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ORIGINAL PAPER

Novel bacteria capable of degrading phenanthrene in activated sludge revealed by stable-isotope probing coupled with high-throughput sequencing

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Abstract The indigenous microorganisms responsible for degrading phenanthrene (PHE) in activated biosludge were identified using DNA-based stable isotope probing. Besides the well-known PHE degraders *Burkholderia*, *Ralstonia*, *Sinobacteraceae* and *Arthrobacter*, we for the first time linked the taxa *Paraburkholderia* and *Kaistobacter* with in situ PHE biodegradation. Analysis of PAH-RHD$_\alpha$ gene detected in the heavy DNA fraction of ^{13}C-PHE treatment suggested the mechanisms of horizontal gene transfer or inter-species hybridisation in PAH-RHD gene spread within the microbial community. Additionally, three cultivable PHE degraders, *Microbacterium* sp. PHE-1, *Rhodanobacter* sp. PHE-2 and *Rhodococcus* sp. PHE-3, were isolated from the same activated biosludge. Among them, *Rhodanobacter* sp. PHE-2 is the first identified strain in its genus with PHE-degrading ability. However, the involvement of these strains in PHE degradation in situ was questionable, due to their limited enrichment in the heavy DNA fraction of ^{13}C-PHE treatment and lack of PAH-RHD$_\alpha$ gene found in these isolates. Collectively, our findings provide a deeper understanding of the

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diversity and functions of indigenous microbes in PHE degradation.

Keywords Indigenous microorganisms · Phenanthrene (PHE) · PHE degraders · DNA-SIP · PAH-RHD$_\alpha$ genes

Introduction

To face the worldwide challenge of increasing contamination of freshwater systems with hundreds of anthropogenic chemicals (Schwarzenbach et al. 2006), a list of the most dangerous compounds has been proposed as target for remediation, based on their toxicological properties and actual occurrence in wastewater (Ledin and Patureau 2010). Among the non-conventional priority pollutants, polycyclic aromatic hydrocarbons (PAHs) are an important class of organic chemicals, which have two or more fused aromatic rings and can affect both human health and ecosystem function (Minai-Tehrani et al. 2009). They are recognised as high-priority pollutants of particular concern in the environment due to their possible toxic, carcinogenic, and mutagenic properties (Keith and Telliard 1979). Thus, it is of paramount importance to reduce PAHs and eliminate their environmental impacts.

Biodegradation is a proved viable option for reducing PAHs contamination in water and soil (Bahr et al. 2015). Using established cultivation-dependent approaches, many microorganisms capable of degrading PAHs have been isolated and evaluated (Jones et al. 2011; Gutierrez et al. 2013). These works have helped to characterise the metabolic pathways and functional genes associated with PAHs degradation, such as PAH-ring hydroxylating dioxygenases (PAH-RHD) (Cebron et al. 2008). However, an important limitation of these approaches is that the majority of microbes are uncultivable (Amann et al. 1995); only a small proportion of the indigenous bacteria present in natural habitats can be cultivated and isolated on laboratory media which cannot reproduce in situ conditions. In addition, cultivation-based approaches greatly underestimate prokaryotic diversity and fail to mimic the complex interactions among individuals present in natural microbial communities (Oren 2004).

In the last decade, cultivation-independent techniques have been developed to evaluate the prokaryotic diversity of complex microbiota (Breznak 2002), and to link microbial identities to their metabolic functions (Dumont and Murrell 2005). These techniques lead to greater complexity than traditional approaches (Gutierrez 2011) and remove the bias imposed by the isolation of microorganisms necessary for cultivation-dependent methods (Lebaron et al. 2001). Stable isotope probing (SIP) is one such method, which employs stable isotopes (^{13}C or ^{15}N) to identify functionally active members within microbial communities by analysing their isotope-enriched intracellular components (DNA, RNA, or proteins) and is especially useful for organisms that are not amenable to cultivation (Singleton et al. 2005; Jiang et al. 2015). High-throughput sequencing (HTS) can provide higher resolution of the structure of complex microbial communities than conventional cloning techniques (Gutierrez 2011). Combined with HTS, DNA-based stable isotope probing (DNA-SIP) is ideally suited to linking microbes within complex microbial communities to their ecological functions (Jameson et al. 2017). Many recent works have begun coupling SIP with HTS to investigate microbial functions in complex environments (Gutierrez et al. 2013; Li et al. 2017; Zhang et al. 2015).

Previous DNA-SIP studies regarding PAHs biodegradation have been limited to contaminated soils and waters, e.g., polluted soils from a former gas manufacturing plant site (Jones et al. 2011) and oil-contaminated seawater from the Deepwater Horizon site (Gutierrez et al. 2013). However, SIP has not yet been employed to examine the potential PAHs-degrading microbes in the activated biosludge of municipal wastewater treatment plants, which contribute significantly to PAHs elimination in water discharge and prevent their release into the environment. In this study, phenanthrene (PHE) was employed as a model PAHs compound, and we applied DNA-SIP to an activated biosludge with the aim of linking the indigenous bacterial taxa to their roles in PHE degradation. Additionally, both cultivation and high-throughput sequencing were used to achieve a deeper understanding of the bacterial communities contributing to PHE degradation. Furthermore, PAH-RHD genes were investigated by analysing the relevant sequences amplified from the ^{13}C-DNA-enriched fraction. We hope to provide novel and useful information regarding the biodegradation of PAHs in municipal wastewater treatment systems.

Materials and methods

Sample collection

Settled activated sludge samples were collected from the activated sludge aeration basin of a municipal wastewater treatment plant (23°11′N, 113°24′E; altitude, 13 m) located in Guangdong Province, China. This plant employs anaerobic/anoxic/oxic (A2/O) activated sludge processes and provides a disposal service for nearby municipal sewage and food industry wastewater. After sampling, the activated sludge was immediately transported to the laboratory at 4 °C. Portions of the activated sludge samples were stored frozen for subsequent DNA extraction, and the remaining samples were immediately utilised in PHE degradation and SIP experiments. PAHs in the activated sludge are listed in Table S1 (Supplementary Material), determined using gas chromatography-mass spectrometry (GC–MS) according to a standard method described below.

SIP microcosms

A 15-mL sample of activated sludge was placed into a 50-mL serum bottle. The bottles were sealed with rubber stoppers and aluminium caps. Stock solutions of PHE were prepared in dimethyl formamide (DMF) and stored at − 20 °C until used. Unlabelled PHE (99%) or ^{13}C-labelled PHE ($^{13}C_{14}$-PHE, 99%; Cambridge Isotope Laboratories Inc., USA) was added to each bottle to reach a final PHE concentration of 10 mg/L. Microcosms without PHE (blank control), and ones using filter-sterilised samples with unlabelled PHE (sterile negative control), were also assembled. Each treatment was conducted in triplicates. All laboratory microcosms were incubated in the dark with 120-rpm shaking at room temperature (∼ 25 °C). To maintain oxic conditions, the serum bottles were opened each day for about 1 h in fume cupboard with sterile condition (Li et al. 2017). On days 3 and 6 after incubation, samples from each treatment were sacrificed for PHE analysis and DNA extraction.

