

Stable isotope probing identifies anthracene degraders under methanogenic conditions

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Abstract Polycyclic aromatic hydrocarbons (PAHs) are common contaminants in groundwater. The remediation of PAH-contaminated groundwater often involves anaerobic biodegradation. The knowledge about the microorganisms responsible for PAH degradation in anaerobic subsurface environment is still lacking. DNA-based stable isotope probing (SIP) was applied to discover the microorganisms responsible for anaerobic anthracene degradation within microcosms inoculated with aquifer sediment from landfill leachate-contaminated site. Three phylotypes were identified as the degraders, all falling within the phylum *Proteobacteria*. Two anthracene degraders were classified within the genera *Methylibium* and *Legionella*, while another one was an unclassified *Rhizobiales* species. They all were first linked to PAH degradation. These findings also provide an illustration of the utility of SIP to discover the roles of uncultured microorganisms in PAH-degrading processes.

Keywords Stable isotope probing (SIP) · *Rhizobiales* · *Methylibium* · *Legionella* · Polycyclic aromatic hydrocarbons (PAHs)

Introduction

The existence of polycyclic aromatic hydrocarbons (PAHs) in the natural environments has caused great concerns, due to their potential threat to environmental ecosystem and human health. PAH compounds are usually present in landfill leachate-contaminated groundwater (Cornelissen et al. 2009; Jiries et al. 2005). Bioremediation is usually an economic and efficient way for PAH reduction at contaminated sites. Up to date, aerobic PAH-degrading bacteria have been well documented (Bamforth and Singleton 2005; Haritash and Kaushik 2009). Species from many genera such as *Pseudomonas*, *Burkholderia*, *Comamonas*, and *Ralstonia* are able to biodegrade an exceptionally wide variety of PAHs, using them as carbon or energy source (Chang et al. 2007). Moreover, numerous studies have investigated PAH biodegradation under different redox conditions, mostly with nitrate or sulfate as terminal electron acceptors (Chang et al. 2005b; Meckenstock et al. 2004). Pure cultures of several anaerobic nitrate- and sulfate-reducing bacteria, capable of degrading PAHs, have also been identified and most of the species are from the genus *Pseudomonas* (Haritash and Kaushik 2009). However, little information is available for anaerobic PAH biodegradation under methanogenic condition (Chang et al. 2005b, 2006). The anaerobic isolate able to degrade PAHs under methanogenic condition is still lacking.

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Although many isolates have been obtained, microorganisms able to transform PAHs in a pure culture may not be able to perform the same function in the field. Different culture conditions or habitats may select for specific PAH degraders. Molecular approaches (e.g., denaturing gradient gel electrophoresis (DGGE), clone libraries, or terminal restriction fragment length polymorphism (TRFLP)) have enabled the identification of community members in a mixed culture sample. For example, clone library analysis showed the *δ-proteobacteria* was the major bacterial group in naphthalene-degrading methanogenic cultures initiated with Baltimore Harbor sediments, but the *Firmicutes* predominated in phenanthrene-degrading methanogenic culture (Chang et al. 2005b). However, these techniques typically do not directly link the ability to degrade a specific contaminant to individual species (Xie et al. 2010). Therefore, little is known about the microorganisms actually responsible for PAH transformation in mixed cultures or in situ at contaminated sites.

Stable isotope probing (SIP) has gained increasing attention as a novel tool for linking function with identity for environmental samples (Radajewski et al. 2000). Recently, SIP has been applied to identify naphthalene degraders in coal tar-contaminated sediment and soil (Jeon et al. 2003; Yu and Chu 2005), organisms capable of degrading naphthalene, phenanthrene, or pyrene in a bioreactor treating soil from a former manufactured-gas plant (Singleton et al. 2005, 2006), pyrene-degrading bacteria in creosote-contaminated soil (Jones et al. 2008). However, all these previous SIP works have been targeted on aerobic PAH-degrading microorganisms.

Due to both the low amount of dissolved oxygen and the slow diffusion of oxygen into subsurface, microorganisms can rapidly deplete oxygen, which suggests that anaerobic biodegradation might be more attractive in the subsurface environment (Chang et al. 2005a). SIP has recently been used to identify the active degraders of contaminants in various anaerobic environments, such as anaerobic benzene degraders in contaminated aquifer (Herrmann et al. 2010), or in freshwater sediment (Liou et al. 2008), sulfate-reducing toluene degrader in a BTEX contaminated aquifer (Bombach et al. 2010), benzoate-utilizing denitrifying bacteria in marine sediments (Gallagher et al. 2005), anaerobic phenol degraders in the treatment of synthetic coke-oven wastewater (Sueoka

et al. 2009), and perchloroethene-respiring microorganisms in anoxic river sediment (Kittelman and Friedrich 2008). These studies all illustrate SIP provides significant potential for understanding in situ anaerobic degradation.

Anthracene is selected as a PAHs model in this study. To obtain a more complete understanding of anthracene degradation in mixed culture, DNA-based SIP was applied here to investigate the microorganisms responsible for anaerobic anthracene degradation within microcosms inoculated with aquifer sediment from landfill leachate-contaminated sites. To the authors' knowledge, this was the first report directly linking anaerobic PAH degradation to specific microorganisms in a mixed community sample using SIP. This work could provide additional insights on the microbial ecology of anaerobic PAH degradation in mixed culture samples.

