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Characterization of 2,2',3-Trihydroxybiphenyl Dioxygenase, an Extradiol Dioxygenase from the Dibenzofuran- and Dibenzo-p-Dioxin-Degrading Bacterium *Sphingomonas* sp. Strain RW1

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A key enzyme in the degradation pathways of dibenzo-p-dioxin and dibenzofuran, namely, 2,2',3-trihydroxybiphenyl dioxygenase, which is responsible for *meta* cleavage of the first aromatic ring, has been genetically and biochemically analyzed. The *dbfB* gene of this enzyme has been cloned from a cosmid library of the dibenzo-p-dioxin- and dibenzofuran-degrading bacterium *Sphingomonas* sp. strain RW1 (R. M. Wittich, H. Wilkes, V. Sinnwell, W. Francke, and P. Fortnagel, Appl. Environ. Microbiol. 58:1005–1010, 1992) and sequenced. The amino acid sequence of this enzyme is typical of those of extradiol dioxygenases. This enzyme, which is extremely oxygen labile, was purified anaerobically to apparent homogeneity from an *Escherichia coli* strain that had been engineered to hyperexpress *dbfB*. Unlike most extradiol dioxygenases, which have an oligomeric quaternary structure, the 2,2',3-trihydroxybiphenyl dioxygenase is a monomeric protein. Kinetic measurements with the purified enzyme produced similar K_m values for 2,2',3-trihydroxybiphenyl and 2,3-dihydroxybiphenyl, and both of these compounds exhibited strong substrate inhibition. 2,2',3-Trihydroxydiphenyl ether, catechol, 3-methylcatechol, and 4-methylcatechol were oxidized less efficiently and 3,4-dihydroxybiphenyl was oxidized considerably less efficiently.

Chlorinated dibenzo-p-dioxins (DBD) and dibenzofurans (DBF) are generated during the commercial production of chloroaromatic compounds as well as the incineration of industrial and domestic wastes. The exceptional toxicity and recalcitrance of these environmental pollutants have generated considerable interest in their possible destruction through microbial degradation, though the considerable efforts invested to isolate organisms with the desired catabolic activities, particularly since the Seveso accident, were signally unsuccessful. Rece ntly, a bacterium, *Sphingomonas* sp. strain RW1 (36), that possesses the capability to completely mineralize DBD and DBF was isolated, which will enable the study of the mechanism of biological breakdown of such substances.

Thus far, studies on DBD and DBF degradation by this strain have revealed that metabolism is initiated by stereospecific dioxygenation of one of the aromatic rings. Following spontaneous chemical conversion of the dihydroxylated intermediates to 2,2',3-trihydroxybiphenyl (a DBF) and its corresponding ether (a DBD), extradiol dioxygenases *meta* cleave the hydroxylated rings (36). The similarity of the pathways raises the question of whether there are one or two sets of enzymes forming one or two pathways for the substrates.

Extradiol (*meta* cleavage) dioxygenases are a family of ferrous iron-containing enzymes that mediate aromatic ring cleavage in a wide variety of aromatic catabolic pathways, including those for naphthalene, benzene, biphenyl, and toluene (11). The pivotal role of these enzymes has been revealed by genetic studies on the TOL pathway, which indicates that its extradiol dioxygenase is a major determinant of substrate specificity of this pathway (27). Another critical aspect of these

enzymes is their inhibition by some products (11), e.g., 3-chlorocatechol, which could be formed during the degradation of 4-chlorodibenzofuran or 1-chlorodibenzo-p-dioxin. Despite their importance, extradiol dioxygenases remain poorly characterized, and to date, no detailed structural information exists for any member of this class of enzymes.

In order to address the question of whether one or two pathways for DBF and DBD exists in strain RW1 and to characterize the crucial *meta* cleavage enzyme(s) of this organism, we have begun to genetically analyze the DBF and DBD phenotypes. We report here the genetic and biochemical characterization of 2,2',3-trihydroxybiphenyl (2,2',3-THB) 1,2-dioxygenase, the second enzyme in the DBD and DBF catabolic pathway.

MATERIALS AND METHODS

Strains, plasmids, and media. *Sphingomonas* sp. strain RW1 was provided by R.-M. Wittich (36) and was grown at 30°C in M9 medium (20) supplemented with 0.1% (wt/vol) DBF (Aldrich) as the sole carbon source. Cloning procedures were performed with *Escherichia coli* CC118 (21). Hyperexpression of *dbfB* was carried out with *E. coli* BL21(DE3) (32). The cosmid library was infected in *E. coli* DK22 (18) and transformed into *E. coli* S17-1 (29). *E. coli* was routinely grown at 37°C in Luria broth (LB) medium (20) containing the appropriate antibiotics. Plasmids used in this study are described with the appropriate methods. 2,3-Dihydroxybiphenyl (2,3-DHB) for enzymatic tests was obtained from Wako Chemicals GmbH (Neuss, Germany), and 3,4-dihydroxybiphenyl (3,4-DHB) was obtained from Ultra Scientific (North Kingston, R.I.). 2,2',3-THB and 2,2',3-trihydroxydiphenyl ether were kindly provided by R.-M. Wittich.