Nucleic acid extraction and ultracentrifugation

Activated sludge samples were collected at the beginning and end of PHE degradation. DNA was extracted from triplicate microcosms (6 mL) of each treatment at different time points using a PowerSoil DNA Isolation Kit (MoBio, USA) according to the manufacturer's instructions. DNA was quantified with an ND-2000 UV–Vis spectrophotometer (NanoDrop Technologies, USA).

Approximately 5 μg of DNA was added to Quick-Seal polypropylene tubes (13 × 51 mm, 5.1 mL; Beckman Coulter, USA) and mixed with Tris–EDTA (TE; 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., USA) and adjusted using CsCl solution or Tris–EDTA buffer. After balancing and sealing, the tubes were transferred to an ultracentrifuge (Optima L-100XP; Beckman Coulter) at 45,000 rpm (20 °C) for 48 h. After centrifugation, DNA was collected from each 400-μL fraction using a fraction recovery system (Beckman Coulter). The BD of each fraction was then measured, and the DNA fractions were purified following our previously published method (Song et al. 2015). The BD and DNA concentration of each fraction are listed in Figs. S1 and S2.

Quantitative polymerase chain reaction (qPCR)

The abundance of bacterial 16S rRNA gene in each fraction was determined by qPCR with bacterial universal primer pairs of Bac519f/Bac907r (Table 1). The PCR reactions were performed in a 20 μL mixture containing 10 μL of SYBR green PCR Premix Ex Taq II (TaKaRa, Japan), 0.2 μL of each primer (10 μm) and 1 μL of DNA template on ABI 7500 real-time PCR system (Applied Biosciences, USA). A 10-fold serial dilution of known copy numbers of the plasmid DNA extracted from *E. coli* were generated to produce the standard curve. The reactions were conducted following the process: an initial denaturation of 10 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, and 15 s at 72 °C. The SYBR green signal was collected after 20 s step at 72 °C in each cycle. The melt curve was obtained from 60 to 95 °C at a rate of 0.2 °C.

Table 1 Primers used for PCR amplification of 16S rRNA and PAH-RHD$_\alpha$ gene

Target	Primer	Sequence (5′–3′)
16S rRNA	515f	GTGCCAGCMGCCGCGGTAA
	806r	AACGCACGCTAGCCGGACTACVSGGGTATCTAAT
	27f	AGAGTTTGATCCTGGCTCAG
	1492r	GGTTACCTTGTTACGACTT
	Bac519f	CA GCMGCCGCGGTAANWC
	Bac907r	CCGTCAATTCMTTTRAGTT
PAH-RHD$_\alpha$	610f	GAGATGCATACCACGTKGGTTGGA
	911r	AGCTGTTGTTCGGGAAGAYWGTGCMGTT
	641f	CGGCGCCGACAAYTTYGTNGG
	933r	GGGGAACACGGTGCCRTGDATRAA
	396f	ATTGCGCTTAYCAYGGBTGG
	696r	ATAGGTGTCTCCAACRAARTT

High-throughput sequencing and computational analyses

PCR amplification of the hypervariable V4 region of the 16S rRNA gene was performed using the DNA fractions derived from ^{12}C-PHE and ^{13}C-PHE microcosms with the 515f/806r primer set (Table 1) (Bates et al. 2011). Each reaction (50 μL) contained 25 μL of rTaq premixed buffer (TaKaRa, Japan), 1 μL of each primer (100 nM), and 10–100 ng of template DNA. Unique 12-base nucleotide sequences were synthesised at the 5′ end of each pair of primers as barcodes to assign sequences to the different fractions. The PCR program was 3 min at 95 °C; 35 cycles of 94 °C for 30 min, 55 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 10 min. Sequencing was conducted using the 2 × 250 bp PE mode on an Illumina MiSeq sequencer in a standard pipeline. Reads were filtered if they contained primer mismatches, uncorrectable barcodes or ambiguous bases, using the QIIME and a custom Perl script. The remaining sequences were analysed using the MOTHUR software package and assigned to operational taxonomic units (OTU) to generate microbiome profiles, according to the protocol described in our previous study (Li et al. 2017).

The relative abundance of each OTU was calculated as described previously (Luo et al. 2009). OTUs of the top 100 relative abundances were selected for analysis. PHE degraders were identified as the OTUs enriched in the heavy DNA fractions of ^{13}C-PHE samples in comparison with ^{12}C-PHE samples. In total, six OTUs (OTU_1, OTU_54, OTU_75, OTU_11, OTU_69 and OTU_108) were selected, which aligned to *Burkholderia* spp., *Paraburkholderia* spp., *Ralstonia* spp., *Sinobacteraceae* spp., *Kaistobacter* spp. and *Arthrobacter* spp. (accession numbers: KY319177-KY319182), respectively, using the Greengenes database. The phylogenetic information for these sequences was obtained using the Basic Local Alignment Search Tool (BLAST) algorithm (National Center for Biotechnology Information, USA) and Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.

Isolation of PHE degraders by enrichment and cultivation

PHE degraders were isolated from the same activated sludge as the SIP microcosms. Activated sludge samples (5 mL) were added to 50 mL of minimal medium (MM) (Table S2, pH = 7.0) with 1000 mg/L PHE (MM-P) as the carbon source. After incubation at 25 °C for 7 days, 5 mL of the cultural suspension was subcultured in 50 mL fresh MM-P and incubated under the same conditions for another 7 days. After three sequential rounds of enrichment, the enriched population was serially diluted and spread onto MM-P agar plates, which were incubated at 25 °C for 4 days. In total, 10 colonies were isolated, purified, and identified. Genomic DNA was extracted and the 16S rRNA gene sequence was PCR-amplified using the universal bacterial primers 27f and 1492r (Table 1). The 16S rRNA gene sequences obtained from isolated strains are available in GenBank under the following accession numbers: KY319174 for *Microbacterium*

sp. PHE-1, KY319175 for *Rhodanobacter* sp. PHE-2 and KY319176 for *Rhodococcus* sp. PHE-3. The PHE degradation capacities of the isolated strains were evaluated using our published method (Jiang et al. 2015) with incubation in MM supplemented with 100 mg/L PHE in the dark for 7 days on a 180-rpm shaking plate at 30 °C. All tests were carried out in triplicates with sterile controls, following the same incubation conditions for enrichment and isolation. PHE degradation efficiency was calculated from the change of PHE concentration, which was determined using the method described in the chemical analysis.

