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Identification of an Alternative 2,3-Dihydroxybiphenyl 1,2-Dioxygenase in *Rhodococcus* sp. Strain RHA1 and Cloning of the Gene

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Gram-positive *Rhodococcus* sp. strain RHA1 possesses strong polychlorinated biphenyl-degrading capabilities. An RHA1 *bphC* gene mutant, strain RDC1, had been previously constructed (E. Masai, A. Yamada, J. M. Healy, T. Hatta, K. Kimbara, M. Fukuda, and K. Yano, Appl. Environ. Microbiol. 61:2079–2085, 1995). An alternative 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD), designated EtbC, was identified in RDC1 cells grown on ethylbenzene. EtbC contained the broadest substrate specificity of any *meta* cleavage dioxygenase identified in a *Rhodococcus* strain to date, including RHA1 BphC. EtbC was purified to near homogeneity from RDC1 cells grown on ethylbenzene, and a 58-amino-acid NH_2-terminal sequence was determined. The NH_2-terminal amino acid sequence was used for the identification of the *etbC* gene from an RDC1 chromosomal DNA 2,3-DHBD expression library. The *etbC* gene was successfully cloned, and we report here the determination of its nucleotide sequence. The substrate specificity patterns of cell extract and native nondenaturing polyacrylamide gel electrophoresis analysis identified the coexpression of two 2,3-DHBDs (BphC and EtbC) in RHA1 cells grown on either biphenyl or ethylbenzene. The possible implication of coexpressed BphC extradiol dioxygenases in the strong polychlorinated-biphenyl degradation activity of RHA1 was suggested.

The microbial degradation of polychlorinated biphenyls (PCBs) has attracted considerable attention because of the stability of the compound and the large amounts which accumulated in the environment prior to their discontinued use in industry. Bacteria capable of utilizing biphenyl as a carbon and energy source are widely distributed in the environment and can cometabolize PCBs to chlorobenzoic acids (1, 6–8, 19, 29). The failure of an organism to utilize any given PCB congener may not be due to limited enzymatic potential but to the inability of the compound to induce the required indigenous microbial catabolic pathway (37). In addition, the initial degradation of PCB congeners by peripheral enzymes may result in the formation of metabolites inhibitive to enzymes in the biphenyl catabolic pathway, such as the accumulation of chlorobenzoates or chlorocatechol and its *meta* cleavage products (2, 19, 43).

Multiple *bphC* extradiol dioxygenases containing different substrate specificities may play an important role in determining the substrate specificities of biphenyl and PCB catabolic operons by preventing intermediate metabolite inhibition of degradative-pathway enzymes. The identification and cloning of multiple BphC extradiol dioxygenases, containing different structural characteristics and/or substrate specificities, in separate *Rhodococcus* biphenyl and PCB degraders have been recently reported (3, 4, 33). Sondossi et al. (43) presented evidence indicating that the *meta* cleavage enzyme, 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD), of the biphenyl pathway may be the most sensitive target of inhibition by PCB cometabolites, suggesting that coexpressed BphC extradiol dioxygenases have an advantage in PCB degradation.

Our laboratory recently isolated a gram-positive *Rhodococcus* sp. strain, RHA1, from hexachlorocyclohexane-contaminated soil (40). *Rhodococcus* sp. strain RHA1 possesses strong PCB-degrading capabilities and can completely degrade PCB48, which consists mainly of tri-, tetra-, and pentachlorobiphenyl (40). RHA1 can utilize both biphenyl and ethylbenzene as its sole carbon source and cometabolizes PCBs under both of these growth conditions (41). A portion of the *bph* operon encoding the enzymes responsible for the utilization of biphenyl is located on a 7.6-kb *Eco*RI-*Bam*HI fragment carrying the *bphA1A2A3A4CB* genes (34). The RHA1 *bphC* gene has been cloned, and its protein product has been purified from *Escherichia coli* (24). A previously constructed *Rhodococcus* sp. strain RHA1 *bphC* mutant, RDC1 (34), maintained the ability to effectively utilize ethylbenzene as its sole carbon source. However, because of the *bphC* gene mutation, RDC1 could no longer utilize biphenyl as its sole carbon source (34).

We thought it would be interesting to determine if multiple 2,3-DHBDs are present in other gram-positive *Rhodococcus* biphenyl and PCB degraders, such as RHA1, and under what growth conditions they are expressed. The goal of this work was to identify and then determine the functional relationships of the multiple BphC extradiol dioxygenases present in *Rhodococcus* sp. strain RHA1. By comparing the BphC extradiol dioxygenase substrate specificity patterns of RHA1 and RDC1, grown on the same or different carbon sources, insight into the functional relationships of different intraspecies extradiol dioxygenases was obtained. All the multiple BphC extradiol dioxygenases identified in *Rhodococcus* strains to date have been

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done with genomic DNA expression libraries, which are unable to provide this information (3, 4, 33). An understanding of the functional relationships of multiple BphC extradiol dioxygenases may aid in addressing the significance of multiple isozymes in the evolution of PCB degradation pathways and possibly help in the development of PCB biodegradation methods. Here we report the first coexpressed 2,3-DHBD isozyme, designated EtbC, to be identified and purified from a single *Rhodococcus* strain by using a *bphC* gene mutant.

