii* LJ-5 had the
247 highest 16S rRNA gene sequence similarity to that of *A. tandoii* DSM 14970^T (KE007359)
248 (97.77%), whereas its similarity levels to other *Acinetobacter* strains were < 97.0%. *A. tandoii*
249 LJ-5 belongs to the genus *Acinetobacter* according to the neighbour-joining (Figure S5)
250 phylogenetic dendrograms based on 16S rRNA gene sequences, and it formed a subclade with *A.*
251 *tandoii* DSM 14970^T. The GC content of *A. tandoii* LJ-5 was 41.0 mol%, which was within the
252 range of that of other *Acinetobacter* spp. (38.1–54.7 mol%). The DNA–DNA hybridisation value
253 for *A. tandoii* LJ-5 with *A. tandoii* DSM 14970^T was 90.11 ± 0.8%, which was significantly above
254 the threshold value of 70%.[56] Taken together, these results indicate that *A. tandoii* LJ-5 belongs to
255 the species *A. tandoii*.

256 As shown in Figure S6, *A. tandoii* LJ-5 grew well in MM-P with PHE concentrations of 100–
257 1,000 mg/L under optimal growing conditions (pH 7.0 and 30°C), suggesting strong tolerance of *A.*
258 *tandoii* LJ-5 to high PHE concentrations. More than 60% of the PHE was biodegraded within 7

259 days at all PHE concentrations. Enrichment of *Acinetobacter* (OUT_4) was detected in the
260 ^{13}C-PHE treatment, indicating that *A. tandoii* LJ-5 is a major PHE degrader *in situ*.

261 **3.5. Presence of PHE metabolism-related genes in *A. tandoii* LJ-5.** To further explore the
262 environmental significance of *A. tandoii* LJ-5, we evaluated its functional genes involved in PHE
263 metabolism. Although one PAH-RHD$_\alpha$ GN gene was detected in the heavy fraction of the
264 ^{13}C-PHE sample, no PAH-RHD$_\alpha$ gene was successfully amplified from *A. tandoii* LJ-5 using the
265 same primer set. However, the genes encoding CATA-1 and CATA-2 and the alpha and beta
266 subunits of PACH-1 and PACH-2 were identified in *A. tandoii* LJ-5 in this study.

267 The CATA-1 (KX364048) and CATA-2 (KX364049) translated amino acid sequences showed
268 high homology with that of CATA from *Acinetobacter junii* (WP_004961950.1, 92%) and
269 *Acinetobacter schindleri* (WP 004809441.1. 93%), respectively, as illustrated in Figure 5a. Figure
270 5b shows the high homologies of *A. tandoii* LJ-5 PACH-1 (KX364050) and PACH-2 (KX364051)
271 at the amino acid level compared with the alpha subunit of PACH from *Acinetobacter bouvetii*
272 DSM 14963 (WP_005011151.1, 99%) and the beta subunit of PACH from *Acinetobacter*
273 *johnsonii* XBB1 (WP_058952216.1, 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 five OTUs directly responsible for *in situ* PHE biodegradation, such as the phylotypes affiliated with *Acinetobacter*, *Sphingobium*, *Kouleothrix*, *Sandaracinobacter* and *Kouleothrixaceae* (genus unclassified) from PAH-contaminated sewage water.

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 PAH-degrading genus in the family *Sphingomonadaceae*.[57,58] Some strains in this genus metabolise 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 *in situ* PHE-degradation capacity of *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 centre.[64] This bacterium tolerates and reduces high levels of tellurite.[64] However, this strain has not been linked previously to PHE degradation; thus, our present results provide strong evidence that some microbes in this genus are primarily responsible for *in situ* PHE degradation in sewage water.