Detection of PAH-RHD$_\alpha$ genes

Three types of PAH-RHD$_\alpha$ gene (PAH-RHD$_\alpha$ L, PAH-RHD$_\alpha$ GP and PAH-RHD$_\alpha$ GN) were investigated in the heavy DNA fraction using the primer sets 396f/696r, 641f/933r and 610f/911r (Table 1), respectively (Ding et al. 2010; Song et al. 2015). PAH-RHD$_\alpha$ L includes a wide range of PAH-RHD$_\alpha$ genes found in both Gram-positive (GP) and Gram-negative (GN) bacteria, while PAH-RHDα GP and PAH-RHDα GN genes are used to detect GP and GN bacterial populations that are capable of degrading PAHs, respectively. Gradient PCR reactions were performed at annealing temperatures of 52–62 °C (Cebron et al. 2008). However, only the PAH-RHD$_\alpha$ GN primer set produced a strong and specific amplicon, and it was selected for this study. In addition, the PAH-RHD$_\alpha$ gene in the DNA extracted from the isolated PHE degraders was also analysed. The amplification reactions were performed using previously described methods (Cebron et al. 2008; Ding

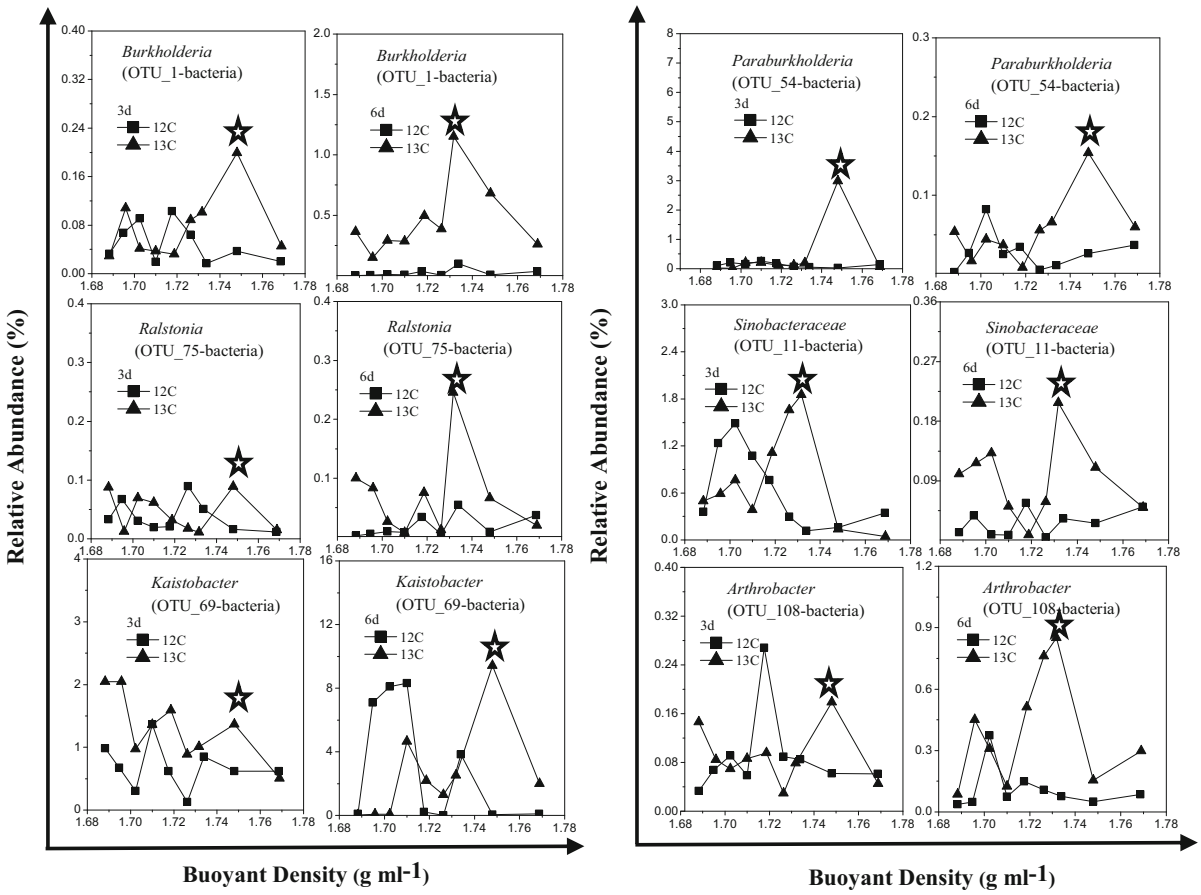


Fig. 1 The relative abundances and shift tendencies of operational taxonomic units (OTUs: OTU_1, OTU_54, OTU_75, OTU_11, OTU_69 and OTU_108) in the fractions with different buoyant densities (BDs) of DNA extracted from activated sludge supplemented with either ^{12}C- or ^{13}C-labelled PHE after 3 and 6 days of incubation

et al. 2010). The GenBank accession number for the partial PAH-RHD$_\alpha$ gene sequence obtained from heavy DNA fraction in the ^{13}C-PHE microcosm is KY319173.

Chemical analysis

Triplicates from each treatment were used for PHE analysis on days 0, 3 and 6. The collected samples were spiked with 1000 ng of deuterated PAHs as a surrogate standard and were then thoroughly extracted twice with dichloromethane (DCM). The organic phase extracts were concentrated to about 0.5 mL after solvent exchange with hexane, then purified with a silica-gel/alumina column (8 mm i.d.) filled with neutral alumina (3 cm, 3% deactivated), neutral silica gel (3 cm, 3% deactivated) and anhydrous Na_2SO_4 (1 cm) from bottom to top, with 15 mL hexane/DCM (1:1, v/v) as eluent. After being concentrated to approximately 50 µL using a gentle stream of N_2, 1,000 ng hexamethylbenzene was added to all samples as an internal standard prior to instrumental analysis. PHE was analysed using gas chromatography (model 7890; Agilent, Santa Clara, CA, USA) with a capillary column (DB-5MS, 30 m, 0.25 mm, 0.25 µm) and a mass spectrometric detector (MSD, model 5975; Agilent) according to previously reported methods (Jiang et al. 2015).

Results

PHE biodegradation in the activated sludge

PHE biodegradation in the ^{12}C-PHE and ^{13}C-PHE treatments is shown in Table S3. The PHE concentration in the sterile treatment decreased slowly from day 3 to 6. In contrast, significantly lower PHE residual concentrations were observed in the biotic treatments after 3 days of incubation, showing the important roles of indigenous microorganisms in PHE degradation. On day 6, residual PHE was only 10–13% and 12–15% in the ^{12}C-PHE and ^{13}C-PHE microcosms, respectively. Throughout the process of biodegradation, no significant difference ($p > 0.05$) was observed between the ^{12}C-PHE and ^{13}C-PHE treatments, consistent with our previous findings (Li et al. 2017).

