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DNA-Stable Isotope Probing Integrated with Metagenomics for Retrieval of Biphenyl Dioxygenase Genes from Polychlorinated Biphenyl-Contaminated River Sediment▽

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Stable isotope probing with [^{13}C]biphenyl was used to explore the genetic properties of indigenous bacteria able to grow on biphenyl in PCB-contaminated River Raisin sediment. A bacterial 16S rRNA gene clone library generated from [^{13}C]DNA after a 14-day incubation with [^{13}C]biphenyl revealed the dominant organisms to be members of the genera *Achromobacter* and *Pseudomonas*. A library built from PCR amplification of genes for aromatic-ring-hydroxylating dioxygenases from the [^{13}C]DNA fraction revealed two sequence groups similar to *bphA* (encoding biphenyl dioxygenase) of *Comamonas testosteroni* strain B-356 and of *Rhodococcus* sp. RHA1. A library of 1,568 cosmid clones was produced from the [^{13}C]DNA fraction. A 31.8-kb cosmid clone, detected by aromatic dioxygenase primers, contained genes of biphenyl dioxygenase subunits *bphAE*, while the rest of the clone's sequence was similar to that of an unknown member of the *Gammaproteobacteria*. A discrepancy in G+C content near the *bphAE* genes implies their recent acquisition, possibly by horizontal transfer. The biphenyl dioxygenase from the cosmid clone oxidized biphenyl and unsubstituted and *para*-only-substituted rings of polychlorinated biphenyl (PCB) congeners. A DNA-stable isotope probing-based cosmid library enabled the retrieval of functional genes from an uncultivated organism capable of PCB metabolism and suggest dispersed dioxygenase gene organization in nature.

Commercially used polychlorinated biphenyls (PCBs), which are mixtures of more than 60 individual chlorinated biphenyl congeners, are among the most persistent anthropogenic chemical pollutants that threaten natural ecosystems and human health (1). Numerous biphenyl-degrading microorganisms have been isolated and studied, especially for the range of PCB congeners that they degrade. Research has been primarily focused on the biodegradative pathways and the biphenyl dioxygenases responsible for initial PCB oxidation by isolated bacteria (14, 27). Knowledge, however, is limited concerning the indigenous microbial populations that metabolize PCBs in the environment. Stable isotope probing (SIP) coupled with metagenomics is one approach to more directly explore which organisms and genetic information may be involved in PCB degradation in PCB-contaminated sites.

SIP was developed to separate and concentrate the nucleic acids or fatty acids of microbial populations that metabolize and, hence, assimilate the isotopically labeled substrates into new cell material (4, 5, 28). Recently, the active PCB degraders in a biofilm community on PCB droplets were revealed as *Burkholderia* species by using DNA-SIP (32). In another DNA-SIP study, 75 different genera that acquired carbon from [^{13}C]biphenyl were found in the PCB-contaminated root zone of a pine tree (22). In addition, that heavy [^{13}C]DNA fraction revealed new dioxygenase sequences and possible PCB degradation pathways from GeoChip (16) results and from PCR-amplified sequences obtained by using primers targeting aromatic-ring-hydroxylating dioxygenase (ARHD) genes (22).

A major hurdle in using DNA-SIP for metagenomic analyses (9) is the very small amount of heavy DNA that is produced and, hence, recovered, making library construction difficult. Two studies have shown the feasibility of DNA-SIP for metagenomic analyses for C-1 compound-utilizing communities, but they first increased the amount of the heavy DNA fraction by multiple-displacement amplification (6, 10) or enriched the community by growth in sediment slurries. (18).

In this study, we used [^{13}C]biphenyl to probe for potential PCB-degrading populations in a PCB-contaminated river sediment and to recover genes potentially involved in the critical first step of PCB degradation, the dioxygenase attack. We found a 31.8-kb cosmid clone that contained a biphenyl dioxygenase sequence (*bphAE*) and demonstrated its activity on PCBs.

MATERIALS AND METHODS

Sample description and SIP microcosms. Sediment historically contaminated with Aroclor 1248 at concentrations of 0.2 to 4.6 mg kg^{-1} was collected in October 2000 from River Raisin at Monroe, MI. The sediment samples were stored at 4°C under river water until use.

