Assessment of Toluene/Biphenyl Dioxygenase Gene Diversity in Benzene-Polluted Soils: Links between Benzene Biodegradation and Genes Similar to Those Encoding Isopropylbenzene Dioxygenases†

Robert Witzig,[1] Howard Junca,[1] Hans-Jürgen Hecht,[2] and Dietmar H. Pieper[1]*

Department of Environmental Microbiology[1] and Division of Structural Biology,[2] GBF—German Research Centre for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany

The PCR–single-strand conformation polymorphism (SSCP) technique was used to assess the diversity and distribution of Rieske nonheme iron oxygenases of the toluene/biphenyl subfamily in soil DNA and bacterial isolates recovered from sites contaminated with benzene, toluene, ethylbenzene, and xylenes (BTEX). The central cores of genes encoding the catalytic α subunits were targeted, since they are responsible for the substrate specificities of these enzymes. SSCP functional genotype fingerprinting revealed a substantial diversity of oxygenase genes in three differently BTEX-contaminated soil samples, and sequence analysis indicated that in both the soil DNA and the bacterial isolates, genes for oxygenases related to the isopropylbenzene (cumene) dioxygenase branch of the toluene/biphenyl oxygenase subfamily were predominant among the detectable genotypes. The peptide sequences of the two most abundant α subunit sequence types differed by only five amino acids (residues 258, 286, 288, 289, and 321 according to numbering in cumene dioxygenase α subunit CumA1 of *Pseudomonas fluorescens* IP01). However, a strong correlation between sequence type and substrate utilization pattern was observed in isolates harboring these genes. Two of these residues were located at positions contributing, according to the resolved crystal structure of cumene dioxygenase from *Pseudomonas fluorescens* IP01, to the inner surface of the substrate-binding pocket. Isolates containing an α subunit with isoleucine and leucine at positions 288 and 321, respectively, were capable of degrading benzene and toluene, whereas isolates containing two methionine substitutions were found to be incapable of degrading toluene, indicating that the more bulky methionine residues significantly narrowed the available space within the substrate-binding pocket.

Aromatic hydrocarbons such as benzene, toluene, and ethylbenzene are common contaminants of soil and groundwater (41) and are listed as priority pollutants by the U.S. Environmental Protection Agency (http://www.epa.gov/waterscience/criteria/wqcriteria.html). One of the most attractive means to remove these compounds from polluted environments is through bioremediation, which can be achieved by natural attenuation, by stimulation of the local microbial activity through the addition of nutrients and/or electron acceptors, or by bioaugmentation (65).

The aerobic degradation of aromatic compounds is frequently initiated by Rieske nonheme iron oxygenases, which catalyze the incorporation of two oxygen atoms into the aromatic ring to form arene *cis*-diols (30), followed by a dehydrogenation reaction catalyzed by a *cis*-dihydrodiol dehydrogenase to give catechol or substituted catechols which serve as substrates for oxygenolytic aromatic ring cleavage. Rieske nonheme iron oxygenases are multicomponent enzyme complexes composed of a terminal oxygenase component (iron-sulfur protein [ISP]) and different electron transport proteins (a ferredoxin and a reductase or a combined ferredoxin-NADH-reductase) (13). The catalytic iron-sulfur proteins are heteromultimers, comprising a large (α) and a small (β) subunit, with the former containing a Rieske-type [2Fe-2S] cluster, a mononuclear nonheme iron oxygen activation center, and a substrate-binding site (13, 27) and being responsible for substrate specificity (30). Comparison of the amino acid sequences of the terminal oxygenase α subunits (ISPα) revealed that they form a family of diverse but evolutionarily related sequences, and four distinct major lineages have been identified (30). Although none of the enzymes is completely specific, a broad correlation between the grouping in toluene/biphenyl, naphthalene, benzoate, or phthalate subfamilies and the native substrates oxidized by the subfamily members can be observed. However, various enzyme-engineering studies on biphenyl, benzene, chlorobenzene, and naphthalene dioxygenases also showed that only slight sequence differences in the amino acid sequences of ISPα can be associated with dramatic changes in substrate specificity or regiospecificity (8, 24, 50, 69). Information on Rieske nonheme iron oxygenases has been obtained mainly from cultured strains. However, various culture-independent studies suggest that distinct and potentially numerically dominant sequence types of as-yet-unrecognized functional genes exist in natural and anthropogenically influenced environments and that the sequences from cultured strains are

* Corresponding author. Mailing address: Department of Environmental Microbiology, GBF—German Research Centre for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany. Phone: (49) 531-6181467. Fax: (49) 531-6181411. E-mail: dpi@gbf.de.
† Supplemental material for this article may be found at http://aem.asm.org/.

TABLE 1. Reference strains used in this study and results of the PCR targeting ISPα genes of the toluene/biphenyl oxygenase subfamily

Organism	Relevant aromatic growth substrate[a]	Reference(s)	Reported ISPα gene(s)	GenBank accession no.	PCR primer set[c]	
					BPDOXF/ BPDOXR	bphAf668-3/ bphAf1153-2
Burkholderia xenovorans LB400	**Bph**[b]	10, 31	*bphA1*	**M86348**	+	+
Cupriavidus necator H850	**Bph**	6	*bphA1*	**AJ544525**	+	+
Ralstonia sp. strain PS12	**Tcb**	7	*tecA1*	**U78099**	+	+
Rhodococcus globerulus P6	**Bph**	2	*bphA1*	**X80041**	(+)	+
Pandoraea sp. strain JB1*	NS				+	+
Pseudomonas sp. strain Cam-1	**Bph**	45	*bphA1*	**AY027651**	+	+
Pseudomonas sp. strain IC	**Bph**, Tt	14	*bphX*	AF077669	+	+
Pseudomonas aeruginosa JI104	**B**	42	*bnzA*	**E04215**	+	+
Pseudomonas fluorescens IP01	**T, Eb, Ipb**	1, 16	*cumA1*	**D37828**	+	+
Pseudomonas pseudoalcaligenes KF707	**Bph**	25	*bphA1*	**M83673**	+	+
Pseudomonas stutzeri AN10	Nah	11	*nahAc*	AF039533	−	−
Pseudomonas stutzeri OM1	Car	49	*carAa*	AB001723	−	−
Pseudomonas stutzeri OX1	B, T, Ph, *o*-X	9			−	−
Pseudomonas putida F1	**B, T, Eb**, *p*-C	21, 29	*todC1, cmtAb*	**J04996**, U24215	+	+
Pseudomonas putida G7	Nah	58	*nahAc*	M83949	−	−
Pseudomonas sp. strain CF600	Ph	56			−	−
Pseudomonas putida mt-2	T, *m*-X, *p*-X, Tt, Bt	12	*xylX, benA*	M64747, AAN68769	−	−
Pseudomonas putida MT53	T, *m*-X, *p*-X, Tt	40	*xylX*		−	−
Pseudomonas putida HS1	T, *m*-X, *p*-X	43	*xylX*	AF134348	−	−
Pseudomonas putida 3,5X	Ph	48			−	−

[a] B, benzene; T, toluene; Eb, ethylbenzene; Ipb, isopropylbenzene; *m*-X, *m*-xylene; *o*-X, *o*-xylene; *p*-X, *p*-xylene; *p*-C, *p*-cumate; Tt, toluate; Bt, benzoate; Ph, phenol; Tcb, tetrachlorobenzene; Bph, biphenyl; Nah, naphthalene; Car, carbazole. NS, not specified.
[b] Boldface type indicates that initial attack proceeds via a Rieske nonheme iron oxygenase of the toluene/biphenyl subfamily (30).
[c] +, PCR signal; (+), weak PCR signal; −, no PCR signal.

not likely to represent the microbial functional gene diversity in the environment (63, 64, 68).

Members of the toluene/biphenyl oxygenase subfamily have been reported to be involved in the degradation of benzene, chlorobenzenes, toluene, ethylbenzene, isopropylbenzene, and biphenyl, and phylogenetic analysis of their ISPα peptide sequences (47) indicated the presence of several distinct sequence clusters, such as benzene/toluene oxygenases, isopropylbenzene oxygenases, and biphenyl oxygenases. The amino acid residues reported to be responsible for the substrate specificity and regioselectivity of members of the subfamily are located within a central core of the α subunit (20, 27, 69). ISPα DNA sequence alignments had identified highly conserved regions for use as primer binding sites in PCR, including conserved regions flanking the central core, thus facilitating molecular studies on the diversity of these catabolic genes in the environment (38, 64, 68). Detection and differentiation of toluene/ biphenyl oxygenases by using PCR-based genetic profiling techniques targeting regions responsible for substrate specificity could provide hints on the overall metabolic network acting cooperatively for in situ biodegradation. Monitoring the structure and diversity of predominant catabolic gene polymorphisms can give indications on functional adaptations, as optimized catabolic enzyme variants are likely to be selected for functioning under the existing environmental conditions.

PCR–single-strand conformation polymorphism (SSCP) analysis has been demonstrated to be a powerful technique for elucidating the structure of complex microbial communities as well as the diversity of catabolic genes (37, 52–54). Based on sequence-dependent folding of single-stranded DNA (ssDNA) into secondary-structure conformations, which exhibit different electrophoretic mobilities in nondenaturing polyacrylamide gels, DNA molecules of the same length but with different nucleotide sequences can be separated (54). Here we describe the application of the PCR-SSCP technique for the simultaneous detection and discrimination of ISPα gene segments retrieved from pure culture strains exhibiting different aromatic hydrocarbon-degradative capabilities and from DNA extracted from soil samples differently contaminated with benzene, toluene, ethylbenzene, and xylenes (BTEX). The translation products of ISPα gene segments recovered from SSCP fingerprints were analyzed on the basis of structural information of related dioxygenase enzymes in order to evaluate the functional significance of structurally important amino acid residues.