The phylogenetic analysis of the microorganisms represented by OTU_57 suggested their close relationship to *Kouleothrix aurantiaca* SCM-E (Figure 2). *K. aurantiaca* SCM-E was first

296 isolated by Kohno from activated industrial waste sludge.[65] The genus *Kouleothrix* belongs to
297 phylum *Chloroflexi* (family *Kouleothrixaceae*, class *Chloroflexi*), which is one of the earliest
298 diverging lineages of bacteria and was first defined by Garrity and Holt in Bergey's Manual of
299 Systematic Bacteriology.[66] Class *Chloroflexi* is one of at least five major *Kouleothrix* subgroups,
300 and all known species in this class have a multicellular filamentous morphology.[67] A number of
301 studies have indicated that microorganisms in the phylum *Chloroflexi* are closely related to PAH
302 degradation. Shahi et al. showed that γ-*Proteobacteria*, *Chloroflexi*, *Firmicutes* and
303 δ-*Proteobacteria* were the most dominant bacterial phyla in petroleum-contaminated soil from a
304 coastal site at an old petroleum sludge storage pit in Turkey.[68] Bacterial species belonging to
305 γ-*Proteobacteria*, δ-*Proteobacteria* and *Chloroflexi* change dramatically after treatment with
306 PAHs, indicating that PAHs play key roles in bacterial community diversity.[69] Muangchinda et al.
307 reported that indigenous microbes from the phylum *Chloroflexi* degrade PAHs and provided
308 bioremediation information for Antarctic soils and sediments,[70] although PAH contaminants such
309 as PHE and pyrene decrease the abundance of *Chloroflexi* during PAH remediation.[71,72] However,
310 *Kouleothrix* and *Kouleothrixaceae* have not been linked previously to PHE metabolism; thus, it is
311 unclear whether these microbes are directly involved in PHE degradation. Our results provide
312 unequivocal evidence that some microorganisms in these taxa are primarily responsible for *in situ*
313 PHE degradation in the complex microbial community of PAH-contaminated sewage water.

314 *Acinetobacter*, belonging to *γ-Proteobacteria* and to the order *Pseudomonadales*, is a GN,
315 non-motile and strictly aerobic bacteria. These bacteria are widespread in natural environments,
316 including hydrocarbon-contaminated sites.[73,74] Members of *Acinetobacter* possess versatile
317 metabolic capabilities, such as pathways for degrading aromatic and hydroxylated aromatic

compounds.[75] 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*.[9,75,76] Hereinto, some strains metabolise PAHs, such as POHE, acenaphthene and pyrene.[77-80] Degradation of PHE by *Acinetobacter* has not been identified using DNA-SIP prior to this study. Our results demonstrate that *A. tandoii* LJ-5 metabolises PHE *in situ*. *A. tandoii* was first described by Emma et al. in 2003 but was not previously associated with PAH degradation.[81] Our results provide *A. tandoii* LJ-5 reference data for application to PAH-contaminated sewage water treatment.

The presence of the distinctive PAH-RHD$_\alpha$ GN gene in the heavy DNA fraction from the ^{13}C-PHE microcosm suggests its functionality associated with PHE-degrading strains of *Acinetobacter*, *Sphingobium*, *Kouleothrix*, *Sandaracinobacter* and *Kouleothrixaceae*, as identified by DNA-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 catabolise aromatic compounds are enriched in five genomic loci within 25% of the genome,[82] whereas the metabolic genes of other aromatic compound degraders, such as microbes in the genus *Sphingomonas* or *Pseudomonas,* are scattered throughout their genome.[83,84] The mechanism is unclear, but some preliminary evidence suggests that syntenic localisation of the genes associated with this metabolic pathway relieves the energy burden on the transcriptional and translational machinery.[85] 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).[86] 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] Successful amplification of CATA also indicates that *A. tandoii* LJ-5 metabolises catechol through the catechol branch of the ortho-cleavage pathway.[87] Previous studies have suggested that the CATA route is preferred under low-contamination conditions.[88,89] The presence of ortho-cleavage for catechol probably helped *A. tandoii* LJ-5 adapt to the low levels of PAHs in the present PAH-contaminated sewage water.

This is the first study to apply a culture-independent DNA-SIP technique to identify the bacterial taxa responsible for PHE degradation in PAH-contaminated sewage. The results provide unequivocal evidence that *Acinetobacter*, *Sphingobium*, *Kouleothrix*, *Sandaracinobacter* and *Kouleothrixaceae* are involved in *in situ* biodegradation of PHE in sewage, none of which has been previously reported as a PHE-degrading microorganism using SIP. *Sandaracinobacter*, *Kouleothrix* and *Kouleothrixaceae* have not been previously linked to PHE degradation. Moreover, given that few bacteria linked to *in situ* 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 identified its functions by characterising its functional PHE metabolic genes and pathways. This is the first report of a role for *A. tandoii* in bioremediation of PAH-contaminated water. These results expand our current knowledge on microorganisms that degrade PHE by combining both cultivation-dependent and cultivation-independent approaches.

