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ORIGINAL ARTICLE

Biphenyl-utilizing bacteria and their functional genes in a pine root zone contaminated with polychlorinated biphenyls (PCBs)

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Bacteria and functional genes associated with biphenyl (BP) degradation in the root zone of an Austrian pine (*Pinus nigra* L.) growing naturally in polychlorinated-BP (PCB)-contaminated soil were identified using stable isotope probing (SIP) integrated with comprehensive functional gene analyses. SIP revealed 75 different genera that derived carbon from ^{13}C-BP, with *Pseudonocardia, Kribella, Nocardiodes* and *Sphingomonas* predominating carbon acquisition. *Rhodococcus* spp. were not detected with SIP, despite being the most abundant BP utilizers isolated from agar plates. Only one organism, an *Arthrobacter* spp., was detected as a BP utilizer by both cultivation and SIP methods. Time-course SIP analyses indicated that secondary carbon flow from BP-utilizing bacteria into other soil organisms may have occurred largely between 4 and 14 days incubation. Functional gene contents of the BP-utilizing metagenome (^{13}C-DNA) were explored using the GeoChip, a functional gene array containing 6465 probes targeting aromatic degradative genes. The GeoChip detected 27 genes, including several associated with catabolism of BP, benzoate and a variety of aromatic ring hydroxylating dioygenase (ARHD) subunits. Genes associated with the β-ketoadipate pathway were also detected, suggesting a potential role for this plant aromatic catabolic pathway in PCB degradation. Further ARHD analyses using targeted polymerase chain reaction primers and sequence analyses revealed novel dioxygenase sequences in ^{13}C-DNA, including several sequences that clustered distantly from all known ARHDs and others that resembled known *Rhodococcus* ARHDs. The findings improve our understanding of BP degradation and carbon flow in soil, reveal the extent of culture bias, and may benefit bioremediation research by facilitating the development of molecular tools to detect, quantify and monitor populations involved in degradative processes.
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Introduction

Aerobic biodegradation of polychlorinated biphenyls (PCBs) and their analog, biphenyl (BP), has been the subject of a large body of research for more than three decades. A fundamental goal has been to identify bacteria and genes involved in the PCB degradation process to optimize bioremediation processes. Numerous phylogenetically diverse bacteria have been isolated that perform BP and/or PCB degradation (Abraham *et al.*, 2002). Genes encoding the BP (upper) degradation pathway and benzoate (lower) pathways (Pieper, 2005) in isolates have been extensively characterized, and some genes have been directly detected in the environment using molecular tools (Erb and Wagner-Dobler, 1993; Ringelberg *et al.*, 2001; Baldwin *et al.*, 2003). However, until recently, methodological limitations

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precluded researchers from ascertaining which bacteria or genes are truly active in BP/PCB degradation in the soil community.

The recent development of stable isotope probing (SIP) methods enables researchers to directly identify microorganisms involved in the degradation and/or assimilation of carbon from a specific compound without the limitations of cultivation (Friedrich, 2006; Whiteley *et al.*, 2006). In DNA-based SIP, microbes within a complex community that grow using a ^{13}C-labeled substrate incorporate ^{13}C into their DNA, permitting it to be separated from the DNA of inactive organisms by isopycnic centrifugation. Subsequent analyses of ^{13}C-DNA reveal the identity of organisms that derived ^{13}C from the substrate, implicating them in the biodegradation of the compound or its metabolites. Previous SIP studies have revealed organisms that derive carbon from a variety of aromatic pollutants in soil, including phenol and polycyclic aromatic hydrocarbons (Madsen, 2006) and soil-derived PCB-degraders in a biofilm growing on PCB droplets (Tillmann *et al.*, 2005). Because DNA-SIP provides entire genomes of organisms that derive ^{13}C from the substrate, it also affords opportunities to investigate their functional gene contents, and degradative genes detected may be implicated in the assimilatory process (Friedrich, 2006).

A previous cultivation-based study at a PCB-contaminated site in the Czech Republic indicated that *Rhodococcus* was the predominant genus of PCB-degrading bacteria (Leigh *et al.*, 2006). Culturable BP utilizers were present throughout the site, including the root zones of trees, and occurred in higher numbers in the root zone of Austrian pine (*Pinus nigra* L.) and goat willow (*Salix caprea*) than other tree species examined or nonrooted soil (Leigh *et al.*, 2006). This finding was consistent with the hypothesis that certain plants biostimulate PCB-degrading bacteria by releasing plant aromatic compounds that function as growth substrates (Donnely *et al.*, 1994; Singer *et al.*, 2004), a hypothesis developed out of the fact that pathways for microbial aromatic degradation are thought to have evolved in response to plant aromatics and are widespread in root-associated soil (Harwood and Parales, 1996). It remains unknown whether the culturable PCB degraders identified accurately reflect active PCB (BP) degraders in the root zone, and which degradative genes or pathways the active organisms may be employing.

We have integrated DNA-SIP with comprehensive functional gene analyses for exploring the PCB-contaminated root zone of pine to identify bacteria that derive carbon from BP and to identify aromatic degradative genes they possess. Functional gene analyses of active BP utilizers were performed by hybridizing ^{13}C-DNA to the GeoChip, a functional gene array containing 6465 probes targeting genes involved in degradation of a wide variety of aromatic compounds, including 388 probes targeting BP degradation pathways, 167 probes targeting benzoate degradation pathways and over 2400 probes targeting degradative pathways for mono-aromatic compounds and plant aromatic compounds. Initial aromatic ring hydroxylating dioxygenases (ARHDs), which catalyze the critical first step in biodegradation of aromatic compounds, were further examined in ^{13}C-DNA by polymerase chain reaction (PCR) and sequence analyses using a specially designed primer set. Culturable BP utilizers were also isolated and compared with SIP results to examine the extent of culture bias.

Materials and methods

Soil sample

Soil was collected from the root zone (20–40 cm depth) of a 24-year-old Austrian pine (*Pinus nigra*) tree growing naturally in a PCB-contaminated site (soil PCB concentration 15 mg/kg) in the Czech Republic. The date of sampling was November 2000, when culturable populations of PCB degraders were at their seasonal maximum as described previously (Leigh *et al.*, 2001, 2006). The sample was stored at 4°C in a plastic bag for 3 years between collection and initiation of this experiment. Fresh samples were unavailable due to capping of the site; however, cultivation of BP-utilizers following protocols applied previously to newly collected soil (Leigh *et al.*, 2006) yielded the same consortium.

Stable isotope probing microcosms

Microcosms containing soil provided with either uniformly ^{13}C-labeled BP (99 atom % ^{13}C; ISOTEC, Miamisburg, OH, USA) or unlabeled BP (Sigma, St Louis, MO, USA) were established to permit destructive harvesting at each time point (1, 4 and 14 days). Three replicate microcosms were established for each substrate and time point. BP was first added to empty, sterile, 160 ml serum bottles by pipetting 100 μl of a 10 mg/ml acetone solution onto the inner wall and then allowing acetone to completely dry for 1–2 h in a laminar flow hood leaving behind BP crystals. A 140 g soil sample was restored to field moisture conditions (Leigh *et al.*, 2006) by moistening with 14 ml sterile distilled water followed by incubation at 25°C for 48 h. Aliquots of homogenized soil (5 g) were then loosely placed in serum bottles containing BP. Microcosms were sealed with Teflon stoppers (Westco, Exton, PA, USA) and aluminum crimp caps to contain the headspace for later $^{13}CO_2$ sampling, and were incubated standing at 25°C in the dark. After 1, 4 and 14 days incubation, a sample of the headspace of one microcosm each containing ^{13}C-BP and unlabeled BP was collected to determine the abundance of ^{13}C in CO_2 (described below) and soil was frozen immediately at −80°C.