Library construction and screening. Genomic DNA from cells of a 500-ml culture of *Sphingomonas* sp. strain RW1 was

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dibenzo-*p*-dioxin   dibenzofuran
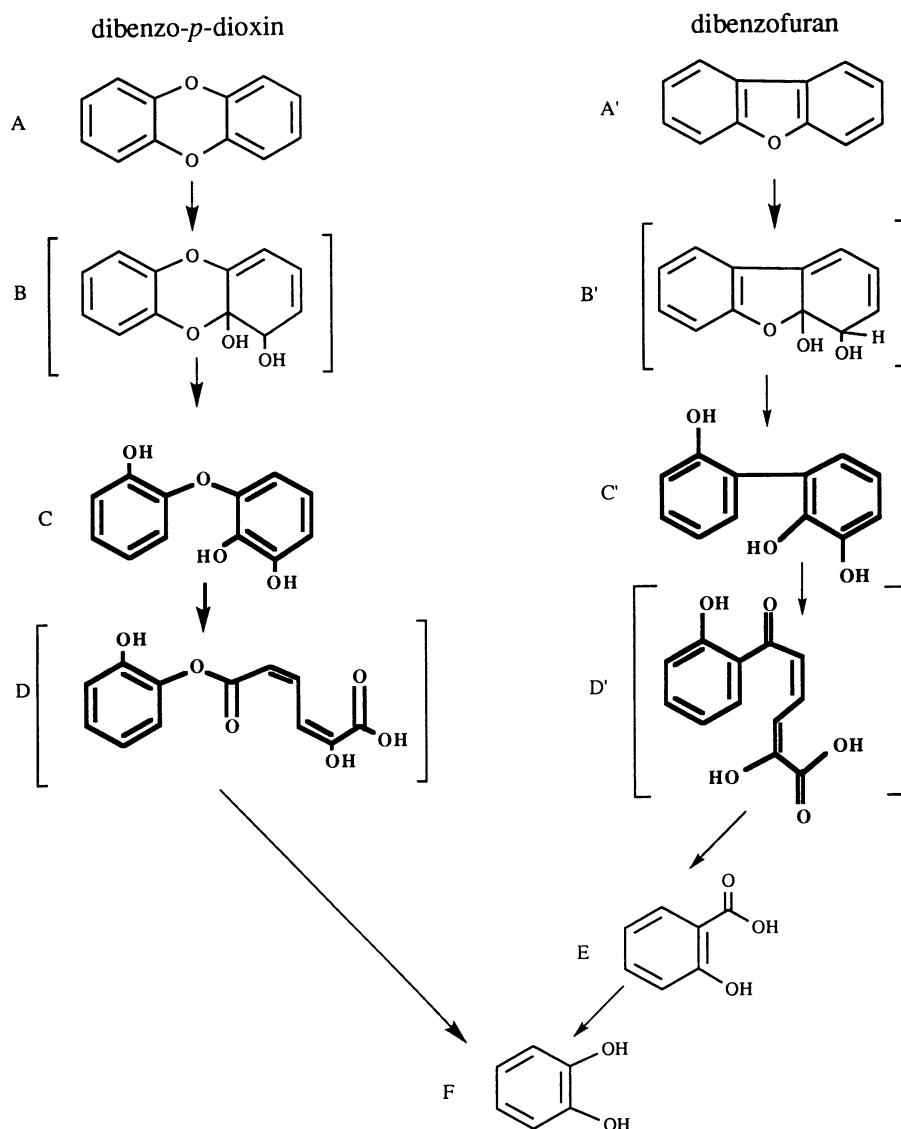

FIG. 1. Proposed pathways for the degradation of DBD (compound A) and DBF (compound A') by *Shingomonas* sp. strain RW1 (36). Compounds: B and B', unstable hemiacetal; C, 2,2',3-THB ether; C', 2,2',3-THB; D, 6-(2-hydroxyphenyl)ester of 2-hydroxymuconic acid; D', 2-hydroxy-6-oxo-(2-hydroxyphenyl)-hexa-2,4-dienoic acid; E, salicylic acid; F, catechol.

prepared by standard procedures (3) and partially digested with *Sau*3A. Fragments between 10 and 30 kb in length were isolated from a sodium chloride gradient and ligated into the broad-host-range cosmid pLAFR3 (30). Recombinant cosmids were packaged in vitro with a packaging kit (Boehringer, Mannheim, Germany) and used for infection of *E. coli* DK22. The gene bank in DK22 was lysed by heat shock and transformed into *E. coli* S17-1. The library was screened by plating it on LB agar plates, incubating the plates overnight, and spraying the colonies the following day with a solution of 0.1% 2,3-DHB in 10% acetone–water.