MATERIALS AND METHODS

Culture conditions. *Rhodococcus* sp. strain RHA1 and its *bphC* gene mutant derivative, strain RDC1, were grown in 200 ml of minimal salt medium (W broth) (40) containing either 0.5% biphenyl or ethylbenzene as the sole carbon source. Ethylbenzene was supplied in vapor form by adding 200 μl to a wire-suspended Eppendorf tube in each 500-ml baffled growth flask. The kanamycin resistance insert used in the RHA1 *bphC* gene mutant to generate RDC1 (34) was maintained by including 50 μg of kanamycin per ml in all RDC1 growth media. Bacto Tryptone and Bacto Yeast Extract were obtained from Difco Laboratories, Detroit, Mich. *E. coli* JM109 was used as a host strain.

Chemicals. 3-Methylcatechol and 4-methylcatechol were obtained from Nacalai Tesque Co., Ltd., Kyoto, Japan. 3-Chlorocatechol and 4-chlorocatechol were purchased from Tokyo Kasai Kogyo Co., Ltd., Tokyo, Japan. All other chemicals were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Extraction and manipulation of DNA. Plasmid DNA was purified by alkaline lysis followed by polyethylene glycol precipitation, as described by Sambrook et al. (38). Restriction enzymes (Takara Shuzo Co. Ltd., Kyoto, Japan) were used according to the supplier's instructions. Restriction enzyme-generated DNA fragments were separated by electrophoresis on 0.8% agarose gels. DNA fragments were purified from agarose gel slices by the Gene Clean procedure (Bio101, LaJolla, Calif.) (45).

Preparation of cell extracts. RHA1 and RDC1 were grown as described above and washed with 20 mM Tris-HCl (pH 7.5) containing 10% acetone. Cell pellets were stored frozen at −70°C. Frozen pellets were thawed on ice and resuspended in cold lysis buffer (20 mM Tris-HCl [pH 7.5], 100 μM $FeSO_4$, 2 mM ascorbate, 10% acetone, 10% glycerol) at a concentration of 1 ml of buffer per 0.7 g (wet weight) of cells. Cells were disrupted in an ice bath by using a Bio-ruptor sonicator (Cosmo Bio, Tokyo, Japan) into aliquots not exceeding 500 μl. Cell debris was removed by centrifugation at 13,000 × *g* for 30 min at 4°C, and the supernatant (cell extract) was carefully decanted and used immediately for enzyme activity measurements.

Activity assays. 2,3-DHBD activity was assayed by the appearance of the yellow *meta* cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid. Enzymatic activity was measured by monitoring the reaction products at 434 nm with a Beckman DU-640 spectrophotometer. Activity assays were performed at 25°C in 20 mM Tris-HCl (pH 7.5) containing 500 μM 2,3-dihydroxybiphenyl. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per min at 25°C. The molar extinction coefficient of the product under assay conditions was taken to be 13,200 cm^{-1} M^{-1} (12). The other substrates used in this study had the following ring fission product absorbances and molar extinction coefficients to determine relative ring cleavage activities: catechol, 375 nm, 36,000 cm^{-1} M^{-1}; 3-methylcatechol, 388 nm, 32,000 cm^{-1} M^{-1}; 4-methylcatechol, 382 nm, 17,000 cm^{-1} M^{-1}; and 4-chlorocatechol, 380 nm, 40,000 cm^{-1} M^{-1} (4). Relative activities were expressed as percentages of the activity observed when 2,3-dihydroxybiphenyl was the substrate, which was defined as 100%. Protein concentrations were determined by the method of Bradford (9), using the Bio-Rad protein assay system (Bio-Rad, Richmond, Calif.) and bovine serum albumin as the standard.

Electrophoretic conditions and protein purification. Sodium dodecyl sulfate (SDS)–15% polyacrylamide gels and corresponding stacking gels were prepared as described by Porzio and Pearson (36). The electrophoresis buffer was that of Laemmli (32). For native nondenaturing gels, SDS was omitted from acrylamide mixtures, loading buffers, and electrophoresis buffers.

EtbC was purified from strain RDC1 with all chromatography steps carried out at 4°C. Buffers were made from distilled water purified on a Millipore MilliQ system. Gel filtration chromatography was performed on a Pharmacia-LKB Biotechnology, Inc., fast protein liquid chromatography (FPLC) system. The chromatographic buffer used consisted of 20 mM Tris-HCl (pH 7.5), 100 μM $FeSO_4$, 2 mM ascorbate, 10% acetone, and 10% glycerol, with the addition of NaCl as indicated. EtbC was labile during the purification steps presumably because of the oxidation of the Fe^{2+} cofactor. To help maintain enzyme stability, all buffers used in chromatographic steps were filtered and degassed, with subsequent immediate N_2 saturation by bubbling nitrogen gas into the solution. Samples collected from each protein purification step were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Broad-range molecular mass standards (Bio-Rad) were used. Gels were stained with Coomassie blue (Bio-Rad) according to published protocols (38).

Purification of EtbC. RDC1 grown in W broth containing ethylbenzene supplied in vapor form was harvested from 200-ml cultures. Cell extracts were prepared as described above. The cytosolic fraction used in enzyme purification was obtained after ultracentrifugation (100,000 × *g*) of the cell extract at 4°C for 1.25 h.

(i) Acetone fractionation. Ice-cold acetone (1.2 volumes) was added slowly to the cytosolic fraction with stirring on ice and transferred into a 50-ml polypropylene centrifuge tube (Nalgene; Nalge Co.). The tube was spun down at 14,000 × *g* for 15 min in an RPR20-2 rotor (Hitachi, Ibaragi, Japan) at 4°C. The pellet was discarded, and the supernatant was collected. An additional 0.3 volume of ice-cold acetone was again added slowly with constant stirring, and the solution was spun down as described above. The pellet was collected and resuspended in chromatographic buffer to approximately 1/10 the volume present after the first acetone precipitation step.