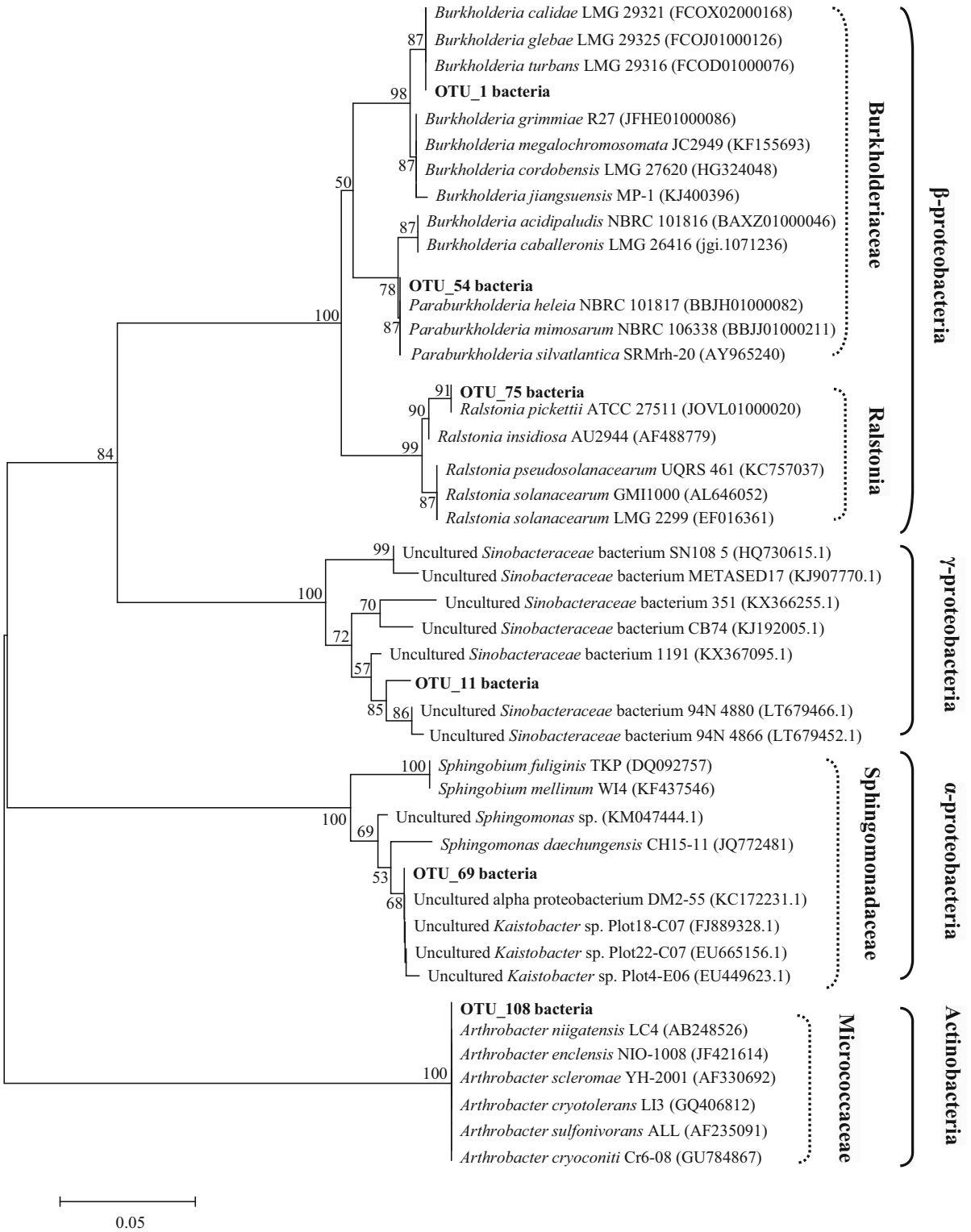
Fig. 2 Phylogenetic tree of OTUs responsible for PHE degradation. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of the bacteria corresponding to OTU_1, OTU_11, OTU_54, OTU_69, OTU_75, OTU_108 and representatives of some related taxa. Bootstrap values (expressed as percentages of 1000 replications) > 50% are shown at the branch points. Bar represents 0.05 substitutions per nucleotide position

In situ PHE degraders as revealed by DNA-SIP

The relative abundance of total 16S rRNA defined at the family and genus levels indicated slight differences in the whole indigenous microbial communities between the ^{12}C-PHE and ^{13}C-PHE treatments (Fig. S3). Meanwhile, most of the abundant bacteria, such as members of unclassified phylotypes within the families *Xanthomonadaceae* and *Isosphaeraceae*, were enriched in both ^{12}C-PHE and ^{13}C-PHE microcosms.

The abundance of bacterial 16S rRNA gene was quantified using qPCR in DNA recovered from each fraction of all samples after ultracentrifugation. As shown in Fig. S4, the fractions with higher BDs (1.7317 or 1.7480 g/mL) for both activated sludge samples after 3 and 6 days of incubation contained the heavy DNA, in which the abundance of the bacterial 16S rRNA gene was significantly higher in ^{13}C-PHE microcosms than those from the corresponding ^{12}C-PHE treatments.

The indigenous microorganisms responsible for ^{13}C assimilation were detected by comparing the relative abundances of specific OTUs between the ^{12}C-PHE and ^{13}C-PHE treatments from each fraction. As shown in Fig. 1, OTU_1, OTU_54, OTU_75, OTU_11, OTU_69 and OTU_108, found at higher BDs (1.7317 or 1.7480 g/mL, marked with a star), were enriched in the ^{13}C-PHE microcosm on days 3 and 6. In contrast, no such enrichment or similar trends were detected in the ^{12}C-PHE treatment. Additionally, there was a concomitant increase in the relative abundance of OTU_1 in the heavy fractions of ^{13}C-PHE treatments from 0.22% on day 3 to 1.25% on day 6. Similarly, the relative abundances of OTU_69, OTU_75 and OTU_108 in the heavy DNA fractions of ^{13}C-PHE treatments on day 6 (9.43, 0.25 and 0.85%, respectively) were significantly higher than those on day 3 (1.4, 0.13 and 0.18%, respectively). Although the relative abundances of OTU_11 and OTU_54 in the heavy DNA fractions of ^{13}C-PHE treatments on


