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Novel aerobic benzene degrading microorganisms identified in three soils by stable isotope probing

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Abstract The remediation of benzene contaminated groundwater often involves biodegradation and although the mechanisms of aerobic benzene biodegradation in laboratory cultures have been well studied, less is known about the microorganisms responsible for benzene degradation in mixed culture samples or at contaminated sites. To address this knowledge gap, DNA based stable isotope probing (SIP) was utilized to identify active benzene degraders in microcosms constructed with soil from three sources (a contaminated site and two agricultural sites). For this, replicate microcosms were amended with either labeled (^{13}C) or unlabeled benzene and the extracted DNA samples were ultracentrifuged, fractioned and subject to terminal restriction fragment length polymorphism (TRFLP). The dominant benzene degraders (responsible for ^{13}C uptake) were determined by comparing relative abundance of TRFLP phylotypes in heavy fractions of labeled benzene (^{13}C) amended samples to the controls (from unlabeled benzene amended samples). Two phylotypes (a *Polaromonas* sp. and an *Acidobacterium*) were the major benzene degraders in the microcosms constructed from the contaminated site soil, whereas one phylotype incorporated the majority of the benzene-derived ^{13}C in each of the agricultural soils ("candidate" phylum TM7 and an unclassified *Sphingomonadaceae*).

Keywords Stable isotope probing · Benzene · TM7 · *Polaromonas* · *Acidobacterium* · *Sphingomonadaceae*

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Introduction

Benzene, toluene, ethylbenzene, and xylenes (BTEX) are common groundwater contaminants largely because of leaking underground gasoline storage tanks. Among them, benzene represents a particular risk to humans due to its carcinogenicity. To understand benzene bioremediation options at contaminated sites, many aerobic benzene degrading isolates have been obtained and examined. Commonly identified isolates appear to fall within two phyla, the *Proteobacteria* (γ and β *Proteobacteria*) and *Actinobacteria*. *Proteobacteria* (γ or β) include, for example, microorganisms within the genera *Pseudomonas* (Alagappan and Cowan 2003; Bertoni et al. 1996; Irie et al. 1987; Kim et al. 2005; Kitayama et al. 1996; Leahy

et al. 2003; Reardon et al. 2002), *Burkholderia* (Alagappan and Cowan 2003; Johnson and Olsen 1997; Leahy et al. 2003; Radway et al. 1998; Reardon et al. 2002), *Ralstonia* (Alagappan and Cowan 2003; Leahy et al. 2003; Olsen et al. 1994), *Achromobacter* (Yeom et al. 1998; Yeom and Yoo 1999), *Pseudoxanthomonas* (Kim et al. 2008), *Hydrogenophaga* (Fahy et al. 2008b), *Alicycliphilus* (Weelink et al. 2008) and *Acinetobacter* (Kim and Jeon 2009). Benzene degraders belonging to the phylum *Actinobacteria* include microorganisms in the genera *Rhodococcus* (Deeb and Alvarez-Cohen 1999; Fahy et al. 2008b; Jung and Park 2004; Na et al. 2005) and *Arthrobacter* (Fahy et al. 2008b). Although many of these microorganisms are present in mixed cultures and at contaminated sites undergoing benzene degradation it remains technically challenging to prove which are the active and dominant in situ benzene degraders within such mixed community samples.

Traditionally, methods for identifying benzene degraders have involved isolations or molecular analyses (e.g. 16S rRNA clone libraries). Studies involving isolations from contaminated sites have also typically reported microorganisms belonging to the phyla *Proteobacteria* and *Actinobacteria* (Cavalca et al. 2004; Fahy et al. 2008b; Hendrickx et al. 2006; Stapleton et al. 2000). For example, one study found that 86.9% of 300 gasoline-degrading isolates, were pseudomonads (Ridgway et al. 1990). In another study, the majority of 81 BTEX degrading isolates from a contaminated site were *Actinobacteria* or *Proteobacteria*, the *Actinobacteria* were mainly *Rhodococcus* and *Arthrobacter* strains and the *Proteobacteria* were mainly *γ-Proteobacteria* (more specifically *Pseudomonas*) (Hendrickx et al. 2006). Similarly, studies involving molecular approaches to investigate benzene degrading communities have also reported the presence of microorganisms in the phyla *Proteobacteria* and *Actinobacteria* at contaminated sites (Aburto et al. 2009; Alfreider and Vogt 2007; Fahy et al. 2008a; Fahy et al. 2006; Hendrickx et al. 2005). For example, in a study involving in situ mesocosms within a BTEX contaminated groundwater plume, 16S rRNA gene analyses of mesocosm and aquifer samples indicated the communities consisted primarily of *Proteobacteria* (mostly fluorescent *Pseudomonas*) (Hendrickx et al. 2005). Likewise, the bacterial community at the edge of a BTEX plume was dominated (based on 16S rRNA gene sequences) by several genera of *β Proteobacteria* (Alfreider and Vogt 2007).