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Captions

Figure 1 The shift tendency of OTU_4, OTU_50, OTU_73, OTU_57 and OTU_107 fragments. The relative abundance of the OTU_4, OTU_50, OTU_73, OTU_57 and OTU_107 fragments over a range of buoyant density (BD) from DNA extracted from the sewage water added with either ^{12}C- or ^{13}C-labeled PHE after 3 days of incubation.

Figure 2 Phylogenetic tree of identified OTUs responsible for *in situ* PHE degradation. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of the bacteria corresponding OTU_4, OTU_50, OTU_57, OTU_73, OTU_107 and their representatives of some other related taxa. Bootstrap values (expressed as percentages of 1200 replications) >50% are shown at the branch points. Bar 0.02 substitutions per nucleotide position.

Figure 3 Phylogenetic tree of amplified PAH-RHD$_\alpha$ GN gene from heavy fraction in ^{13}C-PHE microcosm. PAH-RHD gene showed 97% similarity with *Delftia acidovorans* Eh2-1 clone 5 PhnAc gene.

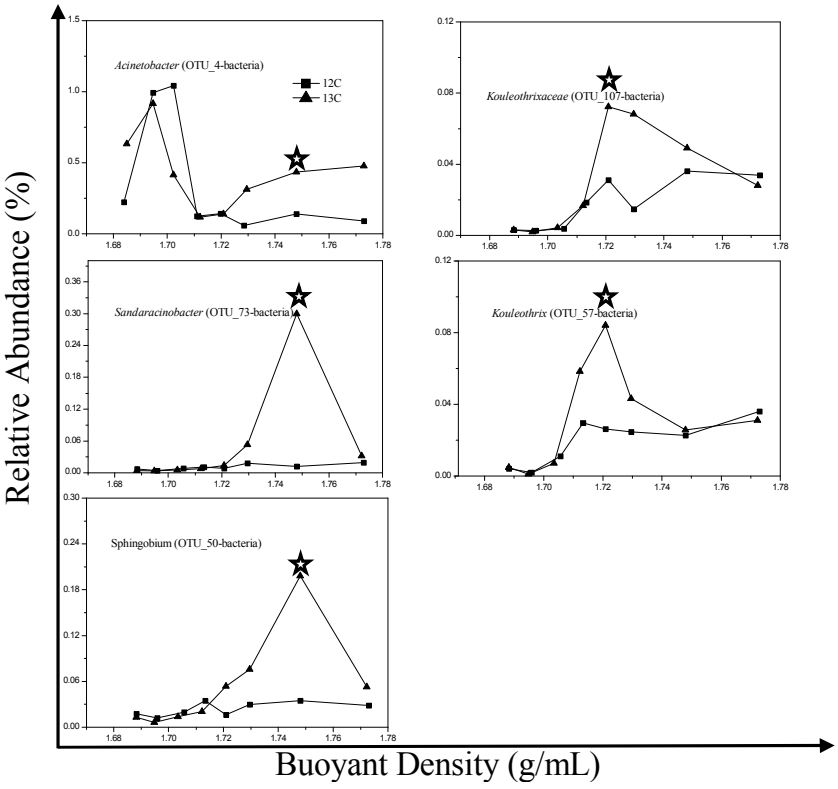
Figure 4 (a) Isolated *A. tandoii* LJ-5 colonies on MM-P agar plate; (b) Transmission electron micrograph of *A. tandoii* LJ-5 cells. Bar, 500 nm (left) and 1000 nm (right).