Burkholderia calidae LMG 29321 (FCOX02000168)
Burkholderia glebae LMG 29325 (FCOJ01000126)
Burkholderia turbans LMG 29316 (FCOD01000076)
OTU_1 bacteria
Burkholderia grimmiae R27 (JFHE01000086)
Burkholderia megalochromosomata JC2949 (KF155693)
Burkholderia cordobensis LMG 27620 (HG324048)
Burkholderia jiangsuensis MP-1 (KJ400396)
Burkholderia acidipaludis NBRC 101816 (BAXZ01000046)
Burkholderia caballeronis LMG 26416 (jgi.1071236)
OTU_54 bacteria
Paraburkholderia heleia NBRC 101817 (BBJH01000082)
Paraburkholderia mimosarum NBRC 106338 (BBJJ01000211)
Paraburkholderia silvatlantica SRMrh-20 (AY965240)
Burkholderiaceae
OTU_75 bacteria
Ralstonia pickettii ATCC 27511 (JOVL01000020)
Ralstonia insidiosa AU2944 (AF488779)
Ralstonia pseudosolanacearum UQRS 461 (KC757037)
Ralstonia solanacearum GMI1000 (AL646052)
Ralstonia solanacearum LMG 2299 (EF016361)
Ralstonia
β-proteobacteria
Uncultured Sinobacteraceae bacterium SN108 5 (HQ730615.1)
Uncultured Sinobacteraceae bacterium METASED17 (KJ907770.1)
Uncultured Sinobacteraceae bacterium 351 (KX366255.1)
Uncultured Sinobacteraceae bacterium CB74 (KJ192005.1)
Uncultured Sinobacteraceae bacterium 1191 (KX367095.1)
OTU_11 bacteria
Uncultured Sinobacteraceae bacterium 94N 4880 (LT679466.1)
Uncultured Sinobacteraceae bacterium 94N 4866 (LT679452.1)
γ-proteobacteria
Sphingobium fuliginis TKP (DQ092757)
Sphingobium mellinum WI4 (KF437546)
Uncultured Sphingomonas sp. (KM047444.1)
Sphingomonas daechungensis CH15-11 (JQ772481)
OTU_69 bacteria
Uncultured alpha proteobacterium DM2-55 (KC172231.1)
Uncultured Kaistobacter sp. Plot18-C07 (FJ889328.1)
Uncultured Kaistobacter sp. Plot22-C07 (EU665156.1)
Uncultured Kaistobacter sp. Plot4-E06 (EU449623.1)
Sphingomonadaceae
α-proteobacteria
OTU_108 bacteria
Arthrobacter niigatensis LC4 (AB248526)
Arthrobacter enclensis NIO-1008 (JF421614)
Arthrobacter scleromae YH-2001 (AF330692)
Arthrobacter cryotolerans LI3 (GQ406812)
Arthrobacter sulfonivorans ALL (AF235091)
Arthrobacter cryoconiti Cr6-08 (GU784867)
Micrococcaceae
Actinobacteria
0.05

day 6 (0.21 and 0.15%, respectively) were lower than those on day 3 (1.85 and 2.97%, respectively), their relative abundances at higher BDs in the ^{13}C-PHE treatments were significantly higher than those in the ^{12}C-PHE microcosms (0.03 and 0.02% on day 6, respectively). Together, these results indicated that the organisms represented by OTU_1, OTU_54, OTU_75, OTU_11, OTU_69 and OTU_108 were incorporated in ^{13}C-PHE assimilation and played central roles in PHE degradation.

Figure 2 shows phylogenetic information for the identified PHE degraders represented by the above OTUs. OTU_1 and OTU_54 belong to the genera *Burkholderia* and *Paraburkholderia* within the family *Burkholderiaceae* (phylum *Proteobacteria*, class *Betaproteobacteria*, order *Burkholderiales*). OTU_1 shares 100% similarity with the partial 16S rRNA gene sequences of strains *Burkholderia calidae* LMG 29321 (FCOX02000168), *Burkholderia glebae* LMG 29325 (FCOJ01000126) and *Burkholderia turbans* LMG 29316 (FCOD01000076), and these strains form a subclade with a high bootstrap value of 87. OTU_54 shares 100% similarity with strains *Paraburkholderia heleia* NBRC 101817 (BBJH01000082), *Paraburkholderia mimosarum* NBRC 106338 (BBJJ01000211) and *Paraburkholderia silvatlantica* SRMrh-20 (AY965240), and also forms a subclade with a bootstrap value of 87. OTU_75 is classified in the genus *Ralstonia* (phylum *Proteobacteria*, class *Betaproteobacteria*, order *Burkholderiales*, family *Ralstonia*) and shares 100% similarity with *Ralstonia pickettii* ATCC 27511 (JOVL01000020), forming a subclade with a high bootstrap value of 91. OTU_69 and OTU_11 are assigned to the genus *Kaistobacter* (phylum *Proteobacteria*, class *Alphaproteobacteria*, order *Sphingomonadales*, family *Sphingomonadaceae*) and family *Sinobacteraceae* (phylum *Proteobacteria*, class *Gammaproteobacteria*, order Nevskiales), respectively. OTU_108 is characterised as genus *Arthrobacter* (phylum *Actinobacteria*, class *Actinobacteria*, order *Micrococcales*, family *Micrococcaceae*) and shares 100% similarity with strains *Arthrobacter niigatensis* LC4 (AB248526), *Arthrobacter enclensis* NIO-1008 (JF421614), *Arthrobacter scleromae* YH-2001 (AF330692), *Arthrobacter cryotolerans* LI3 (GQ406812), *Arthrobacter sulfonivorans* ALL (AF235091) and *Arthrobacter cryoconiti* Cr6-08 (GU784867), and forms a subclade with a bootstrap value of 100.

Isolation and characterisation of PHE-degrading bacteria

To separate the strains classified in the six SIP-identified OTUs and explore their degradation mechanisms and enzymes, PHE-degrading bacteria were isolated from the activated sludge with PHE as the sole carbon source. In total, 10 colonies were isolated, purified and identified as members of the genera *Microbacterium*, *Rhodanobacter* and *Rhodococcus*. Of all PHE-degrading isolates, three strains (*Microbacterium* sp. PHE-1, *Rhodanobacter* sp. PHE-2 and *Rhodococcus* sp. PHE-3) were selected and their near-complete 16S rRNA gene sequences (> 1400 bp) were used to construct a phylogenetic tree with related taxa (Fig. 3). Based on the degradation performance of 100 mg/L PHE listed in Table S4, the most efficient strain was *Microbacterium* sp. PHE-1 (69.7% degradation within 7 days), followed by *Rhodanobacter* sp. PHE-2 and *Rhodococcus* sp. PHE-3, with 52.4 and 45.3% degradation efficiency over the same time period, respectively.