MATERIALS AND METHODS

Bacterial strains. Reference strains used in this study are listed in Table 1 and comprised bacteria which are known to possess genes coding for Rieske nonheme iron oxygenase of the toluene/biphenyl subfamily and bacteria for which the presence of such genes has not been reported. The 16S rRNA gene sequence from the reference strain *Pandoraea* sp. strain JB1*, which we received as *Burkholderia* sp. strain JB1, was reevaluated, since an ISPα gene segment differing from that reported previously (GenBank accession no. AJ010057) was detected (see Results). The nearly complete 16S rRNA gene sequence (1,455 nucleotides) obtained showed 100% sequence identity with 16S rRNA genes from *Pandoraea* spp. but differed from the sequence previously reported for strain JB1 (X92188) by seven nucleotides. Additionally, 36 bacterial isolates obtained from BTEX-contaminated soil samples (37), the majority of which were capable of mineralizing benzene, were analyzed for the presence of ISPα genes of the toluene/biphenyl subfamily.

Soil samples. Soil samples were collected from the unsaturated zone (X, zone above the water table in which the pore spaces are only partially filled with water) and the capillary fringe zone (Y, zone above the water table within which the soil is saturated by water under less than atmospheric pressure) at two sampling sites of a BTEX-contaminated aquifer located in the Czech Republic. The BTEX concentrations in the groundwater (determined by Aquatest a.s., Prague) ranged from 320, 97, 6, and 13 mg/liter of benzene, toluene, ethylbenzene, and xylenes, respectively (site 1), to 55, 2, 0.007, and 0.012 mg/liter of benzene, toluene,

ethylbenzene, and xylenes, respectively (site 3). Further characteristics of the soil samples were described previously (34, 37).

Chemicals. Chemicals were purchased from Aldrich Chemie (Steinheim, Germany), Fluka AG (Buchs, Switzerland), or Merck AG (Darmstadt, Germany). Benzene-*cis*-dihydrodiol (*cis*-3,5-cyclohexadiene-1,2-diol) was obtained from Sigma-Aldrich Chemie (Deisenhofen, Germany).

Oxygen uptake measurements with whole cells. Transformation of benzene, toluene, phenol, and benzene-*cis*-dihydrodiol was determined polarographically by measuring O_2 consumption with a Clark-type oxygen electrode (Bachhofer, Reutlingen, Germany). Benzene-grown cells were harvested during late-exponential-phase growth, washed, and resuspended to a turbidity (A_{546}) of 30 in 50 mM phosphate buffer (pH 7.5). Of this suspension, 0.1 ml was added to 2.9 ml phosphate buffer (50 mM, pH 7.5) saturated with air. After 5 min of constant endogenous oxygen uptake, the reaction was started by adding the substrate (100 mM aqueous stock solutions or solutions in dimethyl sulfoxide in the cases of benzene and toluene) to a final concentration of 0.2 mM. Uptake rates were determined at 25°C and corrected for endogenous O_2 uptake. In the case of benzene-*cis*-dihydrodiol transformation, metabolism was confirmed by high-performance liquid chromatography analysis. Samples were taken during oxygen uptake measurements, and 10 μl of cell-free supernatants was analyzed with a Shimadzu high-performance liquid chromatography system (LC-10AD liquid chromatograph, DGU-3A degasser, SPD-M10A diode array detector, and FCV-10AL solvent mixer) equipped with an SC125/LiChrospher column (Bischoff, Leonberg, Germany) by use of an aqueous solvent system (flow rate, 1 ml/min) containing 0.01% (vol/vol) H_3PO_4 (87%) and 30% (vol/vol) methanol.

DNA extraction. DNA from eight reference strains (*Burkholderia xenovorans* LB400, *Cupriavidus necator* H850, *Pseudomonas* sp. strain Cam-1, *Pseudomonas pseudoalcaligenes* KF707, *Pandoraea* sp. strain JB1*, *Pseudomonas putida* F1, *Ralstonia* sp. strain PS12, and *Rhodococcus globerulus* P6) was extracted using the QIAGEN Genomic DNA Tip 100/G system (QIAGEN, Hilden, Germany). DNA concentrations in the extracts were quantified using a PicoGreen double-stranded DNA (dsDNA) quantitation kit (Molecular Probes, Leiden, The Netherlands) and adjusted to a final concentration of 1 ng/μl with Tris-HCl buffer (10 mM, pH 8.0). To extract DNA from other reference strains and from the BTEX isolates, single colonies were boiled and centrifuged (39) and the supernatants were used either 10-fold or 100-fold diluted in Tris-HCl buffer (10 mM, pH 8.0) as template DNA in PCR. Total DNA from soil samples (wet weight, 500 mg) was extracted using a FastDNA spin kit for soil (Bio101 Systems, Q-BIOgene, Heidelberg, Germany) and DNA concentrations in extracts were determined as described above. The soil extracts, containing approximately 2 ng/μl DNA, were used either directly or 10-fold diluted in Tris-HCl buffer (10 mM, pH 8.0) as template DNA in PCR.

Evaluation of primer binding sites suitable for amplification of ISPα gene segments. DNA and amino acid sequences of the α subunits of Rieske nonheme iron oxygenases (ISPα) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/) and aligned with ClustalW (http://www.ebi.ac.uk/clustalw/). A subset of 31 ISPα genes (GenBank accession numbers AF006691, AJ293587, U53507, D37828, AF049851, AJ010057, U47637, AB086835, M86348, U95054, M83673, AY027651, E04215, D88020, X80041, U27591, D32142, U24277, AF148496, AJ006307, J04996, U15298, U78099, D17319, U51165, AF452376, AB121977, AY554272, AJ536756, AY831463, and AB193045), comprising the majority of currently known members of the toluene/biphenyl subfamily (30), was examined for conserved regions for use as PCR primer binding sites. Amino acid residues which were reported to have an influence on substrate specificity of oxygenases of the toluene/biphenyl subfamily are encoded by ISPα gene segments flanked by conserved sites at approximately positions 670 and 1170 in the *bphA1* gene from *B. xenovorans* LB400 (8, 26, 46, 60, 69). Two degenerate primer sets targeting these conserved regions were used to amplify 525- to 531-bp segments of ISPα genes. The primer set BPDOXF/BPDOXR was designed by Yeates et al. (68); however, DNA sequence alignments revealed a critical Y-A mismatch between reverse primer BPDOXR and the ISPα genes *bphA1* from *R. globerulus* P6 and *Rhodococcus erythropolis* TA421 and *bpdC1* from *Rhodococcus* sp. M5 (GenBank accession numbers X80041, D88020, and U27591, respectively) at position 3 from the primer 3′ end. To increase their specificity for amplification of ISPα genes of the toluene/biphenyl subfamily, these primers were modified. The modified forward and reverse primers (bphAf668-3 [5′-GT TCC GTG TAA CTG GAA RTW YGC-3′] and bphAr1153-2 [5′-CCA GTT CTC GCC RTC RTC YTG HTC-3′], respectively) comprised a pool of degenerate primers in which a minimum of 14 bases at the 3′ end corresponded to the aligned toluene/biphenyl ISPα gene sequences. For PCR-SSCP analysis, ISPα gene segments were amplified with the modified primer set by using a 5′-end-phosphorylated reverse primer (54). The forward primer, bphAf371B (5′-GGC TTT CAC CTG CAS YTA YCA YGG-3′) (targeting positions 348 to 371 in *bphA1* of LB400) was used

together with bphAr1153-2 to amplify 799- to 826-bp ISPα segments, which comprised the primer binding site for bphAf668-3.

PCR amplification of ISPα gene segments. PCR amplification was performed in a final volume of 50 μl containing 1× PCR buffer (QIAGEN, Hilden, Germany) supplemented with 1.5 mM $MgCl_2$, 200 μM of each dNTP (Bioline, Luckenwalde, Germany), 0.5 μM of each primer (MWG-Biotech, Ebersberg, Germany), 2.5 U of HotStarTaq polymerase (QIAGEN), and 2 μl of template DNA. The PCR program comprised an initial denaturation for 15 min at 95°C followed by 32 cycles of 40 s at 94°C, 40 s at 58°C, and 60 s at 72°C and a final elongation for 10 min at 72°C. For amplification of soil DNA, the cycle number was increased to 35 cycles (sample 1Y or 3Y) or 40 cycles (sample 1X). The PCR conditions for amplification with BPDOXF and BPDOXR were as described previously (68). PCR products (2 μl of the PCR mixture) were separated by agarose gel electrophoresis (1.5% agarose, 1× Tris-acetate-EDTA buffer; 1 h at 90 V) and visualized by ethidium bromide staining. PCR yield estimates were obtained by quantitative image analysis of digitized agarose gel images using Scion Image Beta version 4.0.2 (Scion Corporation, Frederick, MD).

PCR-SSCP analysis of ISPα gene segments. PCR products were purified with a MinElute PCR purification kit (QIAGEN) as recommended by the manufacturer and eluted with 20 μl Tris-HCl buffer (10 mM, pH 8.0). The phosphorylated strands were removed by digestion with 12.5 U lambda exonuclease (New England Biolabs, Beverly, MA) in a final volume of 25 μl for 1 h at 37°C (54). The digestion was stopped by the addition of 5 μl sodium acetate (3 M, pH 5.5), and the ssDNA was purified with a MinElute PCR purification kit and eluted with 20 μl sterile water. The ssDNA concentration was assessed by comparing the fluorescence intensities of ssDNA sample bands to the fluorescence intensities of dsDNA standard bands (Low DNA Mass Ladder; Invitrogen) after agarose gel electrophoresis (1.5% agarose, 1× Tris-acetate-EDTA buffer; 1 h at 90 V) and ethidium bromide staining. Prior to the SSCP procedure, samples were diluted with sterile water to yield an ssDNA concentration equivalent to 1.7 ng/μl dsDNA per sample. PCR-negative controls were used without further dilution. Aliquots of 17 μl were evaporated for 15 min at 30°C in a vacuum concentrator to yield a final volume of approximately 4 μl. The samples were mixed with 4 μl SSCP loading buffer (95% [vol/vol] formamide, 10 mM NaOH, 0.025% [wt/vol] bromophenol blue, and 0.025% [wt/vol] xylene cyanol), incubated for 2 min at 95°C in a water bath, and placed on ice water for 5 min. The influence of gel temperature and MDE gel solution (Cambrex Bioscience, Rockland, ME) concentration on the resolution performance of the SSCP method was tested with ssDNA ISPα segments generated from six reference strains (LB400, H850, Cam-1, KF707, JB1*, F1, PS12, and P6) in individual runs using the following conditions (gel temperature/MDE gel solution concentration): (i) 20°C/0.6×, (ii) 24°C/0.6×, (iii) 28°C/0.6×, (iv) 28°C/0.7×, and (v) 30°C/0.7×. Electrophoresis under optimized conditions was performed on 21- by 21-cm 0.7× MDE gels in 1× Tris-borate-EDTA as the running buffer at 400 V and 10 mA for 20 h at 30°C in a Pharmacia Macrophor electrophoresis unit 2010-001 connected to a circulating water bath (Lauda Ecoline RE104 thermostat). The gels were silver stained according to the procedure of Bassam et al. (5) and dried at room temperature. ISPα SSCP fingerprints from pure cultures were reproduced in at least two independent SSCP analyses. Silver-stained gels were scanned on a flatbed scanner (Epson Expression 1600 PRO) to create 8-bit TIFF images, which were then analyzed with GelCompar II software (Applied Maths, Kortrijk, Belgium).