Materials and methods

Soil incubation and chemical analyses

Subsurface aquifer sediment utilized in this study was collected from a landfill leachate-contaminated site. Following sample collection, aquifer sediment was homogenized and sieved through a 0.18-mm screen, and stored at 4°C until use. Sediment microcosms consisted of mineral salt medium (10 ml), as previously described (Yang and McCarty 1998), and sediment (3 g) in serum bottles (150 ml). Three treatments were prepared: sterile control, sample amended with unlabeled anthracene (120 µg, 99%, J&K China Chemical), and sample amended with labeled anthracene (120 µg, ring-¹³C₆ anthracene, 99%, Cambridge Isotope Laboratories). Anthracene stock was first prepared in nonane. A volume of nonane containing the desired mass of anthracene was added to the serum bottle and the solvent was allowed to evaporate. Mineral salt medium and sediment was then added to the anthracene crystals in the bottles. No supplementary electron acceptor was added to the cultures in this study. To obtain anaerobic condition, the headspace in bottles was vacuumed for 10 s, and then refilled with purified N₂ gas by puncturing the septa with a 1 ml syringe. This procedure was repeated three times to ensure that all of the oxygen inside the bottles was replaced by N₂

gas (Li et al. 2010). The bottles were sealed with rubber stoppers and aluminum seals to maintain anaerobic conditions. The sterile controls were obtained by autoclaving repeatedly (three times). All samples exposed to anthracene (labeled or unlabeled) were prepared in duplicate, and thus the SIP investigation occurred in duplicate. Duplicate samples were not pooled, and the entire analysis for each was carried out separately.

Microcosms were incubated on a horizontal shaker (~100 rpm) at 20°C. A 120-day preliminary microcosm experiment (amended with unlabeled anthracene) indicated that percent anthracene remaining in solid phase on day 30, 60, 90, and 120 were 85–88, 42–45, 23–26, and 12–14%, respectively. The active degrader would be more enriched when the target compound was more significantly degraded (Luo et al. 2009). Therefore, on day 120, microcosms were sacrificed and sediment samples in duplicate were dried using a freeze drier (Alpha 1–2 LD plus, Martin Christ, German). Two grams of dried sediment samples were removed for DNA extraction (see below) and the remaining sediment was extracted with acetone for anthracene analysis, as previously described (Zhang et al. 2011). The anthracene analysis in solid phase was conducted using a HPLC apparatus (Shimadzu LC-10Avp, Agilent Technologies) equipped with a LC-10AT pump, a UV-detector, a Venusil PAH column (Agela Technologies). Methanol–water (90:10) was used as the mobile phase at a flow rate of 1 ml min⁻¹. Anthracene was detected by absorbance at 251 nm with the mean recovery rate of 97%. Gas headspace samples were analyzed for methane with a gas chromatograph with a thermal conductivity detector. The operational temperature of the column was 40°C, and the carrier gas (N₂) was at a flow rate of 30 ml min⁻¹ (He et al. 2008).

DNA extraction and ultracentrifugation

Following the depletion of anthracene, DNA was extracted from sediment samples of replicate labeled and replicate unlabeled anthracene amended microcosms with the Powersoil kit (Mobio Laboratories) following the manufacturer's instructions. Separation of the unlabeled and ¹³C-labeled DNA was accomplished by density gradient ultracentrifugation in cesium chloride according to the standard method (Xie et al. 2010, 2011), except that an Optima LE-

80K Preparative Ultracentrifuge (Beckman Instruments) was used. Following ultracentrifugation, the tubes were placed into a fraction recovery system (Beckman Coulter) for fraction (200 µl) collection. The buoyant density (BD) of each fraction was measured (model AR200 digital refractometer, Leica Microsystems Inc.). CsCl was removed by glycogen-assisted ethanol precipitation and purified DNA fractions were stored at -20°C.

TRFLP and 16S rRNA gene sequencing

Each ultracentrifugation fraction for both labeled and unlabeled microcosms was subject to TRFLP analysis. Bacterial 16S rRNA genes were amplified using primers 27F-FAM (5'-GAGTTTGATCMTGGCT-CAG-3', 5' end-labeled with carboxyfluorescein) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Tiangen BioTech, China) (Xie et al. 2010, 2011). The PCR program was run as follows: 94°C (5 min); 94°C (30 s); 55°C (30 s); 72°C (1.5 min) (30 cycles); 72°C (5 min). PCR products (300 ng) were purified with QIAquick PCR purification kit (Qiagen Inc.), following the manufacturer's instructions. Purified PCR products were digested with *Hae*III with a 6 h incubation period at 37°C. Digests *Hha*I and *Msp*I were used to correlate the enriched TRFLP fragment lengths in the heavy fractions to the in silico cut sites of the cloned sequences (Xie et al. 2010). The in silico analysis was performed online (<http://www.restrictionmapper.org/>). The same purified PCR product was used for digestion with *Hae*III, *Hha*I and *Msp*I. The clone sequence was first identified, whose in silico cut site (*Hae*III digest) matched the length of the enriched fragment (*Hae*III digest) in the heavy fractions. If the clone restriction enzyme (*Hha*I and *Msp*I) cut site predicted from sequence also matched the observed fragment length (*Hha*I and *Msp*I digest) in the heavy fractions, the taxonomic identity of the enriched fragment (*Hae*III digest) could be confirmed (Cupples and Sims 2007). The fragment pattern was detected by using an ABI 3730 DNA Analyzer (Applied Biosystems) with an internal lane standard (GeneScan-500 LIZ Size Standard, Applied Biosystems). The percent abundance of each fragment was determined, as previously described (Yu and Chu 2005).