Occurrence of genes involved in PHE metabolism

The PAH-RHD$_\alpha$ genes from GN bacteria were successfully amplified and analysed from the heavy DNA fractions of the ^{13}C-PHE treatment. In the present study, only one type of PAH-RHD$_\alpha$ gene (KY319173) was detected and sequenced, showing 98.4% similarity with the PAH-RHD gene of uncultured *Pseudomonas* sp. (AM743143.1), *Delftia acidovorans* Eh2-1 (AY367788.1) and *Ralstonia* sp. NI1 (AB066442.1) (Fig. 4). Though this PAH-RHD$_\alpha$ gene did not form a subclade with the above PAH-RHD genes, the phylogenetic analyses revealed its close affinities with them. Despite the considerable sequence homology with known PAH-RHD$_\alpha$ genes, multi-sequence alignment illustrated that our identified partial PAH-RHD$_\alpha$ gene has two hypervariable regions in its sequence, including bases 1–58 and 147–152 (Fig. 5A). The nucleotides from 1 to 58 are 100% identical to those from indigenous oil-degrading bacteria (KF155785.1) in the Yellow Sea (China), while those from 59 to 146 show 100% similarity to indigenous PHE-degrading bacteria (KX364042) in wastewater, *Pseudomonas* sp. (AM743143.1) and *Shewanella* sp. (KU372133.1). The final region (nucleotides 147–253) is 100% homologous to the *nagAc* gene encoding naphthalene

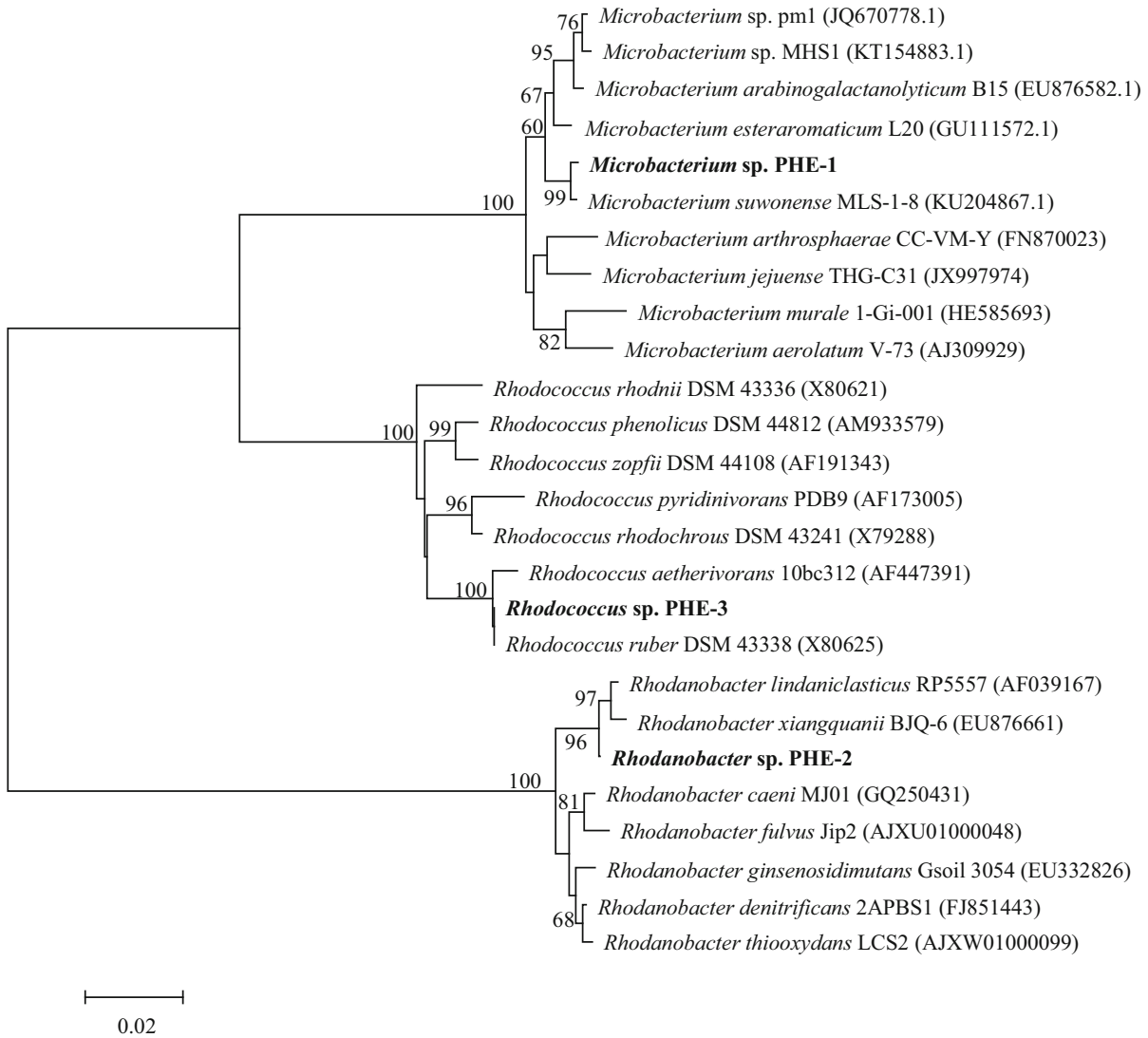


Fig. 3 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of the isolated PHE degraders and representatives of related taxa. Bootstrap values > 50% are shown at the branch points. Bar represents 0.02 substitutions per nucleotide position

1,2-dioxygenase in *Comamonas* sp. (JN655512.1) and the *nahAc* gene encoding naphthalene dioxygenase (NDO) in *Burkholderia* sp. (AF448048.1). However, no PAH-RHD$_\alpha$ gene was successfully amplified from the three isolated PHE degraders using the same primer set.

Discussion

DNA-SIP has been successfully used to demonstrate that native microorganisms collected at field sites are engaged in PHE biodegradation (Gutierrez et al. 2013; Jones et al. 2011). Our work applied ^{13}C-PHE as substrate in DNA-SIP and identified six indigenous PHE-degrading microorganisms, namely phylotypes affiliated with *Burkholderia*, *Paraburkholderia*, *Ralstonia*, *Kaistobacter*, *Arthrobacter* and *Sinobacteraceae* (genus unclassified) from the activated sludge. These microorganisms are indicative of microbial populations directly responsible for PHE biodegradation. It should be noted that all OTUs identified as PHE degraders are rare species, and thus the abundance of

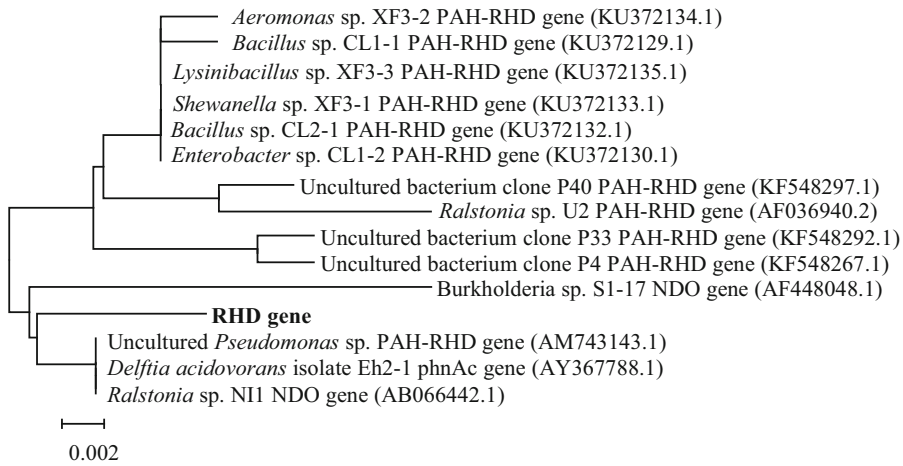


Fig. 4 Phylogenetic tree of PAH-RHD$_\alpha$ GN gene amplified from the heavy DNA fraction of the ^{13}C-PHE microcosms. The identified PAH-RHD$_\alpha$ gene (KY319173) shows 98.4% similarity with PAH-RHD genes from uncultured *Pseudomonas* sp. (AM743143.1), *Delftia acidovorans* Eh2-1 (AY367788.1) and *Ralstonia* sp. NI1 (AB066442.1)