Isolation and reamplification of single-strand products from SSCP profiles. Selected single-strand products were excised from silver-stained polyacrylamide gels and transferred to microtubes containing 50 μl of elution buffer (10 mM Tris-HCl [pH 8.8], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100). The tubes were incubated for 20 min at 95°C, and 3 μl of eluted ssDNA was subjected to PCR reamplification using the same primers and conditions used to generate the original dsDNA segments.

Cloning of PCR-amplified ISPα segments. PCR-reamplified products from selected SSCP bands and ISPα gene segments amplified with the primer set bphAf371B/bphAr1153-2 were purified with a QIAquick PCR purification kit (QIAGEN), ligated into pGEM-T-vector system (Promega), and transformed into *Escherichia coli* JM109 competent cells (Promega) as specified by the manufacturers. Plasmid inserts were amplified by PCR with vector-specific primers M13 forward and M13 reverse (Promega), annealing in the flanking cloning region.

DNA sequencing and sequence analyses. PCR products were purified with a QIAquick PCR purification kit and sequenced using an ABI PRISM BigDye Terminator v1.1 Ready Reaction cycle sequencing kit (Applied Biosystems, Darmstadt, Germany). Primers used for sequencing reactions were the same as those used in the original PCR, and sequencing was performed on an ABI PRISM 3100 genetic analyzer (Applied Biosystems). Raw sequence data from

both strands were assembled with Sequencher software version 4.0.5 (Gene Codes Corporation, Ann Arbor, MI), and consensus sequences were translated in frame (omitting the primer sequences) with BioEdit software version 5.0.9 (http://www.microbiology.ncsu.edu/resources/index.html). For cloned PCR products, only consensus sequences deduced from at least three identical or nearly identical clones (difference of up to four bases) were included in the sequence analysis in order to reduce potential sequence diversity biases due to PCR and/or sequencing errors. DNA similarity searches were performed with the BLASTN algorithm and public nucleotide databases (http://www.ncbi.nlm.nih.gov/). DNA and protein alignments were conducted with ClustalW. Phylogenetic analyses were done with MEGA software version 3.0 (http://www.megasoftware.net/), by using the neighbor-joining method with the p-distance model and pairwise deletion of gaps/missing data. A consensus tree was inferred from a total of 1,000 bootstrap trees generated for each data set.

AFDRA and ARDRA. The presence of a catechol 2,3-dioxygenase (C23O) gene was determined by using PCR amplification with primers C23O-ORF-F and C23O-ORF-R, and C23O types were identified by amplified functional DNA restriction (AFDRA) analysis as reported previously (36). Amplified ribosomal DNA restriction analysis (ARDRA) was performed by using protocols described previously (37).

Nucleotide sequence accession numbers. The ISPα sequences and the 16S rRNA gene sequence of *Pandoraea* sp. strain JB1* are available under GenBank accession numbers DQ166964 to DQ167022 and DQ336933 to DQ336947.

RESULTS

Primer evaluation. Twenty reference strains were chosen to assess the specificity of the primer set BPDOXF/BPDOXR (68), which had been designed for use in studies on the diversity of Rieske nonheme ISPα genes. Using this primer set, single products of the expected size were amplified from 11 reference strains known to possess an ISPα gene of the toluene/biphenyl oxygenase subfamily (Table 1). No products were amplified from reference strains containing α subunits of the naphthalene (*P. putida* G7 [58] and *P. stutzeri* AN10 [11]) or benzoate/toluate (*P. putida* mt-2 [67] and MT53 [40]) oxygenase subfamilies and other reference strains obviously devoid of genes encoding Rieske nonheme iron oxygenase α subunits of the toluene/biphenyl subfamily (Table 1). However, compared to the other positively tested target genes, amplification of *bphA1* from *R. globerulus* P6 resulted in a considerably lower product yield, as judged from quantitative image analysis of ethidium bromide-stained agarose gels (data not shown). To increase the specificity of the primers for amplification of ISPα genes of the toluene/biphenyl subfamily, the primers were modified to match with an updated DNA alignment comprising 31 reference sequences. PCR performed with the modified primers resulted in similar product yields for all reference strains containing target genes.

SSCP analysis of ISPα gene segments. A PCR-SSCP method was established for the detection and discrimination of ISPα genes of the toluene/biphenyl oxygenase subfamily using α subunit gene segments amplified from reference strains with the primer set bphAf668-3/bphAr1153-2. Five different combinations of gel temperature and MDE gel solution concentration were tested to determine the optimum conditions for differentiation of the approximately 530-base single-strand ISPα segments, and best resolution and selectivity of SSCP profiles were achieved using an MDE gel concentration of 0.7× and a gel temperature of 30°C, allowing the discrimination of the predominant bands of 11 reference ssDNA products (Fig. 1). Single-strand ISPα gene segments of identical mobilities were obtained from *B. xenovorans* LB400 and *C. necator* H850, which was expected, as both strains were reported to contain
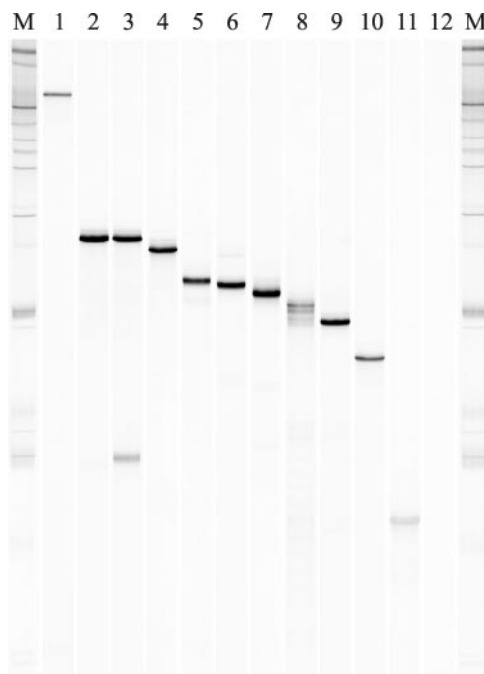

FIG. 1. PCR-SSCP analysis of ISPα gene segments obtained from reference strains. The following strains were analyzed: *P. putida* F1 (*todC1*, lane 1); *B. xenovorans* LB400 (*bphA1*, lane 2); *C. necator* H850 (*bphA1*, lane 3); *Pseudomonas* sp. strain IC (*bphA1*, lane 4); *R. globerulus* P6 (*bphA1*, lane 5); *P. pseudoalcaligenes* KF707 (*bphA1*, lane 6); *P. aeruginosa* JI104 (*bnzA*, lane 7); *Ralstonia* sp. strain PS12 (*tecA1*, lane 8); *Pseudomonas* sp. strain Cam-1 (*bphA1*, lane 9); *Pandoraea* sp. strain JB1* (*bphA1*, lane 10); and *P. fluorescens* IP01 (*cumA1*, lane 11). Lane 12 shows the PCR-negative control. Molecular weight marker III (M) (250 ng per lane; Roche) was used to normalize the band patterns.

ISPα genes identical in sequence in the fragment analyzed here (38). The developed PCR-SSCP method was able to separate even highly homologous ISPα gene segments, as indicated by the different migration patterns of the single strands originating from *Pseudomonas* sp. strain Cam-1 and *P. pseudoalcaligenes* KF707, which differed by only one nucleotide. Sequences retrieved from predominant bands were identical to those previously reported, except that of the *Pandoraea* sp. strain JB1* *bphA1* sequence, which differed in seven nucleotides from the sequence reported for *Burkholderia* sp. strain JB1 (AJ010057). The ISPα gene fragment recovered from the PCR-SSCP profile of the biphenyl degrader *Pseudomonas* sp. strain IC (14) was found to be identical to *bphA1* from *Pseudomonas* sp. strain B4 (U95054). SSCP analysis of the majority of reference strains resulted in the identification of one ISPα gene only; however, a second ISPα gene could be observed with *C. necator* H850 (Fig. 1, lane 3, lower band), the deduced amino acid sequence of which exhibited the highest levels of similarity to putative ISPα proteins from *Bordetella bronchiseptica* strain RB50 (BX640439) and *Variovorax paradoxus* strain E4C (AF209470) (84% to 86% amino acid sequence identity).