Isolating benzene degraders from contaminated sites as well as investigating the microbial community at the molecular level both provide a significant amount of evidence linking such microorganisms to in situ degradation. However, microorganisms able to transform benzene in the laboratory in a pure culture may not be able to perform the same function in the field. In addition, dominant microorganisms at contaminated sites determined via molecular cloning could be dominant for another reason rather than benzene degradation. Here, we take a different methodological approach, called stable isotope probing (SIP), developed in 2000 (Radajewski et al. 2000), to investigate this issue. SIP enables function (e.g. ability to uptake carbon and thus transform a chemical) to be linked with identity within a mixed community sample and involves sample exposure to labeled compounds, separation of heavy (label incorporated) and light (background) nucleic acids using ultracentrifugation, then gene sequencing (16S rRNA or functional genes) to identify the label-consuming microorganisms. To date, SIP has been utilized to study the organisms responsible for the biotransformation of many environmental contaminants, including, for example, polycyclic aromatic hydrocarbons (PAH) (Powell et al. 2008; Singleton et al. 2005, 2006, 2007; Yu and Chu 2005), chlorinated solvents (Kittelmann and Friedrich 2008a, b), 2,4-D (Cupples and Sims 2007), polychlorinated biphenyls (Leigh et al. 2007; Park et al. 2006), phenol (DeRito et al. 2005; Manefield et al. 2002, 2005, 2007), toluene (Luo et al. 2009; Sun et al. 2010), *m*-xylene (Xie et al. 2010), RDX (Roh et al. 2009) and pentachlorophenol (Mahmood et al. 2005). Here, SIP was applied to investigate the microorganisms responsible for benzene degradation within microcosms constructed with sediment from contaminated and uncontaminated sites. The objective was to determine if the commonly recognized aerobic benzene degraders (*Proteobacteria* and *Actinobacteria*) identified via isolations and clone libraries, were responsible for benzene degradation within these mixed community samples. Thus, the work was designed to challenge existing data and provide additional insights on the microbial ecology of aerobic benzene degradation in mixed culture samples. Further, the methods developed here are

being used to investigate the microorganisms responsible for anaerobic benzene degradation in soil microcosms.

Methods

Soil incubations and benzene analyses

Soils utilized in this study were collected from three different locations, including a soil previously exposed to benzene and two soils not previously exposed to benzene. One soil was collected from a gasoline contaminated site and the other two were obtained from agricultural areas previously under alfalfa or corn production. Both agricultural soils had received biosolids from a municipal wastewater treatment plant 2–3 years before sample collection. Following sample collection, soils were homogenized, sieved through 4 mm screen and stored at 4°C until use. Soil microcosms consisted of phosphate-buffered mineral media (25 ml), as previously described (Mu and Scow 1994), and soil (3 g) in serum bottles (150 ml). The bottles were sealed with rubber stoppers and aluminum seals to retain benzene with the microcosms. The treatments include: sterile controls, unlabeled (2 µl, 99.8%, Sigma–Aldrich) and labeled benzene (2 µl, ring-$^{13}C_6$ benzene, 99%, Sigma–Aldrich) amended samples. Sterile controls were obtained by autoclaving repeatedly (three times in 1 day). All samples exposed to benzene (labeled or unlabeled) were prepared in duplicate and thus the SIP investigation (DNA extraction, ultracentrifugation, fractionation and TRFLP) occurred in duplicate for each soil. Duplicate samples were not pooled, the entire analysis for each was carried out separately.

Microcosms were incubated on a horizontal shaker (~100 RPM) at room temperatures (~20°C) and head-space benzene concentrations (200 µl samples) were determined daily with a gas chromatograph (Perkin Elmer) equipped with flame ionization detector and a capillary column (J&W Scientific, DB-624, diameter 0.53 mm). Injector and detector temperature were set at 200°C and the column temperature was 80°C.). The concentration of benzene in the liquid phase was calculated from the measured benzene concentration in the gas phase using Henry's constant.

