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

Jibing Li[1,2], Dayi Zhang[3], Longfei Jiang[1], Mengke Song[1], Chunling Luo[1,*] , Gan Zhang[1]

[1]Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China

[2]University of Chinese Academy of Sciences, Beijing, 100039, China

[3]Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, United Kingdom

***Corresponding author**: Dr. Chunling Luo

E-mail: clluo@gig.ac.cn; Tel.: +86-20-85290290; Fax: +86-20-85290706

Abstract The indigenous microorganisms responsible for degrading phenanthrene (PHE) in activated sludge were identified using DNA-based stable isotope probing (DNA-SIP). Besides the well-known PHE degraders *Burkholderia*, *Ralstonia*, *Sinobacteraceae* and *Arthrobacter,* the taxa *Paraburkholderia* and *Kaistobacter* were found, which were not previously linked with *in situ* PHE biodegradation. Analysis of one PAH-RHD$_\alpha$ gene detected in the heavy fraction of ^{13}C treatment suggested a history of horizontal gene transfer (HGT) or inter-species hybridisation. 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 these, *Rhodanobacter* sp. PHE-2 is the first identified strain in its genus with PHE-degrading abilities. However, the involvement of these strains in PHE degradation *in situ* was questionable, due to their limited enrichment in the heavy DNA fraction of SIP and lack of PAH-RHD$_\alpha$ genes, which were found in the activated sludge. Collectively, our findings provide a more complete understanding of the diversity of indigenous PHE-degrading communities.

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 to target for remediation has been proposed based on their toxicological properties and actual occurrence in wastewater (Ledin and Patureau 2010). Among the non-conventional pollutants targeted, polycyclic aromatic hydrocarbons (PAHs) are an important class of chemical pollutants, with two or more fused aromatic rings, which 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 the environmental impacts of PAHs.

Biodegradation has proven to be a viable option for reducing PAH 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 (Jiang et al. 2015). These works have helped to characterise the metabolic pathways and functional genes associated with PAH degradation, such as PAH-ring hydroxylating dioxygenases (PAH-RHDs) (Cebron et al. 2008). However, an important limitation to this approach is that the majority of microbes are uncultivable (Amann et al. 1995), and thus only a small proportion of the indigenous bacteria present in any environment can be isolated on laboratory media; therefore, cultivation-based methods cannot replicate *in situ* conditions. In addition, cultivation-based approaches greatly underestimate prokaryotic diversity and fail to replicate the complex interactions among individuals that are present in natural microbial communities (Oren 2004).

In the last decade, cultivation-independent techniques have been developed to evaluate the prokaryotic diversity of complex systems (Breznak 2002), and to link the identification of microorganisms with metabolic analyses (Dumont and Murrell 2005). These techniques lead to greater complexity than traditional approaches (Li et al. 2017)

and remove the bias imposed by the isolation of microorganisms necessary for cultivation-based 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 of a microbial community by analysing their isotope-enriched intracellular components (DNA, RNA, or proteins) and is especially useful for organisms that are not amenable to cultivation (Jiang et al. 2015). High-throughput sequencing technologies (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 members of complex microbial communities to functions (Jameson et al. 2017). Microbiologists have recently begun coupling SIP with HTS for the investigation of microbial functions in complex environments (Gutierrez et al. 2013; Li et al. 2017; Zhang et al. 2015).

Previous DNA-SIP studies regarding PAH biodegradation have been limited to contaminated soil and seawater, such as polluted soil 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 potential PAH-degrading microbial groups in the activated sludge of municipal wastewater treatment plants. In this study, phenanthrene (PHE) was employed as a model PAH compound (Jiang et al. 2015). DNA-SIP was applied to an activated sludge sample with the aim of linking the indigenous bacterial taxa with their roles in PHE degradation. In addition, techniques based on cultivation and high-throughput sequencing were used to achieve a more complete understanding of the bacterial communities contributing to PHE degradation. Furthermore, PAH ring-hydroxylating dioxygenase (PAH-RHD) was investigated by analysis of the relevant sequences amplified from the ^{13}C-DNA-enriched fraction. We hope to provide novel and useful information regarding the biodegradation of PAH in municipal wastewater treatment systems with a reliable theoretical basis.