For 16S rRNA gene sequencing, heavy fraction ¹³C-DNA was amplified as above, except the forward primer was unlabeled. PCR products were purified and

cloned into pMD19-T vector (Takara Corp, Japan) following the manufacturer's instruction. *E. coli* clones were grown on Luria–Bertani (LB) medium solidified with 15 g l⁻¹ agar with 50 µg l⁻¹ ampicillin for 16 h at 37°C. The white colonies were verified by PCR with primers M13 F (5'-TGTAACGACGGCCAGT-3') and M13 R (5'-AACAGCTATGACCATG-3'). Clones were sequenced at SinoGenoMax Co., Ltd. (Beijing) and chimerism was checked using the Chimera Check program available at the MSU Center for Microbial Ecology. The closest GenBank sequences to microorganisms identified in this study were searched from the National Center for Biotechnology Information (NCBI) database (Altschul et al. 1990). The Ribosomal Database Project (MSU Center for Microbial Ecology) analysis tool “classifier” was utilized to determine taxonomic identity (Wang et al. 2007). The bacterial partial 16S rRNA gene sequences (800 bp) of microorganisms linked to anthracene degradation were deposited with GenBank under accession numbers HQ888823–HQ888825.

Results

Methane production in microcosms was observed during the whole biodegradation experiment, with an increase from 0 (day 0) to approximately 120 µmol l⁻¹ (day 120), while the amount was negligible in the sterile controls, indicating the occurrence of methanogenesis in microcosms. The degradation pattern was similar for sediment amended with either the labeled or unlabeled anthracene. Approximately half of the anthracene remained after 60 days and a more significant depletion was observed after 120 days, compared with very limited decline in the autoclaved control, confirmed a biological removal mechanism (Table 1).

DNA extracts (day 120) from labeled and unlabeled anthracene amended sediment samples were subject to ultracentrifugation, fractionation, followed by TRFLP on each fraction. Analyses of TRFLP

profiles illustrated the enrichment of three different fragments in the heavy fractions obtained from the ¹³C-anthracene microcosms but not in the unlabeled microcosms (Fig. 1). Relative abundances of the three fragments in TRFLP profiles over a range of BD values are illustrated in Fig. 2. Notably, three TRFLP fragments (193, 198 and 206 bp) were enriched in the heavy fractions. This suggests three different microorganisms were responsible for anthracene biodegradation. Peak relative abundance values in ¹³C-anthracene amended samples were 6.3 or 7.5% (at 1.734 g ml⁻¹), 7 or 8% (at 1.715 g ml⁻¹), and 8.1 or 9.3% (at 1.737 g ml⁻¹) for TRFLP fragments 193, 198 and 206 bp, respectively (Fig. 2).

To determine the identity of these enriched fragments approximately 60 clones were partially sequenced. In addition, abundant TRFLP fragments (relative abundance above 1%) obtained with selected ¹³C fractions with *Hha*I or *Msp*I were used to provide a confirmative identification of each *Hae*III TRFLP enriched fragment (Fig. 3). The abundant fragments obtained from all digests were compared to those obtained from in silico digests to determine the sequence of each enriched fragment. Comparison of TRFLP and in silico cut sites for each identified fragment is presented (Table 2). A slight difference (2–3 bases) between the observed fragment lengths and those predicted using sequence data has also been noted elsewhere (Luo et al. 2009; Xie et al. 2010, 2011). The taxonomic identity of each enriched fragment (cloned sequence) is presented (Table 3). The enriched *Hae*III TRFLP fragments 193, 198 and 206 bp in ¹³C heavy DNA fraction were identified as unclassified *Rhizobiales*, genera *Methylibium* and *Legionella*, respectively.