Concentration and carbon isotopic composition of CO_2
Headspace gas was collected at microcosm initiation (ambient lab air), 1, 4 and 14 day time points in volumes of 1, 1, 0.5 and 0.1 ml, respectively, using an N_2-purged syringe and was stored in 12 ml serum bottles containing ultra-high purity N_2 (Linde gas, Independence, OH, USA) at room temperature. Carbon dioxide in the stored bottles was analyzed for $\delta^{13}C$ within 7 days on a Trace Gas system interfaced to an IsoPrime mass spectrometer (GV Instruments, Manchester, UK). Typical volumes sampled from the 12 ml serum bottles ranged from 200 to 1000 μl. Before analysis, gas tight syringes were purged with ultra-high purity N_2 and a volume equal to the expected sample volume was added to the 12 ml serum bottle to maintain atmospheric pressure in the bottle. An aliquot of the sample was injected using a gas tight syringe into a direct injection port of the Trace Gas. The Trace Gas system automatically concentrates CO_2 cryogenically, removes water by a magnesium perchlorate trap and Nafion drier, and introduces a purified peak of CO_2 to the mass spectrometer following purification on a Poraplot Q gas chromatographic column with He as the carrier gas. The concentration of CO_2 was determined from the area under the mass 44 trace with corrections applied for dilution from added N_2. The carbon isotopic composition of CO_2 is expressed in atom percent.

Cultivation of BP-utilizing bacteria
BP-utilizing bacteria were cultivated by spreading organisms suspended from 0.5 g soil onto basal mineral agar prepared with Difco Noble agar (Becton, Dickinson and Co., Sparks, MD, USA) with BP vapor provided as the sole carbon source (Leigh *et al.*, 2006). Colonies were subjected to a BP clearing zone assay using methods described previously, except that the clearing zone assay was performed with BP rather than 4-bromo-BP (Leigh *et al.*, 2006), and colonies producing clearing zones were enumerated. Eighty-four BP-utilizing colonies were randomly selected from plates of soil at time zero for isolation and further screening. Colonies were isolated on one-eighth strength plate count agar (Leigh *et al.*, 2006), screened again for BP clearing, and then BP utilization further verified by growth in liquid culture with BP crystals as sole carbon source in basal mineral medium.

DNA was extracted from BP-utilizing isolates using the Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA, USA), and 16S rRNA genes were PCR-amplified using universal eubacterial primers 27F and 1392R (Johnson, 1994). PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA) and were submitted to Macrogen Inc. (Seoul, Korea) for single extension sequencing using primer 27F, yielding sequences of approximately 850 bp in length. Sequences were phylogenetically identified using RDPII Classifier and Sequence Match (Cole *et al.*, 2005) and were deposited in GenBank (accession no. EF507168–EF507182).

Soil DNA extraction
DNA was extracted from 4 g aliquots of SIP microcosm soil using a freeze-grinding method modified from Zhou *et al.* (1996). Soil was pretreated for 10 min with 0.5 ml 50 mg/ml lysozyme at 25°C. Following extraction, DNA was precipitated with 30% polyethylene glycol 6000 (Fluka, Buchs, Switzerland) in 1.6 M NaCl (Griffiths *et al.*, 2000) to minimize contamination with humic materials. Pellets were washed with 70% ethanol, resuspended in water, brought to 0.3 M NaCl and were added to spin columns (Bio-Rad, Hercules, CA, USA) containing 1 ml Sephacel (Sigma, St Louis, MO, USA) pre-equilibrated with 4 ml 0.3 M NaCl in TE (10 mM Tris–Cl, pH 8.0), washed with 4 ml 0.3 M TE in NaCl in TE, and eluted with 4 ml 0.5 M CaCl in TE. DNA was then precipitated with isopropanol, pellets washed with 70% ethanol, then resuspended in water (Zhou *et al.*, 1996).

^{13}C-DNA separation
Equilibrium (isopycnic) density gradient centrifugation and fractionation were adapted for DNA-SIP from methods for RNA-SIP by Manefield *et al.* (2002) using cesium trifluoroacetate (CsTFA; Amersham Biosciences, Piscataway, NJ, USA) solution without the addition of formamide and with a starting buoyant density (BD) of 1.60 g/ml. Samples of 1.5 μg DNA were combined with CsTFA solution and then subjected to ultracentrifugation using the same tubes, rotor dimensions and conditions described previously (Manefield *et al.*, 2002). Gradients were fractionated into 100 μl fractions as described previously (Manefield *et al.*, 2002). BD of fractions was determined gravimetrically by weighing aliquots of each fraction from blank gradients run in parallel containing water in place of DNA. Sample DNA was precipitated from fractions with isopropanol for 2 h at 25°C, and then pellets were washed twice with isopropanol and resuspended in Qiagen EB elution buffer (Qiagen, Valencia, CA, USA).

To locate fractions containing ^{13}C-DNA, the distribution of DNA in gradient fractions ranging in BD from 1.561 to 1.684 g/ml was determined by real-time PCR using universal eubacterial primers 1132F (Wilmotte *et al.*, 1993) and 1108R (Amman *et al.*, 1995) targeting 16S rRNA genes. Quantitative PCR (Q-PCR) was performed in triplicate 15 μl reactions containing SYBR green master mix (Applied Biosystems, Foster City, CA, USA), 4.5 pmol each primer and 1 μl template, using a thermocycler program of 40 cycles of 95°C for 15 s and 60°C for 1 min on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA).

Gene copy number was determined in relation to a standard curve constructed with *Pseudomonas stutzeri* JM300 genomic DNA which contains 213 000 16S rRNA copies per ng of DNA (Ginard *et al*., 1997).

After the range of fractions containing ^{13}C-labeled DNA or unlabeled DNA were identified, they were combined to constitute compiled 'heavy' and 'light' fractions from each sample for downstream molecular analyses. To control for background contamination of DNA throughout the density gradient, the equivalent heavy fractions from a control unlabeled sample (total community DNA at time 0) were also compiled, subjected to the same analyses, and genes detected in the background subtracted from those found in ^{13}C-DNA.

Community profiling

Terminal restriction-fragment length polymorphism (T-RFLP) analyses were performed on total community DNA, density gradient fractions representing unlabeled (light) DNA, ^{13}C-labeled (heavy) DNA and the background control. DNA samples were PCR-amplified using primers 27F and 1392R targeting eubacterial 16S rRNA genes (Johnson, 1994). Primer 27F was labeled with 6-carboxyfluorescien (6-FAM) on the 5′ end. PCR was performed by initially amplifying 1 ng template with 5 pmol primer in a 25 μl reaction using a thermocycler program of 95°C for 9 min, then 25 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 1 min 40 s and final extension at 72°C for 10 min, followed by a reconditioning step in which 5 μl aliquots of initial PCR product were transferred to new reactions and amplified for three cycles under the same PCR conditions except in 50 μl volumes with 10 pmol primers (Thompson *et al*., 2002). PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA), and then 40 ng was digested with *Hha*I endonuclease (New England Biolabs, Beverly, MA, USA) following the manufacturer's instructions in reaction volumes of 15 μl for 4 h at 37°C. Digests were precipitated and resuspended in 10 μl as described previously (Gallagher *et al*., 2005), and then the entire volume was loaded on an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) and run using capillary injection time of 10 s. T-RFLPs were analyzed using GeneScan and Genotyper software systems (Applied Biosystems, Foster City, CA, USA).