(ii) Gel filtration chromatography. The acetone-fractionated enzyme solution was applied directly to a Hiload Superdex 200-pg FPLC gel filtration column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with chromatographic buffer containing 0.15 M NaCl. The same buffer was used to elute proteins from the column at a flow rate of 0.5 ml/min. Fractions (2 ml) were collected and assayed for 2,3-DHBD activity after 1 column void volume of buffer had passed through the column.

(iii) Ion-exchange column chromatography. The pooled gel filtration fractions containing EtbC activity were applied directly to a HiTrap Q 1-ml ion-exchange column (Pharmacia Biotech) equilibrated with the chromatography buffer described above, except with a pH of 8.5. After the column was washed with 3 column volumes of the same buffer, the proteins were eluted with 10 column volumes of a linear NaCl gradient (0 to 1 M) in chromatographic buffer at a flow rate of 0.2 ml/min. Fractions (350 μl) were collected and assayed for 2,3-DHBD activity.

NH_2-terminal amino acid sequencing. The NH_2-terminal amino acid sequence of EtbC was determined by automated Edman degradation on an Applied Biosystems 477A pulsed liquid-phase sequencer, using approximately 30 μg of protein.

Electrophoresis and activity staining of coexpressed BphC extradiol dioxygenases. Nondenaturing 15% polyacrylamide gels were loaded with purified EtbC, RHA1 cell extracts from biphenyl- and ethylbenzene-grown cells, and RDC1 cell extract from ethylbenzene-grown cells by use of native sample buffer (5). The gels were run at 20 mA for approximately 6 h with a Mini-Protean II electrophoresis system (Bio-Rad) at 4°C. After electrophoresis, gels were soaked for approximately 5 min in 20 mM Tris-HCl (pH 7.5) containing 10% acetone. Electrophoretically separated proteins were then assayed for BphC extradiol dioxygenase activity by incubating the native gels in 10 ml of a 20 mM Tris-HCl (pH 7.5)–10% acetone solution containing approximately 0.01 g of either 2,3-dihydroxybiphenyl, catechol, or 4-methylcatechol (substrate concentrations of 5, 9, and 8 mM, respectively) per ml. BphC extradiol dioxygenase activity resulted in the formation of *meta* cleavage products which were clearly visible on polyacrylamide gels as yellow bands.

Cloning and nucleotide sequence of the *etbC* gene. Genomic DNA from RDC1 was prepared by using hexadecyltrimethyl ammonium bromide for selective precipitation of proteins as described previously (5). RDC1 chromosomal DNA was digested with *Sau*3AI, and the entire mixture was ligated into pUC119, which was digested with *Bam*HI and treated with alkaline phosphatase (Boehringer Mannheim Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The resulting plasmids were transformed into $CaCl_2$-prepared competent *E. coli* JM109 cells (38) and plated onto LB agar plates containing 100 μM ampicillin and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Clones that expressed 2,3-DHBD activity were identified by spraying colonies with a solution containing 2 mM 2,3-dihydroxybiphenyl in diethyl ether. Positive clones were identified by their yellow color resulting from the formation of the yellow *meta* cleavage metabolite 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (18). DNA fragments were subcloned into pUC119. A Kilo-Sequence deletion kit (Takara Shuzo), utilizing exonuclease III and mung bean nuclease, was used to generate unidirectional deletion derivatives (21). The nucleotide sequences of deletion derivatives were determined by the dideoxy termination method (39), using a Pharmacia-LKB Alf*red* DNA sequencer. Sequence analyses and homology searches were done with GeneWorks (IntelliGenetics, Inc., Mountain View, Calif.). The unweighted pair group method was employed for dendrogram construction.

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. D76438.

RESULTS

Substrate specificities of RHA1 and RDC1 BphC extradiol dioxygenases. *Rhodococcus* sp. strain RDC1 was derived from strain RHA1 by insertional mutagenesis of the *bphC* gene (34). Whereas strain RHA1 can grow on both biphenyl and ethylbenzene, mutant strain RDC1 can grow only on ethylbenzene. The extradiol dioxygenase substrate specificities of RHA1 and RDC1 cell extracts were determined to see if the expression of

TABLE 1. Substrate specificities of RHA1 and RDC1 cell extracts compared with that of EtbC purified to near homogeneity

Substrate[a]	Relative activity[b]			
	RHA1 (bp)	RHA1 (eb)	RDC1 (eb)	EtbC[c]
2,3-Dihydroxybiphenyl	100 (2.05)	100 (4.00)	100 (1.80)	100
Catechol	39.5	33.5	19.0	17.0
3-Methylcatechol	65.0	37.0	145	132
4-Methylcatechol	19.0	53.0	155	142
4-Chlorocatechol	1.2	2.2	7.0	7.0

[a] Each substrate was used at 500 μM.
[b] Activities are expressed as percentages of the activity observed when 2,3-dihydroxybiphenyl was the substrate, which was defined as 100%. A parenthetical abbreviation indicates the carbon source used for cell growth. bp, biphenyl; eb, ethylbenzene. Parenthetical values are the V_{max} values (in micromoles min^{-1} mg^{-1}) of extradiol dioxygenase in cell extracts. For the EtbC V_{max}, refer to Table 2.
[c] EtbC purified from ethylbenzene-grown RDC1 cells.

TABLE 2. Purification of EtbC to near homogeneity from RDC1 cells grown on ethylbenzene

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)[a]	Recovery (%)	Purification factor
Cytosolic fraction	16.4	33.5	40	1.2	100	1.0
Acetone fractionation	3.2	6.7	38	5.6	93	4.7
Gel filtration	7.0	0.8	19	22.5	48	18.7
HiTrap ion exchange	0.6	0.1	3	22.2	7	18.5

[a] Units are expressed in micromoles of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid formed per minute.

multiple BphC extradiol dioxygenases could be detected during biphenyl and ethylbenzene growth.