Diversity of ISPα genes in isolates from BTEX-contaminated soil samples. Recently, a large set of bacterial strains was isolated from BTEX-contaminated soil samples based on their ability to convert catechol by *meta*-cleavage to the yellow product 2-hydroxymuconic semialdehyde (37). A high proportion of

TABLE 2. Substrate utilization profiles and genotypic characteristics of BTEX isolates[a]

Isolate name	Growth substrate[b]			C23O of subfamily I.2.A	C23O type[c]	ISPα type[d]	ISPα/C23O combination[f]	ARDRA type[g]
	Benzene	Toluene	Ethylbenzene					
IA1YICDA	−	+	−	+	D	−		PS2
IA1YICDB	−	+	−	+	D	−		PS2
3YC1b	+	+	−	−		−		ND
3YC2	+	+	+	−		A[e]	I	PS2●
3YC4	+	+	+	−		A	I	PS2●
3YBTEX2b	+	+	+	−		A	I	PS2
1XXyl1	+	+	+	−		A	I	ND
1YC1	+	+	+	+	ND	A[e]		PS1●
1XC2	+	+	−	+	ND	A[e]		PS2
1YXyl2	+	+	+	+	A●	A	II	PS1
1YXyl3	+	+	+	+	A●	A	II	PS1
1XBTEX3	+	+	+	+●	A●	A	II	PS1
1XBTEX4	+	+	+	+	A●	A	II	PS1
1YXyl1	+	+	+	+	A2	A[e]	II	PS1
1YdBTEX2	+	+	+	+	A2	A	II	PS1
1XBTEX1	+	+	+	+	B	A	III	PS1
1XB2	+	+	+	+	B	A	III	PS1
1XC1	+	+	+	+●	C●	A[e]	IV	PS1
3YXyl2	+	+	−	+●	C●	A[e]	IV	PS2
3YC5	+	+	−	+	C●	A[e]	IV	PS2
3YdBTEX2	+	+	+	+	C	E[e]	V	PS2●
1YB1	+	−	−	+	A●	C	VI	PS1
1YB2	+	−	−	+	A1	C[e]	VI	PS1
1YB3	+	−	−	+	A1	C	VI	PS1
1YC2	+	−	−	+	A1	C[e]	VI	PS1
1YC3	+	−	−	+	A1	C	VI	PS1
1YBTEX1	+	−	−	+	A1	C	VI	PS1
1YBTEX3	+	−	−	+	A1	C	VI	PS1
1YdBTEX3	+	−	−	+	A1	C	VI	PS1
3YdBTEX1b	+	−	−	+	A1	C	VI	PS2
3YXyl2b	+	−	−	+	A1	C[e]	VI	PS2
3YXyl3	+	−	−	+	A1	C	VI	PS2
3YXyl1	+	+	−	+	A1	C[e]	VI	PS2
1XB1	+	−	−	+	A●	C**[e]	VII	PS3
3YC3	+	−	−	−		B[e]	VIII	AR●
1XXyl1b	+	−	−	−		D[e]	IX	SP●

[a] Data on substrate utilization, presence/type of C23O gene, and ARDRA type were reported previously (37); data indicated by ● were obtained in this study by using previously reported procedures (36, 37).

[b] +, growth on substrate vapor; −, no growth on substrate vapor.

[c] C23O types marked by ● were identified by AFDRA as reported previously (36), whereas all other C23O types were previously identified by using both AFDRA and DNA sequencing (37). ND, not determined.

[d] ISPα type was determined by sequencing of reamplified SSCP products; −, no PCR product.

[e] ISPα sequence was also determined by sequencing of cloned PCR products amplified with primers bphAf371B and bphAr1153-2.

[f] Roman numerals are used to distinguish between different combinations of C23O and ISPα genotypes observed in a given isolate.

[g] The major portion of isolates from the highly contaminated site 1 was closely related to *Pseudomonas veronii* CIP 104663 (AF064460, ARDRA profile PS1), whereas isolates from the slightly contaminated site 3 were predominantly related to *Pseudomonas mandelii* CIP 105273 (AF058286, ARDRA profile PS2). Strain 3YC3 exhibited an ARDRA fingerprint identical to those of previously analyzed *Arthrobacter* isolates (e.g., AY512644) and was thus considered to be a member of the genus *Arthrobacter*. Strain 1XXyl1b had previously been identified as a member of the genus *Sphingomonas* (AY512601) (34).

these isolates was found to be capable of mineralizing benzene and/or toluene and harbored C23O subfamily I.2.A variants, which were also observed to be abundant in the study sites (37). In this study, a subset (36 isolates) of these bacterial strains was screened for the presence of ISPα genes of the toluene/biphenyl oxygenase subfamily using primers bphAf668-3 and bphAr1153-2, and products of the expected size could be amplified with DNA extracted from 33 out of the 36 bacterial isolates (Table 2). ISPα gene segments could not be amplified from the isolates IA1YICDA and IA1YICDB, which were capable of mineralizing toluene, as well as *m*- and *p*-xylene, but not benzene (37). This phenotype is similar to that of strains harboring the well-studied toluene degradation pathway encoded by TOL plasmids (12, 67), where degradation is initiated

by oxidation of the methyl group. Additionally, isolate 3YC1b failed to give a PCR product. Its capability to grow on benzene, however, suggests that it either harbors an ISPα gene exhibiting low gene sequence homology with the primers used here or possibly initiates the degradation by means of two successive monooxygenation reactions.

PCR-SSCP analysis of the ISPα gene segments revealed the presence of only one predominant single-strand product per isolate and allowed five different electrophoretic mobilities to be distinguished (Fig. 2A, band positions A, B, C, D, and E). The nucleotide sequence of the most frequently occurring band, designated ISPα type A, was, as verified by sequencing, identical in 17 out of the 33 positively tested isolates, and the derived translation product showed highest identity to the
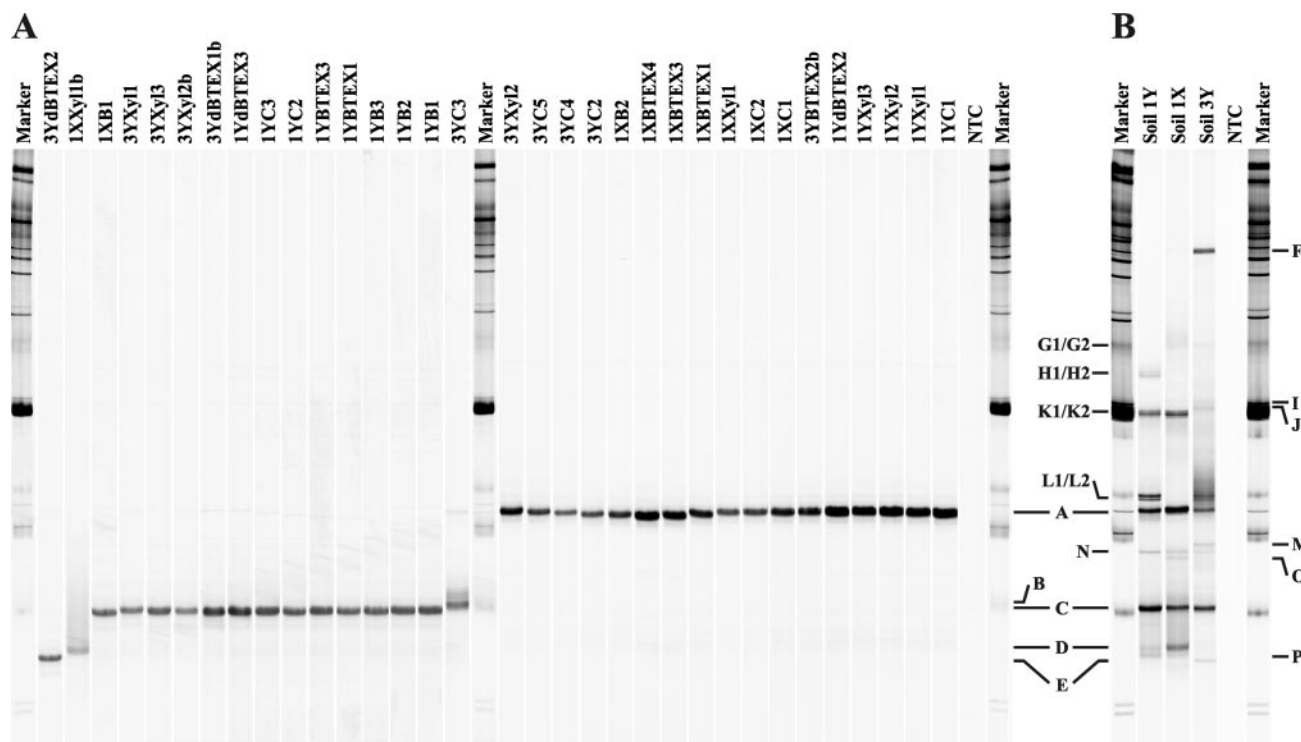
FIG. 2. SSCP profiles of ISPα gene segments obtained from BTEX-degrading isolates (A) and BTEX-contaminated soil samples (B). Bands selected for reamplification (by PCR), cloning, and sequencing are indicated by letters. Molecular weight marker III (Roche) (250 ng per lane) was used to normalize the band patterns. ISPα types were named according to their band positions as types A through E for the isolate profiles and types A through P for the soil profiles. NTC, PCR nontemplate control.

ISPα peptide (IpbAa) of *P. putida* RE204 isopropylbenzene dioxygenase (22) (Fig. 3). The second predominant ISPα type, referred to as ISPα type C, was observed in 13 isolates. All but one of these isolates contained an ISPα gene sequence that differed from sequence type A by only six nucleotides, corresponding to a difference of five amino acids in the deduced peptide sequence. The ISPα sequence retrieved from isolate 1XB1, designated ISPα type C**, differed from sequence type C by a single nucleotide, resulting in one amino acid difference. Three further ISPα sequence types, detectable in one isolate each, were found to be either highly homologous to ISPα types A and C (sequence type E) or more closely related to the ISPα sequence cluster encoding biphenyl dioxygenases of gram-positive strains and (chloro-)benzene/toluene dioxygenases (ISPα types B and D) (Fig. 3). ISPα PCR-SSCP analysis performed on artificial mixtures of genomic DNA extracted from three strains harboring different ISPα types showed that each component was amplified by PCR, and product yields reflected the abundance of each ISPα gene in the template mixture (see Fig. S1 in the supplemental material).