Figure 5 Phylogenetic tree based of CATA and PACH sequences from strain LJ-5. (a) High homology at amino acid level (92% and 93%) was detected with catechol 1,2-dioxygenase of *Acinetobacter junii* (WP_004961950.1) and *Acinetobacter schindleri* (WP_004809441.1), respectively. (b) High homology at amino acid level (99% and 99%) was detected with subunit alpha of protocatechuate 3,4-dioxygenase of *Acinetobacter bouvetii* DSM 14963 (WP_005011151.1), and subunit beta of protocatechuate 3,4-dioxygenase of *Acinetobacter johnsonii* XBB1 (WP_058952216.1), respectively.

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

Targets	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

650 **Figure 1**



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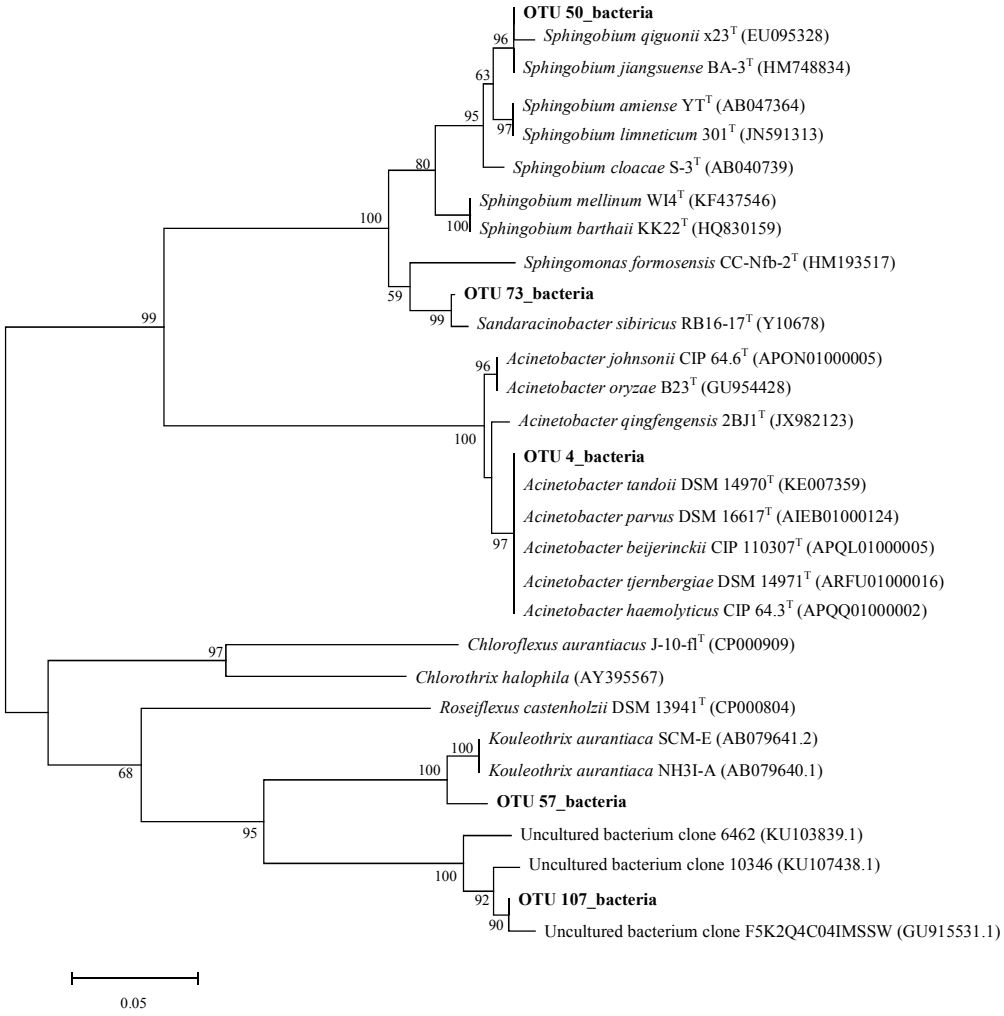
653 **Figure 2**