Cell extracts were prepared from RHA1 cells harvested at log phase with ethylbenzene or biphenyl as the sole carbon source. RHA1 cell extracts prepared under both growth conditions exhibited the same substrate range. Both contained good extradiol dioxygenase relative activities toward catechol, 3-methylcatechol, and 4-methylcatechol and weak relative activities toward 4-chlorocatechol (Table 1). In contrast, purified BphC from RHA1 contains greater relative activities toward catechol and 3-methylcatechol, 95 and 125% respectively, compared with activity toward 2,3-dihydroxybiphenyl, while it contains no activity toward 4-methylcatechol or 4-chlorocatechol (24). The substrate pattern of the RHA1 cell extract from cells grown on biphenyl differed from that of purified BphC, suggesting that an alternative extradiol dioxygenase which possessed activities toward 4-methylcatechol and 4-chlorocatechol was coexpressed with BphC in RHA1. In addition, the similar substrate patterns of RHA1 grown on either biphenyl or ethylbenzene suggested the possibility of the coexpression of BphC and an alternative extradiol dioxygenase under both growth conditions, indicating induction of the *bph* operon by ethylbenzene.

Cell extracts were prepared from RDC1 cells harvested at log phase with ethylbenzene as the sole carbon source. Although the RDC1 cell extract exhibited significantly lower extradiol dioxygenase activity toward catechol, it possessed activities toward 3-methylcatechol and 4-methylcatechol greater than that toward 2,3-dihydroxybiphenyl and a low relative activity of 7% toward 4-chlorocatechol (Table 1). In addition, RHA1 and RDC1 cell extracts from ethylbenzene-grown cells also exhibited different amounts of 3-chlorocatechol inhibition (data not shown), further suggesting the predominance of an alternative extradiol dioxygenase, designated EtbC, expressed in RDC1 cells grown on ethylbenzene. Since the 3-chlorocatechol *meta* cleavage product has no color, we were unable to measure the rate of conversion of this substrate (43).

Purification of EtbC to near homogeneity from strain RDC1. EtbC was purified to near homogeneity by the procedures explained in Materials and Methods. RDC1 cells grown on ethylbenzene supplied in vapor form were harvested and disrupted, and cell extracts were prepared as described in Materials and Methods. The protein purification scheme is presented in Table 2. SDS-PAGE analysis of the purification steps were visualized by Coomassie blue staining (Fig. 1). A predominant protein band, corresponding to EtbC, along with a few faint contaminating-protein bands was present after HiTrap ion-exchange chromatography. Although no significant increase in specific activity was achieved by ion-exchange chromatography, some contaminating-protein bands were removed. Preliminary characterization of EtbC in the RDC1 cell extract showed that it was stable at pH 8.5, which was used in the ion-exchange chromatographic step (data not shown). Although EtbC was purified to near homogeneity (Fig. 1), the low specific activity and fold purification achieved in the purification scheme suggested enzyme lability (Table 2). We included 10% acetone, 100 μM ferrous sulfate (Fe^{2+}), and 2 mM ascorbate in all degassed and nitrogen-purged chromatography buffers to help stabilize EtbC, since some extradiol dioxygenases have been shown to be oxygen sensitive and to be stabilized by incubation with acetone, ferrous iron (Fe^{2+}), and ascorbate (10, 11).

The molecular mass of EtbC determined by SDS-PAGE was approximately 34 kDa (Fig. 1). The substrate specificity of EtbC purified to near homogeneity from RDC1 cells matched the substrate specificity pattern present in the RDC1 cell extract, suggesting that this is the predominant extradiol dioxygenase expressed in RDC1 cells grown on ethylbenzene (Table 1). The purified EtbC sample was determined to have an approximate native molecular mass of 300 kDa by gel filtration chromatography with known molecular mass standards (data not shown). The 34-kDa subunit molecular mass determined by SDS-PAGE suggests that the EtbC holoenzyme could contain eight or nine subunits. However, since most other 2,3-DHBDs contain eight subunits, we believe that EtbC contains the usual octameric structure (12, 24, 44).

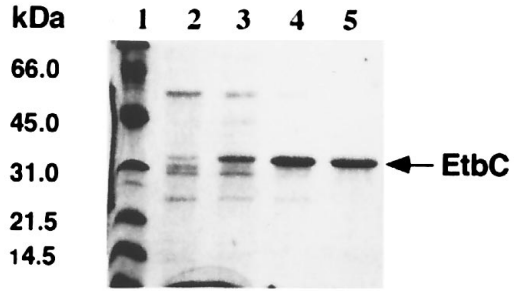


FIG. 1. SDS-PAGE of EtbC, a 2,3-DHBD, after chromatographic purification to near homogeneity from RDC1 cells grown on ethylbenzene. Coomassie blue-stained SDS-PAGE gel of EtbC from various stages of purification to near homogeneity. Approximately 1 μg of protein was loaded for each stage of purification. Lane 1, molecular mass standards β-galactosidase (116 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.5 kDa); lane 2, cytosolic fraction; lane 3, acetone fractionation; lane 4, gel filtration chromatography; and lane 5, HiTrap ion-exchange chromatography fraction.