Sequencing of DNA. Manipulation of the DNA was performed by standard procedures (20). DNA sequencing was performed by the method of Sanger et al. (28), using the T7 sequencing kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Overlapping subclones were generated by *Bal* 31 exonuclease (New England Biolabs) partial

digests. Gel sequences were aligned and assembled with PC/ GENE (IntelliGenetics Inc., Mountain View, Calif.). Homology searches were carried out on GENMON (GBF), using the EMBL and SWISS-PROT data bases.

Hyperexpression of 2,2',3-THB dioxygenase. The plasmid pT7-5RW, constructed by ligating a 1.4-kb *Pst*I-*Sal*I fragment containing the *dbfB* gene into the pT7-5 vector (33), was transformed into BL21(DE3). Cells were grown in LB containing 50 μg of ampicillin per ml to an optical density at 600 nm of 1. Expression of the T7 polymerase gene was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested 3 h after induction.

Preparation of crude extracts. Crude extracts were prepared either aerobically and anaerobically at 4°C. Anaerobic procedures and buffer preparations were performed as described below. Cells were harvested by centrifugation for 10 min at 6,000 rpm in a Sorvall GSA rotor. Approximately 43 g (wet
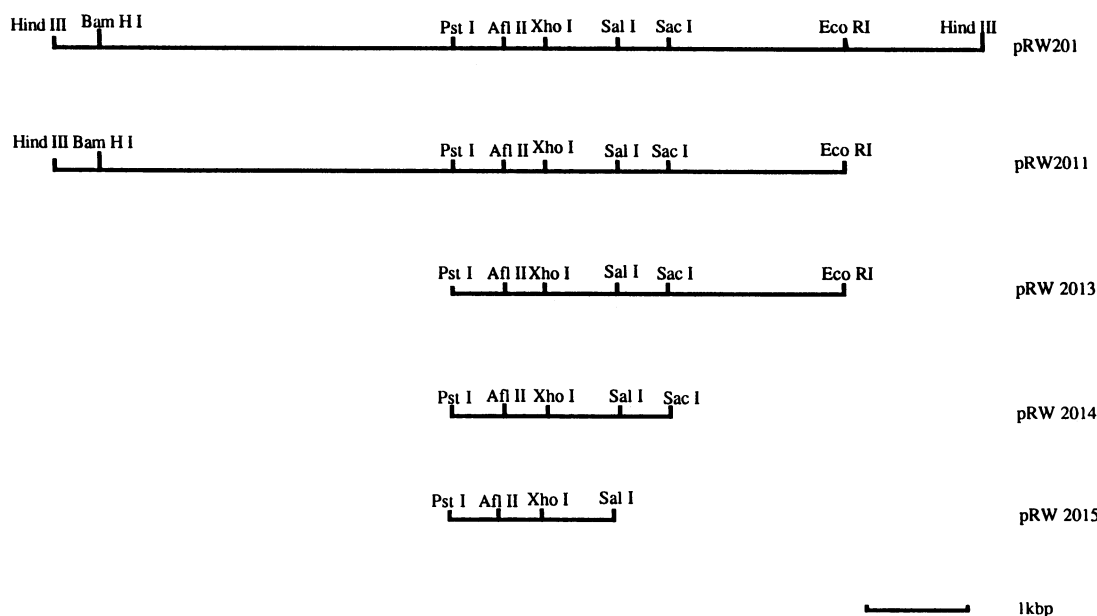
FIG. 2. Subcloning of the 1.4-kb PstI-SalI fragment carrying the 2,2',3-THB dioxygenase gene. Only clones exhibiting the 2,2',3-THB dioxygenase activity are presented.

weight) of cells was resuspended in 20 ml of 10 mM Tris–10% acetone, pH 7.5, and disrupted in a French pressure cell (Aminco Corp.) operated at 20,000 lb/in². Cell debris was removed by centrifugation at 60,000 rpm for 45 min (TLA 100.3; Beckman) at 4°C. Protein concentrations were determined by the method of Bradford (6), using bovine serum albumin (BSA) as the standard.

Protein purification. Purification of the 2,2',3-THB dioxygenase was performed under strict anaerobic conditions. All buffers used were made oxygen free by boiling under a stream of nitrogen. The buffers were supplemented with 0.1 mM ferrous ammonium sulfate and 1 mM dithiothreitol and stored under N_2. During the preparation of crude extracts, all manipulations were carried out in the anaerobic chamber (Coy, Ann Arbor, Mich.) or in a N_2 atmosphere. Column chromatography was performed on a Pharmacia LKB Biotechnology fast protein liquid chromatography system in an anaerobic chamber. The chamber was filled with 95% N_2 and 5% H_2. Traces of oxygen were removed by a palladium catalyst. Protein was concentrated in an anaerobic chamber with an Amicon ultrafiltration cell containing a YM 10 membrane.