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 ℃. 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 Supplementary Table S1, and were 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 an aluminium cap. Unlabelled PHE (99%) or ^{13}C-labelled PHE ($^{13}C_{14}$-PHE, 99%; Cambridge Isotope Laboratories, Inc., Tewksbury, MA, 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 triplicate. All laboratory microcosms were incubated in the dark with 120-rpm shaking at room temperature (~25 ℃). To maintain oxic conditions, the serum bottles were opened each day for about 1 hour (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. Total nucleic acids were extracted from the cell pellets harvested via

centrifugation of 15 mL of each sample from the ^{12}C-PHE and ^{13}C-PHE treatments, using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was quantified with an ND-2000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Approximately 5 μg of DNA was added to Quick-Seal polypropylene tubes (13 × 51 mm, 5.1 mL; Beckman Coulter, Pasadena, CA, 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., Buffalo Grove, IL, 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 g (20 ℃) 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 a published method (Song et al. 2015). The relationships between BD and the fraction number or DNA concentration are listed in Fig. S1 and S2, respectively.

High-throughput sequencing and computational analyses

Polymerase chain reaction (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 Bio, Otsu, japan), 1 μL (100 nM) of each primer, 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 ℃; 35 cycles of 94 ℃ for 30 min, 55 ℃ for 1 min, and 72 ℃ for 1 min; and final extension at 72 ℃ 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. The remaining sequences were analysed using the MOTHUR software

package and sequences were 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 determined as described previously (Luo et al. 2009). OTUs of the top 100 relative abundances were selected for analysis. Bacteria represented by OTUs that were enriched in the heavy fractions of ^{13}C-PHE samples compared with ^{12}C-PHE samples were identified as PHE degraders. Finally, 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 algorithm (National Center for Biotechnology Information, Bethesda, MD, USA) and Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.

Isolation of PHE degraders by enrichment and cultivation

Activated sludge samples (5 mL) were added to 50 mL of minimal medium (MM) (Table S2, pH =7.0) with 1,000 mg/L PHE (MM-P) as the carbon source. After incubation at 25 ℃ for 7 days, 5 mL of this culture 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 ℃ 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 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 ℃. Sterile controls were also incubated. All tests were carried out in triplicate with the same standard techniques and incubation conditions

used in enrichment and isolation. PHE degradation was determined using the method described in the chemical analysis.

Detection of PAH-RHD genes

Three PAH-RHD$_\alpha$ genes (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 were 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 ℃ (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 et al. 2010).

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

Chemical analysis

Three samples from each treatment were used for PHE analysis on days 0, 3 and 6. The activated sludge sample was spiked with 1,000 ng of deuterated PAHs as a surrogate standard and was 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 N2, 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, significant PHE biodegradation was observed in the biotic treatments after 3 days of incubation. On day 6, residual PHE was 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).

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 indigenous microbial communities of the ^{12}C-PHE and ^{13}C-PHE treatments (Fig. S3). These results also indicated that the majority of the abundant bacteria, such as members of unclassified phylotypes within the families *Xanthomonadaceae* and *Isosphaeraceae*, were enriched in both the ^{12}C-PHE and ^{13}C-PHE microcosms.