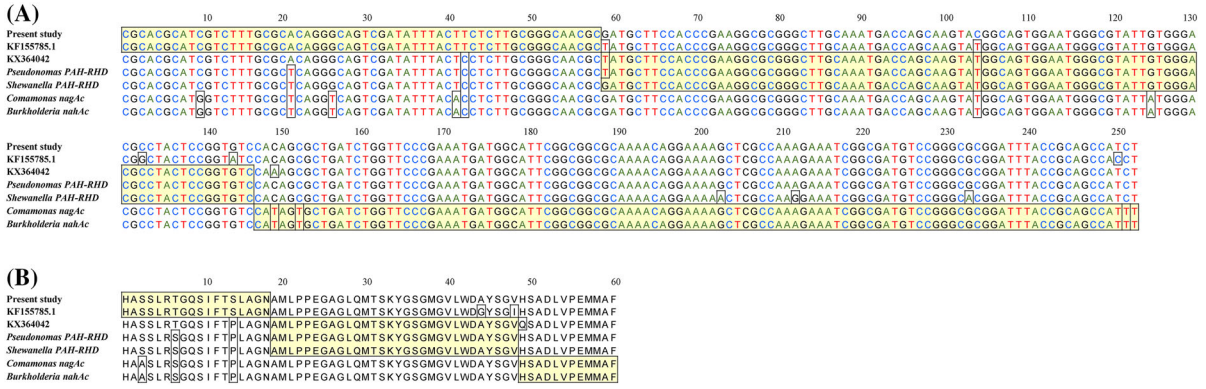


Fig. 5 **A** Multi-sequence alignment of PAH-RHD$_\alpha$ GN genes amplified from the heavy DNA fractions of the ^{13}C-PHE treatment. The identified PAH-RHD$_\alpha$ gene (KY319173) shows a mosaic or hybridised pattern of alignment to other PAH-RHD genes in *Shewanella* sp. (KU372133.1), *Pseudomonas* sp. (AM743143.1) and two uncultured bacteria (KF155785.1 and KX364042.1), and with naphthalene dioxygenase (NDO) genes in *Comamonas* sp. (JN655512.1) and *Burkholderia* sp. (AF448048.1). **B** Multi-sequence alignment of translated partial PAH-RHD from the amplified PAH-RHD gene. Yellow-boxed nucleotides and residues (square) represent contiguous consensus sequences between the PAH-RHD gene in this study and other PAH-RHD or NDO genes. Nucleotides and residues marked with an unfilled box (square) represent point mutations. This alignment was made using the CLUSTAL W-based optimal alignment tool. (Color figure online)

bacteria does not reflect their roles in pollutant assimilation owing to the occurrence of various carbon sources in their natural habitats. In the present study, the dominant bacteria, including members of unclassified phylotypes in the families *Xanthomonadaceae* and *Isosphaeraceae*, were not directly linked to PHE degradation. They might play roles in degrading other organic pollutants existing in the activated sludge and PHE addition can only promote the rare PHE-degrading species in this study.

The genera *Burkholderia* and *Paraburkholderia* belong to the family *Burkholderiaceae* within the order *Burkholderiales* of the *Betaproteobacteria*. The genus *Burkholderia* was first described in 1992 (Yabuuchi et al. 2013). Members of this genus are characterised as GN, aerobic, non-spore-forming, straight or curved rod-shaped bacteria (Guentas et al. 2016). These bacteria are widespread in natural environments, including soils, plants, animals, and humans (Coenye and Vandamme 2003). *Burkholderia* are known to degrade various environmental contaminants, such as polychlorobiphenyls (Cámara et al. 2004) and PAHs (Kumar et al. 2008). Degradation of PHE by *Burkholderia* has been reported previously

(Kim et al. 2005). However, to our knowledge, in situ PHE degradation by indigenous *Burkholderia* has not been identified using DNA-SIP prior to this study. The genus *Paraburkholderia* has been proposed following the demarcation of the genus *Burkholderia* based on phylogenetic studies of 16S rRNA and several housekeeping genes (Sawana et al. 2014). According to the International Journal of Systematic and Evolutionary Microbiology (IJSEM) validation lists 164 and 165, the genus *Paraburkholderia* comprises at least 46 species (Gao et al. 2016). Some strains affiliated to *Paraburkholderia* possess the ability to metabolise caffeine, which is a key ingredient in many popular drinks, in particular tea and coffee (Gao et al. 2016). Nevertheless, *Paraburkholderia* has not been previously linked with PHE mineralization and thus their role in PAHs metabolism remains unclear. Our results provide unequivocal evidence that microorganisms in this taxon are among the primary indigenous organisms responsible for PHE degradation in the complex community of activated sludge.

The genus *Ralstonia* also belongs to the order *Burkholderiales*, but differs from *Burkholderia* spp. in its morphological/physiological characteristics, GC content and DNA-DNA hybridisation (Yabuuchi et al. 1995). This genus is highly unusual in that it contains strains that are opportunistic human pathogens able to survive in oligotrophic environments (Chen et al. 2001). Sequence analysis of the SIP-identified OTU_57 suggested its close relationship to *Ralstonia pickettii* ATCC 27511 (Fig. 2). *Ralstonia pickettii* is the typical strain of the genus *Ralstonia*, and is considered the only representative of clinical importance (Ryan et al. 2006). Although *R. pickettii* is not viewed as a major pathogen and its virulence is relatively low, it has been verified as a risk factor associated with infection and mortality. Microbes in this genus are also of considerable biotechnological interest due to their potential for biodegrading organophosphorus insecticides (Li et al. 2010) and aromatic compounds (Wongwongsee et al. 2013). *Ralstonia* strain BPH, which was isolated from mangrove sediments, can degrade 50–76% of 100 mg/L PHE within 2 weeks (Wongwongsee et al. 2013). Some DNA-SIP evidence from PAHs-contaminated soil revealed that *Ralstonia* is directly responsible for naphthalene biodegradation (Singleton et al. 2005). However, no studies have used SIP to demonstrate the PHE-degradation capacity of indigenous *Ralstonia*. Thus, our present results provide strong evidence that members of this genus are responsible for PHE degradation in activated sludge.