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656 **Figure 3**

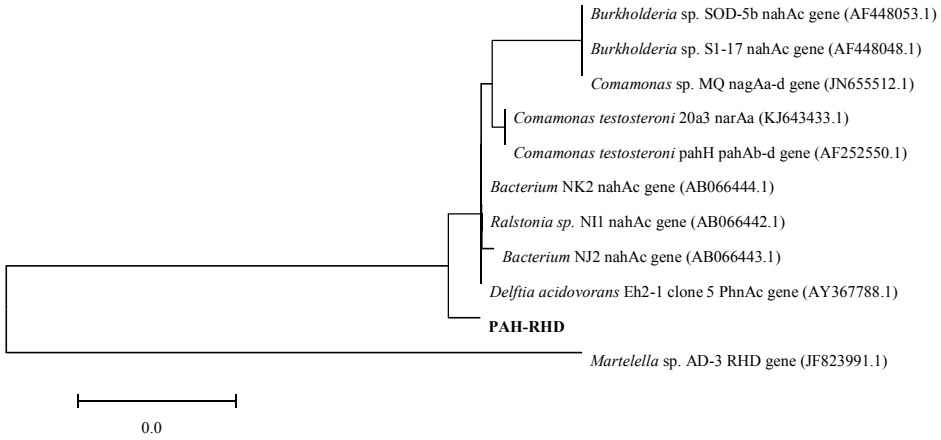