Five replicate microcosms, each containing 5 g of sediment amended with 10 mg of uniformly labeled [^{13}C]biphenyl (99 atom % ^{13}C) (Sigma-Aldrich) and 10 ml of K1 minimal medium (34) were placed in 160-ml serum bottles. The sample bottles were sealed with Teflon stoppers and aluminum crimp caps and

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incubated at room temperature in the dark on a horizontal shaker at 150 rpm. The microcosms were aerated by opening the flasks in sterile conditions for 10 min every 3 to 4 days, and after 14 days, DNA was extracted from all microcosms.

To monitor biphenyl metabolism, nine microcosms amended with 10 mg of unlabeled biphenyl and three sterile microcosms with twice-autoclaved sediment and unlabeled biphenyl were established in parallel and incubated as described above. After 0, 7, and 14 days of incubation, triplicate microcosms were sacrificed for biphenyl extraction by the addition of 10 ml of saturated KCl and 10 ml of dichloromethane. Biphenyl concentrations were determined by gas chromatography with flame ionization detection. Split injections (50:1) were made on a J&K Scientific ICB-PAH capillary column (15-m length, 0.25-mm inner diameter, and 0.15-μm film thickness). Temperature conditions were inlet at 220°C, oven at 80°C for 1 min and then ramped at 40°C min^{-1} to 220°C, and detector at 325°C. Colony counts at each time point were obtained using R2A (29) agar plates and counting after 3 weeks of incubation.

DNA extraction and [^{13}C]DNA separation. DNA was extracted following a previous protocol (35) but modified as follows to recover high-molecular-weight DNA. All sediment slurries were centrifuged at 3,500 × *g*, and 4 g of sediment pellet was transferred to a disposable 50-ml polypropylene centrifuge tube where 13.5 ml of extraction buffer containing 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 100 mM EDTA, 1.5 M NaCl, and 1% cetyltrimethylammonium bromide (CTAB) was added. Tubes were amended with 1.5 ml of 20% sodium dodecyl sulfate (wt/vol) and incubated in a 65°C water bath for 2 h with gentle inversion every 10 min. Supernatant without whitish material was collected after centrifugation at 3,000 × *g* for 5 min, transferred into another 50-ml polypropylene tube, and extracted with an equal volume of chloroform. DNA was precipitated with isopropanol, washed with ethanol, and dissolved in water at 50°C. For removing humic substances, the DNA solution was adjusted to 0.3 M NaCl by adding 1 M NaCl in TE (10 mM Tris-Cl and 1 mM EDTA, pH 8.0) and placed into 1-ml DEAE Sephacel (Sigma-Aldrich) columns pre-equilibrated with 0.3 M NaCl in TE. The columns were washed with 4 ml of 0.3 M NaCl in TE, and DNA was eluted with 4 ml of 0.5 M NaCl in TE. DNA was again precipitated with isopropanol, washed with ethanol, and dissolved in water at 50°C.

Totals of 70 μg of DNA at 0 days and 14 days (D_0, DNA from sediment at zero time, and D_{14}, DNA from sediment in microcosms incubated with [^{13}C]biphenyl for 14 days) were loaded into 18.5 ml of cesium trifluoroacetate (CsTFA) (Amersham, Piscataway, NJ) solution without the addition of ethidium bromide and with a starting buoyant density of 1.60 g ml^{-1}. The CsTFA solution with DNA was transferred to 18.5-ml Ultracrimp tubes (Sorvall, Waltham, MA). The tubes were centrifuged in a TV-865B vertical rotor (Sorvall) at 179,000 × *g* (43,500 rpm) for 40 h at 20°C. The gradients were fractionated into 500-μl fractions (up to 37 fractions) by displacement with water using a syringe pump at a flow rate of 1 ml min^{-1}. The buoyant density of each fraction was measured at 25°C with a refractometer. DNA fractions were precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and isopropanol. The DNA pellets were then washed and resuspended in EB elution buffer (Qiagen, Valencia, CA) and incubated at 50°C for 1 h. Fractionated DNA was quantified with an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). Secondary isopycnic density gradient centrifugation of combined DNA and quantitative PCR were conducted as described previously (22).