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 (1.73172 or 1.74801g/mL), 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 (marked with a star in Fig. 1) 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 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 (0.13%, 0.13% and 0.18%, respectively). Although the relative abundances of OTU_11 and OTU_54

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

Fig. 2 shows phylogenetic information for the 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 isolate members of the strains classified in the six SIP-identified OTUs, and to explore their degradation mechanisms and enzymes, PHE-degrading bacteria were isolated from the activated sludge with enrichment of PHE. 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 (> 1,400 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 analysed from the heavy fractions of the ^{13}C-PHE treatment. In the present study, only one type of PAH-RHD$_\alpha$ gene (KY319173) was detected and sequenced, which showed 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). 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 to 58 and 147 to 152 (Fig. 5). 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, *Pseudonomas* sp. (AM743143.1) and *Shewanella* sp. (KU372133.1). The

final region (nucleotides 147 to 253) is 100% homologous to the *nagAc* gene encoding naphthalene 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$_{\alpha}$ genes were 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 also used DNA-SIP and identified six indigenous PHE-degrading organisms, namely phylotypes affiliated with *Burkholderia*, *Paraburkholderia*, *Ralstonia*, *Kaistobacter*, *Arthrobacter* and *Sinobacteraceae* (genus unclassified) from the activated sludge. These organisms 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 bacteria does not reflect their roles in pollutant assimilation in the culture. In the present study, the dominant bacteria, including members of unclassified phylotypes in the families *Xanthomonadaceae* and *Isosphaeraceae*, are not directly linked to PHE degradation. However, these species may have roles in degrading other organic pollutants, since many organic compounds exist in activated sludge and PHE addition promoted only 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 in soil, 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, degradation of PHE 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 house-keeping 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). However, *Paraburkholderia* has not been previously linked with PHE degradation and thus their role in PAH 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*. It differs from *Burkholderia* spp. in its morphological and 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 a close relationship to *Ralstonia pickettii* ATCC 27511 (Fig. 2). *R. pickettii* is the type strain of the genus *Ralstonia*, and is considered the only representative of clinical importance (Ryan et al. 2006). Although *R. pickettii* is not considered 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 biodegradation of 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 PAH-contaminated soil revealed that *Ralstonia* was 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 PAH 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 *Alphaproteobacteria*. It is usually detected in 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 its possible function in PAH 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 PAH-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 bacteria (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 described 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* has been successfully used to build a consortium with *Achromobacter* sp. and *Marinobacter* sp. that was 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. These dramatically contradictory results of cultivation-dependent and cultivation-independent approaches provide some insight into the reasons why constructed systems are often not as efficient as expected under field conditions. The likely explanation for this inefficiency is that the inoculated strains face intensive competition from a series of native 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, amplification of the PAH-RHD$_\alpha$ gene from the isolated PHE degraders failed, which might be attributed to incompatibility of the primers used in this study or the presence of a different PHE degradation mechanism in these strains. Failure to amplify the PAH-RHD$_\alpha$ GN gene in these strains provided further evidence for their non-participation in PHE degradation in activated sludge.

Notably, the PAH-RHD$_\alpha$ GN gene identified in the heavy DNA fractions exhibited 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. 5). The corresponding peptide residues (Fig. 5) showed 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 were 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 and hybridisation. Our results indicate that such evolutionary processes have occurred in the present activated sludge, leading to formation of a novel PAH-RHD 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 fraction indicated that HGT or species hybridisation occurred in the activated sludge. Moreover, one isolated PHE degrader, *Rhodanobacter* PHE-2, was identified as the first example of PHE-degrading ability in the genus *Rhodanobacter*. However, this strain did not participate in indigenous

PHE degradation. This work has added to our current understanding of indigenous microorganisms with the ability to degrade PHE during the wastewater treatment process.

Acknowledgements

Financial support was provided by the National Natural Science Foundation of China (Nos. 41322008 & 41673111) and the Doctoral Scientific Research Foundation of Guangdong Province (No. 2016A030310118).