There was a strong correlation between the ISPα type and the substrate utilization profile observed with the respective isolates (Table 2), as all isolates harboring ISPα type A could grow on benzene and toluene as a sole source of carbon and energy, while all but 1 of the 13 isolates containing ISPα type C were able to grow on benzene but not on toluene. To provide evidence that degradation of benzene by the isolates is initiated by dioxygenation rather than two successive monooxygenations, oxygen uptake rates by two representative isolates con-

taining ISPα type A and two isolates containing ISPα type C as well as that by the isolate containing ISPα type B were assessed (Table 3). All isolates exhibited a significant oxygen uptake with benzene *cis*-dihydrodiol, indicating that this compound is dehydrogenated and subsequently subject to ring cleavage, whereas oxygen uptake with phenol was absent. While all tested isolates showed significant oxygen uptake with benzene, which had been used as growth substrate, only the two isolates harboring ISPα type A were active with toluene.

To obtain additional sequence information in an approximately 300-bp stretch of the ISPα genes upstream of the region used for PCR-SSCP, the isolates were subjected to a second PCR analysis using the primer set bphAf371B/bphAr1153-2. PCR amplification of ISPα gene segments was observed with the same 33 isolates described above (data not shown), and sequencing of the cloned products from seven strains harboring ISPα type A, four strains harboring ISPα type C, and the one strain harboring ISPα type C** (Table 2) revealed that they were identical in the 300-bp DNA stretch upstream of the sequence derived from SSCP analysis, indicating that sequence differences were confined to a region of the carboxy-terminal portion of the α subunits that was previously identified to be of crucial importance for determining substrate specificity (8, 24, 26, 60, 69).

ISPα gene diversity in BTEX-contaminated soil samples. The developed PCR-SSCP technique was used to assess the diversity and distribution of Rieske nonheme iron oxygenases of the toluene/biphenyl subfamily in differently contaminated soil samples from a BTEX-contaminated site. Comparison of
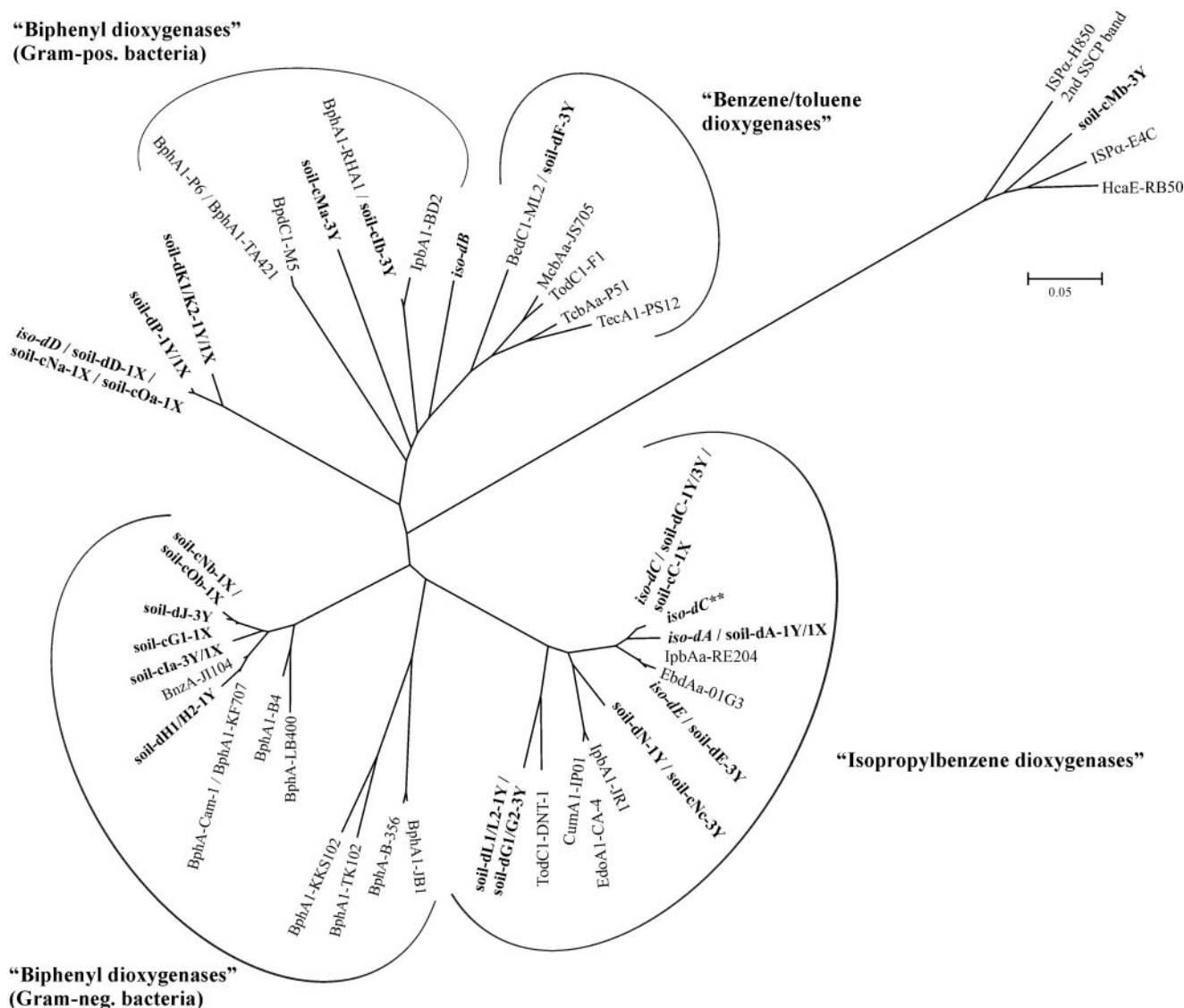
FIG. 3. Phylogenetic analysis of deduced peptide sequences from ISPα gene segments recovered from SSCP profiles (comprising 158 to 161 amino acids, corresponding to positions 224 to 384 of BphA1 from *B. xenovorans* LB400). Characterized ISPα peptides of the toluene/biphenyl oxygenase subfamily (IpbAa, *Pseudomonas putida* RE204 [AF006691]; EbdAa, *Pseudomonas putida* 01G3 [AJ293587]; IpbA1, *Pseudomonas* sp. strain JR1 [U53507]; CumA1, *Pseudomonas fluorescens* IP01 [D37828]; EdoA1, *Pseudomonas fluorescens* CA-4 [AF049851]; BphA1, *Burkholderia* sp. strain JB1 [AJ010057]; BphA1, *Comamonas testosteroni* B-356 [U47637]; BphA1, *Comamonas testosteroni* TK102 [AB086835]; BphA1, *Pseudomonas* sp. strain KKS102 [D17319]; BphA1, *Burkholderia xenovorans* LB400 [M86348]; BphA1, *Pseudomonas* sp. strain B4 [U95054]; BphA1, *Pseudomonas pseudoalcaligenes* KF707 [M83673]; BphA, *Pseudomonas* sp. strain Cam-1 [AY027651]; BnzA, *Pseudomonas aeruginosa* JI104 [E04215]; BphA1, *Rhodococcus erythropolis* TA421 [D88020]; BphA1, *Rhodococcus globerulus* P6 [X80041]; BpdC1, *Rhodococcus* sp. strain M5 [U27591]; BphA1, *Rhodococcus* sp. strain RHA1 [D32142]; IpbA1, *Rhodococcus erythropolis* BD2 [U24277]; BedC1, *Pseudomonas putida* ML2 [AF148496]; McbAa, *Ralstonia* sp. strain JS705 [AJ006307]; TodC1, *Pseudomonas putida* F1 [J04996]; TcbAa, *Pseudomonas* sp. strain P51 [U15298]; TecA1, *Ralstonia* sp. strain PS12 [U78099]; ISPα, *Variovorax paradoxus* E4C [AF209469]; HcaE, *Bordetella bronchiseptica* RB50 [NC_002927]; ISPα, *Cupriavidus necator* H850 [2nd SSCP band]) and ISPα peptides from bacterial isolates (bold italics) and soil samples (bold) are shown. The ISPα peptides obtained from soil (soil-) were designated according to the procedure of sequence recovery, i.e., direct sequencing of reamplified SSCP products (d) or sequencing of cloned PCR-amplified products (c), followed by band position (A through P) and sample origin (1X, 1Y, or 3Y). In cases where more than one consensus sequence was retrieved by cloning, the different sequence types are indicated by lowercase letters (a, b, or c) after the band position designation. The ISPα peptide sequences from isolates (iso-) were deduced from directly sequenced reamplification products. The tree was constructed by using the neighbor-joining method in MEGA with the p-distance model and pairwise deletion of gaps/missing data. The scale bar indicates the percent divergence. The names of ISPα peptide clusters were assigned according to the native substrates used for isolation of their hosts and characterization of their activity. pos., positive; neg., negative.

the SSCP profiles revealed significant differences in the catabolic gene structure between the three soil samples (Fig. 2B). Bands that were detectable against a low surrounding background could be sequenced directly from reamplified ssDNA, resulting in unambiguous identification of the single-strand products. The majority of bands with high relative intensities and identical mobilities, like bands A and C, were found to contain identical single-stranded DNA species. Several of the

TABLE 3. Oxygen uptake rates of selected benzene-degrading isolates

Isolate name	ARDRA type	Phenotype[a]	Genotype					Oxygen uptake rate (μmol of O_2 min^{-1}) with[b]:			
			C23O subfamily I.2.A	ISPα	$xylM$[c]	$tbmD$[c]	$tmoA$[c]	Benzene	Toluene	Phenol	DHD[d]
1YdBTEX2	PS1	BT	+	A	+	+	−	5 ± 1	14 ± 4	<1	90 ± 15
1XB2	PS1	BT	+	A	+	−	−	4 ± 1	8 ± 2	<1	65 ± 10
3YC3	AR	B	−	B	ND	ND	ND	15 ± 3	<1	<1	35 ± 5
1YB2	PS1	B	+	C	+	+	+	4 ± 1	<1	<1	35 ± 5
3YdBTEX1b	PS2	B	+	C	+	−	−	4 ± 1	<1	<1	50 ± 10

[a] BT, benzene and toluene degradation; B, benzene degradation.
[b] Oxygen uptake rates were determined with whole cells grown on benzene and are expressed as the rate of O_2 uptake measured polarographically using cell densities (A_{546}) of 1. Rates given are means of three independent determinations.
[c] PCR result was reported by Hendrickx et al. (34). ND, not determined.
[d] DHD, benzene *cis*-dihydrodiol.

closely migrating single-strand products represented identical ssDNA sequence types (bands G1/G2, H1/H2, K1/K2, and L1/L2), suggesting that the same single strand formed two conformers migrating with slightly different mobilities. In the case of faint bands, direct sequencing of reamplified single-strand products did not result in conclusive sequences. To reveal the identity of the ISPα types contained in these bands, the reamplification products were cloned and sequenced. For each band, one (or two distinct) consensus sequence(s) could be inferred from at least three clones containing nearly or completely identical sequence types.