DNA extraction and ultracentrifugation

Following removal of benzene (days 4–6), DNA was extracted from both labeled and unlabeled benzene amended soil samples with the Powersoil DNA extraction kit (Mobio Laboratories) following the manufacturer's instructions. The percentage of added benzene remaining was approximately 31% (contaminated soil), 40% (alfalfa soil) and 49% (corn soil) when DNA was extracted for SIP analysis. Approximately 10 µg DNA (quantified with Nanodrop, ND-1000) was added to the Quick-Seal polyallomer tubes (13 × 51 mm, 5.1 ml, Beckman Coulter) along with a Tris–EDTA/CsCl solution. The Tris–EDTA/CsCl solution was prepared by adding 6.5 g CsCl into 4.5 ml Tris–EDTA (TE, pH 8.0). Prior to sealing the tubes (cordless quick-seal tube topper, Beckman Coulter), the buoyant density (BD) was determined with a model AR200 digital refractometer (Leica Microsystems Inc.) and adjusted by adding small volumes of CsCl solution or Tris–EDTA buffer (final density ranged from 1.717 to 1.728 g ml^{-1}). The tubes were centrifuged at 178,000*g* (20°C) for 48 h in a Stepsaver 70 V6 Vertical Titanium Rotor (8 × 5.1 ml capacity) within a Sorvall WX 80 Ultra Series Centrifuge (Thermo Scientific). Following ultracentrifugation, the tubes were placed into a fraction recovery system (Beckman Coulter) for fraction (150 µl) collection. The buoyant density (BD) of each fraction was measured, and CsCl was removed by glycogen-assisted ethanol precipitation. Purified DNA fractions were resuspended in water and stored at −20°C.

PCR, TRFLP and 16S rRNA gene sequencing

Each ultracentrifugation fraction was analyzed by terminal restriction fragment length polymorphism (TRFLP). Briefly, the fractions were amplified with 27F-FAM (5′-AGAGTTTGATCMTGGCTCAG, 5′ end-labeled with carboxyfluorescine) and 1492R (5′-GGTTACCTTGTTACGACTT) (Operon Biotechnologies) using the following PCR program: 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 (150 ng) were purified with QIAquick PCR purification kit (Qiagen Inc.), following the manufacturer's instructions and digested with *Hae III* (New England Biolabs) with a 6 h incubation period. Additional digests (*Hha*I, *Msp*I,

*Bsp1286*I, *BsrB*I) for TRFLP analyses were included to correlate the TRFLP fragment lengths to the in silico cut sites of the cloned 16S rRNA gene sequences and thus determine the identity of the enriched fragments. DNA fragments were separated by capillary electrophoresis (ABI Prism 3100 Genetic Analyzer, Applied Biosystems) at the Research Technology Support Facility (RTSF) at Michigan State University. Data were analyzed with GeneScan software (Applied Biosystems) and the percent abundance of each fragment was determined.

For 16S rRNA gene sequencing, heavy fraction ^{13}C-DNA was amplified as above, except the forward primer was unlabeled (27F 5′-AGAGTTTGATC MTGGCTCAG) and the final extension period was extended (72°C for 15 min). The PCR products were purified with QIAquick PCR purification kit (Qiagen Inc.) and cloned into *Escherichia coli* TOP10 vector supplied with a TOPO TA cloning kit (Invitrogen Corporation). *E. coli* clones were grown on Luria–Bertani (LB) medium solidified with 15 g agar l^{-1} with 50 μg ampicillin l^{-1} for 16 h at 37°C. Colonies with inserts were verified by PCR with primers M13 F (5′-TGTA AAACGACGGCCAGT-3′) and M13 R (5′-AACAG CTATGACCATG-3′), plasmids were extracted from the positive clones with a QIAprep miniprep system (Qiagen, Inc.) and the insertions were sequenced at RTSF. The Ribosomal Database Project (Center for Microbial Ecology, Michigan State University) analysis tool "classifier" (Wang et al. 2007) was utilized to assign taxonomic identity. The partial 16S rRNA gene sequences of organisms linked to benzene degradation were deposited with GenBank under accession numbers HM020703, HM020702 and HM020701.