16S rRNA and ARHD gene clone libraries. Amplifications of 16S rRNA genes for clone libraries were conducted using primers 27F (17) and 529R (33) on D_0 and 27F and 1392R (17) on the heavy DNA fraction from D_{14} (D_{14}H). Cycling conditions were as follows: denaturation for 5 min at 94°C; 25 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min (for D_0) or 1 min 40 s (for D_{14}H) at 72°C; and an additional 7-min extension at 72°C. PCR amplification of ARHD genes was performed using primers ARHD1F (5′-TTYRYNTGYANNTAYCAYGGNTGGG-3′) and ARHD2R (5′-AANTKYTCNGCNGSNRMYTTCCA-3′) with D_{14}H as previously described (22). PCR amplicons of both 16S rRNA and ARHD genes were gel purified using a QIAquick gel extraction kit (Qiagen) and cloned using a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). Clone libraries were sequenced using primers T7 or T3 at the Michigan State University, Research Technology Support Facility, with an ABI 3730 genetic analyzer (Applied Biosystems, Inc., Foster City, CA). The phylogenetic identification of 16S rRNA gene consensus sequences was performed by using the RDP-II Classifier (7).

Cosmid library construction and screening library with ARHD primers. Size-selected D_{14}H (25 to 40 kb) was obtained by electrophoresis on 1% (wt/vol) low-melting-point agarose Tris-acetate-EDTA gel, and the desired size of DNA was recovered by using Gelase (Epicentre, Inc., Madison, WI) without UV irradiation, end repaired with T4 DNA polymerase, and then inserted into the pWEB cosmid (Epicentre, Inc.) at the SmaI site. A cosmid library was constructed by using a pWEB cosmid cloning kit. All cosmid clones were stored at −80°C. PCR amplification with ARHD primers was used for cosmid library screening as described above. Every 96 cosmid clones were pooled as templates for PCR screening.

Sequencing cosmid clone and genomic analysis. The cosmid clone L11E10 was sheared into approximately 4-kb fragments by using a GeneMachines HydroShear device (Genomic Solutions, Ann Arbor, MI). The fragments were end repaired with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase (Epicentre). The DNA fragments were then ligated into the vector pCR-Blunt (Invitrogen) and transformed into *Escherichia coli* TOP10. A total of 192 colonies were picked and then grown in LB broth plus 50 μg ml^{-1} kanamycin in deep-well microtiter plates. Plasmid DNA was isolated by using the Invitrogen PureLink 96-well lysis technique. The two ends of the inserted DNA fragment were sequenced by using either primer BL (5′-TCGGATCCACTAGTAACGGC-3′) or primer BR (5′-CCAGTGTGATGGATATCTGC-3′). The sequences were trimmed and assembled using Lasergene software (DNAStar, Madison, WI).

PCB transformation by expression in *E. coli*. *bphAE* of *Burkholderia xenovorans* LB400 ($bphAE_{LB400}$) was amplified from genomic DNA by using primers 5′-CACCATGAGTTCAGCAATCAAGAA-3′ (underlined sequences were for directional cloning described below) for the forward sequence of *bphA* and 5′-CTAGAAGAACATGCTCAGGTT-3′ for the reverse sequence of *bphE*. PCR for $bphAE_{LB400}$ was performed with Platinum *Pfx* polymerase (Invitrogen) and 30 pmol of each primer for 25 cycles of 1 min at 94°C, 1 min at 55°C, and 4 min at 72°C. The *bphAE* genes of L11E10 ($bphAE_{L11E10}$) were amplified from the cosmid clone DNA by using 5′-CACCATGAATACTTTGATCAAAGAA-3′ for the forward sequence of *bphA*, with modification of the start codon GTG to ATG, and 5′-TTAGAAGAACATGCTCAGGTT-3′ for the reverse sequence of *bphE*. PCR for L11E10 was performed for 25 cycles of 1 min at 94°C, 1 min at 55°C, and 6 min at 68°C. Both pET101[LB400-*bphAE*] and pET101[L11E10-*bphAE*] were generated by using a Champion pET101 directional TOPO expression kit (Invitrogen). pET101[LB400-*bphAE*] or pET101[L11E10-*bphAE*] and pDB31[LB400-*bphFGBC*] (2) were cotransformed into *Escherichia coli* BL21 Star(DE3).