The family *Sinobacteraceae* is within the class *Gammaproteobacteria* and order *Nevskiales*. Members of the family *Sinobacteraceae* have been linked with PAHs degradation. Nogi reported that a cultivated bacterial strain, *Povalibacter uvarum,* possesses the ability to metabolise PAHs such as anthracene and PHE (Nogi et al. 2014). Strains in the genus *Polycyclovorans* exhibit a narrow spectrum of substrate metabolism, utilising various aromatic compounds including benzene, toluene, biphenyl, naphthalene, anthracene, and PHE as their sole or principal sources of carbon and energy (Gutierrez et al. 2012). To our knowledge, PHE degradation by indigenous *Sinobacteraceae* using DNA-SIP has not been documented previously.

Sequence analysis of the microorganism represented by OTU_69 suggested a close relationship to the genus *Kaistobacter*. Like *Sphingobium, Kaistobacter* belongs to the family *Sphingomonadaceae* within the class *Alphaproteobacteria*. It is usually detected at uranium mining and milling sites (Radeva et al. 2013), and in soils contaminated with heavy metals (Navarro-Noya et al. 2010). *Kaistobacter* has been reported to have the ability to biodegrade EPTC (S-ethyl dipropylthiocarbamate) and atrazine in soils (Liu et al. 2016). Moreover, *Kaistobacter* may supress the disease tobacco bacterial wilt (Liu et al. 2016). No information about the possible function of *Kaistobacter* in PAHs degradation is available, although *Sphingobium* is a well-known PAHs-degrading genus in the family *Sphingomonadaceae*. The taxon *Kaistobacter* has not been linked with PHE degradation previously, and thus their role at PAHs-contaminated sites remains unclear. The results from the present study provide strong evidence that microbes in this genus are among the primary indigenous organisms responsible for PHE degradation, which expands our knowledge of this genus.

The indigenous PHE-degrading bacteria in activated sludge were found to be diverse, with representatives affiliated with *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, as well as *Actinobacteria* (Fig. 2). *Arthrobacter*, a genus of GP belonging to the *Micrococcaceae* family of *Actinobacteria*, is a catalase-positive, aerobic and asporogenous rod-shaped bacterium (Hu et al. 2016).

Arthrobacter species are known to have versatile metabolic capabilities, including degradation of organophosphorus pesticides (Singh and Walker 2006) and PAHs (Cebron et al. 2011). In addition, these bacteria are highly competitive in the presence of root exudates (Kozdrój and Elsas 2000). Some studies have reported that *Arthrobacter* species are the dominant PHE degraders in the presence of root exudates (Cebron et al. 2011; Kozdrój and Elsas 2000). Here, our results provide strong evidence that microbes in this genus are the dominant active PHE degraders in activated sludge.

Microbacterium and *Rhodococcus* have previously been confirmed as PHE degraders (Cebron et al. 2011; Liu et al. 2016). Unlike *Microbacterium* and *Rhodococcus*, no species in the genus *Rhodanobacter* has been directly linked to PHE degradation. *Rhodanobacter* is successfully used to build a consortium with *Achromobacter* sp. and *Marinobacter* sp. that are capable of efficiently degrading a wide range of PAHs (Arulazhagan et al. 2014). To our knowledge, this is the first example of *Rhodanobacter* exhibiting the ability to degrade PHE. However, results from the SIP experiment indicated that these strains did not contribute to in situ PHE degradation in the same activated sludge. The dramatic contradiction of cultivation-dependent and cultivation-independent approaches provide some insight into the reasons why constructed microconsortiums are often not as efficient as expected in the fields, that this inefficiency is caused by intensive competition of the inoculated strains face from a series of indigenous organisms (Jiang et al. 2015).

The presence of the distinctive PAH-RHD$_\alpha$ GN gene in the heavy DNA suggests it may be the functional gene employed by the PHE degraders identified by DNA-SIP in *Burkholderia*, *Paraburkholderia*, *Ralstonia*, *Sinobacteraceae*, *Kaistobacter* and *Arthrobacter*. It should be also noted that these PHE degraders might contain other functional genes that were not targeted by the primers used in this study. Additionally, the failure in amplifying PAH-RHD$_\alpha$ gene from the isolated PHE degraders might be attributed to incompatibility of the primers used in this study with some novel PAH-RHD genes or the presence of a different PHE degradation mechanism in these strains. Combined with the results that the isolated PHE degraders were not enriched in the heavy DNA fraction of ^{13}C-PHE treatment, we provided indirect evidence for their non-participation in PHE degradation in activated sludge.

Notably, the PAH-RHD$_\alpha$ GN gene identified in the heavy DNA fractions exhibits a mosaic pattern, with alignment hybridised to different known genes encoding PAH-RHDs from *Shewanella* sp. (KU372133.1), *Pseudomonas* sp. (AM743143.1) and uncultured bacterium (KF155785.1 and KX364042.1) and NDOs from *Comamonas* sp. (JN655512.1) and *Burkholderia* sp. (AF448048.1) (Fig. 5A). The corresponding peptide residues (Fig. 5B) show that the first histidine (H) might be the Rieske-type [2Fe–2S] cluster binding site, which is the active site responsible for non-heme iron oxygenase targeting aromatic compounds (Ferraro et al. 2005). The region following this H has been identified as a conserved sequence in many studies, but a combination of several PAH-RHD and *nagAc/nahAc* genes are identified in the present work. Horizontal gene transfer (HGT) and hybridisation, which allow for creation of novel genes by homologous recombination of exogenous DNA, are two major mechanisms of reticulate evolution (Makarenkov et al. 2014). Bacteria acquire heterologous genes from foreign species by HGT, which help them adapt to changing environmental conditions (Zhaxybayeva et al. 2004). Hybridisation is a very common phenomenon in nature, especially among plants, amphibians and reptiles, but is rather rare among microorganisms (Makarenkov et al. 2014). Previous studies have shown that hybridisation plays an important role in generating adaptive variation and functional novelty (Zhang et al. 2017). Due to the high sequence similarity and homology in the function of oxygenating aromatic rings between the PAH-RHD and NDO genes, they may be shared and recombined extensively between genera via HGT or hybridisation. Our results suggest that the above evolutionary processes may have occurred in the present activated sludge, leading to the formation of the novel PAH-RHD$_\alpha$ gene.

Overall, DNA-SIP was first applied to identify the indigenous bacterial taxa responsible for PHE degradation in activated sludge from a municipal wastewater treatment plant. Besides the four well-known PHE degraders, *Paraburkholderia* and *Kaistobacter* were linked to PHE degradation for the first time. Analysis of one PAH-RHD$_\alpha$ gene detected in the heavy DNA fraction suggested that HGT or hybridisation may have occurred in the activated sludge and helped with the formation and spread of PAH-RHD genes within

the microbial community. Moreover, one isolated PHE degrader, *Rhodanobacter* PHE-2, was identified as the first example of PHE-degrading ability in the genus *Rhodanobacter*, although this strain did not participate in PHE degradation in situ. This work has added to our current understanding of indigenous microorganisms with the ability to degrade PHE during the wastewater treatment process.

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