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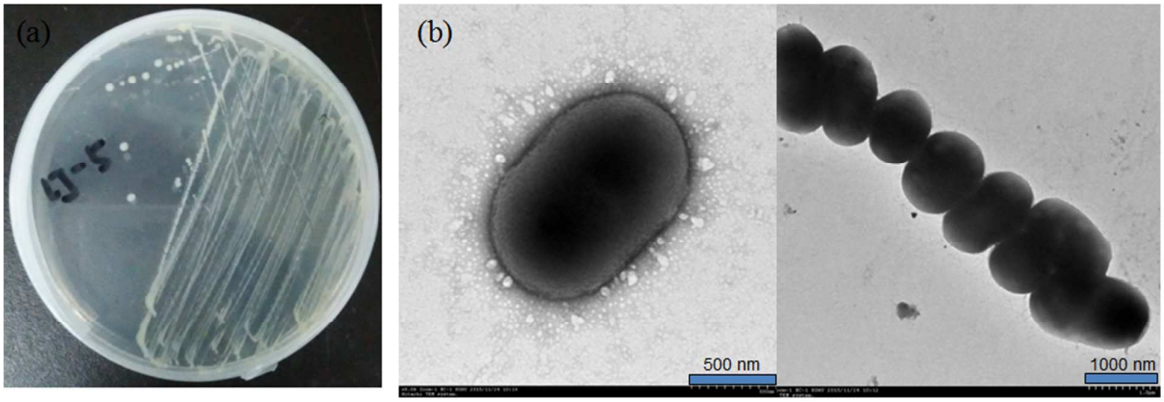
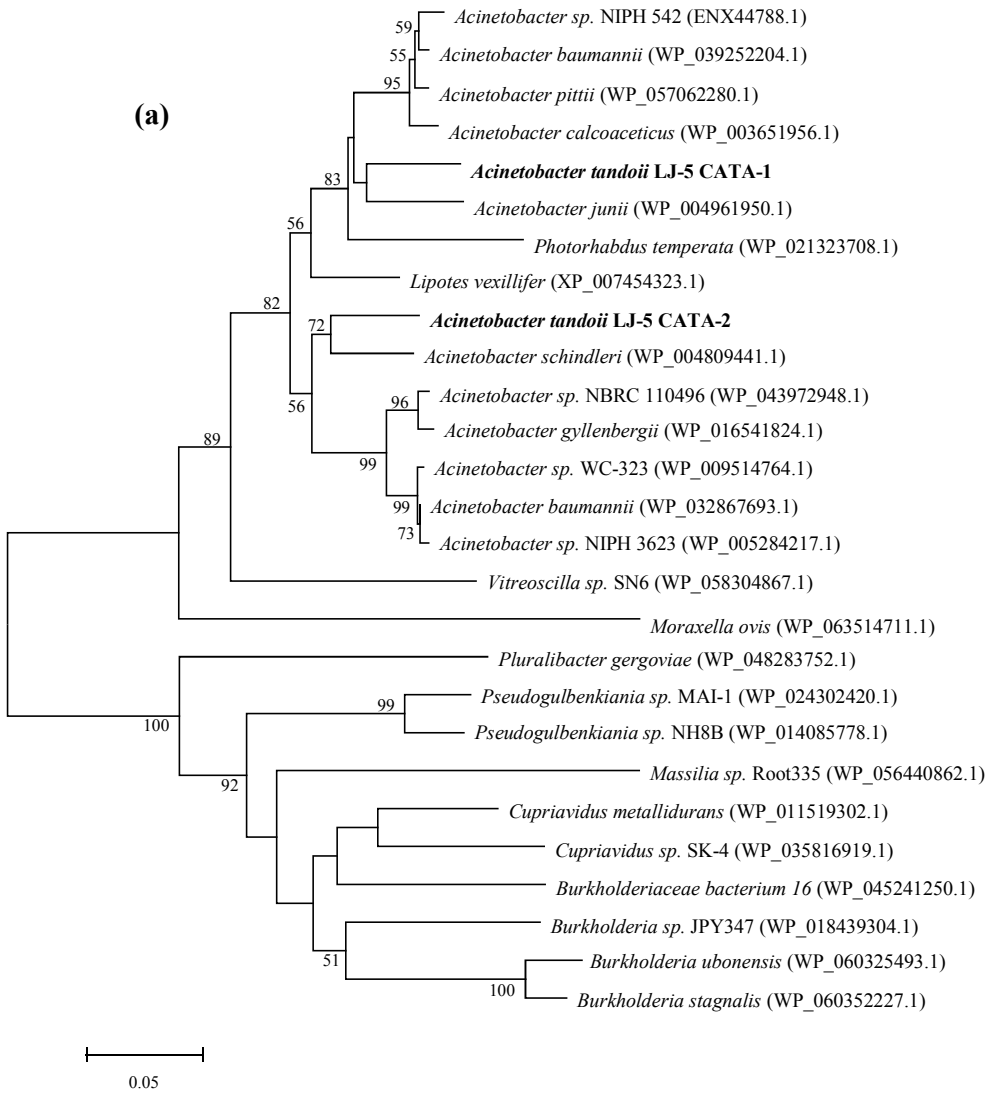
Figure 4