Phylogenetic analysis of the peptide sequences deduced from directly sequenced PCR-amplified SSCP products revealed the majority of the soil peptides to be related to ISPα sequences of ethylbenzene/isopropylbenzene dioxygenases (Fig. 3), with the nucleotide sequences retrieved from bands A, C, and E being identical to the corresponding ISPα segments retrieved from isolates. The ISPα gene sequences retrieved from bands G1/G2 and L1/L2, respectively, differed by nine nucleotides; however, none of them caused a difference in the deduced peptide sequences, which were most closely related to TodC1 from the toluene-degrading bacterium *Thauera* sp. strain DNT-1 (57). The ISPα sequence types soil-dK, soil-dP, and soil-dD, which were, among the soil samples tested, detectable only in the highly contaminated soil samples 1X and 1Y, grouped together in a divergent branch of the toluene/biphenyl oxygenase subfamily and exhibited less than 75% identity to previously described ISPα peptide sequences. The peptide sequence inferred from an intense band, which was detected only in the SSCP profile of soil 3Y, was found to be identical to benzene dioxygenase ISPα from *P. putida* ML2 (62). ISPα sequences closely related to *P. pseudoalcaligenes* KF707 (61) and *Pseudomonas* sp. strain Cam-1 (45) biphenyl dioxygenase ISPα peptides were inferred from bands H1/H2 and J.

DISCUSSION

In this study, the PCR-SSCP technique was used for detection and discrimination of ISPα gene homologues amplified from bacterial isolates and environmental samples. Several primer sets suitable for amplification of Rieske nonheme iron oxygenase ISPα gene segments have been reported previously (4, 34, 38, 64, 68). Since it has been noted that amino acid residues important for the substrate specificity of Rieske non-

heme iron oxygenases of the toluene/biphenyl subfamily are concentrated in the C-terminal portion of the α subunits (26, 51, 69), primer sets enabling amplification of this region are of special interest. SSCP analysis has been shown to be a sensitive method for the discrimination of gene homologues that is able to distinguish sequence differences as small as one base substitution in several hundred bases (33, 37, 54). However, it has also been demonstrated that the sensitivity of PCR-SSCP in the detection of single base substitutions decreased with segments larger than 300 bases (33, 55). Thus, the primer binding sites previously selected by Yeates et al. (68) were chosen, as these produced the shortest possible gene segment encoding the major portion of amino acids crucial for substrate specificity. Adjusting the reported primer sequences to match the toluene/biphenyl dioxygenase subfamily members together with careful optimization of the PCR-SSCP conditions allowed detection and differentiation of all reference ISPα gene segments down to single base differences.

PCR-SSCP analysis of ISPα gene segments obtained from the three differently contaminated soil samples showed that there was a high degree of catabolic genotype diversity. Sequence analysis indicated that the detectable polymorphisms were dominated by ISPα sequence types related to the ethylbenzene/isopropylbenzene oxygenase branch rather than by sequences related to the expected toluene/benzene oxygenase branch (Fig. 3). In particular, ISPα types A and C not only were observed in all three soil samples, suggesting that the environmental conditions were selective for strains possessing these ISPα types, but also were predominant in benzene- and/or toluene-degrading bacterial isolates previously isolated from these soil samples (Fig. 2A and B).

This finding was unexpected as, to our knowledge, Rieske nonheme iron oxygenases closely related to the ethylbenzene/isopropylbenzene oxygenase branch were previously not observed to be prevalent in BTEX-contaminated environments. Previous investigations, however, have concentrated on the presence and abundance of ISPα genes more closely related to the toluene/benzene and biphenyl dioxygenase branches by using DNA:DNA hybridization (59) or PCR-based assays (15, 44). The use of specific DNA probes and oligonucleotide primers may have failed to detect genes exhibiting lower levels of homology with the targeted genes. More-detailed studies on the diversity of Rieske nonheme iron oxygenases in pristine soil and soil with a history of aromatic hydrocarbon pollution

(68) as well as in soil and groundwater at an aromatic hydro-
carbon-contaminated site (63, 64) have been performed by
analyzing clone libraries of PCR products which were ampli-
fied with degenerate primers targeting the same primer bind-
ing sites as used here (68) or primer binding sites flanking the
ISPα gene region which encodes the Rieske-type [2Fe-2S] clus-
ter (63, 64). In these studies, amplification of sequence types
showing no clear affiliation to previously described ISPα pro-
teins (68) or of a significant portion of gene segments which
did not code for Rieske nonheme iron oxygenase ISPα pro-
teins (63) was reported. In contrast to these findings, the vast
majority of the peptide sequences retrieved from the BTEX-
contaminated soil samples analyzed here showed a clear mem-
bership to the toluene/biphenyl oxygenase subfamily, with only
a small fraction of the clones (less than 10%) prepared from
faint SSCP bands exhibiting no clear pattern of relationship to
previously described Rieske nonheme iron oxygenase ISPα
proteins. A comparison of the ISPα peptide sequences ob-
tained in this study with those reported by Taylor et al. (63, 64)
suggests that the structures of dioxygenases putatively involved
in pollutant degradation are clearly different in the organic
waste landfill soil and groundwater samples and the industrial
site analyzed here. Common to both environments seems to be
the low abundance of genes closely related to the toluene/
benzene dioxygenase branch of the toluene/biphenyl subfam-
ily, suggesting that the previously characterized dioxygenases
may not play significant roles in the sites under investigation.

As indicated above, the ISPα sequence types A and C were
not only abundant in all three contaminated soil samples an-
alyzed in this study but also predominant among the benzene-
degrading isolates recovered from these soil samples. Overall,
ISPα gene segments of the toluene/biphenyl subfamily were
amplifiable from 33 out of 34 isolates that were capable of
growth on benzene, and biochemical analysis supported the
notion that, at least in the five representative strains analyzed,
benzene metabolism is initiated by dioxygenation, yielding
benzene dihydrodiol as an intermediate, rather than monooxy-
genation, which would result in phenol as intermediate (Table
3). In a recent study, the presence of alternative modes of
benzene/toluene activation has been suggested for a subset of
the isolates analyzed here, whereas genes encoding Rieske
nonheme iron oxygenases were not detected (34). Evidently,
the primers designed by Hendrickx et al. (34) for detection of
toluene/biphenyl ISPα genes failed to amplify the genes re-
ported here. Moreover, there was no correlation between the
growth phenotype and the catabolic genotypes detected by
Hendrickx et al. (34) (Table 3). As benzene lacks a side chain,
this substrate cannot be transformed by xylene monooxygen-
ases (genotype *xylM*). Furthermore, neither the strains harbor-
ing a gene coding for a phenol hydroxylase α subunit (genotype
tbmD) nor strain 1YB2, which contained a gene fragment
probably encoding an α subunit of aromatic monooxygenases
(genotype *tmoA*), showed significant oxygen uptake with phe-
nol. Therefore, degradation of benzene via phenol as the in-
termediate seems rather unlikely.

The fact that all isolates harboring genes similar to those
encoding isopropylbenzene dioxygenases were capable of min-
eralizing benzene but only a subset was able to grow on toluene
was on the first view astonishing, as all previously described
isolates harboring ethylbenzene/isopropylbenzene dioxygenase

genes (*P. putida* RE204, *P. putida* 01G3, *Pseudomonas* sp.
strain JR1, *Pseudomonas fluorescens* IP01, *P. fluorescens* CA-4,
and *Thauera* sp. strain DNT-1) were reported to mineralize
toluene (1, 16–19, 22, 57). However, there was a strong corre-
lation between the growth phenotype and the ISPα type de-
tected (Table 2), with only isolates containing ISPα type C, B,
or D unable to grow on toluene, suggesting that the differences
in substrate specificity are related to structural differences in
the α subunits of the terminal oxygenase components. Align-
ments of ISPα peptides retrieved from BTEX-contaminated
soil and isolates to those of related proteins (see Fig. S2 in the
supplemental material) revealed that the residues His234 and
His240 (numbered according to *P. fluorescens* IP01 cumene
dioxygenase [IP01-CumDO]), which are involved in the coor-
dination of the mononuclear iron in the active site of IP01-
CumDO and RHA1-BphDO (20, 27), as well as Asp231, which
was postulated to play the role of an electron transfer bridge
(20, 27), were completely conserved among the ISPα peptides
retrieved from bacterial isolates and environmental samples.
Among the 14 residues proposed to define the inner surface of
the substrate-binding pocket in IP01-CumDO (20), 8 were
found to be strictly conserved among the ISPα sequences re-
trieved in this study (Gln227, Phe228, Met232, Ala235, His323,
Leu333, and Phe378, in addition to the above-mentioned
His240). The isopropylbenzene dioxygenase-like ISPα sequences
derived from the soil samples and isolates in this study differed
from the IP01-CumDO sequence at up to three residues de-
fining the inner surface of the substrate-binding pocket (see
Fig. S2 in the supplemental material). Strikingly, isolates har-
boring an A321L ISPα variant (ISPα types A and E) were
capable of mineralizing both toluene and benzene, whereas
those harboring I288M and A321M variants (ISPα type C and
C**) were capable of mineralizing benzene only. Modeling
performed on the basis of the IP01-CumDO crystal structure
(pdb-id 1WQL [20]), using "O" (35) for manual replacement
of side chains and choice of the best-fitting rotamer, revealed
that replacing Ile288 and Ala321 in IP01-CumDO with bulky
methionine significantly narrowed the available space in the
substrate-binding pocket (data not shown). This gives a possi-
ble explanation for the different phenotypes and the negligible
oxygen uptake of isolates 1YB2 and 3YdBTEX1b with toluene
as the substrate. Also, ISPα types B and D as well as K1/K2 and
P contained bulky residues (methionine and phenylalanine,
respectively) in positions 288 and 321, and the two single iso-
lates containing ISPα type B and D were also capable of
mineralizing benzene only. Taken together, these results indi-
cate a significant contribution of the amino acid side chains in
positions 288 and 321 to the shape and size of the substrate-
binding pocket. Bagnéris et al. (3) also had suggested that less
bulky amino acids in position 321 should facilitate access to
toluene, which, as a substrate, is larger than benzene.