References

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143-169

Arulazhagan P, Sivaraman C, Kumar SA, Aslam M, Banu JR (2014) Co-metabolic degradation of benzo(e)pyrene by halophilic bacterial consortium at different saline conditions. J Environ Biol 35:445-452

Bahr A, Fischer A, Vogt C, Bombach P (2015) Evidence of polycyclic aromatic hydrocarbon biodegradation in a contaminated aquifer by combined application of in situ and laboratory microcosms using (13)C-labelled target compounds. Water Res 69:100-109

Bates ST, Berglyons D, Caporaso JG, Walters WA, Knight R, Fierer N (2011) Examining the global distribution of dominant archaeal populations in soil. ISME J 5:908

Breznak JA (2002) A need to retrieve the not-yet-cultured majority. Environ Microbiol 4:4–5

Cámara B, Herrera C, González M, Couve E, Hofer B, Seeger M (2004) From PCBs to highly toxic metabolites by the biphenyl pathway. Environ Microbiol 6:842–850

Cebron A, Louvel B, Faure P, France-Lanord C, Chen Y, Murrell JC, Leyval C (2011) Root exudates modify bacterial diversity of phenanthrene degraders in PAH-polluted soil but not phenanthrene degradation rates. Environ Microbiol 13:722-736

Cebron A, Norini MP, Beguiristain T, Leyval C (2008) Real-Time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-RHDalpha) genes from Gram positive and Gram negative bacteria in soil and sediment samples. J Microbiol Methods 73:148-159

Chen WM, Laevens S, Lee TM, Coenye T, De VP, Mergeay M, Vandamme P (2001) Ralstonia taiwanensis sp. nov., isolated from root nodules of Mimosa species and sputum of a cystic fibrosis patient. Int J Syst Evol Microbiol

51:1729-1735

Coenye T, Vandamme P (2003) Diversity and significance of Burkholderia species occupying diverse ecological niches. Environ Microbiol 5:719-729

Ding GC, Heuer H, Zuhlke S, Spiteller M, Pronk GJ, Heister K, Kogel-Knabner I, Smalla K (2010) Soil type-dependent responses to phenanthrene as revealed by determining the diversity and abundance of polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenase genes by using a novel PCR detection system. Appl Environ Microbiol 76:4765-4771

Dumont MG, Murrell JC (2005) Stable isotope probing - linking microbial identity to function. Nat Rev Microbiol 3:499-504

Ferraro DJ, Gakhar L, Ramaswamy S (2005) Rieske business: Structure-function of Rieske non-heme oxygenases. Biochem Biophys Res Commun 338:175-190

Gao Z, Yuan Y, Xu L, Liu R, Chen M, Zhang C (2016) Paraburkholderia caffeinilytica sp. nov., isolated from the soil of a tea plantation. Int J Syst Evol Microbiol 66:4185-4190

Guentas L, Gensous S, Cavaloc Y, Ducousso M, Amir H, De Georges de Ledenon B, Moulin L, Jourand P (2016) Burkholderia novacaledonica sp. nov. and B. ultramafica sp. nov. isolated from roots of Costularia spp. pioneer plants of ultramafic soils in New Caledonia. Syst Appl Microbiol 39:151-159

Gutierrez T (2011) Identifying polycyclic aromatic hydrocarbon-degrading bacteria in oil-contaminated surface waters at Deepwater Horizon by cultivation, stable isotope probing and pyrosequencing. Rev Environ Sci Bio 10:301-305

Gutierrez T, Green DH, Nichols PD, Whitman WB, Semple KT, Aitken MD (2012) Polycyclovorans algicola gen. nov., sp. nov., an Aromatic-Hydrocarbon-Degrading Marine Bacterium Found Associated with Laboratory Cultures of Marine Phytoplankton. Appl Environ Microbiol 79:205-214

Gutierrez T, Singleton DR, Berry D, Yang T, Aitken MD, Teske A (2013) Hydrocarbon-degrading bacteria enriched by the Deepwater Horizon oil spill identified by cultivation and DNA-SIP. ISME J 7:2091-2104

Hu QW, Chu X, Xiao M, Li CT, Yan ZF, Hozzein WN, Kim CJ, Zhi XY, Li WJ (2016) Arthrobacter deserti sp. nov., isolated from a desert soil sample. Int J Syst Evol Microbiol 66:2035-2040

Jameson E, Taubert M, Coyotzi S, Chen Y, Eyice Ö, Schäfer H, Murrell JC, Neufeld JD, Dumont MG (2017) DNA-, RNA-, and Protein-Based Stable-Isotope Probing for High-Throughput Biomarker Analysis of Active Microorganisms. Springer New York