The PCB degradation capabilities of transformants were assessed by using a resting-cell assay. *E. coli* BL21 containing pET101[LB400-*bphAE*] or pET101 [L11E10-*bphAE*] plus pDB31[LB400-*bphFGBC*] was grown in LB medium containing 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin, in addition to 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG), at 37°C. Log-phase cells were washed and resuspended to an optical density of 1.75 at 600 nm in M9 medium containing 0.8 mM IPTG and 0.1% (wt/vol) sodium acetate. Portions (2 ml) were pipetted into glass vials, amended separately with one of two PCB mixtures in 10 μl of acetone, and sealed with Teflon-lined stoppers and aluminum crimp caps. The PCB mixtures were identical to mixtures 1B and 2B (3) except that 2,2′,4,4′,6,6′-chlorinated biphenyl (CB) was used as the internal standard instead of 2,2′,4,4′,6-CB; the final concentration of each congener was 1 μg ml^{-1}. The tubes were then incubated at 37°C with shaking at 200 rpm for 18 h. Following incubation, the contents of the tubes were acidified with three to four drops of concentrated HCl, and the PCBs were extracted three times with 1 ml of hexane/acetone (1:1, vol/vol). The extracts from each sample were combined and analyzed for PCBs by using a gas chromatograph fitted with an electron capture detector and a DB-5 capillary column (30-m length, 0.32-mm ID, 0.25-μm film thickness). The oven temperature program was 140°C for 1 min, followed by an increase at 2°C min^{-1} to 260°C. The inlet and detector temperatures were 220°C and 325°C, respectively. PCBs were quantified using a four-point calibration curve and the internal standard method. In a separate experiment, the accumulation of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) by transformants was determined at 434 nm (19) with a UV-Vis spectrophotometer (Varian, Inc., Palo Alto, CA) after the addition of biphenyl.

Nucleotide sequence accession numbers. The sequences obtained were deposited in GenBank with accession numbers as follows: ARHD of D_{14}H, accession no. GQ231323 to GQ231332; 16S rRNA clone libraries of D_{14}H, accession no. GQ231333 to GQ231378; D_0, accession no. GQ231379 to GQ231433; and cosmid clone L11E10, accession no. GQ231434.

RESULTS

Disappearance of biphenyl during the incubation. To confirm the feasibility of this sediment for the SIP experiment, biphenyl disappearance was measured in microcosms incubated with unlabeled biphenyl. Only 0.6% of the biphenyl remained after a 14-day aerobic incubation, whereas none of

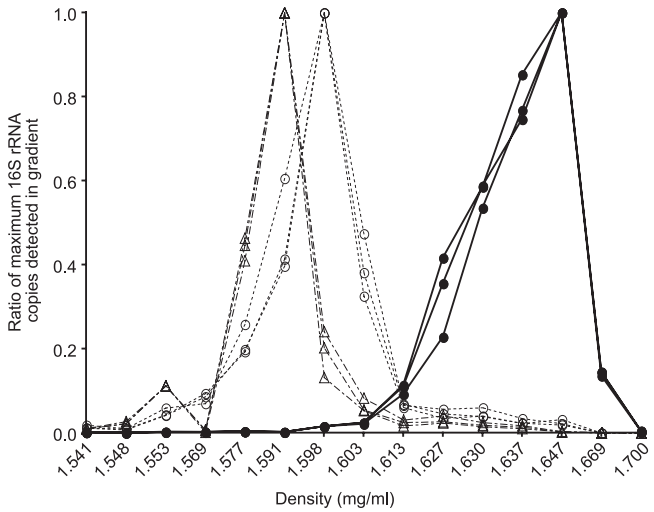

FIG. 1. [^{12}C]- and [^{13}C]DNA were separated by small-scaled secondary isopycnic centrifugation and quantified by quantitative PCR of 16S rRNA genes in triplicate samples. Solid circles and lines, $D_{14}H$; open circles and dashed lines, D_0; open triangles and dashed lines, unlabeled D_{14}.

the biphenyl disappeared in the sterile microcosms. During this period, the total culturable bacteria increased from 4.6×10^5 to 1.79×10^8 CFU g^{-1} dry sediment as determined by plate counts.