The dioxygenase activity was precipitated from crude extracts by the addition of 1 volume of saturated ammonium sulfate solution in Tris-EDTA buffer (3). The mixture was stirred gently on ice for 30 min. The precipitate was collected by centrifugation at 50,000 rpm (Beckman, Ti 75 rotor) (4°C) under N_2, and redissolved in 20 ml of 50 mM potassium phosphate, pH 7.5. This solution was loaded on a Phenyl Sepharose Hiload HR 16/10 column equilibrated with 50 mM potassium phosphate–1.7 M ammonium sulfate, pH 7.5. The enzyme was eluted with a linear gradient of 70 to 100% 50 mM potassium phosphate, pH 7.5. Fractions containing more than 400 U/ml were pooled and loaded onto a Mono Q HR 10/10 column equilibrated with 10 mM Tris–10% isopropanol, pH 7.5. The enzyme was eluted with a linear gradient of 4 to 20% 10 mM Tris–10% isopropanol–1 M sodium chloride, pH 7.5. Active fractions were combined, concentrated, and loaded onto a HiLoad 26/6 Superdex 200 gel filtration column equil-

ibrated with a solution containing 10 mM Tris, 10% isopropanol, and 500 mM NaCl, pH 7.5. Fractions containing activity of more than 130 U/ml were combined and concentrated to a protein concentration of 9.5 mg/ml. 2,2',3-THB dioxygenase was stored in liquid nitrogen and showed no loss of activity over a period of 4 months.

NH₂-terminal sequencing. The NH₂-terminal sequence was determined by automated Edman degradation on an Applied Biosystems model 470A protein sequencer.

Electrophoresis and gel filtration. The purity and size of the denatured protein were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on a Bio-Rad Miniprotein II essentially by the method of Laemmli (19). Gels were silver stained with the Bio-Rad (München, Germany) silver stain kit according to the manufacturer's instructions. The molecular mass of the native protein was estimated by loading samples of purified protein (90 μg in 200 μl) and crude extracts of Shingomonas sp. strain RW1 and E. coli onto a Superose 6 HR10/30 column equilibrated with a solution containing 10 mM Tris, 0.5 M sodium chloride, and 10% isopropanol, pH 7.5. The following proteins were used as standards: tyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa). A_{280} and enzymatic activity were used to monitor the column fractions.

Enzyme assays. Dioxygenase activity was assayed by monitoring the formation of reaction products on a Beckman DU-70 spectrophotometer equipped with a Haake circulating water bath. The assay was performed in a total volume of 3 ml (pH 7.5, 25°C) containing 50 mM potassium phosphate and 1 μmol of 2,3-DHB. The reaction was initiated by the addition of 10 μl of appropriately diluted protein solution. The enzyme was diluted in anaerobic 50 mM potassium phosphate buffer supplemented with 0.5 mg of BSA, 0.1 mM ferrous ammonium sulfate, and 1 mM dithiothreitol to prevent oxidation. The reaction was monitored by following the production of HOPDA (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid) at

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ATCCTCTTCGAGATACTCGATGGTGATTCGGATCTAGCCGGTAGCTTTGCTACGAAGCAT  60
GTCCGCCTTACGAATAGTCGCTACCAACGCATGTCCAATGATCAGTTCGAGGCCATATAC  120
AACTCGACTGGACCTCAATTCATTCCGAGTGAAAGACCAACATCGGCGACGGCGCTCAAC  180
TCGAAGGACCAGTCTAGGCCTTCCTCGTCAACGTAGCGTGACTTCAATCGCCTTTAGTCA  240
ATCGGACAAATTGTAAAACAGTTAAGCCACAATTGTTTTTGGAGCAAAAGGAGAGTTATC  300
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```
GTCATGTCAGTAAAACAACTTGGCTACCTTATTTTTGAATGTAGGGCTGATGTTCTGGAG  360
        MetSerValLysGlnLeuGlyTyrLeuIlePheGluCysArgAlaAspValLeuGlu  19
```

```
CAAATGGTAGTCGTATACCAAGATATCATCGGTGCTGTGGTGGAGCGTGATGAAGGCGGA  420
GlnMetValValValTyrGlnAspIleIleGlyAlaValValGluArgAspGluGlyGly  39
```

```
CGCGCTCTTGTTCGCCTTGATGGCCGACCTTTCCGTATCAGGCTCGATCCTGGCCCCGCA  480
ArgAlaLeuValArgLeuAspGlyArgProPheArgIleArgLeuAspProGlyProAla  59
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```
AACCGCCTTGCGGCGATCGGTTGGAATGTAGACCCTAGTGATCTGGCGGCGATAGCGGAA  540
AsnArgLeuAlaAlaIleGlyTrpAsnValAspProSerAspLeuAlaAlaIleAlaGlu  79
```

```
CAGGTGGAGAAGGCATGTTATTCGGTCGTAACGGCTGATGCGGAACTGGCCGCAGATCGC  600
GlnValGluLysAlaCysTyrSerValValThrAlaAspAlaGluLeuAlaAlaAspArg  99
```