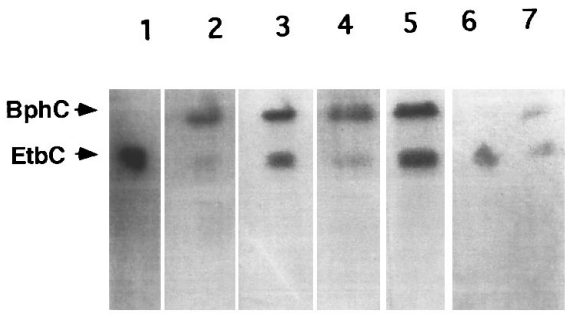
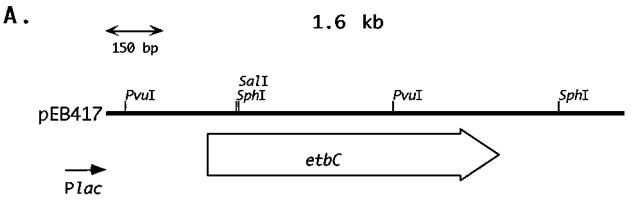


FIG. 2. Native nondenaturing PAGE and 2,3-DHBD activity stain of RHA1 and RDC1 cell extracts. Lane 1, EtbC (ca. 0.25 μg) purified to near homogeneity; lane 2, RHA1 cell extract (ca. 180 μg) from ethylbenzene-grown cells; lane 3, RHA1 cell extract (ca. 180 μg) from ethylbenzene-grown cells; lane 4, RHA1 cell extract (ca. 180 μg) from biphenyl-grown cells; lane 5, RHA1 cell extract (ca. 180 μg) from biphenyl-grown cells; lane 6, RDC1 cell extract (ca. 80 μg) from ethylbenzene-grown cells; and lane 7, RHA1 cell extract (ca. 60 μg) from biphenyl-grown cells. Lanes 6 and 7 were from a separately run gel with different cell extract samples. Activities were stained with 2,3-dihydroxybiphenyl (lanes 1, 3, 5, 6, and 7) or with catechol (lanes 2 and 4).

An EtbC protein sample was prepared for NH_2-terminal amino acid sequencing as described in Materials and Methods. The 58-amino-acid EtbC NH_2-terminal sequence was determined by automated Edman degradation to be Met-Ala-Lys-Val-Thr-Glu-Leu-Gly-Tyr-Leu-Gly-Leu-Ser-Val-Ser-Asn-Leu-Asp-Ala-Trp-Arg-Asp-Tyr-Ala-Ala-Gly-Ile-Met-Gly-Met-Gln-Val-Val-Asp-Asp-Gly-Glu-Asp-Asp-Arg-Ile-Tyr-Leu-Arg-Met-Asp-Arg-Trp-His-His-Arg-Ile-Val-Leu-His-Ala-Asp-Gly. The NH_2-terminal amino acid sequence of EtbC had only 33% similarity with BphC from RHA1 (24, 34). The greatest sequence similarities were found with BphC from *Pseudomonas* (*Sphingomonas*) *paucimobilis* Q1 (69%), NahC from the naphthalene-sulfonic acid-degrader strain BN6 (60%), and NahC from *P. putida* PpG7 (55%), which are extradiol dioxygenases that prefer bicyclic aromatic compounds as substrates (23, 31, 44).

Native PAGE analysis of RHA1 and RDC1 cell extracts. Native nondenaturing PAGE and BphC extradiol dioxygenase activity staining analysis were carried out as described in Materials and Methods. Two yellow 2,3-dihydroxybiphenyl activity staining bands were detected in RHA1 cell extracts from biphenyl- and ethylbenzene-grown cells, suggesting the coexpression of two BphC extradiol dioxygenases, BphC and EtbC (Fig. 2). The lower yellow staining band migrated with the yellow activity staining band of purified EtbC, suggesting that it was produced by EtbC activity. Duplicate native gels containing the same RHA1 cell extract but incubated with catechol and 4-methylcatechol showed that the appearance of the upper and lower yellow activity staining bands correlated with the substrate specificities of BphC and EtbC, respectively. Native gel activity staining with catechol produced a faint EtbC *meta* cleavage product band because of its low-level specificity for this substrate, while the BphC *meta* cleavage product band was much stronger (Fig. 2). Incubation with 4-methylcatechol produced only the lower activity staining band because of EtbC's high-level substrate specificity for this compound and the lack of BphC substrate specificity for 4-methylcatechol (data not shown). In the case of RDC1 cell extract from ethylbenzene-grown cells, only one yellow 2,3-dihydroxybiphenyl activity staining band was detected (Fig. 2). This single yellow staining band migrated with the lower RHA1 activity staining band, suggesting that it also was produced by EtbC activity. The lack of an upper yellow activity staining band in this cell extract showed that BphC was not expressed, consistent with the *bphC* insertional mutation in strain RDC1 (34).

Cloning and sequencing of the *etbC* gene. An expression library of RDC1 genomic DNA was constructed in pUC119 as described in Materials and Methods. Approximately 30,000 colonies were tested for 2,3-DHBD activity by spraying plates with a solution of 2,3-dihydroxybiphenyl. Ten colonies which turned bright yellow when sprayed were isolated and purified. Plasmid DNAs from these clones were prepared and digested with various restriction enzymes. Two of the clones positive for 2,3-DHBD activity contained identical restriction patterns and were designated clone pEB417 (Fig. 3A). Clone pEB417 contained a DNA insert of approximately 1.6 kb. The preliminary DNA sequence of pEB417 contained an NH_2-terminal nucle-



B.