DNA extraction and isopycnic centrifugation. D_0 and D_{14} were extracted by our high-molecular-weight DNA extraction method. Both D_0 and D_{14} were separately loaded, in amounts of approximately 70 μg each, for 18.5-ml-scaled isopycnic centrifugation. [^{13}C]DNA fractions of D_{14} were collected for the buoyant densities from 1.634 to 1.656 g ml^{-1}, where DNA was detected in D_{14} but not in D_0. For confirmation that this fraction had [^{13}C]DNA, the collected DNAs from the heavy fraction ($D_{14}H$), from the unlabeled-biphenyl-incubated microcosms at 14 days (unlabeled D_{14}), and from D_0 were subjected to 2-ml-scaled isopycnic centrifugation, followed by quantitative PCR of 16S rRNA genes in the separated fractions (Fig. 1). These results confirmed that $D_{14}H$ consisted of only [^{13}C]DNA and was clearly separated from either D_0 or unlabeled D_{14}. The approximately 3 μg of $D_{14}H$ was enough to construct a 16S rRNA gene clone library, a metagenomic library, and a PCR-based ARHD library.

Analysis of 16S rRNA and ARHD genes in clone libraries. Fifty-five 16S rRNA gene clones from D_0 and 46 clones from $D_{14}H$ were sequenced. The two libraries exhibited distinct microbial community compositions and diversities (∫-LIBSHUFF *P* values for comparison of the two libraries were <0.001) (30). The $D_{14}H$ clone library, which should include active biphenyl-degrading microorganisms, contained members of the genera *Achromobacter*, *Pseudomonas*, *Acidovorax*, *Ramlibacter*, *Azoarcus*, and *Hydrogenophaga*, which were not found in the D_0 clone library (Table 1).

A library of ARHD gene sequences in $D_{14}H$ yielded five unique ARHD sequences from 10 clones, which could be divided into two groups based on the translated amino acid sequences (99 to 106 amino acids). Clones 8, 13 (number of identical sequences [*n*] = 3), and 17 (*n* = 2) exhibited, respectively, 92%, 94%, and 94% amino acid identity to a large subunit of the biphenyl dioxygenase of *Comamonas testosteroni* strain B-356 (31) (now *Pandoraea pnomenusa* [15]). Another group that included clones 11 (*n* = 2) and 12 (*n* = 2) were similar to a large subunit of dioxygenase of the gram-positive *Rhodococcus* sp. strain RHA1 (24), with amino acid identities of 82% and 77%, respectively.

Screening for and analysis of biphenyl dioxygenases. A library of 1,568 cosmid clones which contained DNA inserts from $D_{14}H$ averaging 30 to 40 kb (data not shown) was constructed and screened for genes encoding large subunits of biphenyl dioxygenases (*bphA*s) using primers to detect ARHD-encoding DNA. Five of the clones yielded ARHD amplicons of 300 to 330 bp, but sequencing of the amplicons showed that only one clone, L11E10, actually contained a *bphA* sequence.

TABLE 1. Phylogenetic classification of 16S rRNA genes in clone libraries from D_0 and $D_{14}H$

Phylogenetic group[a]	No. of clones found in:		Genus(era)[b] (no. of clones)
	D_0	$D_{14}H$	
Actinobacteria			
Intrasporangiaceae[c]	2		
Propionibacteriaceae[c]		1	
Unclassified *Actinobacteria*	1		
Acidobacteria	4		
Bacteroidetes	3		
Chloroflexi			
Caldilineaceae[c]	7		*Levilinea* (1[d]), *Leptolinea* (1[d])
Unclassified *Anaerolineae*	10		
Firmicutes	1		
Planctomycetes	1		*Pirellula* (1[d])
Proteobacteria			
Alphaproteobacteria			
Rhodobacteraceae[c]	2		*Rhodobacter* (1[d])
Unclassified *Alphaproteobacteria*	1		
Betaproteobacteria			
Rhodocyclaceae[c]		1	*Azoarcus* (1)
Gallionellaceae[c]	1		*Gallionella* (1[d])
Comamonadaceae[c]	1	9	*Acidovorax* (6), *Ramlibacter* (2), *Hydrogenophaga* (1), *Rhodoferax* (1[d])
Alcaligenaceae[c]		22	*Achromobacter* (22)
Hydrogenophilaceae[c]	2		*Thiobacillus* (2[d])
Unclassified *Betaproteobacteria*	5	1	
Gammaproteobacteria			
Pseudomonadaceae[c]		9	*Pseudomonas* (9)
Xanthomonadaceae[c]		1	
Deltaproteobacteria	2		*Smithella* (1[d]), *Pelobacter* (1[d])
Unclassified *Proteobacteria*		1	
OP10	1		
Unclassified bacteria	11	1	
Total	55	46	

[a] The taxonomic assignment was based on the lowest taxonomic level that gave a >80% confidence level for assignment by the RDP-II Classifier, release 9.50 (7).
[b] Genus is indicated when the confidence level was more than 80%.
[c] The indicated taxonomy unit is the family.
[d] Indicated genus was found in D_0.