In the well-characterized isopropylbenzene-degradative path-
ways of *P. putida* RE204 and *P. fluorescens* IP01 (1, 22), the genes
coding for the oxygenase subunits are organized in operons to-
gether with genes encoding a *cis*-dihydrodiol dehydrogenase
and an extradiol dioxygenase (C23O) of the subfamily I.3.A
(23). In the isolates 1YB2 and 1YdBTEX2, which had been
analyzed in more detail and obviously do not express any
3-isopropylcatechol 2,3-dioxygenase (subfamily I.3.A) during
growth on benzene (37), benzene mineralization seems to in-

volve an isopropylbenzene dioxygenase-like Rieske nonheme oxygenase for initial activation and a subfamily I.2.A C23O (36, 37) for catechol cleavage. As shown in Table 2, in addition to strain 1YB2, 12 further independently selected isolates (affiliated to three distinct *Pseudomonas* lineages) harbored the same combination of a type C (or C**) ISPα gene and a type A C23O gene. While the isolates harboring ISPα type C contained the C23O type A exclusively, the strains harboring ISPα type A were found to contain different C23O subfamily I.2.A genes or failed to yield a PCR product with primers targeting C23O subfamily I.2.A genes; the latter result indicated that these strains obviously contained a C23O of other subfamilies. The high degree of sequence homology between ISPα type A and C on the one hand and C23O type A2 and A1 on the other suggests a close evolutionary relationship between the two gene variants. The flexible combination of ISPα type A genes with different types of C23O genes indicates that the pathways were assembled in a modular fashion, in which the C23O genes were retrieved from different sources, possibly driven by the selective conditions of the environment, and the occurrence of identical or nearly identical genes and pathway module combinations in different hosts indicates that both the ethylbenzene/isopropylbenzene dioxygenase and the catechol 2,3-dioxygenase are localized on mobile genetic elements and have been spread through horizontal gene transfer.

C23Os of subfamily I.2.A have previously not been reported to be involved in the degradation of benzene or toluene via a dioxygenolytic route. They have, however, been commonly described as involved in phenol (56), toluate (12, 67), and salicylate (28) degradation. The respective catabolic operons comprising the C23O I.2.A gene have a rather stable structure and comprise genes coding for both the hydrolytic branch and the oxalocrotonate branch of the *meta*-cleavage pathway (66). Recruitment of both branches of the *meta*-cleavage pathway can be assumed to be advantageous to the presence of the hydrolytic branch only (as present in strains like RE204), particularly for benzene degradation (32). Both branches were expressed in isolates 1YB2 and 1YdBTEX2 (37).

In summary, bacteria from the examined BTEX-contaminated soil samples seem to possess a genotypic flexibility, which is important for their adaptation and evolution while facing challenging and continuously changing conditions in these ecosystems. Screening methods targeting genes encoding key enzymes in pollutant degradation as molecular markers, such as PCR-SSCP analyses, can be utilized to determine both the biodegradative potential and predominant catabolic pathways in an environment. Further studies on catabolic gene diversity and genetic flexibility will certainly be important elements in our understanding of natural bioremediation processes.

ACKNOWLEDGMENTS

REFERENCES

1. **Aoki, H., T. Kimura, H. Habe, H. Yamane, T. Kodama, and T. Omori.** 1996. Cloning, nucleotide sequence, and characterization of the genes encoding enzymes involved in the degradation of cumene to 2-hydroxy-6-oxo-7-methyl-octa-2,4-dienoic acid in *Pseudomonas fluorescens* IP01. J. Ferment. Bioeng. **81:**187–196.

2. **Asturias, J. A., E. Diaz, and K. N. Timmis.** 1995. The evolutionary relationship of biphenyl dioxygenase from gram-positive *Rhodococcus globerulus* P6 to multicomponent dioxygenases from gram-negative bacteria. Gene **156:**11–18.

3. **Bagnéris, C., R. Cammack, and J. R. Mason.** 2005. Subtle difference between benzene and toluene dioxygenases of *Pseudomonas putida*. Appl. Environ. Microbiol. **71:**1570–1580.

4. **Baldwin, B. R., C. H. Nakatsu, and L. Nies.** 2003. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Appl. Environ. Microbiol. **69:**3350–3358.

5. **Bassam, B. J., G. Caetano-Anolles, and P. M. Gresshoff.** 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. **196:**80–83.

6. **Bedard, D. L., R. E. Wagner, M. J. Brennan, M. L. Haberl, and J. F. Brown, Jr.** 1987. Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53:**1094–1102.

7. **Beil, S., B. Happe, K. N. Timmis, and D. H. Pieper.** 1997. Genetic and biochemical characterization of the broad spectrum chlorobenzene dioxygenase from *Burkholderia* sp. strain PS12—dechlorination of 1,2,4,5-tetrachlorobenzene. Eur. J. Biochem. **247:**190–199.

8. **Beil, S., J. R. Mason, K. N. Timmis, and D. H. Pieper.** 1998. Identification of chlorobenzene dioxygenase sequence elements involved in dechlorination of 1,2,4,5-tetrachlorobenzene. J. Bacteriol. **180:**5520–5528.

9. **Bertoni, G., M. Martino, E. Galli, and P. Barbieri.** 1998. Analysis of the gene cluster encoding toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1. Appl. Environ. Microbiol. **64:**3626–3632.

10. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. **1:**23–29.

11. **Bosch, R., E. Garcia-Valdes, and E. R. Moore.** 1999. Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from *Pseudomonas stutzeri* AN10. Gene **236:**149–157.

12. **Burlage, R. S., S. W. Hooper, and G. S. Sayler.** 1989. The TOL (pWW0) catabolic plasmid. Appl. Environ. Microbiol. **55:**1323–1328.

13. **Butler, C. S., and J. R. Mason.** 1997. Structure-function analysis of the bacterial aromatic ring-hydroxylating dioxygenases. Adv. Microb. Physiol. **38:**47–84.

14. **Carrington, B., A. Lowe, L. E. Shaw, and P. A. Williams.** 1994. The lower pathway operon for benzoate catabolism in biphenyl-utilizing *Pseudomonas* sp. strain IC and the nucleotide sequence of the *bphE* gene for catechol 2,3-dioxygenase. Microbiology **140:**499–508.

15. **Cavalca, L., E. Dell'Amico, and V. Andreoni.** 2004. Intrinsic bioremediability of an aromatic hydrocarbon-polluted groundwater: diversity of bacterial population and toluene monoxygenase genes. Appl. Microbiol. Biotechnol. **64:**576–587.

16. **Chablain, P. A., G. Philippe, A. Groboillot, N. Truffaut, and J. F. Guespin-Michel.** 1997. Isolation of a soil psychrotrophic toluene-degrading *Pseudomonas* strain: influence of temperature on the growth characteristics on different substrates. Res. Microbiol. **148:**153–161.

17. **Chablain, P. A., A. L. Zgoda, C. O. Sarde, and N. Truffaut.** 2001. Genetic and molecular organization of the alkylbenzene catabolism operon in the psychrotrophic strain *Pseudomonas putida* 01G3. Appl. Environ. Microbiol. **67:**453–458.

18. **Corkery, D. M., and A. D. W. Dobson.** 1998. Reverse transcription-PCR analysis of the regulation of ethylbenzene dioxygenase gene expression in *Pseudomonas fluorescens* CA-4. FEMS Microbiol. Lett. **166:**171–176.

19. **Dabrock, B., J. Riedel, J. Bertram, and G. Gottschalk.** 1992. Isopropylbenzene (cumene)—a new substrate for the isolation of trichloroethene-degrading bacteria. Arch. Microbiol. **158:**9–13.

20. **Dong, X. S., S. Fushinobu, E. Fukuda, T. Terada, S. Nakamura, K. Shimizu, H. Nojiri, T. Omori, H. Shoun, and T. Wakagi.** 2005. Crystal structure of the terminal oxygenase component of cumene dioxygenase from *Pseudomonas fluorescens* IP01. J. Bacteriol. **187:**2483–2490.

21. **Eaton, R. W.** 1996. *p*-Cumate catabolic pathway in *Pseudomonas putida* F1: cloning and characterization of DNA carrying the *cmt* operon. J. Bacteriol. **178:**1351–1362.

22. **Eaton, R. W., and K. N. Timmis.** 1986. Characterization of a plasmid-specified pathway for catabolism of isopropylbenzene in *Pseudomonas putida* RE204. J. Bacteriol. **168:**123–131.

23. **Eltis, L. D., and J. T. Bolin.** 1996. Evolutionary relationships among extradiol dioxygenases. J. Bacteriol. **178:**5930–5937.

24. **Erickson, B. D., and F. J. Mondello.** 1993. Enhanced biodegradation of polychlorinated biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. Appl. Environ. Microbiol. **59:**3858–3862.

25. **Furukawa, K., and T. Miyazaki.** 1986. Cloning of a gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **166:**392–398.