Results

Benzene concentration in microcosms declined rapidly (4–6 days) in all three soil microcosms, but was limited in the autoclaved controls (~20–40% decrease, likely due to sorption), confirming a biological removal mechanism (Fig. 1). DNA extracts from labeled and unlabeled benzene amended soil samples were subject to ultracentrifugation, fractionation, followed by TRFLP on each fraction. Analyses of TRFLP profiles illustrated the enrichment of different fragments in each soil in the heavy fractions

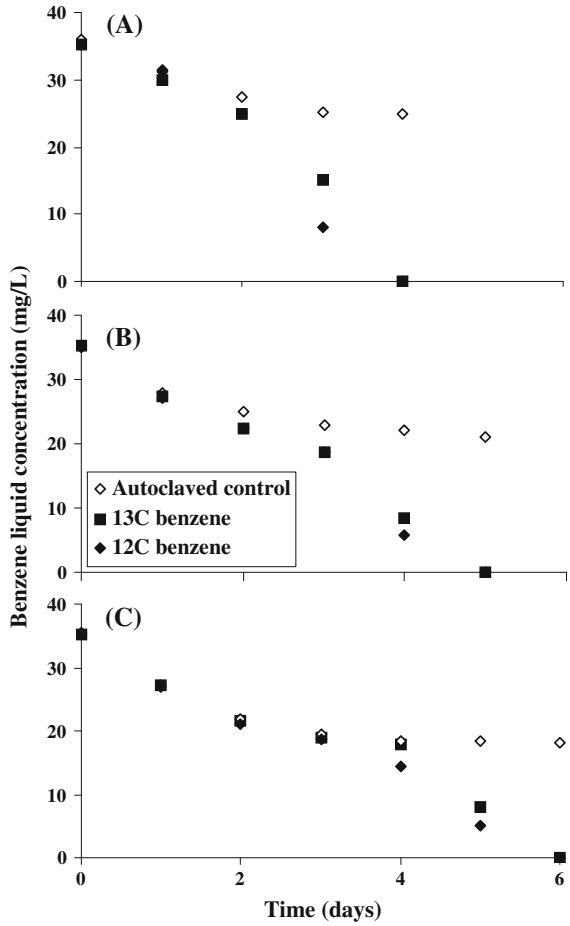

Fig. 1 Benzene concentration with time in controls and samples amended with labeled or unlabeled benzene in microcosms constructed with contaminated site soil (**a**), or two different agricultural soils (alfalfa soil, **b** and corn soil, **c**)

obtained from the ^{13}C benzene microcosms but not in the controls (unlabeled) (Fig. 2). This pattern suggests a different organism was responsible for benzene degradation in each soil. Two TRFLP fragments (313 and 214 bp) were enriched in the microcosms constructed with soil from a gasoline contaminated site, whereas only one TRFLP fragment (291 or 209 bp) was enriched in each of the two agricultural soils. DNA extracted from replicate microcosms for each soil produced similar trends. Peak relative abundance values in ^{13}C benzene amended samples were 29.4% (at 1.727 g ml^{-1}), 25.4% (at 1.723 g ml^{-1}), 58.0% (at 1.734 g ml^{-1}), and 84.1% (at 1.739 g ml^{-1}) for TRFLP fragments 313, 214, 291 and 209 bp, respectively (Fig. 2).