```
GCGGCCGCCCAAGTTCGACAGTTCGCCGATAACGATGGCTTTACCCATGAACTGTATGTC  660
AlaAlaAlaGlnValArgGlnPheAlaAspAsnAspGlyPheThrHisGluLeuTyrVal  119
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GAATCGTCGTTTCCCACCGACCCTGTGCTCGAGTCTCTATTCGTCTGTGGTGAAGAAGCG  720
GluSerSerPheProThrAspProValLeuGluSerLeuPheValCysGlyGluGluAla  139
```

```
AACGGCATCTTCGGCTTGGGGCACCTCGTAGTGATCGTGGCTGATCGGGCGAAGACTCAA  780
AsnGlyIlePheGlyLeuGlyHisLeuValValIleValAlaAspArgAlaLysThrGln  159
```

```
TCTTTTTTCACTGATGTTCTGGGCTTCGGACTCAGTGATCGGGTAACTTGCCTGAAGCCG  840
SerPhePheThrAspValLeuGlyPheGlyLeuSerAspArgValThrCysLeuLysPro  179
```

```
ACATCTTCTTCCTTCACTGCAACCAGCGTCATCACACCGTTGCACTTTCGGCACCGGCGC  660
ThrSerSerSerPheThrAlaThrSerValIleThrProLeuHisPheArgHisArgArg  199
```

```
TCGGTCTTAAGCCAGGTATGGTTCAATCATCTGATGCTAGAAGCCAAGAGCAAAGAGCAG  720
SerValLeuSerGlnValTrpPheAsnHisLeuMetLeuGluAlaLysSerLysGluGln  219
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GTTGATCGCCGGTTCGCAGCCGTCAAGCGCTTGGGCTATGATGTCCTCATGACAATTGGC  1020
ValAspArgArgPheAlaAlaValLysArgLeuGlyTyrAspValLeuMetThrIleGly  239
```

```
CAGCACTCCAACGATAAGGTCTACTCCTTCTACATGATGGCACCGGCTGGTTTTGCAGTT  1080
GlnHisSerAsnAspLysValTyrSerPheTyrMetMetAlaProAlaGlyPheAlaVal  259
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```
GAGTTGGGCTTTGGTGGCCAGGTGATTGGAGATTTGGAAAGTTGGCATGTTGGATTCTAT  1140
GluLeuGlyPheGlyGlyGlnValIleGlyAspLeuGluSerTrpHisValGlyPheTyr  279
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GACGCACCGAGCATTTGGGGCCACGAGTTGCAGTTGCCGGCGCATTGAATTATTAAGGCC  1200
AspAlaProSerIleTrpGlyHisGluLeuGlnLeuProAlaHis***                294
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ATACACCTAACATTGTCCGCCGGTCAGCGCCAATGGCACAGATTTGAGGTTGTGATTTAA  1260
GGAGGATTTGGGCTTCGTCGTAGTGACGAAGGAACGAAGATGAAGCCCAAATCCTCCTTG  1320
AAAAAATCGGCGCGAAGGCCCCAGCTGAGCGGGTGGTGAAGGACATACGGGTGGACGCGC  1380
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FIG. 3. Nucleotide sequence of the 1.4-kb PstI-SalI fragment. The deduced amino acid sequence of the open reading frame is shown. The putative Shine-Dalgarno sequence is underlined. The totally conserved amino acids are printed in bold type.

434 nm (extinction coefficient $[\varepsilon] = 13.2 \text{ mM}^{-1} \text{ cm}^{-1}$) (pH 7.5). One unit of activity is defined as the amount of enzyme that converts 1 μmol of substrate per min. Initial velocity determinations were based on three trials.

The pH optimum of the purified 2,2′,3-THB dioxygenase was determined over a pH range from 7.0 to 9.0 with extinction coefficients corrected for pH (8). The kinetic constants of 2,3-DHB cleavage by 2,2′,3-THB dioxygenase were determined by measuring the initial rate of cleavage over a concentration range of 5 μmol to 2 mmol. The reaction was initiated with approximately 0.3 μmol of purified enzyme. Data were fitted to the substrate inhibition equation (1) with MINSQ (Micromath Inc.), a least-squares fitting routine.

The cleavage of catechol (375 nm; $\varepsilon = 36 \text{ cm}^2/\mu\text{mol}$), 3-methylcatechol (382 nm; $\varepsilon = 16.8 \text{ cm}^2/\mu\text{mol}$), and 4-methylcatechol (388 nm; $\varepsilon = 31.5 \text{ cm}^2/\mu\text{mol}$) (35) were assayed in a similar manner, using the wavelengths and extinction coefficients shown in the parentheses.