16S rRNA gene sequence analyses

Cloning and sequence analyses were performed on 16S rRNA gene amplicons from total community DNA from time 0, ^{13}C-DNA from each time point and the background control. PCR was performed using the same conditions as for T-RFLPs, but without the reconditioning step or the 6-FAM label on the 27F primer. For each sample, 12 replicate 25 μl PCR reactions were created to generate abundant PCR products and to span variations in template and amplification bias, and then were compiled, purified and concentrated using the Qiaquick PCR cleanup kit (Qiagen, Valencia, CA, USA). Products were gel-purified using the Qiaquick gel purification kit (Qiagen, Valencia, CA, USA). Immediately before cloning, 3′ poly-A overhangs were added by incubating 25 μl reactions containing 20 μl purified amplicon, 25 μmol dATP, 0.5 U taq polymerase and 75 μmol $MgCl_2$ in 1 × taq buffer at 72°C for 10 min. Cloning was performed using the TOPO-TA cloning kit for sequencing (Invitrogen, Carlsbad, CA, USA), and clones were provided to Macrogen Inc. (Seoul, Korea) for plasmid purification and sequencing by single extension with primer 27F. Resulting sequences of approximately 650 bp in length were trimmed for a base score of >20 (error probability <0.1). Sequences from ^{13}C-DNA that shared $\geqslant 99\%$ sequence identity to any sequence detected in the background control library (heavy fractions from nonincubated soil) were identified using FastGroup-II, a web-based program designed for dereplicating clone libraries (Yu *et al*., 2006). In this way, we ensured that any DNA contamination in density gradients was not misinterpreted as ^{13}C-DNA. Phylogenetic analyses were performed using RDPII Classifier and Sequence Match (Cole *et al*., 2005) and were deposited in GenBank (accession no. EF506948–EF507159).

Aromatic ring hydroxylating dioxygenase gene sequence analyses

ARHDs were PCR-amplified in total community DNA, ^{13}C-DNA and the background control using a primer set previously developed to universally target ARHDs catalyzing the initial step in polyaromatic hydrocarbon (including BP) degradation (Bellicanta and Pellizari, 2004). The specificity of primers ARHD1F and ARHD1R for BP dioxygenase genes was tested *in silico* using the Fungene bphA1 Stephan Gantner database (http://flyingcloud.cme.msu.edu/fungene/). The primers ARHD1F (5′-TTYRYNTGYANNTAYCAYGGNTGGG) and ARHD1R (5′-CCCANCCRTGRTANNTRCANRYRAA) yield PCR products ranging in size from 308 to 329 bp in length.

PCR reactions were performed with 30 pmol of each primer in a total volume of 25 μl, using a thermocycler program of 97°C for 3 min, 40 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, with final extension for 5 min at 72°C. PCR products from ^{13}C-DNA were gel-purified and cloned as described above for 16S rRNA gene amplicons. Plasmids were extracted and subjected to single-extension sequencing using the primer ARHD1F by the Research Technology Support Facility (Michigan State University, East Lansing, MI, USA). For BP-utilizing isolates, ARHDs were PCR-amplified from genomic DNA extracts and sequenced using the

same methods as for ^{13}C-DNA. ARHD sequences were subjected to GenBank BLASTn searches to identify nearest matching sequences in the database. For ARHD phylogenetic tree reconstruction, partial gene sequences were translated to their amino-acid sequences and alignment was made using the CLUSTALW program, version 1.82 (Thompson *et al.*, 1994) of the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/#). Alignment was visually inspected and manually corrected when necessary. Alignment was exported and the parsimony algorithm was run in PAUP* for bootstrap values with 1000 replicates (Swofford, 2000). ARHD sequences obtained from clones (accession no. EF507160–EF507167) and isolates (accession no. EF507183–EF507184) were deposited in GenBank.

GeoChip

A functional gene array, or GeoChip, containing over 24 000 probes targeting numerous functional genes important to microbial ecological processes (He *et al.*, 2007), was used to explore the functional genes present in SIP samples. The GeoChip contains 6465 probes targeting genes involved in degradation of a wide variety of aromatic compounds, including 388 probes targeting BP, 167 targeting benzoate degradation pathways and over 2400 targeting conserved monoaromatic degradative pathways (i.e. β-ketoadipate, homogentisate) and catabolic genes for plant aromatic compounds (i.e. vanillin, cymene). The GeoChip was constructed with three different 50-mer probes targeting each gene of interest designed with the highest specificity to individual organisms and sequences possible (He *et al.*, 2007).

Three replicate hybridizations were performed for each of the following samples: total community DNA, ^{13}C-DNA (14 days incubation) and the background control (heavy fractions from nonincubated soil). Whole community genome amplification (WCGA) (Wu *et al.*, 2006) was used to amplify approximately 1 ng aliquots of each DNA sample to produce $>3\,\mu$g DNA for each array hybridization. WCGA was performed using a TempliPhi Amplification kit (Amersham Biosciences, Piscataway, NJ, USA). Reaction mixtures consisted of approximately 1 ng DNA plus 10 μl sample buffer, 10 μl reaction buffer, 1 μl enzyme mix, 1 μl single-stranded binding protein (260 ng/μl) and 1 μl spermidine (1 mM). Reactions were incubated at 30°C for 4 h and then inactivated by heating to 65°C for 10 min. Approximately 4 μl of WCGA was analyzed by 1% agarose gel electrophoresis to confirm successful amplification.

WCGA-amplified DNA was Cy5-labeled using a Bioprime Array CGH Genomic Labeling System (Invitrogen, Carlsbad, CA, USA). Aliquots of WCGA products (3 μg) were dried completely, combined with 20 μl random octamer (3 mg/ml) and 3 μl 1 mM spermidine in a 40 μl reaction, heated to 99.9°C for 5 min and then chilled on ice. A 20 μl aliquot of master mix was added, containing 2.5 μl of dNTP mix (2.5 mM dTTP, 5 mM dATP, 5 mM dCTP and 5 mM dGTP), 1 μl Cy5 dUTP (Amersham Biosciences, Piscataway, NJ, USA), 0.7 μl recA (490 ng/ul), 2 μl exo-Klenow and 13.8 μl nuclease-free H_2O. Labeling reactions were incubated at 37°C for 6 h, and then were purified using a Qiaquick PCR purification kit. Dye incorporation was evaluated using a NanoDrop (NanoDrop Technologies, Wilmington, DE, USA), producing typical base to dye ratios of 85–100. Cy5-labeled DNA was dried completely and stored at −20°C until hybridization.

Arrays were preybridized at 50°C in 50% formamide, 5 × sodium chloride and sodium citrate (SSC) buffer, 0.1% sodium dodecyl sulfate (SDS) and 0.1 mg/ml bovine serum albumin for 30–60 min, followed by three washings with ddH_2O, one wash in isopropanol and drying by centrifugation. For hybridization, Cy5-labeled DNA was suspended in 50% formamide, 5 × SSC, 0.1% SDS, 0.1 μg/μl herring sperm DNA, 1.6 μl recA (490 ng/μl) and water for a final volume of 40 μl. Hybridization was conducted at 50°C for approximately 18 h. After hybridization, arrays were washed twice in 1 × SSC with 0.1% SDS at 50°C for 5 min, twice in 0.1 × SSC and 0.1% SDS at 25°C and five times in 0.1 × SSC and then arrays were dried by centrifugation.

Arrays were scanned using a GenePix 4000B scanner and analyzed with GenePix software (Molecular Devices, Sunnyvale, CA, USA), and then data were further analyzed using a Perl script (He *et al.*, 2007). Briefly, spots with >2 of signal-to-noise ratio (SNR), defined as the mean fluorescence minus mean background fluorescence divided by the standard deviation (s.d.) of background fluorescence, were filtered for further analysis. SNR values for each spot were normalized by the sum of all spots' mean signals (excluding positive control spots) on the same slide. Data from the three replicate slides for each sample were combined and outliers removed (>2 s.d.). Gene detection was considered positive when a positive hybridization signal was obtained from $\geqslant 33\%$ of spots targeting the gene on three replicate arrays combined.