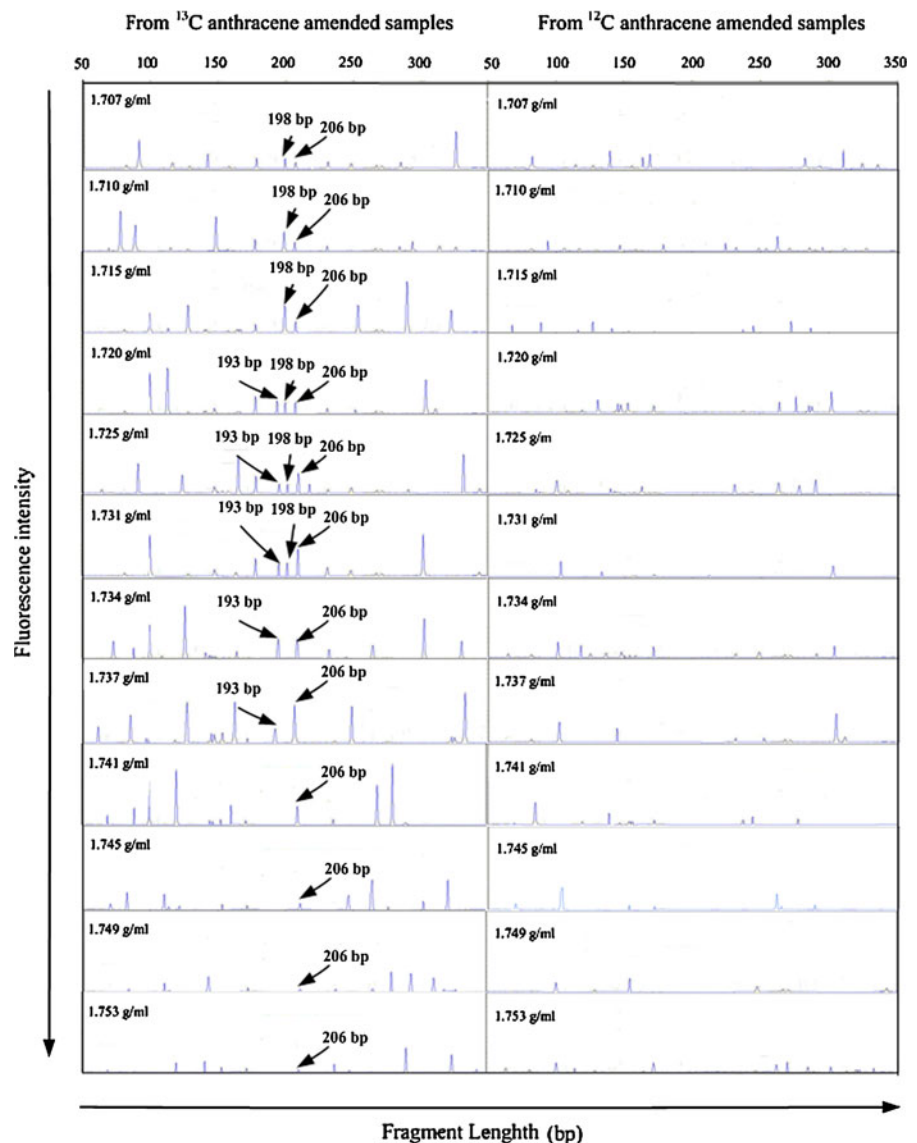
Discussion

A field-based SIP work has shown that a *β-proteobacterium* strain was responsible for naphthalene degradation in coal tar-contaminated sediment (Jeon et al. 2003). *β-* and *γ-Proteobacteria* were responsible for naphthalene degradation in coal tar-contaminated soil (Yu and Chu 2005). The primary pyrene degraders identified in a bioreactor treating soil from a former manufactured-gas plant site were uncultivated *β-* and *γ-proteobacteria* (Singleton et al. 2006). The *γ-proteobacteria* responsible for pyrene degradation

Table 1 Ranges for percentages of anthracene remaining in solid phase over the experimental period

Time (days)	60	120
Sterile controls	93–97	94–95
Anthracene (¹² C)	40–46	14–18
Anthracene (¹³ C)	40–50	12–15

Fig. 1 Comparison of heavy fraction TRFLP profiles from unlabeled (^{12}C) and labeled (^{13}C) anthracene amended sediments to illustrate the enriched fragments in labeled fractions compared to the unlabeled fractions control. The duplicate illustrated the same trend