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PvuI
CGATCGTCCCAACGAATACAGGGTCTATTCCAACTTTTTCGCGTTCCGTAGCCGCAACGAGAGGGATGAAGACAG     75

GCCGCTGGTGGGCAGTCGGGAAGACACCTGGCGTCACAAGGACGGCGACTATCTCCTCGTGAAGCGGATCATCAC    150

ACCCAAATGGAACACGCTGCTCAGCAAGAACCTCAACATCTTCCTCTGAGCATTCGGGCATACCGGCTGGCCAAG    225

CCGGTATGCCCGGATGCGGAAAGGAGTAACCAATGGCAAAAGTGACCGAACTCGGCTATCTTGGACTATCCGTA    300
                  RBS(etbC) M  A  K  V  T  E  L  G  Y  L  G  L  S  V
                                                SphI      SalI
TCCAATCTCGACGCCTGGCGAGACTATGCCGCCGGAATCATGGGCATGCAGGTCGTCGACGACGGCGAGGACGAC    375
S  N  L  D  A  W  R  D  Y  A  A  G  I  M  G  M  Q  V  V  D  D  G  E  D  D

CGCATCTATCTCCGGATGGACAGGTGGCACCACCGAATCGTGCTGCACGCGGACGGCAGCGACGATCTCGCCTAC    450
R  I  Y  L  R  M  D  R  W  H  H  R  I  V  L  H  A  D  G  S  D  D  L  A  Y

ATCGGCTGGCGCGTGGCGGGCCCAGTGGAACTCGACGAACTCGCCGAGCAACTGAAGAACGCCGGTATCCCCTTC    525
I  G  W  R  V  A  G  P  V  E  L  D  E  L  A  E  Q  L  K  N  A  G  I  P  F

GAGGTCGCCTCCGACGCCGACGCGGCCGAGCGACGCGTGCTTGGCCTGGTCAAATTGCATGATCCAGGCGGCAAC    600
E  V  A  S  D  A  D  A  A  E  R  R  V  L  G  L  V  K  L  H  D  P  G  G  N

CCGACGGAGATCTTCTATGGTCCCCAAGTGGACACCTCCTCCCCGTTTCACCCCGGACGGCCGATGTTCGGCAAG    675
P  T  E  I  F  Y  G  P  Q  V  D  T  S  S  P  F  H  P  G  R  P  M  F  G  K

TTCGTCACCGAAGGCCAGGGACTCGGCCACATAATCATTCGCGAGGACGACGTGGAAGAGGCGACTCGCTTCTAC    750
F  V  T  E  G  Q  G  L  G  H  I  I  I  R  E  D  D  V  E  E  A  T  R  F  Y

CGACTGCTCGGACTGGAGGGTGCGGTCGAGTACAAATTCGCGCTACCCAACGGCGCGGTTGGGACCCCGGTCTTC    825
R  L  L  G  L  E  G  A  V  E  Y  K  F  A  L  P  N  G  A  V  G  T  P  V  F
          PvuI
ATGCACTGCAACGATCGGCACCACTCCCTTGCCTTCGGGGTCGGTCCGATGGACAAGCGCATCAACCACCTGATG    900
M  H  C  N  D  R  H  H  S  L  A  F  G  V  G  P  M  D  K  R  I  N  H  L  M

ATCGAATACACCCACCTCGATGACCTCGGCTACGCACACGATCTAGTACGGCAGCAAAAGATCGACGTGACCCTG    975
I  E  Y  T  H  L  D  D  L  G  Y  A  H  D  L  V  R  Q  Q  K  I  D  V  T  L

CAAATCGGCAAACACTCCAACGACGAAGCACTCACGTTCTACTGCGCCAACCCATCCGGCTGGCTGTGGGAACCA   1050
Q  I  G  K  H  S  N  D  E  A  L  T  F  Y  C  A  N  P  S  G  W  L  W  E  P

GGCTGGGGCTCCCGCCCCGCACCAGCCCAGCAGGAACACTACCTACGCGACATCTTCGGTCACGACAACGAGGTT   1125
G  W  G  S  R  P  A  P  A  Q  Q  E  H  Y  L  R  D  I  F  G  H  D  N  E  V

GAAGGCTATGGCCTGGACATCCCCCTCAAAGGCCTCGACATCCCCGCATAAAGAAGCAGGTGGGCGGTGGGGAGG   1200
E  G  Y  G  L  D  I  P  L  K  G  L  D  I  P  A  *