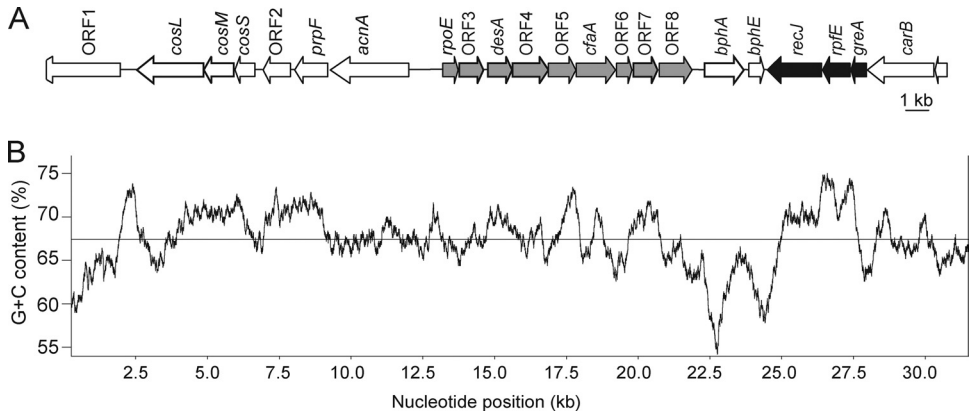

FIG. 2. (A) Schematic diagram of gene order in clone L11E10. Following are gene product descriptions: *cosL*, large subunit of carbon monoxide dehydrogenase; *coxM*, middle subunit of carbon monoxide dehydrogenase; *coxS*, small subunit of carbon monoxide dehydrogenase; *prpF*, *AcnD* (the homolog of *acnA*)-accessory protein; *acnA*, aconitate hydratase; *rpoE*, RNA polymerase sigma 70 factor; *desA*, fatty acid desaturase; *cfaA*, cyclopropane fatty acyl phospholipid synthase; *bphA*, large subunit of biphenyl dioxygenase; *bphE*, small subunit of biphenyl dioxygenase; *recJ*, single-stranded DNA-specific exonuclease; *rpfE*, regulatory protein; *greA*, transcription elongation factor; and *carB*, large subunit of carbamoyl phosphate synthase. The order of genes *rpoE*-ORF3-*desA*-ORF4-ORF5-*cfaA*-ORF6-ORF7-ORF8 (gray arrows) and *recJ-rpfE-greA* (black arrows) in L11E10 was identical to that in six sequenced *Xanthomonas* genomes. (B) G+C contents of window size 500 bp. The line in the middle refers to the average G+C content of insert DNA in L11E10.

The $bphA_{L11E10}$ sequence was not an exact match with any of the PCR-amplified ARHD sequences found in $D_{14}H$.

The clone L11E10 contained an insert of 31,850 bp with 67.38% G+C content. Seventeen of 22 open reading frames (ORFs) in L11E10 gave top BlastX hits against ORFs in the genera *Xanthomonas* and *Stenotrophomonas*. Genes for subunits of the biphenyl dioxygenase (*bphA* and *-E*) were found in L11E10. L11E10 contained no other genes directly relevant to the known biphenyl degradation pathway (Fig. 2A). The *bphA* was highly similar to *bphA* in *Pseudomonas* sp. strain Cam-1 (90%) and *bphA1* in *Pseudomonas pseudoalcaligenes* KF707 (89.5%) (13). The *bphA* also encoded the motif Cys-X-His-X17-Cys-X2-His that forms the Rieske-type [2Fe-2S] cluster of iron-sulfur proteins. $bphE_{L11E10}$ (*bphE* is a small subunit of biphenyl dioxygenase) was 93% identical to $bphE_{LB400}$ and *bphA2* in *P. pseudoalcaligenes* KF707.