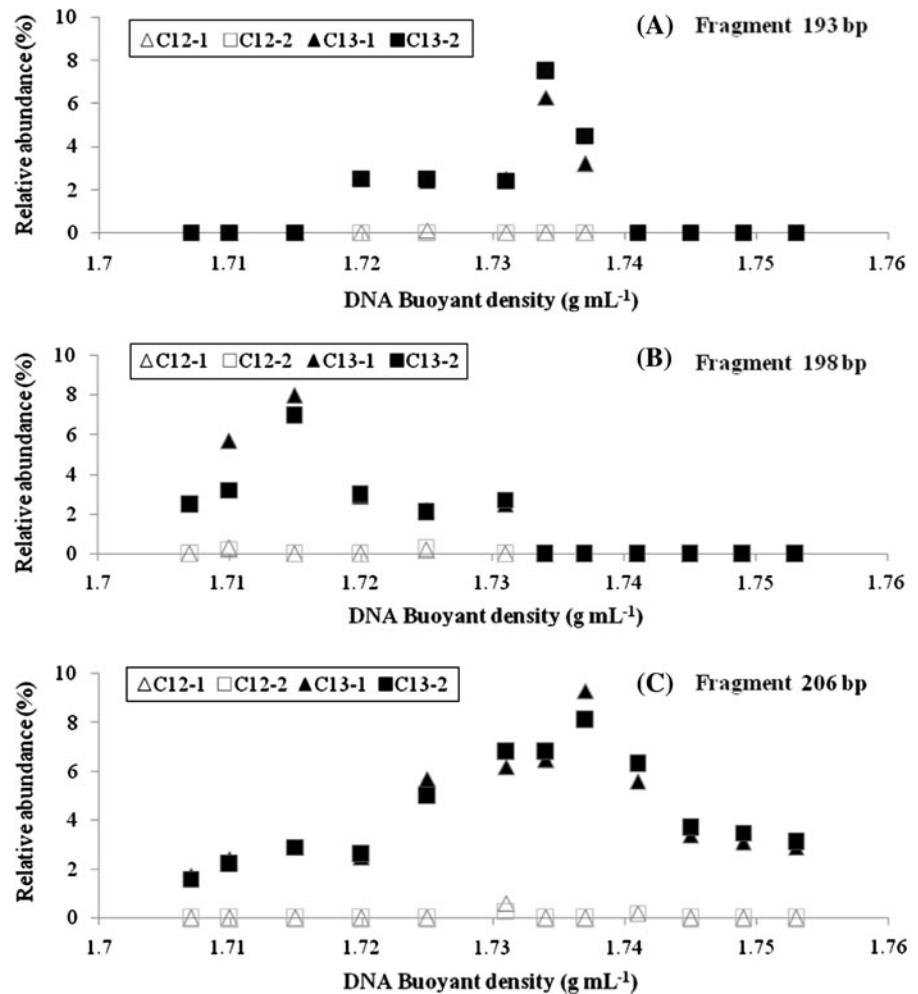


was the most prominent sequences recovered in ^{13}C heavy DNA fraction from creosote-contaminated soil treated either by the addition of inorganic nutrients or by slurring (Jones et al. 2008). These previous reports provide strong evidence that phylum *Proteobacteria* is widely involved in PAH degradation. The current study was to investigate the active anaerobic PAH degraders in microcosm inoculated with subsurface sediment a landfill leachate-contaminated site. DGGE analysis showed the dominant bacterial species in groundwater contaminated with landfill leachate were also α -, β - or γ -*proteobacteria* (Röling et al. 2001). Sequence analysis of 16S rDNA clone library indicated

the presence of β -, γ -, δ -, ϵ -*proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Cyanobacteria* in leachate-polluted aquifer (Tian et al. 2005). In this study, members of α -, β - and γ -*proteobacteria* were directly linked to anthracene degradation under methanogenic condition. This finding sustained the role of phylum *Proteobacteria* in PAH degradation under methanogenic condition. However, given the slow anthracene degradation rates observed, the possibility of label cross-feeding can not entirely be ruled out.

Clone libraries of ^{13}C labeled heavy DNA from the aerobic phenanthrene and naphthalene incubations were composed primarily of genus *Acidovorax*, and

Fig. 2 Relative abundance of the three enriched TRFLP fragments (a–c) in fractions of three ^{13}C -anthracene amended sediment compared to unlabeled anthracene amended sediment (day 120)



genera *Pseudomonas* and *Ralstonia*, respectively (Singleton et al. 2005). Another two previous SIP works showed genus *Polaromonas* or *Intrasporangium* was responsible for aerobic naphthalene degradation in coal tar-contaminated sites (Jeon et al. 2003; Yu and Chu 2005). In this study, genera *Methylibium* and *Legionella*, and unclassified *Rhizobiales* were responsible for anaerobic anthracene degradation.

Many different bacteria able to degrade anthracene aerobically, previously isolated from PAH-contaminated site, belong to genera *Mycobacterium* (Pizzul et al. 2007), *Pseudomonas* (Al-Thani et al. 2009), *Achromobacter* (Al-Thani et al. 2009), *Janibacter* (Yamazoe et al. 2004), and *Rhodococcus* (Tongpim and Pickard 1996). It is of interest to be noted that any previously reported anthracene degraders were not identified here. The active anthracene degraders might be site-dependent. In this study, the microcosms were

inoculated with aquifer sediment in landfill leachate-contaminated site. To the authors' knowledge, no isolate of PAH degrader has been obtained from the kind of habitat.

The enriched *Hae*III TRFLP fragment 193 bp in ^{13}C heavy DNA fraction was identified here as an unclassified *Rhizobiales* species within the α -proteobacteria. The 16S rRNA gene sequence of the *Rhizobiales* (HQ888823) illustrated high similarity to reported sequences within Genbank. The three most similar partial 16S rRNA gene sequences (99% similarity) (CU918797.1, CU925735.1 and CU927124.1) originated from anaerobic sludge digesters (Rivière et al. 2009). The closest cultivated match was a *Rhizobiales* species (AJ810382.1, 96% identity) from legume (Lafay and Burdon 2006). Some members of the order *Rhizobiales* could degrade benzene, toluene, ethylbenzene and xylene (Cavalca et al. 2004), hydroxy- and

Fig. 3 TRFLP profiles from fractions (1.715 and 1.737 g ml⁻¹) of ¹³C labeled anthracene amended sediment with two different restriction enzymes