Results

^{13}C-BP incorporation

Excess $^{13}CO_2$ was detected in microcosm headspaces following 1 day incubation, and increased in abundance throughout the 14 days experiment (Table 1), whereas no change in $^{13}CO_2$ was detected in control microcosms containing unlabeled BP or no BP. On the basis of previous reports that cells growing on BP mineralize 60–80% of BP to CO_2 (Bailey *et al.*, 1983), the amount of ^{13}C-BP utilized by the microbial community in microcosms containing 5 g soil was calculated to be 0.9–1.2 μg within 1 day and then reached 59–79 μg by 14 days.

Table 1 Mineralization of ^{13}C-BP as determined by $^{13}CO_2$ evolution

SIP incubation time (days)	*$^{13}CO_2$ excess in headspace (nmol)*	*^{13}C-BP mineralized (μg)*[a]
1	56	0.9–1.2
4	1636	26–35
14	3694	59–79

Abbreviation: BP, biphenyl.
[a]Calculated as a range assuming 60–80% of BP utilized is mineralized to CO_2 (Bailey *et al.*, 1983).

Q-PCR targeting 16S rRNA genes in density gradient fractions indicated that significant quantities of ^{13}C-DNA were present on days 4 and 14, whereas a lesser but detectable amount of labeled DNA was detected at day 1 (Figure 1). Unlabeled DNA formed a peak at BD of 1.599 g/ml, whereas ^{13}C-DNA occupied fractions ranging in BD from approximately 1.617 to 1.671 g/ml. Duplicate density gradients run for each same sample were nearly identical in DNA quantity and distribution. Heavy fractions (BD of 1.632–1.671 g/ml) and light fractions (BD of 1.591–1.602 g/ml) were compiled from the two replicate gradients for each sample for further analyses. In parallel with the ^{13}C-BP-incubated samples, equivalent heavy fractions from background control gradients were also analyzed.

Community and phylogenetic analyses

T-RFLP analyses of the background control fractions revealed the presence of five major and several minor T-RFs contaminating the heavy fractions of density gradients, and these peaks were thus omitted in subsequent analyses of T-RFLPs of ^{13}C-DNA samples (Figure 2). As incubation time increased, ^{13}C-DNA samples produced larger numbers of T-RFs (Figure 3). Some T-RFs associated with ^{13}C-labeled populations were also detected in the total community profile, whereas others were only found in ^{13}C-DNA samples. The number of T-RFs shared among ^{13}C-DNA and the total community was similar between 1 and 4 days, but then increased between 4 and 14 days incubation (Figure 3).

Clone libraries of 16S rRNA gene amplicons were constructed from total community DNA at time 0, ^{13}C-DNA from 1, 4 and 14 days, and from the heavy background control. Subtraction of sequences detected in the background that shared 99% sequence identity from ^{13}C-DNA libraries resulted in the removal of 5, 5 and 3 sequences from the libraries from 1, 4 and 14 days SIP incubation, resulting in valid libraries of 50, 77 and 86 sequences, respectively. Rarefaction curves indicated that full community coverage was not achieved in any library, and Chao1 richness estimates were 785, 1000 and 1123 ribotypes for 1, 4 and 14 days valid ^{13}C-DNA libraries. Seventy-five different genera were found to

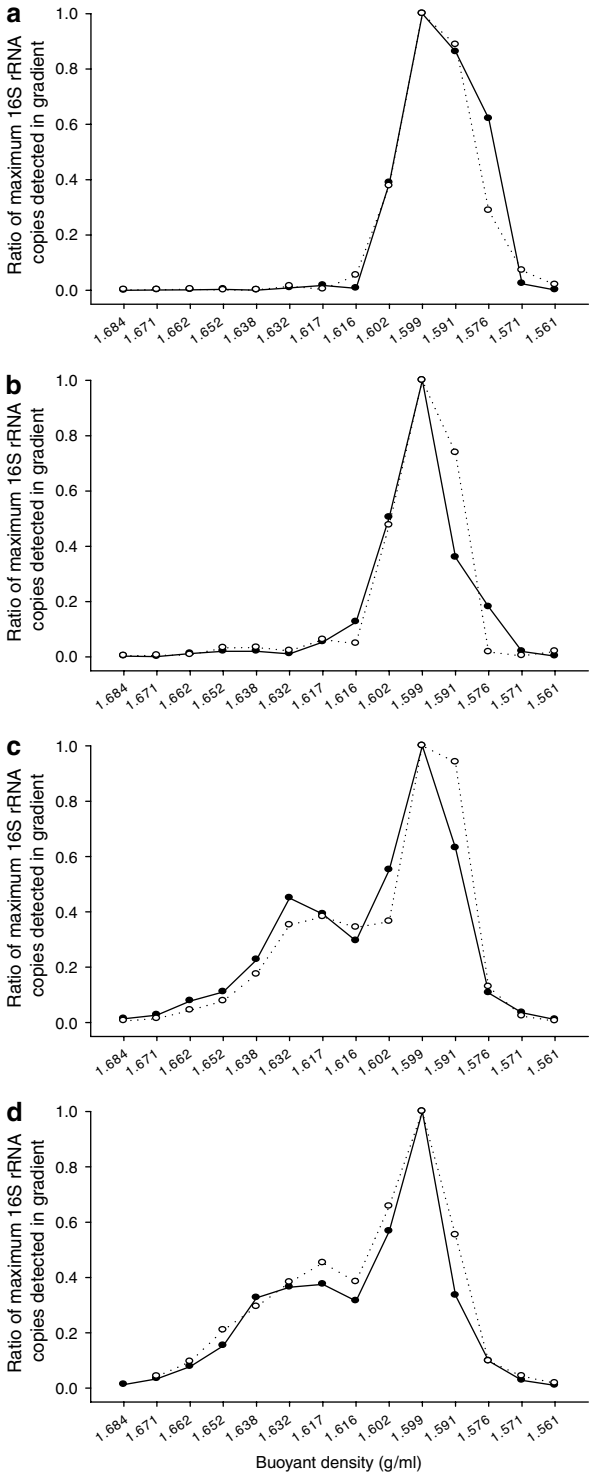

Figure 1 Detection of ^{13}C-DNA in density gradient fractions derived from DNA at (**a**) the initiation of the experiment ($t=0$) and following incubation times of (**b**) 1 day; (**c**) 4 days; and (**d**) 14 days using Q-PCR targeting 16S rRNA genes. (**a**) (solid symbols) and (**b**) (open symbols) denote replicate density gradients run for each sample.

derive carbon from BP, with the majority of ^{13}C-labeled organisms being Actinomycetes and Proteobacteria (Table 2). The most abundant sequences detected in ^{13}C-DNA libraries were members of the