Functional analysis of biphenyl dioxygenases. To determine the activity of BphAE encoded in L11E10 ($bphAE_{L11E10}$) toward biphenyl and PCBs, $bphAE_{L11E10}$ was expressed in *E. coli* BL21 along with *bphFGBC* from *B. xenovorans* LB400 ($bphFGBC_{LB400}$). $bphFGBC_{LB400}$ encodes ferredoxin (BphF), ferredoxin reductase (BphG), biphenyl-2,3-dihydrodiol 2,3-dehydrogenase (BphB), and 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), involved in the upper pathway of biphenyl catabolism. In this pathway, biphenyl is transformed to HOPDA, producing a yellow color (23). When *E. coli* BL21 transformants containing $bphAE_{L11E10}$ were induced with IPTG and incubated with biphenyl, they produced the yellow color indicative of HOPDA within 2 h. In resting-cell assays with PCB mixtures, the same transformants metabolized 2,3-CB, 2,4′-CB, 4,4′-CB, 2,4,4′-CB, and 2,4′,5-CB; the 4,4′-CB and 2,4,4′-CB were metabolized to a greater extent than by similar transformants containing $bphABFG_{LB400}$ genes. These results are consistent with the activities in resting-cell assays of *P. pseudoalcaligenes* KF707 (11), with the exceptions that KF707 also exhibited some transformation of 2,2′,3,3′-CB and 2,3′,4,4′-CB (Table 2).

DISCUSSION

A major hurdle in DNA-SIP-based metagenomics is the recovery of [^{13}C]DNA in sufficient quantity for cosmid library construction and the production of a target number of clones. Due to these constraints, we used sediment slurries that were able to increase biphenyl consumption compared to the level of consumption in our SIP study using [^{13}C]biphenyl in rhizosphere soil (22), thus enhancing the incorporation of labeled carbon into cell material and obtaining sufficient [^{13}C]DNA to produce a cosmid library. The resulting community, $D_{14}H$, seems to have less bacterial diversity than the heavy fraction from using [^{13}C]biphenyl in rhizosphere soil (22), as would be expected from the addition of the larger amount of biphenyl.

TABLE 2. Depletion of PCB congeners by $BphA_{L11E10}$ and $BphA_{LB400}$

PCB congener	% Depletion with BphA of:			
	L11E10[a]	LB400[a]	LB400[b]	KF707[b]
2,2′	<10	100	100	5
2,3	100	100	100	100
2,4′	100	100	100	100
4,4′	100	<10	15	100
2,2′,5	0	100	100	0
2,4,4′	92	22	45	93
2,5,4′	89	99	94	83
2,2′,3,3′	<10	96	94	60
2,2′,3,5′	0	96	96	0
2,2′,4,4′	0	16	38	0
2,2′,5,5′	0	99	95	0
2,3′,4,4′	0	0	16	24
2,3′,4′,5	<10	94	83	0
3,3′,4,4′	0	0	0	0
2,2′,3′,4,5	0	<10	38	0
2,2′,3,4,5′	0	29	58	0
2,2′,4,5,5′	0	64	73	0

[a] Data from this study.
[b] Resting-cell assay data were obtained from a previous study (11).

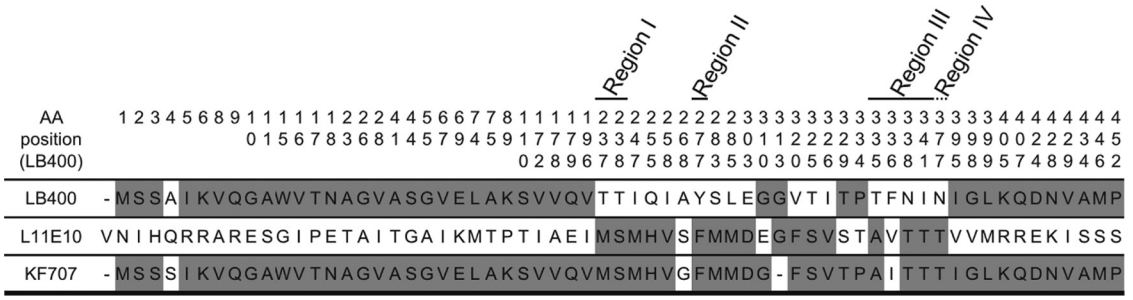

FIG. 3. Amino acid sequence alignment of large subunit of $BphA_{LB400}$, $BphA_{L11E10}$, and $BphA_{KF707}$. Only the positions that are not identical among the three BphAs are shown in the alignment. The numbers of amino acid positions are for $BphA_{LB400}$. The shaded areas represent conserved amino acid sequences. AA, amino acid.