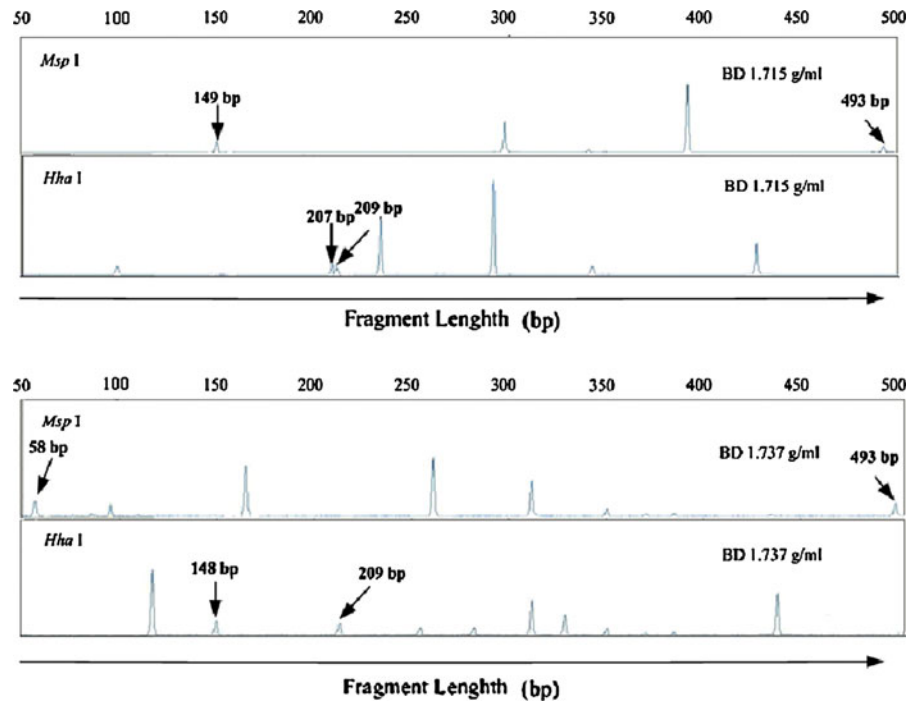


Table 2 Comparison of enriched fragments (bp) in heavy fraction TRFLP to clone restriction enzyme cut sites predicted from sequence data to confirm the identity of the enriched TRFLP fragments

Fragments	Restriction enzyme	TRFLP	Sequence data
Fragment 1	<i>Hae</i> III	193	191
	<i>Msp</i> I	58	61
	<i>Hha</i> I	148	148
Fragment 2	<i>Hae</i> III	198	198
	<i>Msp</i> I	149	149
	<i>Hha</i> I	207	205
Fragment 3	<i>Hae</i> III	206	206
	<i>Msp</i> I	493	496
	<i>Hha</i> I	209	213

Table 3 Phylogenetic affiliation of each enriched fragment (*Hae*III digest) as determined with the RDP analysis tool “classifier”

Fragment	Phylum	Class	Order	Family	Genus
Fragment 193 bp	<i>Proteobacteria</i>	α - <i>Proteobacteria</i>	<i>Rhizobiales</i>	<i>Unclassified_Rhizobiales</i>	
Fragment 198 bp	<i>Proteobacteria</i>	β - <i>Proteobacteria</i>	<i>Burkholderiales</i>	<i>Burkholderiales_incertain_sedis</i>	<i>Methylibium</i>
Fragment 206 bp	<i>Proteobacteria</i>	γ - <i>Proteobacteria</i>	<i>Legionellales</i>	<i>Legionellaceae</i>	<i>Legionella</i>

dihydroxy-benzoates, halogenated aromatic compounds (Vela et al. 2002), and phenol (Baek et al. 2003). However, to the authors’ knowledge, this was also the first study to directly link the order *Rhizobiales* to the degradation of any PAH compounds. Moreover,

although some α -*proteobacteria* species are well known for their ability to transform PAHs (Lafortune et al. 2009), the uniqueness of the 16S rRNA gene sequence identified here appears to represent a truly novel PAH degrader within the class.

The microorganism (with representative *HaeIII* fragment 198 bp) was classified within the β -*proteobacteria* as a *Methylibium* species. The three closest matches of the sequence (HQ888824) of *Methylibium* strain identified here were obtained from a restored grassland (EU300364.1, 100%, unpublished GenBank data), trichloroethene-contaminated groundwater with propane-stimulated bioremediation (AY435511.1, 98%) (Connon et al. 2005), and tallgrass prairie soil (FJ479220.1, 97%). Moreover, the 16S rRNA gene sequence of the *Methylibium* obtained in this study was closely related to a *Methylibium* strain (DQ664244.1, 97%) and an *Azonexus* strain (DQ664241.1, 97%) isolated from a eutrophic freshwater pond (Chou et al. 2008). Some species of *Methylibium* have usually been linked to aerobic methyl tert-butyl ether (MTBE) degradation (Kane et al. 2007; Nakatsu et al. 2006). However, a *Methylibium* species could also degrade aromatic (benzene, toluene, and xylene) and straight-chain hydrocarbons present in petroleum products (Kane et al. 2007). To the authors' knowledge, this was also the first study to directly link a microorganism within this genus to the degradation of any PAH compounds.

The third microorganism (with representative *HaeIII* fragment 206 bp) responsible for anthracene degradation under methanogenic condition was classified within the γ -*proteobacteria* as a *Legionella* species. The 16S rRNA gene sequence (HQ888825) of the *Legionella* strain identified in this study had a low similarity (95%) to the sequences in GenBank, representing a novel PAH degrader within the class γ -*proteobacteria*. Surprisingly, little information exists on microorganisms within the genus *Legionella*. Two members of genus *Legionella* have been linked to be opportunistic pathogen or pathogen, respectively (Jjemba et al. 2010; Solomon et al. 2000). However, to the authors' knowledge, there has been no report concerning the role of genus *Legionella* in degradation of environmental contaminants. Therefore, the result obtained in the current study could contribute to the limited pool of knowledge on the function of this novel organism within mixed microbial communities.