GTGACCGCAAGAGGTGGGGCCCCTTCGATTGGATCGCCCCGCCACGCTTCGGGCGTAACGGTAAGAAATGTCGTAT   1275

CGAATCTTGTAGGAGAAGATAATGGCGAAGACTGTCGAAATCATCGAGAAGCGTTTCCCGTCTGGCACCTTGGCC   1350

  SphI
TCGCATGC                                                                       1358
```

FIG. 3. Cloning of the RHA1 *etbC* gene from the *bphC* gene mutant RDC1. (A) The restriction sites of relevant enzymes on clone pEB417, containing the *etbC* gene, are shown. The location and direction of the *lac* promoter (P*lac*) on pUC119 are indicated by a solid arrow. (B) Nucleotide sequence of the 2,3-DHBD gene (*etbC*) of *Rhodococcus* sp. strain RHA1. The possible ribosome-binding sequence (RBS) is boxed, and the restriction sites depicted in panel A are underlined. The termination codon for the *etbC* gene is indicated by an asterisk.

otide sequence whose translation was an identical match with the 58-amino-acid NH_2-terminal sequence of RHA1 EtbC, identifying clone pEB417 as containing the *etbC* structural gene. Deletion derivatives from both the right and left ends of the pEB417 1.6-kb DNA insert were generated and used as sequencing templates to determine the nucleotide sequences of both the forward and reverse strands of the *etbC* gene. The DNA sequence of the *etbC* gene is shown in Fig. 3B. The *etbC* gene contains 915 bp coding for a 305-amino-acid protein with a molecular mass of 33,989 Da. This is in agreement with the approximate molecular mass of EtbC determined by SDS-PAGE, 34 kDa (Fig. 1). The *etbC* structural gene has 71 and 61% identities with *bphC* from *P. paucimobilis* Q1 and *nahC* from *P. putida* PpG7, respectively (23, 44).

DISCUSSION

EtbC is the first rhodococcal 2,3-DHBD to possess high relative activities toward both 3-methylcatechol and 4-methylcatechol (4, 33) (Table 1). In addition, no rhodococcal 2,3-DHBD cloned so far contains any activity toward 4-chlorocatechol. Perhaps EtbC's substrate specificity toward 4-methylcatechol allows its relative activity toward 4-chlorocatechol. Interestingly, whereas *Rhodococcus globerulus* P6 BphC2 and BphC3 were identified as belonging to a new class of extradiol dioxygenases (3), EtbC apparently belongs to the same class as do RHA1 BphC and *R. globerulus* P6 BphC1, since it contains the usual octameric structure of other 2,3-DHBDs (12, 24, 44).

The following observations suggest that EtbC is functional in the degradation of ethylbenzene: (i) RDC1 cannot grow on biphenyl, but it grows on ethylbenzene; (ii) EtbC is the predominant *meta* cleavage enzyme in RDC1 cells grown on ethylbenzene; (iii) EtbC is expressed in RHA1 cells grown on ethylbenzene; (iv) EtbC has higher specific activities on alkylcatechols, 3-methylcatechol, and 4-methylcatechol than does RHA1 BphC; and (v) RDC1 and RHA1 cells growing on ethylbenzene accumulate a yellow compound in their supernatants characteristic of *meta* cleavage product formation.

The amino acid sequence of EtbC deduced from its nucleotide sequence was compared with those of 14 other extradiol dioxygenases. The dendrogram resulting from these identity comparisons is depicted in Fig. 4. The high-level amino acid sequence identity of RHA1 EtbC with BphC from *P. paucimobilis* Q1 (70%), coupled with its high-level activity against 2,3-dihydroxybiphenyl, qualifies EtbC as a 2,3-DHBD. As described previously by Asturias et al. (3), the dendrogram shows two subfamilies within the extradiol dioxygenases, those that preferentially cleave monocyclic aromatic compounds (XylE grouping) and those that preferentially cleave bicyclic aromatic compounds (BphC grouping). Two exceptions in the bicyclic group are the 3-methylcatechol dioxygenase (TodE) of the toluene degradation pathway from *P. putida* F1 and EtbC from RHA1. Although the deduced amino acid sequences of TodE and EtbC have only 32% identity, both of these isofunctional monocyclic aromatic extradiol dioxygenases have the distinction of being grouped by similarity into different extradiol dioxygenase subgroups specific for bicyclic aromatic compounds (Fig. 4). In addition, they both have high-level specific activity on 3-methylcatechol, a toluene degradation metabolite, and poor catechol substrate specificity, which distinguishes them from the rest of the extradiol dioxygenases specific for bicyclic and monocyclic aromatic compounds, respectively (12, 15, 28, 44). These are interesting distinctions since TodE is also able to cleave 2,3-dihydroxybiphenyl (17).

RHA1 cells grown on biphenyl and ethylbenzene resulted in the coexpression of extradiol dioxygenases BphC and EtbC.

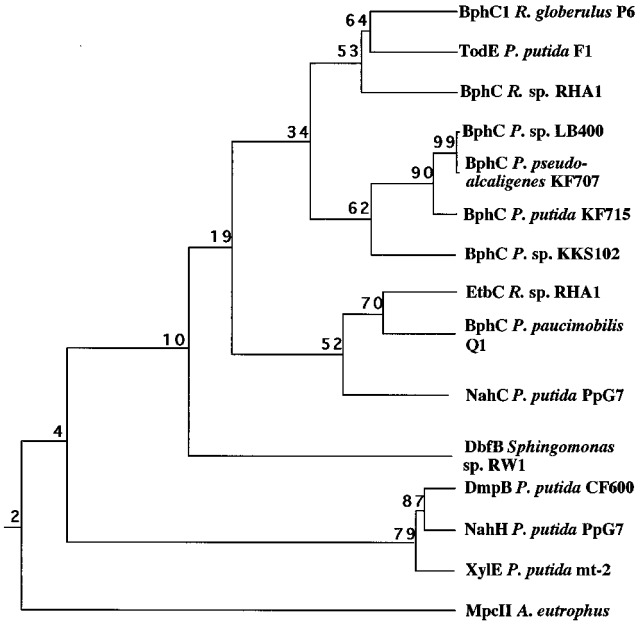

FIG. 4. Dendrogram resulting from the comparison of different extradiol dioxygenase amino acid sequences. The 2,3-DHBD (BphC1) from *R. globerulus* P6 (3), 3-methylcatechol dioxygenase (TodE) from *P. putida* F1 (46), BphC from *Rhodococcus* sp. strain RHA1 (34), BphC from *Pseudomonas* sp. strain LB400 (13), BphC from *P. pseudoalcaligenes* KF707 (16), BphC from *P. putida* KF715 (25), BphC from *Pseudomonas* sp. strain KKS102 (30), EtbC from *Rhodococcus* sp. strain RHA1, BphC from *P. paucimobilis* Q1 (44), NahC from *P. putida* PpG7 (23), DbfB from *Sphingomonas* sp. strain RW1 (22), catechol dioxygenases DmpB, NahH, and XylE from *P. putida* CF600 (42), PpG7 (20), and mt-2 (35), respectively, and MpcII from *Alcaligenes eutrophus* (27) were compared. Numbers indicate percentages of identity.