This approach is useful for recovering functional genes from potentially unculturable populations and for analyzing their natural genetic context, but would not be useful for recovering genes from populations that might be specialists for low substrate concentrations. The dioxygenase clone we recovered did not overlap with the sequences amplified by the ARDH primers. The most likely explanation is that PCR bias favored genes not recovered in the cosmid.

The $D_{14}H$ community analysis showed that the dominant bacterial groups were closely related to previously known PCB- and biphenyl-utilizing bacteria. The most dominant group, members of the genus *Achromobacter*, includes *Achromobacter xylosoxidans* KF701, which can grow on biphenyl, 4-methylbiphenyl, 2-hydroxybiphenyl, benzoate, and salicylate (12). Seven sequences in the family *Comamonadaceae*, classified as *Acidovorax* and *Hydrogenophaga* by the RDP classifier, are most similar to PCB- and biphenyl-degrading *Acidovorax* sp. (formerly *Pseudomonas* sp.) strain KKS102 (20, 26) and biphenyl-utilizing and PCB-cometabolizing psychrotrophic *Hydrogenophaga taeniospiralis* IA3-A (21). Also, the genus *Pseudomonas* includes *P. pseudoalcaligenes* KF707, a well-known biphenyl- and PCB-degrading microorganism.

It is interesting that L11E10 had only the *bphAE* genes of the biphenyl pathway and that the genetic organization differs from the upper *bph* operons of known biphenyl-degrading microorganisms (27). In addition, the G+C content around *bphAE* was lower than the average for the clone (Fig. 2B). Furthermore, the order of genes *rpoE*-ORF3-*desA*-ORF4-ORF5-*cfaA*-ORF6-ORF7-ORF8 and *recJ-rpfE-greA* (Fig. 2A) in L11E10 was identical to that in six sequenced *Xanthomonas* genomes, none of which have the upper *bph* operons. Therefore, *bphAE* in L11E10 could have been recently acquired from another microorganism, perhaps as an outcome of the at-least-40-year exposure to Aroclor 1248 in these sediments. It is possible that the gene organization of *bph* operons in nature is dispersed, while the *bph* operons found in biphenyl-degrading microorganisms typically isolated by enrichment culture are less common but better arranged for rapid growth and, hence, isolation.

Analysis of the origin of L11E10 suggests that the insert DNA came from a gammaproteobacterium, because the L11E10 *recJ*, encoding a single-stranded DNA-specific exonuclease required for efficient recovery of DNA synthesis (8), had high homology to those in *Gammaproteobacteria*.

$BphAE_{L11E10}$ showed a PCB congener transformation spectrum similar to but narrower than that of the KF707 biphenyl dioxygenase ($BphA_{KF707}$). It appeared to transform only PCB congeners without chlorines at the 2,3 positions. This is consistent with BphA protein sequences in which regions I, II, III, and IV of L11E10, responsible for substrate specificity (25), are identical to these regions in $BphA_{KF707}$ except for having Val-337 (L11E10) instead of Ile-335 (KF707) at LB400 position 336 (Fig. 3). As such, Val-337 (L11E10) may effect a narrow specificity toward 2,2′,3,3′-CB and 2,3′,4,4′-CB. Even though the differences in the N terminus (31 amino acid differences before position 196) and C terminus (11 amino acid differences after position 395) of $BphA_{L11E10}$ and $BphA_{KF707}$ or $BphA_{LB400}$ are greater than these differences in $BphA_{LB400}$ and $BphA_{KF707}$ (only one amino acid difference), this does not appear to affect PCB substrate specificity (14).

Combining DNA-SIP and metagenomic analyses should increase our understanding of genomic features of microbial populations in nature since it avoids cultivation bias and minimizes interference from nonfunctional genes. The efficiency of the methods, particularly the sufficient recovery of labeled nucleic acids of high molecular weight, and its use under conditions that typify the natural environment, e.g., little disturbance and natural substrate concentrations, need further development.

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