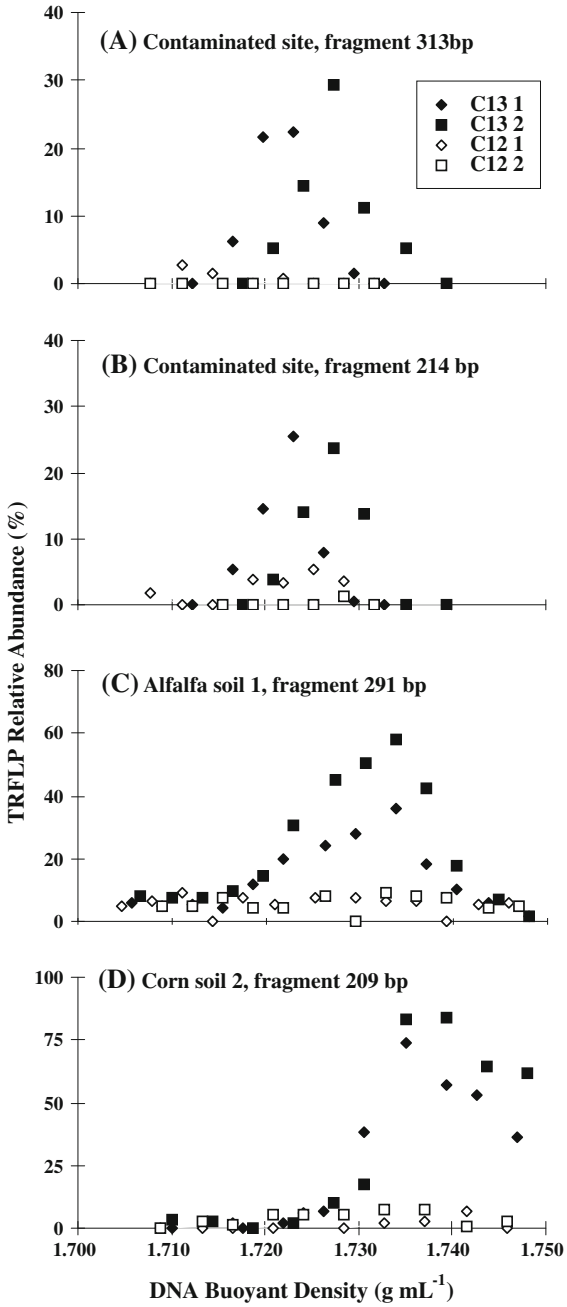

Fig. 2 Relative abundance of the four highly enriched TRFLP fragments (**a**–**d**) in fractions of three ^{13}C benzene amended soils compared to the controls (unlabeled benzene amended soils), note the *y*-axis have different scales

To determine the identity of these enriched fragments approximately 10 plasmids were partially sequenced for each microcosm. In addition, dominant TRFLP fragments obtained with ^{13}C enriched heavy fractions with two additional restriction enzymes were used to provide a positive identification of the Hae III TRFLP enriched fragments (313, 214, 291 and 209 bp). The dominant fragments obtained from all TRFLP restriction enzymes were compared to those obtained from in silico digests to determine the 16S rRNA sequence of the enriched fragments. A comparison of TRFLP cut sites and in silico cut sites for each identified phylotype is presented (Table 1). The slight differences (2–3 bases) between the measured fragment lengths and those predicted using sequence data have also been noted by others (Clement et al. 1998; Liu et al. 1997; Osborn et al. 2000). The taxonomic identity, as determined by the RDP analysis tool "classifier" (Wang et al. 2007), of each enriched fragment (cloned 16S rRNA sequence), thus benzene degrading microorganism is presented (Table 2).

Discussion

Although many aerobic benzene degrading microorganisms have been isolated and examined in detail, the actual organisms responsible for benzene degradation within the community of a mixed culture have been more difficult to identify. However, the recently developed molecular method, SIP, has enabled microbial ecologists to link function with identity within mixed communities. The current study is the first to use SIP to focus specifically on aerobic benzene degradation and to investigate aerobic benzene degraders in both contaminated and uncontaminated soil. It is important to note that label cross-feeding can be a concern in SIP studies. In the current investigation we are confident this was not a major limitation because benzene degradation occurred rapidly (rapid loss in 2 days) and the enriched fragments were all highly enriched (~30–80% relative abundance). However, we did not conduct SIP over time and cannot entirely rule out the possibility of label cross-feeding.

A number of other studies have used SIP to identify benzene degraders, however, all have primarily targeted anaerobic benzene degradation (Herrmann et al. 2010; Kasai et al. 2006; Kunapuli et al. 2007; Liou et al. 2008; Oka et al. 2008; Sakai et al. 2009). RNA-based SIP enabled researchers to link *Azoarcus* spp. (*Azoarcus* strains DN11 and AN9) with benzene

Table 1 Comparison of dominant fragments in heavy fraction TRFLP to clone restriction enzyme cut sites predicted from sequence analyses to confirm the identity of the enriched TRFLP fragments