Jiang L, Song M, Luo C, Zhang D, Zhang G (2015) Novel Phenanthrene-Degrading Bacteria Identified by DNA-Stable Isotope Probing. PloS one 10:e0130846

Jones MD, Crandell DW, Singleton DR, Aitken MD (2011) Stable-isotope probing of the polycyclic aromatic hydrocarbon-degrading bacterial guild in a contaminated soil. Environ Microbiol 13:2623-2632

Keith LH, Telliard WA (1979) Priority Pollutants: I. A Perspective View. Environ Sci Technol 13:416-423

Kim J-D, Shim S-H, Lee C-G (2005) Degradation of phenanthrene by bacterial strains isolated from soil in oil refinery fields in Korea. J Microbiol Biotechnol 15:337-345

Kozdrój J, Elsas JDV (2000) Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. Soil Biol Biochem 32:1405-1417

Kumar W, Lakshmi C, Khanna S (2008) Biodegradation and bioremediation of endosulfan contaminated soil. Bioresour Technol 99:3116-3122

Lebaron P, Servais P, Agogue H, Courties C, Joux F (2001) Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? Appl Environ Microbiol 67:1775-1782

Ledin A, Patureau D (2010) Xenobiotics in the Urban Water Cycle. Springer Netherlands

Li J, Liu J, Shen W, Zhao X, Hou Y, Cao H, Cui Z (2010) Isolation and characterization of 3,5,6-trichloro-2-pyridinol-degrading Ralstonia sp. strain

T6. Bioresour Technol 101:7479-7483

Li J, Luo C, Song M, Dai Q, Jiang L, Zhang D, Zhang G (2017) Biodegradation of Phenanthrene in Polycyclic Aromatic Hydrocarbon-Contaminated Wastewater Revealed by Coupling Cultivation-Dependent and -Independent Approaches. Environ Sci Technol 51:3391-3401

Liu X, Zhang S, Jiang Q, Bai Y, Shen G, Li S, Ding W (2016) Using community analysis to explore bacterial indicators for disease suppression of tobacco bacterial wilt. Sci Rep 6:36773

Luo CL, Xie SG, Sun WM, Li XD, Cupples AM (2009) Identification of a novel toluene-degrading bacterium from the candidate phylum TM7, as determined by DNA stable isotope probing. Appl Environ Microbiol 75:4644-4647

Makarenkov V, Boc A, Legendre P (2014) A New Algorithm for Inferring Hybridization Events Based on the Detection of Horizontal Gene Transfers Clusters, Orders, and Trees: Methods and Applications. Springer, pp 273-293

Minai-Tehrani D, Minoui S, Herfatmanesh A (2009) Effect of salinity on biodegradation of polycyclic aromatic hydrocarbons (PAHs) of heavy crude oil in soil. Bull Environ Contam Toxicol 82:179-184

Navarro-Noya YE, Jan-Roblero J, del Carmen González-Chávez M, Hernández-Gama R, Hernández-Rodríguez C (2010) Bacterial communities associated with the rhizosphere of pioneer plants (Bahia xylopoda and Viguiera linearis) growing on heavy metals-contaminated soils. Anton Leeuw Int J G 97:335-349

Nogi Y, Yoshizumi M, Hamana K, Miyazaki M, Horikoshi K (2014) Povalibacter uvarum gen. nov., sp. nov., a polyvinyl-alcohol-degrading bacterium isolated from grapes. Int J Syst Evol Microbiol 64:2712-2717

Oren A (2004) Prokaryote diversity and taxonomy: current status and future challenges. Philosophical transactions of the Royal Society of London Series B, Biological sciences 359:623-638

Radeva G, Kenarova A, Bachvarova V, Flemming K, Popov I, Vassilev D, Selenska-Pobell S (2013) Bacterial diversity at abandoned uranium mining and milling sites in Bulgaria as revealed by 16S rRNA genetic diversity study.