The rate of cleavage of 2,2′,3-THB was monitored at 434 nm. The extinction coefficient of 2′-OH-HOPDA was determined by incubating 50 μmol of 2,2′,3-THB in 3 ml of potassium phosphate buffer containing 1.2 μmol of dioxygenase. This amount represents a sufficient quantity of enzyme to effect the complete cleavage of THB in less than 1 s. The initial velocity determinations were corrected for the spontaneous hydrolysis of 2′-OH-HOPDA to metabolite M (31). The rate

TABLE 1. Comparison of the amino acid sequences of extradiol dioxygenases to that of 2,2′,3-THB dioxygenase

Sequence	Enzyme	Species (reference)	% Homology
BPHC	2,3-DHB dioxygenase	*Pseudomonas* sp. strain LB400 (15)	32.3
BPHC$PSEPS	2,3-DHB dioxygenase	*P. pseudoalcaligenes* (10)	30.6
BPHC$PSEPU	2,3-DHB dioxygenase	*P. putida* KF715 (14)	30.5
TODE$PSEPU	3-Methylcatechol 2,3-dioxygenase	*P. putida* (37)	29
BPHC$PSES1	2,3-DHB dioxygenase	*Pseudomonas* sp. strain KKS102 (17)	27.6
BPHC$PSEPA	2,3-DHB dioxygenase	*P. paucimobilis* (34)	21.8
NAHH$PSEPU	Catechol 2,3-dioxygenase	*P. putida* (13)	21.8
MCP2$ALCEU	Catechol 2,3-dioxygenase	*Alcaligenes eutrophus* (16)	16
DMPB$PSEPU	Catechol 2,3-dioxygenase	*P. putida* (4)	15.6
XYLE	Catechol 2,3-dioxygenase	*P. putida* (15)	15.6
XYLE$PSEPU	Catechol 2,3-dioxygenase	*P. putida* (25)	15
NAHC$PSEPU	1,2-Dihydroxynaphthalene dioxygenase	*P. putida* (12)	11.9

of hydrolysis of 2′-OH-HOPDA was determined with different concentrations of this compound. The reaction was monitored at 434 nm, and the data were fitted to the appropriate equations. The delay time between the starting of the reaction and the collection of data was measured and used to improve the estimate of the extinction coefficient of 2′-OH-HOPDA.

The cleavage of 2,2′,3-trihydroxydiphenyl ether was monitored by monitoring the oxygen consumption during the dioxygenase reaction with an oxygen electrode (Bachhofer, Reutlingen, Germany).

Nucleotide sequence accession number. The nucleotide sequence of the 1.4-kb *PstI-SalI* fragment (SPDBFB) was deposited in EMBL under accession number X72850.

RESULTS

Cloning and sequencing of *dbfB*. Screening of the cosmid library yielded 10 distinct clones that could convert 2,3-DHB to the bright yellow product HOPDA and that were classified according to the restriction patterns of their recombinant plasmids and the substrate specificities of the encoded dioxygenase activities. Four of the clones exhibited the ability to *meta* cleave 2,2′,3-THB. The recombinant cosmids of these clones possessed a common 10-kb *Hin*dIII fragment which was subcloned into pUC18, yielding pRW201 (Fig. 2). Further restriction analysis and subcloning of this fragment resulted in the identification of a 1.4-kbp *PstI-SalI* fragment that encoded the 2,2′,3-THB *meta* cleavage activity (pRW2015).

Sequencing of this 1.4-kb fragment revealed an 885-bp open reading frame coding for a 32.46-kDa protein (Fig. 3) with an estimated pI of 4.96. The pI determined by electrofocussing of the purified enzyme (see below) was 4.55, a difference which could be due in part to the ferrous iron in the native enzyme. A purine-rich region, 5′-AAGGAGA-3′, upstream of the putative start codon showed 100% identity to the Shine-Dalgarno sequences of two other extradiol dioxygenases (17)

and is assumed to be the ribosome binding site. The presence of a promoter on the *PstI-SalI* fragment is indicated by the fact that this fragment could be cloned in either orientation with respect to the p*lac* promoter of the pUC vector to give constructs exhibiting 2,2′,3-THB dioxygenase activities.

Homology of 2,2′,3-THB dioxygenase to other extradiol dioxygenases. No significant nucleotide sequence homologies between *dbfB* and other genes encoding extradiol dioxygenases were found: 55% homology to the 2,3-DHB dioxygenase of *Pseudomonas* sp. strain LB400 (15) was the best example. Comparison of amino acid sequences, however, revealed significant homologies (>30% of total identity) to the 2,3-DHB dioxygenases of *Pseudomonas* sp. strain LB400 (15), *Pseudomonas pseudoalcaligenes* (10), and *Pseudomonas putida* KF715 (14) (Table 1). Inclusion of the amino acid sequence of 2,2′,3-THB dioxygenase in the alignment of 12 related extradiol dioxygenases performed by Hofer et al. (15) decreased the number of strictly conserved amino acids from 18 to 14. Of these 14 residues, 4 histidines (positions 146, 197, 208, and 241), 1 tyrosine (position 250), and 1 glutamate (position 260) (Fig. 3) are of particular interest, because they could function as ligands to the ferrous iron in the catalytic center.