This was clearly illustrated by native nondenaturing PAGE analysis of RHA1 cell extracts from biphenyl- and ethylbenzene-grown cells, which showed the presence of two coexpressed BphC extradiol dioxygenases when activity was stained with 2,3-dihydroxybiphenyl (Fig. 2). The lower yellow activity staining band was produced by EtbC since it contained the substrate specificities of EtbC and migrated with purified EtbC. The upper yellow activity staining band was produced by BphC since it contained the substrate specificities of BphC and was not present in the ethylbenzene-grown RDC1 cell extract. The intensities of the BphC and EtbC 2,3-dihydroxybiphenyl activity staining bands for RHA1 cell extracts on native gels were almost identical, suggesting their coexpression at approximately the same levels. In addition, their catechol activity staining band intensities correlated with the relative catechol activities of purified BphC and EtbC, lending more support to their coexpression at similar levels. However, EtbC did not complement the *bphC* mutation for the growth of RDC1 on biphenyl. The polar effect on the dihydrodiol dehydrogenase gene (*bphB*), resulting from insertional mutagenesis of the RHA1 *bphC* gene (34), seemed to prevent the growth of RDC1 on biphenyl.

Further evidence for the coexpression of BphC and EtbC was suggested by the substrate specificity patterns of RHA1 cell extracts from biphenyl- and ethylbenzene-grown cells. RHA1 cell extracts from biphenyl- and ethylbenzene-grown cells contained hybrid substrate specificity patterns that encompassed the substrate specificities of both purified BphC and EtbC. RHA1 cell extracts had relative activities on catechol which were lower than that of purified BphC (24) but higher than that of purified EtbC (Table 1). In addition, RHA1

cell extracts had relative activities toward 4-methylcatechol and 4-chlorocatechol which were lower than that of purified EtbC but not present in purified BphC. However, these hybrid substrate specificity patterns don't seem to correlate with the relative expression of BphC and EtbC in RHA1, as suggested by the native PAGE *meta* cleavage product activity staining described above. In addition, purified BphC and EtbC have greater activities on 3-methylcatechol than on 2,3-dihydroxybiphenyl, but the relative activity on 3-methylcatechol was significantly reduced for RHA1 cell extracts from biphenyl- and ethylbenzene-grown cells when the two extradiol dioxygenases were coexpressed. In contrast, the cell extract of RDC1 cells grown on ethylbenzene, which contains only EtbC, possessed a substrate specificity pattern identical to that of purified EtbC, suggesting that the presence of both BphC and EtbC in the activity assay inhibits their relative activities toward catechol substrates compared with that of 2,3-dihydroxybiphenyl. Alternatively, the lower pathway enzyme (*meta* cleavage compound hydrolase), expressed in RHA1 but not in RDC1 because of the polar effect of the *bphC* insertional mutation, may cause the lower relative activities on catechol substrates. Another possible explanation for the skewed hybrid substrate specificity patterns of the coexpressed BphC and EtbC enzymes is the presence of an inhibitory substance in the RHA1 cell extract but not in the RDC1 cell extract, resulting in competitive inhibition of extradiol dioxygenase activities on catechol substrates in relation to 2,3-dihydroxybiphenyl.

The identification and cloning of multiple BphC extradiol dioxygenases, containing different structural characteristics and/or substrate specificities, in separate *Rhodococcus* biphenyl and PCB degraders have been recently reported (3, 4, 33). However, analyses of their functional relationships, such as those presented here, are necessary to gain some insight into their possible role in the degradation of PCBs. Cell extract of the strong PCB-degrader *R. globerulus* P6 grown on biphenyl had two 2,3-DHBD activity peaks, corresponding to BphC1 and BphC2 or BphC3, detected after gel filtration chromatography (3). This was the first indication of coexpressed BphC extradiol dioxygenases and their possible implication in biphenyl and PCB degradation. Using an RHA1 *bphC* insertional mutant, strain RDC1, we more conclusively identified coexpressed BphC-like extradiol dioxygenases, BphC and EtbC, in RHA1 grown on biphenyl and an alternative carbon source, ethylbenzene. RHA1 cells grown on either biphenyl or ethylbenzene strongly cometabolize PCBs (40, 41).

Although the BphA terminal dioxygenase is believed to determine biphenyl and PCB substrate recognition (14, 17, 26), the importance of lower pathway coexpressed *meta* cleavage dioxygenases, such as BphC and EtbC in the strong PCB-degrader RHA1, should not be overlooked. Their importance is supported by the finding that 2,3-DHBD may be the most sensitive target of the biphenyl pathway to catechol and chlorocatechol metabolite inhibition, resulting from PCB cometabolism (43). Catechol and chlorocatechols are transformed by 2,3-DHBD, and their presence in the reaction medium inhibits the activity of this enzyme toward its own pathway substrate, 2,3-dihydroxybiphenyl (43). The resulting accumulation of dihydroxylated metabolites may slowly inhibit biphenyl dioxygenase (BphA) activity (43), suggesting that multiple BphC-like extradiol dioxygenases (BphC and EtbC) containing different substrate specificities have an advantage in determining the substrate recognition of the biphenyl and PCB catabolic operon in RHA1 by preventing the PCB intermediate metabolite inhibition of degradative-pathway enzymes and thus allowing better growth and subsequent strong PCB cometabolism in RHA1 cells grown on biphenyl and ethylbenzene. The activity of EtbC toward 4-chlorocatechol, albeit low, may also aid in growth and PCB cometabolism in RHA1 by removing some of this potent inhibitor of biphenyl pathway oxygenation steps (43).

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