26. **Furukawa, K., H. Suenaga, and M. Goto.** 2004. Biphenyl dioxygenases: functional versatilities and directed evolution. J. Bacteriol. **186:**5189–5196.

27. **Furusawa, Y., V. Nagarajan, M. Tanokura, E. Masai, M. Fukuda, and T. Senda.** 2004. Crystal structure of the terminal oxygenase component of biphenyl dioxygenase derived from *Rhodococcus* sp. strain RHA1. J. Mol. Biol. **342:**1041–1052.

28. **Ghosal, D., I. S. You, and I. C. Gunsalus.** 1987. Nucleotide sequence and expression of gene *nahH* of plasmid NAH7 and homology with gene *xylE* of TOL pWW0. Gene **55:**19–28.

29. **Gibson, D. T., M. Hensley, H. Yoshioka, and T. J. Mabry.** 1970. Formation of (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida.* Biochemistry **9:**1626–1630.

30. **Gibson, D. T., and R. E. Parales.** 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr. Opin. Biotechnol. **11:**236–243.

31. **Goris, J., P. De Vos, J. Caballero-Mellado, J. Park, E. Falsen, J. F. Quensen, J. M. Tiedje, and P. Vandamme.** 2004. Classification of the biphenyl- and polychlorinated biphenyl-degrading strain LB400(T) and relatives as *Burkholderia xenovorans* sp. nov. Int. J. Syst. Evol. Microbiol. **54:**1677–1681.

32. **Harayama, S., N. Mermod, M. Rekik, P. R. Lehrbach, and K. N. Timmis.** 1987. Roles of the divergent branches of the *meta*-cleavage pathway in the degradation of benzoate and substituted benzoates. J. Bacteriol. **169:**558–564.

33. **Hayashi, K.** 1991. PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. PCR Methods Appl. **1:**34–38.

34. **Hendrickx, B., H. Junca, J. Vosahlova, A. Lindner, I. Ruegg, M. Bucheli-Witschel, F. Faber, T. Egli, M. Mau, M. Schlomann, M. Brennerova, V. Brenner, D. H. Pieper, E. M. Top, W. Dejonghe, L. Bastiaens, and D. Springael.** 2006. Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site. J. Microbiol. Methods **64:**250–265.

35. **Jones, T. A., J. Y. Zou, S. W. Cowan, and Kjeldgaard.** 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr. A **47:**110–119.

36. **Junca, H., and D. H. Pieper.** 2003. Amplified functional DNA restriction analysis to determine catechol 2,3-dioxygenase gene diversity in soil bacteria. J. Microbiol. Methods **55:**697–708.

37. **Junca, H., and D. H. Pieper.** 2004. Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. Environ. Microbiol. **6:**95–110.

38. **Kahl, S., and B. Hofer.** 2003. A genetic system for the rapid isolation of aromatic-ring-hydroxylating dioxygenase activities. Microbiology **149:**1475–1481.

39. **Kanakaraj, R., D. L. Harris, J. G. Songer, and B. Bosworth.** 1998. Multiplex PCR assay for detection of *Clostridium perfringens* in feces and intestinal contents of pigs and in swine feed. Vet. Microbiol. **63:**29–38.

40. **Keil, H., S. Keil, R. W. Pickup, and P. A. Williams.** 1985. Evolutionary conservation of genes coding for *meta* pathway enzymes within TOL plasmids pWW0 and pWW53. J. Bacteriol. **164:**887–895.

41. **Keith, L. H., and W. A. Telliard.** 1979. Priority pollutants. I. A perspective view. Environ. Sci. Technol. **13:**416–423.

42. **Kitayama, A., E. Suzuki, Y. Kawakami, and T. Nagamune.** 1996. Gene organization and low regiospecificity in aromatic-ring hydroxylation of a benzene monooxygenase of *Pseudomonas aeruginosa* JI104. J. Ferment. Bioeng. **82:**421–425.

43. **Kunz, D. A., and P. J. Chapman.** 1981. Isolation and characterization of spontaneously occurring TOL plasmid mutants of *Pseudomonas putida* HS1. J. Bacteriol. **146:**952–964.

44. **Luz, A. P., V. H. Pellizari, L. G. Whyte, and C. W. Greer.** 2004. A survey of indigenous microbial hydrocarbon degradation genes in soils from Antarctica and Brazil. Can. J. Microbiol. **50:**323–333.

45. **Master, E. R., and W. W. Mohn.** 1998. Psychrotolerant bacteria isolated from Arctic soil that degrade polychlorinated biphenyls at low temperatures. Appl. Environ. Microbiol. **64:**4823–4829.

46. **Mondello, F. J., M. P. Turcich, J. H. Lobos, and B. D. Erickson.** 1997. Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. Appl. Environ. Microbiol. **63:**3096–3103.

47. **Nam, J. W., H. Nojiri, T. Yoshida, H. Habe, H. Yamane, and T. Omori.** 2001. New classification system for oxygenase components involved in ring-hydroxylating oxygenations. Biosci. Biotechnol. Biochem. **65:**254–263.

48. **Ng, L. C., V. Shingler, C. C. Sze, and C. L. Poh.** 1994. Cloning and sequences of the first eight genes of the chromosomally encoded (methyl) phenol degradation pathway from *Pseudomonas putida* P35X. Gene **151:**29–36.

49. **Ouchiyama, N., S. Miyachi, and T. Omori.** 1998. Cloning and nucleotide sequence of carbazole catabolic genes from *Pseudomonas stutzeri* strain OM1, isolated from activated sludge. J. Gen. Appl. Microbiol. **44:**57–63.

50. **Parales, R. E., S. M. Resnick, C. L. Yu, D. R. Boyd, N. D. Sharma, and D. T. Gibson.** 2000. Regioselectivity and enantioselectivity of naphthalene dioxygenase during arene *cis*-dihydroxylation: control by phenylalanine 352 in the α subunit. J. Bacteriol. **182:**5495–5504.

51. **Pieper, D. H.** 2005. Aerobic degradation of polychlorinated biphenyls. Appl. Microbiol. Biotechnol. **67:**170–191.

52. **Schmalenberger, A., F. Schwieger, and C. C. Tebbe.** 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Appl. Environ. Microbiol. **67:**3557–3563.

53. **Schmalenberger, A., and C. C. Tebbe.** 2003. Bacterial diversity in maize rhizospheres: conclusions on the use of genetic profiles based on PCR-amplified partial small subunit rRNA genes in ecological studies. Mol. Ecol. **12:**251–261.

54. **Schwieger, F., and C. C. Tebbe.** 1998. A new approach to utilize PCR-single-strand-conformation polymorphism for 16s rRNA gene-based microbial community analysis. Appl. Environ. Microbiol. **64:**4870–4876.

55. **Sheffield, V. C., J. S. Beck, A. E. Kwitek, D. W. Sandstrom, and E. M. Stone.** 1993. The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. Genomics **16:**325–332.

56. **Shingler, V., F. C. Franklin, M. Tsuda, D. Holroyd, and M. Bagdasarian.** 1989. Molecular analysis of a plasmid-encoded phenol hydroxylase from *Pseudomonas* CF600. J. Gen. Microbiol. **135:**1083–1092.

57. **Shinoda, Y., Y. Sakai, H. Uenishi, Y. Uchihashi, A. Hiraishi, H. Yukawa, H. Yurimoto, and N. Kato.** 2004. Aerobic and anaerobic toluene degradation by a newly isolated denitrifying bacterium, *Thauera* sp. strain DNT-1. Appl. Environ. Microbiol. **70:**1385–1392.

58. **Simon, M. J., T. D. Osslund, R. Saunders, B. D. Ensley, S. Suggs, A. Harcourt, W. C. Suen, D. L. Cruden, D. T. Gibson, and G. J. Zylstra.** 1993. Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. Gene **127:**31–37.

59. **Stapleton, R. D., and G. S. Sayler.** 1998. Assessment of the microbiological potential for the natural attenuation of petroleum hydrocarbons in a shallow aquifer system. Microb. Ecol. **36:**349–361.

60. **Suenaga, H., M. Mitsuoka, Y. Ura, T. Watanabe, and K. Furukawa.** 2001. Directed evolution of biphenyl dioxygenase: emergence of enhanced degradation capacity for benzene, toluene, and alkylbenzenes. J. Bacteriol. **183:**5441–5444.

61. **Taira, K., J. Hirose, S. Hayashida, and K. Furukawa.** 1992. Analysis of bph operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem. **267:**4844–4853.

62. **Tan, H.-M., H. Y. Tang, C. L. Joannou, N. H. Abdel-Wahab, and J. R. Mason.** 1993. The *Pseudomonas putida* ML2 plasmid-encoded genes for benzene dioxygenase are unusual in codon usage and low in G+C content. Gene **130:**33–39.

63. **Taylor, P. M., and P. H. Janssen.** 2005. Variations in the abundance and identity of class II aromatic ring-hydroxylating dioxygenase genes in groundwater at an aromatic hydrocarbon-contaminated site. Environ. Microbiol. **7:**140–146.

64. **Taylor, P. M., J. M. Medd, L. Schoenborn, B. Hodgson, and P. H. Janssen.** 2002. Detection of known and novel genes encoding aromatic ring-hydroxylating dioxygenases in soils and in aromatic hydrocarbon-degrading bacteria. FEMS Microbiol. Lett. **216:**61–66.

65. **Timmis, K. N., and D. H. Pieper.** 1999. Bacteria designed for bioremediation. Trends Biotechnol. **17:**201–204.

66. **Williams, P. A., and J. R. Sayers.** 1994. The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas.* Biodegradation **5:**195–217.

67. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124:**7–13.

68. **Yeates, C., A. J. Holmes, and M. R. Gillings.** 2000. Novel forms of ring-hydroxylating dioxygenases are widespread in pristine and contaminated soils. Environ. Microbiol. **2:**644–653.

69. **Zielinski, M., S. Backhaus, and B. Hofer.** 2002. The principal determinants for the structure of the substrate-binding pocket are located within a central core of a biphenyl dioxygenase alpha subunit. Microbiology **148:**2439–2448.