Hyperexpression of *dbfB* in *E. coli*. Several different expression systems and strains were tested for hyperexpression of *dbfB*. The highest levels of activity were obtained from pT7-5RW in strain BL21 (data not shown). The highest specific activity was found 3 h after induction with IPTG and was approximately 30-fold higher than that of *Sphingomonas* sp. strain RW1 grown on DBF. SDS-PAGE analysis revealed most of the hyperexpressed protein present as inclusion bodies (data not shown). Attempts to solubilize the inclusion bodies did not yield active protein.

Enzyme purification and biochemical characterization. Purification of 2,2′,3-THB dioxygenase in the soluble fraction resulted in an approximately 60-fold increase in the specific activity of the enzyme with a 15% yield. Essential details of the

TABLE 2. Anaerobic purification of 2,2′,3-THB dioxygenase from *E. coli* BL21(DE3)

Purification step	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)[a]	Recovery (%)
None (crude extract)	18,616	139,619	7.5	100
Ammonium sulfate precipitation	3,448	113,390	32.8	81
Phenyl Sepharose column	2,090	101,619	48.6	73
Mono Q column	50	22,090	442	16
Superdex 200 column	45	20,455	454	15

[a] One unit is defined as the amount of protein that converts 1 mmol of 2,3-DHB per min.
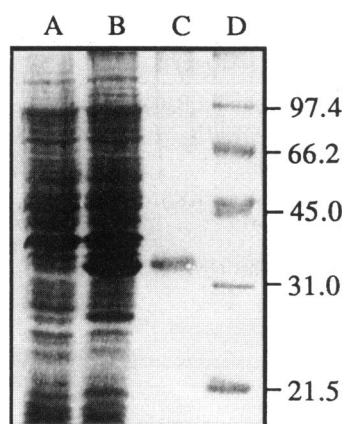
FIG. 4. Silver-stained SDS-PAGE loaded with proteins. Lanes: A, strain BL21(DE3) pT7-5 3 h after induction with IPTG; B, strain BL21(DE3) carrying pT7-5RW 3 h after induction with IPTG, as described in Materials and Methods; C, 4 μg of purified 2,2′,3-THB dioxygenase; D, molecular size standards from Bio-Rad. The molecular masses (in kilodaltons) of the standards are shown on the right.

TABLE 3. Kinetic parameters of the 2,2′,3-THB dioxygenase with different substrates[a]

Compound	K_m	V_{max}[b] (10^6)	K_{ss}
2,3-DHB	8.5 (2) μM	9.6 (0.3)	914 (30)
2,2′,3-THB	11 (0.9) μM	2.9 (0.3)	830 (170)
Catechol	83 (4) mM	4 (1.5)	
3-Methylcatechol	5.3 (0.6) mM	2.8 (0.1)	
4-Methylcatechol	5.7 (1.1) mM	0.3 (0.03)	
3,4-DHB	+[c]		
2,2′,3-Trihydroxydiphenyl ether	+[d]		

[a] Values in parentheses indicate standard errors of the means.
[b] V_{max} values correspond to nanomoles of substrate per milligram of enzyme per minute.
[c] +, too low to be determined accurately.
[d] +, activity determined by oxygen electrode.

purification are summarized in Table 2. The enzyme was purified to greater than 95% homogeneity, as indicated by silver-stained SDS-PAGE analysis (Fig. 4). The protein size was estimated to be about 32 kDa. The first 63 amino acids from the amino terminus, as determined by Edman degradation, were identical to those deduced from the amino acid sequence of *dbfB* (Fig. 3), except that the first methionine was absent, indicating that this N-terminal residue is posttranscriptionally removed.

The size of the native protein was calculated to be 34 ± 3.4 kDa by gel filtration of the purified protein, 30 ± 3 kDa from crude extracts of the *E. coli* clone, and 31.3 ± 3 kDa from extracts of *Sphingomonas* sp. strain RW1. These results indicate that the native enzyme is monomeric. The purified enzyme was observed to form higher-molecular-weight aggregates, corresponding to dimers and hexamers, within an hour of aerobic storage at 0°C. These oligomeric forms of the dioxygenase were considerably less active. Whether this lower activity is a function of the aggregation of the protein or oxidation of the active-site iron is not clear. However, because care was taken to handle the dioxygenase anaerobically and all kinetic measurements were performed within 25 min of thawing the enzyme, we believe that the kinetic parameters reported here reflect the properties of the native, monomeric form of the dioxygenase.

Attempts to purify the enzyme aerobically yielded preparations with specific activities of 0.9 U/mg of protein, which was less than 2 orders of magnitude lower than that of anaerobically purified enzyme (454 U/mg of protein), even with the addition of buffers containing various organic solvents, such as ethanol, glycerol, and acetone, known to stabilize other extradiol dioxygenases. Anaerobic reconstitution of aerobically purified material with dithiothreitol and ferrous ammonium sulfate was possible but could raise the yield only from 0.02% of the initial activity to 0.1%.