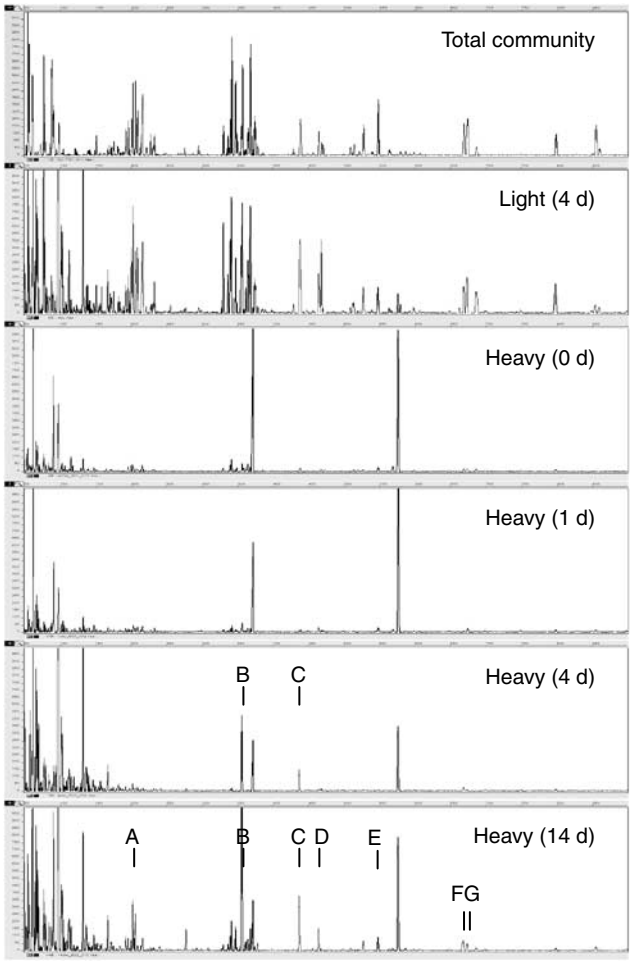

Figure 2 T-RFLP profiles of 16S rRNA gene amplicons digested with *Hha*I. Labeled peaks correspond to predicted T-RF size from sequenced clones (A) *Variovorax, Polaromonas, Anaerolinea*; (B) *Pseudonocardia, Kribbella*; (C) *Nocardiodes, Actinoplanes, Streptomyces*; (D) *Streptomyces*; (E) *Anaerolinea*; (F) *Nocardiodes*; (G) unclassified Actinobacteria.

genera *Pseudonocardia*, *Nocardiodes*, *Kribbella* and *Sphingomonas*, whereas many other organisms were detected in lower abundance. Fourteen percent of sequences were not classified by RDPII classifier at the 80% confidence threshold and may be novel organisms (Table 2).

Twenty-two of the isolates were capable of growth on BP as a sole carbon source on agar plates, clearing BP sprayed over colonies and growth on BP as sole carbon source in liquid medium. Sequence analyses of 16S rRNA genes revealed that the majority (73%) of isolates were members of the genus *Rhodococcus*, whereas *Arthobacter*, *Pseudomonas*, *Staphylococcus* and *Stenotrophomonas* were also isolated (Table 2). All BP-utilizing isolates produced 16S rRNA gene sequences that differed from those obtained from ^{13}C-DNA, with the exception of one sequence that was obtained from two *Arthobacter* isolates and two *Arthrobacter* clones. Plate counts of BP utilizers showed no significant change over time.

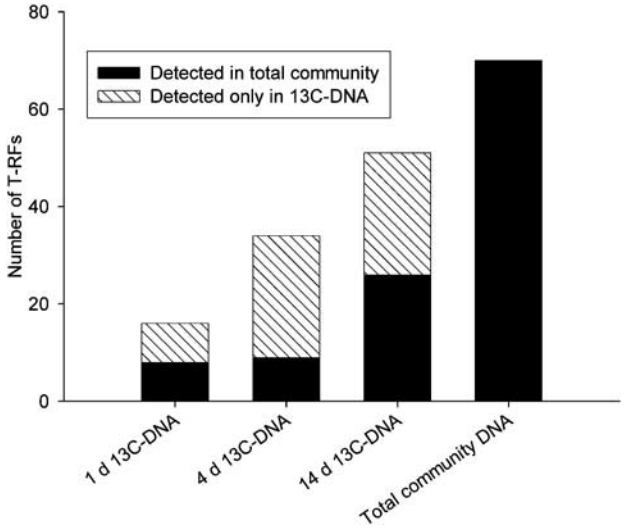

Figure 3 Number of T-RFs detected in ^{13}C-DNA following SIP incubation of 1, 4 and 14 days (after subtraction of background peaks) and in total community DNA from time 0.

Mean numbers with s.d.s in parentheses detected at time 0, 1, 4 and 14 days were 1.42×10^5 (6.48×10^4), 2.18×10^5 (3.37×10^4), 1.47×10^5 (7.88×10^4) and 1.44×10^5 (1.55×10^5) organisms per gram of fresh soil, respectively.

T-RFs were predicted by *in silico* digestion of sequences obtained from clones and were matched with major peaks in T-RFLPs (Figure 2). Sequences that were detected with highest frequency in ^{13}C-DNA clone libraries were associated with many of the major T-RFs. The dominant peak in ^{13}C-DNA was 356 bp in size, and matched T-RFs predicted from *Pseudonocardia* clones, with a secondary overlapping peak attributed to *Kribbella* (357–358 bp). The *Pseudonocardia* peak was present at low intensity at 1 day, and then increased in height to become the dominant peak at 4 and 14 days. Some *Pseudonocardia* clones also produced predicted T-RFs below the 50 bp cutoff for accurate T-RF measurement. *Nocardiodes* sequences produced T-RFs of several different sizes, including two T-RFs that matched detectable peaks (Figure 2). *Sphingomonas* sequences produced T-RFs of both 80 bp and below 50 bp.

Functional gene analyses

PCR amplification using ARHD primers produced amplicons of appropriate size (308–329 bp) visible on agarose gels from ^{13}C-DNA fractions after 4 and 14 days incubation. No PCR products were visible from total community DNA, ^{13}C-DNA following 1 day incubation and background control samples. When DNA from BP-utilizing isolates was amplified, only *Rhodococcus* spp. produced amplicons.

Sequences obtained from isolates and cloned amplicons of ^{13}C-DNA (14 days) were subjected to BLASTn searches, and along with nearest matches

Table 2 Phylogenetic identification of organisms based on 16S rRNA gene sequences using RDP Classifier

Identity (PHYLUM, family)	*Number of sequences detected*							
	Total DNA clone library		*^{13}C-DNA clone libraries*				*BPl-utilizing isolates*	
	t = 0	*Genera*	*1 d*	*4 d*	*14 d*	*Genera*	*t = 0*	*Genera*
ACIDOBACTERIA								
Acidobacteriaceae	0		0	0	2	*Geothrix*[a]	0	
ACTINOBACTERIA								
Acidimicrobiaceae	1	*Acidimicrobium*	0	1	0	*Acidimicrobium*	0	
Geodermatophilaceae	0		1	1	0	*Blastococcus*	0	
Intrasporangiaceae	3	*Janibacter*[a]	1	1	0	*Tetraspaera*[a], *Terrabacter*	0	
Microbacteriaceae	4	*Agromyces, Leifsonia*	1	0	0	*Leifsonia*[b]	0	
Micrococcaceae	0		0	1	2	*Arthrobacter*	2	*Arthrobacter*
Micromonosporaceae	0		0	4	1	*Actinoplanes, Spirilliplanes*[a], *Asanoa*[a]	0	
Nocardiaceae	3		0	0	0		16	*Rhodococcus*
Nocardioidaceae	3	*Aeromicrobium, Nocardioides, Kribbella*	4	4	6	*Kribbella, Nocardioides*	0	
Pseudonocardiaceae	1	*Pseudonocardia*	0	9	8	*Pseudonocardia*	0	
Rubrobacteraceae	1	*Solirubrobacter*	1	3	0	*Solirubrobacter*	0	
Streptomycetaceae	2	*Streptomyces*	0	0	2	*Streptomyces*	0	
Unclassified[a]	11	*Acidimicrobium*[a], *Acidothermus*[b], *Micropruina*[b], *Modestobacter*[b], *Solirubrobacter*[b], *Unclassified*[c]	10	7	6	*Acidimicrobium*[b], *Solirubrobacter*[b], *Spirillospora*[b], *Unclassified*[c]	0	
BACTERIODETES								
Crenotrichaceae	5	*Chitinophaga*	0	2	1	*Chitinophaga*	0	
Flavobacteriaceae	1	*Flavobacterium*	0	0	0	0		
Flexibacteraceae	3	*Dyadobacter, Reichenbachia*[b], *Sporocytophaga*[b]	0	0	0	0		
Sphingobacteriaceae	0		1	0	0	*Pedobacter*		
Unclassified	2	*Chitinophaga*[b], *Ulvibacter*[b]	0	2	0	*Gramella*[b]	0	
CHLOROFLEXI								
Anaerolinaeceea	0		0	0	2	*Anaerolinea*		
FIRMICUTES								
Staphylococcaceae	0		0	0	0		1	*Staphylococcus*
GEMMATIMONADETES								
Gemmatimonadaceae	2	*Gemmatimonas*	0	3	3	*Gemmatimonas*	0	
PLANCTOMYCETES								
Planctomycetaceae	0		2	2	2	*Isospaera, Pirellula*	0	
PROTEOBACTERIA								
α-Acetobacteraceae	1	*Rhodopila*	0	0	1	*Stella*	0	
Bradyrhizobiaceae	2	*Afipia*[b], *Bradyrhizobium*	2	1	2	*Afipia*[b], *Agromonas*[b], *Bradyrhizobium, Nitrobacter*[b]	0	
Caulobacteraceae	0		0	1	0	*Caulobacter*	0	
Hyphomicrobiaceae	1	*Rhodoplanes*	1	1	0	*Blastochloris, Rhodoplanes*	0	
Phyllobacteriaceae	0		0	0	1	*Mesorhizobium*	0	
Rhodobacteraceae	0		0	1	0	*Roseivirga*	0	
Rhodobiaceae	0		1	0	0	*Rhodobium*	0	