Conclusions

SIP was used to identify the microorganisms responsible for anaerobic anthracene degradation within

microcosms inoculated with aquifer sediment from landfill leachate-contaminated sites. Three phylogenetic types within the phylum *Proteobacteria* were identified as the degraders. Two were classified within the genera *Methylibium* and *Legionella* respectively, and another one was an unclassified *Rhizobiales*. They all were first linked to PAH degradation. These findings have the potential to add important new knowledge to PAH degradation under methanogenic conditions.

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References

- Al-Thani RF, Abd-El-Haleem DAM, Al-Shammri M (2009) Isolation and characterization of polyaromatic hydrocarbons-degrading bacteria from different Qatari soils. *Afr J Microbiol Res* 3:761–766
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Baek SH, Kim KH, Yin CR, Jeon CO, Im WT, Kim KK, Lee ST (2003) Isolation and characterization of bacteria capable of degrading phenol and reducing nitrate under low-oxygen conditions. *Curr Microbiol* 47:462–466
- Bamforth SM, Singleton L (2005) Bioremediation of polycyclic aromatic hydrocarbons, current knowledge and future directions. *J Chem Technol Biotechnol* 80:723–736
- Bombach P, Chatzinotas A, Neu TR, Kästner M, Lueders T, Vogt C (2010) Enrichment and characterization of a sulfate-reducing toluene degrading microbial consortium by combining in situ microcosms and stable isotope probing techniques. *FEMS Microbiol Ecol* 71:237–246
- Cavalca L, Dell'Amico E, Andreoni V (2004) Intrinsic bioremediability of an aromatic hydrocarbon-polluted groundwater: diversity of bacterial population and toluene monooxygenase genes. *Appl Microbiol Biotechnol* 64:576–587
- Chang W, Um YS, Holoman TRP (2005a) Molecular characterization of anaerobic microbial communities from benzene-degrading sediments under methanogenic conditions. *Biotechnol Prog* 21:1789–1794
- Chang W, Um YS, Holoman TRP (2005b) Molecular characterization of polycyclic aromatic hydrocarbon (PAH)-degrading methanogenic communities. *Biotechnol Prog* 21:682–688
- Chang W, Um YS, Holoman TRP (2006) Polycyclic aromatic hydrocarbon, PAH degradation coupled to methanogenesis. *Biotechnol Lett* 28:425–430
- Chang YT, Lee JF, Chao HP (2007) Variability of communities and physiological characteristics between free-living bacteria and attached bacteria during the PAH biodegradation in a soil/water system. *Eur J Soil Biol* 43:283–296
- Chou JH, Jiang SR, Cho JC, Song J, Lin MC, Chen WM (2008) *Azonexus hydrophilus* sp. nov., a nifH gene-harboring bacterium isolated from freshwater. *Int J Syst Evol Microbiol* 58:946–951