Water, Air, Soil Pollut 224:1748

Ryan MP, Pembroke JT, Adley CC (2006) Ralstonia pickettii: a persistent gram-negative nosocomial infectious organism. J Hosp Infect 62:278-284

Sawana A, Adeolu M, Gupta RS (2014) Molecular signatures and phylogenomic analysis of the genus Burkholderia: proposal for division of this genus into the emended genus Burkholderia containing pathogenic organisms and a new genus Paraburkholderia gen. nov. harboring environmental species. Front Genet 5:429

Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, Gunten UV, Wehrli B (2006) The challenge of micropollutants in aquatic systems. Science 313:1072-1077

Singh BK, Walker A (2006) Microbial degradation of organophosphorus compounds. FEMS Microbiol Rev 30:428–471

Singleton DR, Powell SN, Sangaiah R, Gold A, Ball LM, Aitken MD (2005) Stable-Isotope Probing of Bacteria Capable of Degrading Salicylate, Naphthalene, or Phenanthrene in a Bioreactor Treating Contaminated Soil. Appl Environ Microbiol 71:1202

Song M, Luo C, Jiang L, Zhang D, Wang Y, Zhang G (2015) Identification of Benzo[a]pyrene-Metabolizing Bacteria in Forest Soils by Using DNA-Based Stable-Isotope Probing. Appl Environ Microbiol 81:7368-7376

Wongwongsee W, Chareanpat P, Pinyakong O (2013) Abilities and genes for PAH biodegradation of bacteria isolated from mangrove sediments from the central of Thailand. Mar Pollut Bull 74:95-104

Yabuuchi E, Kosako Y, Oyaizu H, Yano I, Hotta H, Hashimoto Y, Ezaki T, Arakawa M (2013) Proposal of Burkholderia gen. nov. and Transfer of Seven Species of the Genus Pseudomonas Homology Group II to the New Genus, with the Type Species Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov. Microbiol Immunol 36:1251-1275

Yabuuchi E, Kosako Y, Yano I, Hotta H, Nishiuchi Y (1995) Transfer of two Burkholderia and an Alcaligenes species to Ralstonia gen. Nov.: Proposal of

Ralstonia pickettii (Ralston, Palleroni and Doudoroff 1973) comb. Nov., Ralstonia solanacearum (Smith 1896) comb. Nov. and Ralstonia eutropha (Davis 1969) comb. Nov. Microbiol Immunol 39:897–904

Zhang D, Berry JP, Zhu D, Wang Y, Chen Y, Jiang B, Huang S, Langford H, Li G, Davison PA, Xu J, Aries E, Huang WE (2015) Magnetic nanoparticle-mediated isolation of functional bacteria in a complex microbial community. ISME J 9:603-614

Zhang J-l, Ma Y-p, Wu Z-k, Dong K, Zheng S-l, Wang Y-y (2017) Natural hybridization and introgression among sympatrically distributed Rhododendron species in Guizhou, China. Biochem Syst Ecol 70:268-273

Zhaxybayeva O, Lapierre P, Gogarten JP (2004) Genome mosaicism and organismal lineages. Trends Genet 20:254-260

Tables and Figure Legends

Table 1 Primers used for the PCR of 16S rRNA and PAH-RHD 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
	396f	ATTGCGCTTAYCAYGGBTGG
	696r	ATAGGTGTCTCCAACRAARTT

Figure Captions

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

Fig. 2 Phylogenetic tree of OTUs responsible for phenanthrene (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 1,000 replications) > 50% are shown at the branch points. Bar represents 0.05 substitutions per nucleotide position.

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

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

Fig. 5 Multi-sequence alignment of PAH-RHD$_\alpha$ GN genes amplified from the heavy fractions of the ^{13}C-PHE treatment. The PAH-RHD gene 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-RHDs from the amplified PAH-RHD gene. Yellow-boxed nucleotides and residues (□) 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 (□) represent point mutations. This alignment was made using the CLUSTAL W-based optimal alignment tool.