Table 2 Continued

Identity (PHYLUM, family)	*Number of sequences detected*							
	Total DNA clone library		*^{13}C-DNA clone libraries*				*BPl-utilizing isolates*	
	t = 0	*Genera*	*1 d*	*4 d*	*14 d*	*Genera*	*t = 0*	*Genera*
Sphingomonadaceae	6	*Sphingomonas*	3	7	10	*Sphingomonas*	0	
Unclassified	4	*Blastochloris*[b], *Hyphomicrobium*[b], *Nitrobacter*[b], *Unclassified*[c]	9	2	3	*Afipia*[b], *Blastochloris*[b], *Devosia*[b], *Filomicrobium*[b], *Methylocystis*[b], *Methylosinus*[b], *Microvirg*[b], *Rhodobium*[b], *Rhodoplanes*[b], *Rhodopseudomonas*[b], *Stella*[b], *Teichococcus*[b]	0	
β-Burkholderiaceae	0		1	2	0	*Ralstonia, Cupriavidus*[b]	0	
Comamonadaceae	0		0	5	8	*Comamonas, Hydrogenophaga, Polaromonas, Variovorax*	0	
Hydrogenophilaceae	0		0	0	1	*Thiobacillus*	0	
Oxalobacteraceae	0		0	1	0	*Herbaspirillum*[b]	0	
Rhodocyclaceae	0		0	0	2	*Sterolibacterium*	0	
Unclassified	1	*Caenibacterium*[b]	1	2	3	*Caenibacterium*[b], *Thiobacter*[b], *Variovorax*[b], *Zoogloea*[b]	0	
δ-Bdellovibrionaceae	0		0	0	1	*Bdellovibrio*	0	
Unclassified	1	*Anaeromyxobacter*	0	1	2	*Desulforhabdus*[b], *Haliangium*[b], *Smithella*[b], *Unclassified*[c]	0	
γ-Coxiellaceae	0		1	0	0	*Rickettsiella*	0	
Pseudomonadaceae	0		0	1	1	*Pseudomonas*	2	*Pseudomonas*
Xanthomonadaceae	3	*Pseudoxanthomonas*[b], *Thermomonas*	0	0	2	*Lysobacter*	1	
Stenotrophomonas								
Unclassified	4	*Alkalispirillum*[a], *Balneatrix*[b], *Rhabdochromatium*[b]	1	4	1	*Alkanindiges*[b], *Methylococcus*[b], *Thiorhodospira*[a], *Unclassified*[c]	0	
UNCLASSIFIED	20		9	8	13		0	
Total sequences analyzed	85		50	77	86		22	

Abbreviation: BP, biphenyl.
[a]Unclassified denotes that phylogenetic assignment by RDP Classifier has bootstrap value below 80%.
[b]RDP Classifier bootstrap value below 80% for genus assignment.
[c]RDP Classifier bootstrap value below 20% for genus.

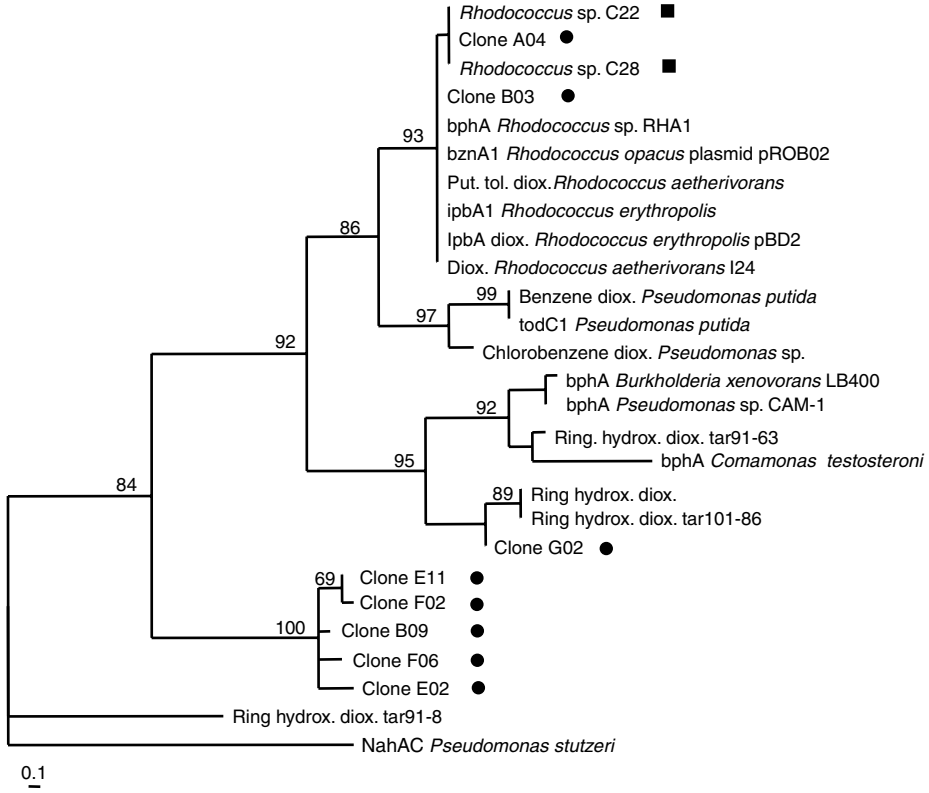

Figure 4 Phylogenetic tree based on shared amino acids of translated sequences of ADHRs genes. Sequences generated in this study for isolates (square) and PCR amplification from ^{13}C-DNA (circles) are marked. The scale is the expected number of substitutions per position. Numbers at the nodes are shown only for percentage bootstrap values above 50% of 1000 resamplings using the parsimony method. The NahAC sequence from *Pseudomonas stutzeri* was treated as the outgroup.

and reference sequences were translated and aligned to produce a phylogenetic tree (Figure 4). The most abundant ARHD sequence obtained from ^{13}C-DNA (25 out of 39 clones sequenced) is represented by Clone A04 in Figure 4. This sequence shared 100% amino-acid sequence similarity to *Rhodococcus* isolates C28 and C22, respectively, within the 77 amino-acid residues compared, whereas the nucleotide sequence similarity was 99 and 100%, respectively. The sequence represented by Clone B03 was detected only once in the library, and produced nearest GenBank matches of ARHDs targeting BP, isopropylbenzene, benzene and toluene associated with *Rhodococcus* spp., however, did not match any Genbank sequence exactly. When the 56 amino-acid residues available for Clone B03 were compared to the nearest GenBank matches that shared the same clade on the phylogenetic tree (Figure 4), amino-acid sequence similarities of 98% were obtained in all cases.