- Connon SA, Tovanaboot A, Dolan M, Vergin K, Giovannoni SJ, Semprini L (2005) Bacterial community composition determined by culture-independent and -dependent methods during propane-stimulated bioremediation in trichloroethene-contaminated groundwater. *Environ Microbiol* 7:165–178
- Cornelissen G, Okkenhaug G, Breedveld GD, Sorlie JE (2009) Transport of polycyclic aromatic hydrocarbons and polychlorinated biphenyls in a landfill: a novel equilibrium passive sampler to determine free and total dissolved concentrations in leachate water. *J Hydrol* 369:253–259
- Cupples AM, Sims GK (2007) Identification of in situ 24-dichlorophenoxyacetic acid-degrading soil microorganisms using DNA-stable isotope probing. *Soil Biol Biochem* 39:232–238
- Gallagher E, McGuinness L, Phelps C, Young LY, Kerkhof LJ (2005) ^{13}C -carrier DNA shortens the incubation time needed to detect benzoate-utilizing denitrifying bacteria by stable-isotope probing. *Appl Environ Microbiol* 71:5192–5196
- Haritash AK, Kaushik CP (2009) Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *J Hazard Mater* 169:1–15
- He R, Ruan AD, Jiang CJ, Shen DS (2008) Responses of oxidation rate and microbial communities to methane in simulated landfill cover soil microcosms. *Bioresour Technol* 99:7192–7199
- Herrmann S, Kleinstueber S, Chatzinotas A, Kuppardt S, Lueders T, Richnow HH, Vogt C (2010) Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing. *Environ Microbiol* 12:401–411
- Jeon CO, Park W, Padmanabhan P, DeRito C, Snape JR, Madsen EL (2003) Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ biodegradation in contaminated sediment. *Proc Natl Acad Sci USA* 100:13591–13596
- Jiries A, Rimawi O, Lintelmann J, Batarseh M (2005) Polycyclic aromatic hydrocarbons (PAH) in top soil, leachate and groundwater from Ruseifa solid waste landfill, Jordan. *Int J Environ Pollut* 23:179–188
- Jjemba PK, Weinrich LA, Cheng W, Giraldo E, LeChevallier MW (2010) Regrowth of potential opportunistic pathogens and algae in reclaimed-water distribution systems. *Appl Environ Microbiol* 76:4169–4178
- Jones MD, Singleton DR, Carstensen DP, Powell SN, Swanson JS, Pfaender FK, Aitken MD (2008) Effect of incubation conditions on the enrichment of pyrene-degrading bacteria identified by stable-isotope probing in an aged, PAH-contaminated soil. *Microb Ecol* 56:341–349
- Kane SR, Chakicherla AY, Chain PSG, Schmidt R, Shin MW, Legler TC, Scow KM, Larimer FW, Lucas SM, Richardson PM, Hristova KR (2007) Whole-genome analysis of the methyl tert-butyl ether-degrading beta-proteobacterium *Methylibium petroleiphilum* PM1. *J Bacteriol* 189:1931–1945
- Kittlmann S, Friedrich MW (2008) Identification of novel perchloroethene-respiring microorganisms in anoxic river sediment by RNA-based stable isotope probing. *Environ Microbiol* 10:31–46
- Lafay B, Burdon JJ (2006) Molecular diversity of rhizobia nodulating the invasive legume *Cytisus scoparius* in Australia. *J Appl Microbiol* 100:1228–1238
- Lafortune I, Juteau P, Deziel E, Lepine F, Beaudet R, Villemur R (2009) Bacterial diversity of a consortium degrading high-molecular-weight polycyclic aromatic hydrocarbons in a two-liquid phase biosystem. *Microb Ecol* 57:455–468
- Li CH, Wong YS, Tama NFY (2010) Anaerobic biodegradation of polycyclic aromatic hydrocarbons with amendment of iron III in mangrove sediment slurry. *Bioresour Technol* 101:8083–8092
- Liou JSC, DeRito CM, Madsen EL (2008) Field-based and laboratory stable isotope probing surveys of the identities of both aerobic and anaerobic benzene-metabolizing microorganisms in freshwater sediment. *Environ Microbiol* 10:1964–1977
- 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* 75:4644–4654
- Meckenstock RU, Safinowski M, Griebler C (2004) Anaerobic degradation of polycyclic aromatic hydrocarbons. *FEMS Microbiol Ecol* 49:27–36
- Nakatsu CH, Hristova K, Hanada S, Meng XY, Hanson JR, Scow KM, Kamagata Y (2006) *Methylibium petroleiphilum* gen. nov., sp. nov., a novel methyl tert-butyl ether-degrading methylotroph of the Betaproteobacteria. *Int J Syst Evol Microbiol* 56:983–989
- Pizzul L, Sjogren A, Castillo MD, Stenstroem J (2007) Degradation of polycyclic aromatic hydrocarbons in soil by a two-step sequential treatment. *Biodegradation* 18:607–616
- Radajewski S, Ineson P, Parekh NR, Murrell JC (2000) Stable-isotope probing as a tool in microbial ecology. *Nature* 403:646–649
- Rivière D, Desvignes V, Pelletier E, Chaussonnerie S, Guermazi S, Weissenbach J, Li T, Camacho P, Sghir A (2009) Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *ISME J* 3:700–714
- Röling WFM, Van Breukelen BM, Braster M, Lin B, Van Verseveld HW (2001) Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer. *Appl Environ Microbiol* 67:4619–4629
- 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* 71:1202–1209
- Singleton DR, Sangaiah R, Gold A, Ball LM, Aitken MD (2006) Identification and quantification of uncultivated proteobacteria associated with pyrene degradation in a bioreactor treating PAH contaminated soil. *Environ Microbiol* 8:1736–1745
- Solomon JM, Rupper A, Cardelli JA, Isberg RR (2000) Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. *Infect Immun* 68:2939–2947
- Sueoka K, Satoh H, Onuki M, Mino T (2009) Microorganisms involved in anaerobic phenol degradation in the treatment

- of synthetic coke-oven wastewater detected by RNA stable-isotope probing. *FEMS Microbiol Lett* 291:169–174
- Tian YJ, Yang H, Wu XJ, Li DT (2005) Molecular analysis of microbial community in a groundwater sample polluted by landfill leachate and seawater. *J Zhejiang Univ Sci B* 23:165–170
- Tongpim S, Pickard MA (1996) Growth of *Rhodococcus* S1 on anthracene. *Can J Microbiol* 42:289–294
- Vela S, Haggblom MM, Young LY (2002) Biodegradation of aromatic and aliphatic compounds by rhizobial species. *Soil Sci* 167:802–810
- Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73:5261–5267
- Xie SG, Sun WM, Luo CL, Cupples AM (2010) Stable isotope probing identifies novel *m*-xylene degraders in soil microcosms from contaminated and uncontaminated sites. *Water Air Soil Pollut* 212:113–122
- Xie SG, Sun WM, Luo CL, Cupples AM (2011) Novel aerobic benzene degrading microorganisms identified in three soils by stable isotope probing. *Biodegradation* 22:71–81
- Yamazoe A, Yagi O, Oyaizu H (2004) Degradation of polycyclic aromatic hydrocarbons by a newly isolated dibenzofuran-utilizing *Janibacter* sp strain YY-1. *Appl Microbiol Biotechnol* 65:211–218
- Yang Y, McCarty PL (1998) Competition for hydrogen within a chlorinated solvent dehalogenating anaerobic mixed culture. *Environ Sci Technol* 32:3591–3597
- Yu CP, Chu KH (2005) A quantitative assay for linking microbial community function and structure of a naphthalene-degrading microbial consortium. *Environ Sci Technol* 39:9611–9619
- Zhang SY, Wang QF, Xie SG (2011) Microbial community changes in contaminated soils in response to phenanthrene amendment. *Int J Environ Sci Technol* 8:321–330