667 **Figure 5**



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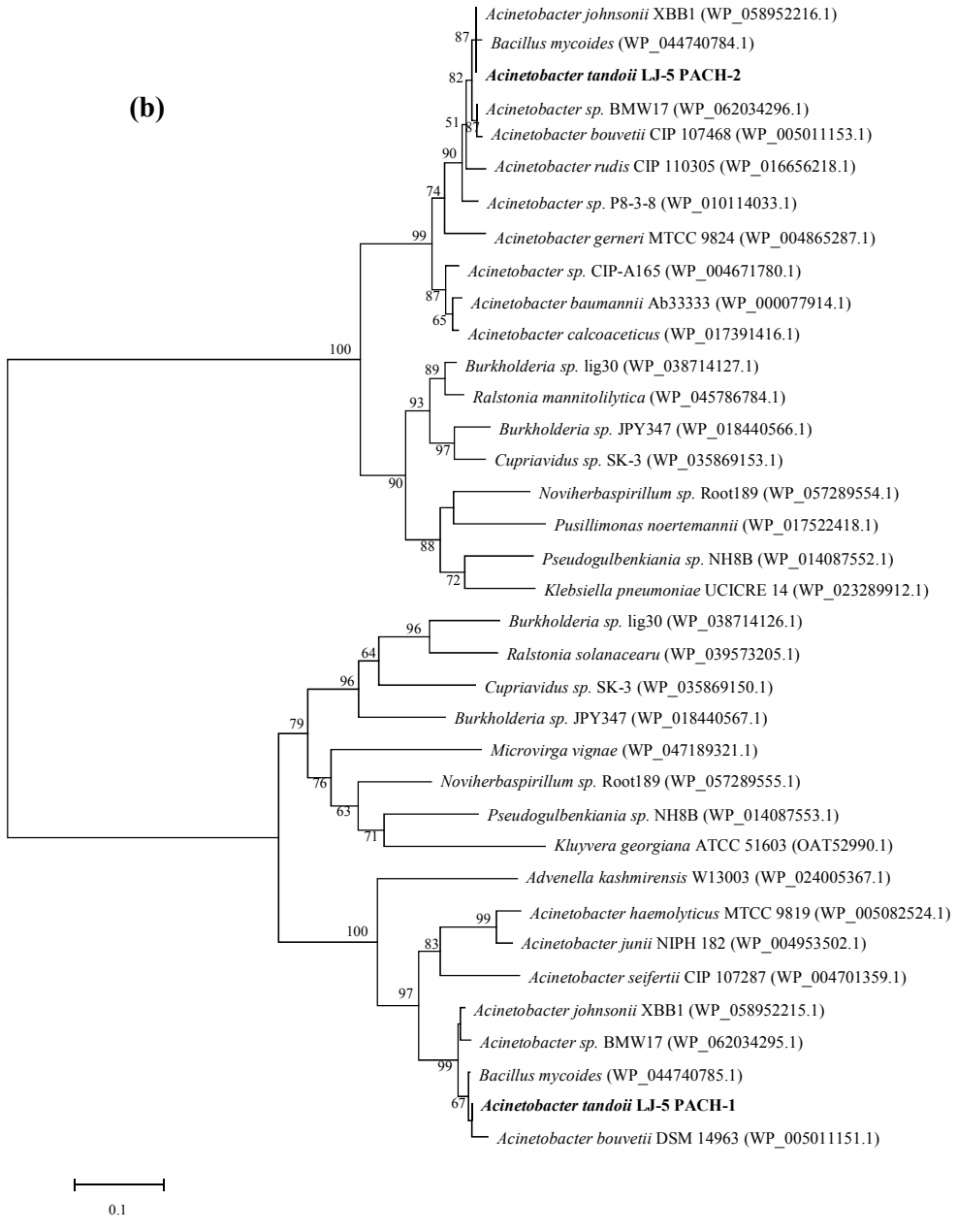
(b)
Acinetobacter johnsonii XBB1 (WP_058952216.1)
Bacillus mycoides (WP_044740784.1)
Acinetobacter tandoii LJ-5 PACH-2
Acinetobacter sp. BMW17 (WP_062034296.1)
Acinetobacter bouvetii CIP 107468 (WP_005011153.1)
Acinetobacter rudis CIP 110305 (WP_016656218.1)
Acinetobacter sp. P8-3-8 (WP_010114033.1)
Acinetobacter gerneri MTCC 9824 (WP_004865287.1)
Acinetobacter sp. CIP-A165 (WP_004671780.1)
Acinetobacter baumannii Ab33333 (WP_000077914.1)
Acinetobacter calcoaceticus (WP_017391416.1)
Burkholderia sp. lig30 (WP_038714127.1)
Ralstonia mannitolilytica (WP_045786784.1)
Burkholderia sp. JPY347 (WP_018440566.1)
Cupriavidus sp. SK-3 (WP_035869153.1)
Noviherbaspirillum sp. Root189 (WP_057289554.1)
Pusillimonas noertemannii (WP_017522418.1)
Pseudogulbenkiania sp. NH8B (WP_014087552.1)
Klebsiella pneumoniae UCICRE 14 (WP_023289912.1)
Burkholderia sp. lig30 (WP_038714126.1)
Ralstonia solanacearu (WP_039573205.1)
Cupriavidus sp. SK-3 (WP_035869150.1)
Burkholderia sp. JPY347 (WP_018440567.1)
Microvirga vignae (WP_047189321.1)
Noviherbaspirillum sp. Root189 (WP_057289555.1)
Pseudogulbenkiania sp. NH8B (WP_014087553.1)
Kluyvera georgiana ATCC 51603 (OAT52990.1)
Advenella kashmirensis W13003 (WP_024005367.1)
Acinetobacter haemolyticus MTCC 9819 (WP_005082524.1)
Acinetobacter junii NIPH 182 (WP_004953502.1)
Acinetobacter seifertii CIP 107287 (WP_004701359.1)
Acinetobacter johnsonii XBB1 (WP_058952215.1)
Acinetobacter sp. BMW17 (WP_062034295.1)
Bacillus mycoides (WP_044740785.1)
Acinetobacter tandoii LJ-5 PACH-1
Acinetobacter bouvetii DSM 14963 (WP_005011151.1)
0.1

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