Whereas clones A04 and B03 nearly matched known ARHDs in GenBank along their full (~300 bp) nucleotide sequence length, 12 sequences obtained from ^{13}C-DNA matched only short (~30 bp) segments of other initial aromatic dioxygenases in GenBank. ARHDs were the only gene type represented in the nearest GenBank matches with *E* values below 1 for these sequences. In our phylogenetic tree (Figure 4), the sequences clustered distantly from the Gram-positive or Gram-negative groups of ARHDs. Sequences were analyzed using a Rieske protein Hidden Markov model (HMM) to determine if the Rieske center was present in the sequences obtained. Because one ARHD primer is within the Rieske center, only approximately one-third of the Rieske region (5′ end) could be evaluated. This region in the sequences obtained matched closely to the Rieske HMM model constructed by Pfam database (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00355).

GeoChip analyses, after subtraction of all genes also detected in background, positively detected 30 genes associated with organic contaminant degradation in the ^{13}C-DNA (Table 3). The majority (90%) of organic contaminant degradation genes detected were related to aromatic degradation. Four BP degradation genes were detected in ^{13}C-DNA; *bpdB* and *bpdF* from *Rhodococcus* sp. M5 and *bphD* and *bphB* from *Bacillus* sp. JF8. Genes encoding components of oxygenase enzymes catalyzing benzoate, dibenzofuran, phenol, phenylpropionate and protocatechuate degradation were also detected, as were several genes associated with the *β*-ketoadipate pathway.

Table 3 Organic contaminant degradation genes detected in ^{13}C-DNA following 14 days incubation with ^{13}C-BP and subtraction of background contamination

Target compound	*Gene description*	*Short gene name or locus tag*	*Species*	*GenBank ID*		*Detect. in total comm. DNA*
				Nucleotide	*Protein*	
Aromatic						
Benzoate (aerobic)	Possible oxygenase		*Mycobacterium tuberculosis* H37Rv	38490288	1806225	–
Benzoate (anaerobic)	Acetyl-CoA acetyltransferase	*phbA*	*Wautersia eutropha*	141953	135754	–
Benzoate (anaerobic)	Benzoyl-CoA reductase subunit	*badD*	*Rhodopseudomonas palustris* CGA009	3243084	2190579	–
Benzonitrile	Nitrile hydratase beta subunit		*Pseudomonas putida*	3172137	1877505	+
Benzonitrile	Putative amidase		*Synechocystis* sp. PCC 6803	1001200	2492835	+
Benzonitrile	Probable amidase amid (acylamidase) (acylase)	*amiD*	*Mycobacterium bovis* AF2122/97	31791177	31794557	–
Biphenyl	Ferredoxin	*bpdB*	*Rhodococcus* sp. M5	927231	927234	–
Biphenyl	2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase	*bpdF*	*Rhodococcus* sp. M5	1177720	1177721	+
Biphenyl	HOPDA hydrolase	*bphD*	*Bacillus* sp. JF8	32562909	32562911	–
Biphenyl	Biphenyl dihydrodiol dehydrogenase	*bphB*	*Bacillus* sp. JF8	32562909	32562914	+
Carbazole	Subunit of meta-cleavage enzyme	*carBa*	*Janthinobacterium* sp. J3	28201193	28201208	–
Catechol-meta derivative	2-hydroxy-6-oxohepta-2,4-dienoate hydrolase		*Helicobacter pylori* 26695	15644634	15645359	+
Catechol-ortho derivative	Muconate cycloisomerase		*Oceanobacillus iheyensis* HTE831	23097455	23100298	–
Catechol-ortho derivative	COG4188: Predicted dienalactone hydrolase	Npun6278	*Nostoc punciforme*	30581878	23129978	–
Catechol-ortho derivative	COG4188: Predicted dienalactone hydrolase	Ddes0990	*Desulfovibrio desulfuricans* G20	28877457	23474082	–
Dibenzofuran	Alpha subunit of dibenzofuran dioxygenase	*dfdAI*	*Terrabacter* sp.YK3	22036071	22036072	–
Dibenzofuran	Transcriptional activator protein	*dodR2*	*Rhodococcus opacus*	38524449	38524452	–
Naphthalene	2-hydroxychromene-2-carboxylate isomerase		*Bordetella parapertussis* 12822	33594723	33595692	–
Naphthalene	Naphthalene cis-dihydrodiol dehydrogenase	*nahB*	*Pseudomonas* sp. ND6	42632299	38638604	–
Phenol-aerobic	Phenol hydroxylase		*Uncultured soil bacterium*	22759909	22759910	–
Phenol-aerobic	Phenol hydroxylase		*Uncultured soil bacterium*	22759925	22759926	–
Phenoxybenzoate	Phenoxybenzoate dioxygenase	*orf pobA*	*Pseudomonas pseudoalcaligenes*	473249	473250	+
Phenylpropionate	Ring hydroxylating dioxygenase, alpha subunit:Rieske [2Fe-2S] domain	Avin4673	*Azotobacter vinelandii*	67087429	67088289	–
Phenylpropionate	COG4638: Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases, large terminal subunit		*Pseudomonas aeruginosa* UCBPP-PA14	32040668	32042180	–
Protocatechuate	Protocatechuate 3,4-dioxygenase beta chain	*pcaH*	*Bradryhizobium japonicum* USDA 110	27375111	27376038	–
Toluene-anaerobic	Sensor kinase	*tdiS*	*Azoarcus* sp. T	17223649	17223651	+
Xylene	Regulatory protein	*xylS*	*Pseudomonas putida*	18150858	18150921	–
Other organic						
g-hexachlorocyclohexane	Reductive dechlorinase of 2,5-dichlorohydroquinone	*linD*	*Sphingomonas paucimobilis*	1731851	1731852	+
Dichloroethane	Haloalkane dehalogenase		*Xylella fastidiosa* 9a5c	15836605	15838559	–
Nitrilotriacetate	Nitrilotriacetate monooxygenase component B	*ntaB*	*Agrobacterium tumefaciens* str. C58	17938588	17938814	–

Abbreviations: BP, biphenyl; CoA, coenzyme A.

Discussion

SIP revealed a wealth of bacteria in the root zone of pine that derived carbon from BP, either directly through BP degradation or indirectly via growth on metabolic intermediates released from BP utilizers or their detritus (Table 2). *Pseudonocardia*, *Nocardiodes, Kribella*, *Sphingomonas*, *Variovorax and Polaromonas* were the predominant genera in the pine root zone that derived carbon from BP, as evidenced by early (1–4 days), sustained and frequent detection in 16S rRNA clone libraries constructed from ^{13}C-DNA over the incubation period. Because clone libraries did not achieve full coverage based on rarefaction curves, T-RFLP peak heights are more reliable indicators of relative abundance than clone frequency although as in all PCR-based analyses amplification bias cannot be ruled out. On the basis of both abundance in clone libraries and associated T-RFLP peak height, *Pseudonocardia* was the most abundant utilizer of BP-derived carbon detected in this study.