Soil	Restriction enzyme	TRFLP	Sequence data
Contaminated soil			
Enriched fragment 1	*Hae*III	313	315
	*Msp*I	489	486
	*Hha*I	204	203
Enriched fragment 2	*Hae*III	214	213
	*Msp*I	147	149
	*Bsp1286*I	207	208
Alfalfa soil 1			
Dominant enriched fragment	*Hae*III	291	293
	*Msp*I	401	404
	*Hha*I	85	82
Corn soil 2			
Dominant enriched fragment	*Hae*III	209	212
	*Msp*I	509	510
	*BsrB*I	59	59

degradation under denitrification conditions (Kasai et al. 2006). Others have used DNA-based SIP to investigate benzene degradation under iron-reducing conditions, reporting that Gram-positive *Peptococcaceae* were important members of the benzene degrading community (Kunapuli et al. 2007). Another study using DNA-based SIP reported that microorganisms from the family *Desulfobacteraceae* were involved in benzene degradation under sulfate-reducing conditions (Oka et al. 2008). A fourth study involved DNA-based SIP to target benzene degrading microorganisms under aerobic and anaerobic conditions both in the field and in laboratory microcosms (Liou et al. 2008). Key findings included evidence for anaerobic benzene degradation by both *Pelomonas* (*β Proteobacteria*) and *Pseudomonas*. Clone sequences from heavy fractions of the aerobic microcosms included *γ Proteobacteria*, *Cyanobacteria*, *Bacteroidetes* and *Firmicutes*, with the latter dominating the clone library (49%) (Liou et al. 2008). Two recent manuscripts indicated benzene was degraded by a consortium of microorganisms under sulfate (syntrophs, hydrogenotrophic sulfate reducers and acetoclastic methanogens) (Herrmann et al. 2010) or methanogenic conditions (fermenting bacteria, hydrogen-producing acetogens, and methanogens) (Sakai et al. 2009). These studies all illustrate SIP provides significant potential for understanding in situ degradation.

In the current study, the key benzene degrading species identified were all different from those in the SIP studies described above. Specifically, two phylotypes (a *Polaromonas* sp. and an *Acidobacterium*) were the major benzene degraders in the microcosms constructed from the contaminated site soil, whereas only one phylotype was primarily responsible for benzene degradation in each of the agricultural soils ("candidate" phylum TM7 and an unclassified *Sphingomonadaceae*). The current literature on each phylotype is discussed in turn below and, when appropriate, previous reports of contaminant transformation are also included.

Polaromonas species have previously been linked to the degradation of organic compounds. A recent SIP study with the same soil indicated the same *Polaromonas* strain could grow using toluene (Sun et al. 2010). Further, a recent molecular investigation

Table 2 Phylogenetic affiliation of each enriched fragment (thus benzene degrading microorganism) as determined with the RDP analysis tool "classifier" (Wang et al. 2007)

Soil/fragment	Phylum	Class	Order	Family	Genus
Contaminated soil					
Fragment 313 bp	Proteobacteria	β Proteobacteria	Burkholderiales	Comamonadaceae	Polaromonas
Fragment 214 bp	Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	Subgroup 3
Alfalfa soil					
Fragment 291 bp	Proteobacteria	α Proteobacteria	Sphingomonadales	Sphingomonadaceae	Unclassified Sphingomonadaceae
Corn soil					
Fragment 209 bp	TM7	–	–	–	TM7 genera incertae sedis

on a benzene contaminated aquifer reported the dominance of *Polaromonas* species in groundwater samples (Aburto et al. 2009), however for that study, SIP was not used to definitively link function with identity. Further, *Polaromonas* sp. strain JS666 can grow using the environmental contaminant *cis*-dichloroethene as the sole carbon and energy source (Coleman et al. 2002; Mattes et al. 2008) and *P. naphthalenivorans* can use naphthalene as a sole carbon and energy source (Jeon et al. 2004), and was, in fact, first identified via SIP (Jeon et al. 2003). The other three well established *Polaromonas* species include the type strain *P. vacuolata*, isolated from Antarctic marine waters (Irgens et al. 1996), *P. aquatica* isolated from tap water (Kampfer et al. 2006) and *P. hydrogenivorans*, a psychrotolerant hydrogen oxidizing bacterium obtained from soil over permafrost in Alaska (Sizova and Panikov 2007). Previous researchers have suggested *Polaromonas* species display traits (slow growing, oligotrophic) that likely limit their isolation by standard methods (Mattes et al. 2008). Therefore, the current study illustrates the significant potential of SIP for discovering the functional abilities of microorganisms previously not targeted by traditional methods. In addition, these results suggest a *Polaromonas* strain can efficiently degrade benzene within a mixed community sample and may therefore be an efficient in situ benzene degrader.

The other major benzene degrading microorganism from the contaminated soil classified within subgroup three of the phylum *Acidobacteria*. To our knowledge, organisms within this subgroup have not previously been linked to benzene degradation and although *Acidobacteria* are widespread and abundant in soils, little is known about these organisms (Zhang and Xu 2008). The phylum was first identified in 1997 (Ludwig et al. 1997), contains a number of soil isolates obtained with various new cultivation strategies (Joseph et al. 2003; Sait et al. 2002; Stevenson et al. 2004) as well as seven species with validly published names (*Acidobacterium capsulatum*, *Holophaga foetida*, *Geothrix fermentans*, *Terriglobus roseus*, *Edaphobacter modestus*, *Edaphobacter aggregans*, *Acanthopleuribacter pedis*) (Coates et al. 1999; Eichorst et al. 2007; Fukunaga et al. 2008; Kishimoto et al. 1991; Koch et al. 2008; Liesack et al. 1994). However, the vast majority of *Acidobacteria,* for which 16S rRNA gene sequences have been obtained, still remain uncultured, and consequently, their role in the environment is poorly understood (Meisinger et al. 2007). Interestingly, based on 16S rRNA clone libraries, it appears that members of this phylum typically represent a significant portion ($\sim$20%, but up to 80%) of soil bacterial communities (Chan et al. 2006; Dunbar et al. 1999; Janssen 2006; Lee et al. 2008). Further, these organisms appear to be genetically diverse, with the branching depth being nearly as great as the *Proteobacteria* phylum (Hugenholtz et al. 1998). In addition, they are also metabolically diverse, with 16S rRNA genes being found in a wide range of environmental samples including a deep sea ecosystem (Quaiser et al. 2008), uranium contaminated subsurface sediments (Barns et al. 2007), chromium contaminated river system (Branco et al. 2005) an acidic mining lake (Kleinsteuber et al. 2008), a lead–zinc mine tailing site (Mendez et al. 2008) and wastewater treatment systems (Crocetti et al. 2002). Our results contribute to the limited pool of knowledge on the function of these organisms within mixed microbial communities and therefore their possible role in the environment.

The benzene degrader in agricultural soil one classified within a phylum that has previously been linked to benzene degradation. Specifically, this organism classified with the family *Sphingomonadaceae* (genus unclassified *Sphingomonadaceae*), found within α *Proteobacteria*. Microorganisms within this family can degrade a wide variety of both natural and anthropogenic compounds, including PAHs, BTEX and various pesticides (Balkwill et al. 1997; Cho et al. 2009; Greene et al. 2000; Mueller et al. 1997; Shi et al. 2001; Stolz 2009). However, previous studies have involved either isolations (with testing for degradation) or clone libraries (from a contaminated site or sample), therefore uncertainty remains as to whether these organisms are responsible for contaminant degradation in situ or in a mixed culture. Our results provide strong evidence that a microorganism in this family is the primary organism involved in benzene degradation in the mixed community of this agricultural soil.

The benzene degrading microorganism in agricultural soil two classified within "candidate" phylum TM7. This phylum has been described as biology's "dark matter" problem (Marcy et al. 2007), because although representatives (based on 16S rRNA genes) have been found in a diverse variety of ecosystems, no stable culture of any species exists and so their

roles in these environments as well as their general metabolic capabilities are both largely unknown. Recently we reported that an organism classifying with this phylum (GenBank accession number FJ629383) was responsible for in situ toluene degradation in an agricultural soil (Luo et al. 2009). The agricultural soil used in the toluene study was different from agricultural soil two and, correspondingly, the 16S rRNA gene sequences of the toluene degrader and the current benzene degrader (three clone sequences) are different (93–95%). These findings indicate microorganisms within "candidate" phylum TM7 could be useful organisms for BTEX bioremediation. Additional research is needed to uncover the complete functional abilities of such bacteria in mixed microbial systems.

Conclusions

Stable isotope probing was used to identify the microorganisms responsible for benzene degradation within microcosms inoculated with sediment from contaminated and uncontaminated sites. Although two of the benzene degrading microorganisms identified belonged a phylum (*Proteobacteria*) previously linked to benzene degradation, neither represented commonly reported aerobic benzene degraders (*Pseudomonas*, *Burkholderia*, *Ralstonia*) within this phylum. Such data could indicate previous studies involving isolations and clone libraries may not accurately represent the active benzene degraders in the mixed community of an environmental sample. The other two benzene degraders identified classified within phyla of few (*Acidobacterium*) or no stable cultured representatives ("candidate" phylum TM7). These finding provide an excellent illustration of the utility of SIP to discover the role of uncultured microorganisms in environmental processes.

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