PCB degradation capabilities have previously been reported for *Sphingomonas* spp. (Pieper, 2005), whereas the other dominant genera that derived carbon from BP were not known to degrade PCBs or BP although they were known to degrade other aromatics. *Pseudonocardia* spp. are known to degrade the monoaromatics benzoate, quinate and hydroxybenzoate (Grund and Kutzner, 1998; Hammann and Kutzner, 1998), toluene (Juteau *et al.*, 1999) and tetrachlorobenzene (Kampfer and Kroppenstedt, 2004). *Nocardiodes* spp. are capable of degrading phenanthrene (Saito *et al.*, 2000) and monoaromatics 2,4-dichlorophenoxyacetate (2,4-D) (Ferraroni *et al.*, 2005) and nitrophenols (Ebert *et al.*, 1999). A previous SIP experiment identified *Variovorax* as a naphthalene degrader (Padmanabhan *et al.*, 2003), and it is also known to degrade plant aromatics vanillylamine and capsaicin (Flagan and Leadbetter, 2006) and other aromatics including linuron (Dejonghe *et al.*, 2003) and 2,4-D (Kamagata *et al.*, 1997). *Polaromonas* has been reported to degrade naphthalene (Jeon *et al.*, 2004). The numerous other organisms identified as likely BP utilizers by virtue of acquiring ^{13}C within 1 day were not previously known to degrade BP (Table 2).

A pronounced disparity was observed between BP utilizers identified using SIP and the cultivation method. One *Arthobacter* strain was the only organism identified as a BP utilizer using both methods. *Rhodococcus* spp. represented 73% of BP-utilizing isolates, however, were not detected in any 16S rRNA gene clone libraries produced by SIP. This discrepancy may be due to the slow growth rate of rhodococci, which required up to 21 days to form colonies on agar plates, whereas the SIP incubation only extended for 14 days. This is supported by the apparent lack of growth of culturable BP utilizers in soil over the course of the study as determined by plate counts. The possibility of poor PCR priming of rhodococci was also considered; however, when primers used to generate 16S rRNA gene clone libraries (27F and 1392R) were compared to sequences of closely related rhodococci obtained from the RDP database, no sequence mismatches were found. If the cloning process was biased against rhodococci, then large T-RFs attributable to rhodococci would be expected on T-RFLP profiles; however, this was not the case. T-RFs predicted from 16S rRNA gene sequences of *Rhodococcus* isolates overlapped with very small peaks also assigned to the close relatives, *Nocardiodes* and *Kribella,* which were present in ^{13}C-DNA clone libraries.

Our findings differ from the only previously published SIP study aimed at identifying PCB-degrading bacteria (Tillmann *et al.*, 2005). In the previous study, *Burkholderia* were found to be the only species that degraded ^{13}C-labeled 2,2′-dichlorobiphenyl in a biofilm that was formed from a soil inoculum but was growing on PCB droplets. Culture-based studies of the biofilm were in agreement with the SIP results. The difference in taxa and species richness identified in our work are likely a result of differences in dominant PCB degraders in the soils studied as well as the distinct difference in community growth conditions between the two studies.

The increase in the number of ^{13}C-associated T-RFs observed over the course of the incubation period (Figure 3) suggests that carbon flowed through BP utilizers into other organisms in the soil community. Secondary carbon flow is most evident at the 14 days time point, when an increased number of T-RFs detected in the total community became ^{13}C-labeled (Figure 3), indicating that abundant soil heterotrophs began scavenging ^{13}C. Clone libraries from ^{13}C-DNA (Table 2) revealed eight different genera detected only after 14 days incubation, including *Lysobacter*, which was previously identified using SIP as a micropredator of ^{13}C-labeled *Escherichia coli* cells added to soil (Lueders *et al.*, 2006). Slower growing BP utilizers may also be expected to appear late in the incubation period. Unfortunately, after significant secondary carbon flow is underway, primary substrate degraders cannot be distinguished from secondary feeders with confidence.

When the metagenome of organisms that derived carbon from BP was explored using the GeoChip functional gene array, 28 different genes associated with aromatic degradation were detected. Four genes in the BP degradation pathway associated with *Rhodococcus* sp. M5 (*bpdB*, *bpdF*) and *Bacillus* sp. JF8 (*bphD*, *bphB*) were ^{13}C-enriched. However, known genes associated with other steps in BP degradative pathways were not detected in ^{13}C-DNA, nor were ARHDs that target BP or other polyaromatic compounds. Probing the pine rhizosphere with an array constructed from sequences of known degradative genes likely significantly under-

samples the variety of aromatic degradative genes present and involved in BP utilization.

The latter hypothesis is supported by sequences obtained from ^{13}C-DNA using primers designed to target the ARHDs, all of which were novel compared to known sequences present in GenBank. Some sequences clustered among known ARHDs, whereas five of the sequences were highly distinct from any known ARHD sequences although they possessed the portion of the Rieske center explored by the primers and matched short regions (30 bp) of other known aromatic dioxygenases. The novelty of these sequences invites further inquiry to obtain full sequences and examine their substrate specificity to evaluate their potential value to bioremediation.

The most frequently detected ARHD sequences (25 of 39 clones) were most closely related to *Rhodococcus* aromatic dioxygenases, and shared 99–100% nucleotide sequence similarity to ARHDs amplified from *Rhodococcus* isolates. This suggests two possible hypotheses. First, rhodococci may have derived carbon from BP, despite the absence of *Rhodococcus* sequences in 16S rRNA gene clone libraries. Alternatively, *Rhodococcus*-like dioxygenases may be more phylogenetically widespread than previously known, and could be present in related Actinobacteria that were frequently detected with SIP, such as *Pseudonocardia* or *Nocardiodes*, or other more distant organisms as a result of lateral gene transfer.

Benzoate (chlorobenzoate) is a major intermediate produced during BP (PCB) degradation and is subject to mineralization via various monoaromatic degradative pathways, including the β-ketoadipate pathway (Harwood and Parales, 1996; Pieper, 2005). The β-ketoadipate pathway is a convergent pathway common among soil bacteria that serves to mineralize a variety of monoaromatic xenobiotics as well as plant aromatics by funneling them through the central intermediate β-ketoadipate via either protocatechuate or catechol branches (Harwood and Parales, 1996). Organisms that derived carbon from BP in the pine root zone possessed several genes encoding enzymes of the β-ketoadipate pathway, including protocatechuate 3,4-dioxygenase and four different enzymes involved in the catechol branch of the pathway (Table 3). Thus, organisms active in BP mineralization in the root zone of pine, in theory, possess the capability to derive carbon and energy from plant aromatics. This provides further support for the hypothesis that plant-released aromatics have the capacity to support the growth of bacteria involved in PCB degradation (Singer *et al.*, 2004).

SIP successfully concentrated genes of interest associated with organisms truly involved in BP mineralization that were not detectable in the total community due to their low abundance. Sequences of novel ARHD genes, many other functional genes and 16S rRNA genes detected in ^{13}C-DNA were not detected directly in the total community DNA (Table 3, Figure 3). These findings underscore the utility of SIP for facilitating the discovery of genes of value to bioremediation and biocatalysis.

Together, this novel phylogenetic and functional gene information provides an improved understanding of BP degradation and carbon flow in soil communities and sheds new light on culture bias. Knowing the identity of bacteria and genes involved in biodegradation also enables the development of molecular tools to detect, quantify and monitor populations truly active in bioremediation processes. These tools could be applied to identify effective plant species for rhizoremediation by quantifying active PCB degraders in spatial association with different plants with much greater accuracy than cultivation methods used previously (Leigh *et al.*, 2006). Future SIP studies could also provide new insights into the mechanisms of rhizoremediation by determining whether plant aromatics function as growth substrates for pollutant-degrading bacteria in the root zone.

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