Kinetic characterization. At a substrate concentration of 0.33 mM, the pH optimum for the *meta* cleavage of 2,3-DHB by 2,2′,3-THB dioxygenase was determined to be 7.5. All subsequent kinetic measurements were performed at this pH. The K_m of the dioxygenase for 2,3-DHB was determined to be 8.5 ± 2 μM (Table 3). The enzyme displayed strong substrate inhibition (K_{ss} = 914 ± 30 μM).

The ε at 434 nm of 2′-OH-HOPDA at pH 7.5 was calculated to be 22.4 ± 1.4 mM^{-1} cm^{-1}. The nonenzymatic rate of conversion of 2′-OH-HOPDA to the colorless metabolite M (31) was determined to be first order with respect to 2′-OH-HOPDA over a concentration range of 5 to 200 μM. The half-life was determined to be 10.9 ± 0.08 s. This rate permitted the estimation of initial velocities of cleavage of 2,2′,3-THB by the dioxygenase (Fig. 5). The K_m of the enzyme for this substrate was similar to that observed for 2,3-DHB.

The K_m values of 2,2′,3-THB 1,2-dioxygenase for catechol, 3-methylcatechol, and 4-methylcatechol were found to be approximately 1,000-fold higher than those of 2,3-DHB and 2,2′,3-THB (Table 3). No substrate inhibition was observed with the monocyclic substrates. Cleavage of 3,4-DHB was observed but occurred too slowly for reliable data to be collected. Cleavage of 2,2′,3-trihydroxydiphenyl ether by the purified dioxygenase could be detected by monitoring the oxygen consumption and showed a specific activity of approximately 15% of the specific activity for 2,3-DHB.

DISCUSSION

Sphingomonas sp. strain RW1 exhibits the interesting properties of degrading DBF and DBD and of cometabolizing
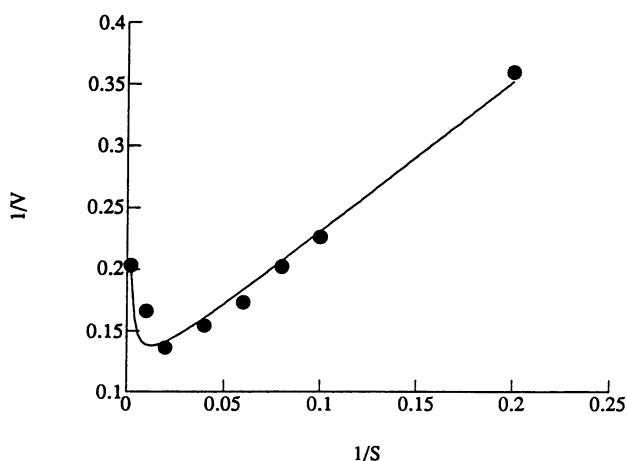

FIG. 5. Lineweaver-Burk representation of 2,2′,3-THB cleavage by 2,2′,3-THB dioxygenase (50 mM phosphate, pH 7.5, 25°C). The curve represents a fit of the data to the substrate inhibition equation with the following parameters: K_m = 11 ± 2 μM; K_{ss} = 546 ± 140 μM; and V_{max} = 9.4 ± 0.3 × 10^6 nmol/min/mg of protein.

monochlorinated derivatives thereof. The genetical and bio-chemical analysis of this catabolic activity will undoubtly advance efforts to develop biodegradative strategies for this class of serious environmental pollutants. Degradation of both DBF and DBD is initiated by a dioxygenase yielding 2,2′,3-THB and 2,2′,3-trihydroxydiphenyl ether (7). The following *meta*-cleavage step is also performed by a single enzyme, the 2,2′,3-THB dioxygenase, which was characterized in this study. It seems that the broad growth substrate specificity of the strain reflects not the existence of multiple narrowly specific enzymes, but rather the existence of enzymes with broad substrate specificity.

Inspection of the polypeptide sequence of 2,2′,3-THB dioxygenase revealed its primary structure to be that of a typical extradiol dioxygenase. In particular, strong homologies to 2,3-DHB 1,2-dioxygenases from biphenyl-degrading strains were found, which is perhaps not surprising, given the structural relatedness of the substrates of these enzymes. The additional hydroxyl substituent of 2,2′,3-THB is present on the other aromatic ring which is not *meta* cleaved and should thus minimally influence the steric and electronic interactions of the substrate with the enzyme. This is also consistent with the similar K_m values of 2,2′,3-THB dioxygenase for 2,2′,3-THB and 2,3-DHB (Table 3). Thus, 2,2′,3-THB dioxygenase may have been acquired from a biphenyl pathway. Although *Sphingomonas* sp. strain RW1 does not grow on biphenyl, it can be readily adapted to do so within an few generations (36). Whether the *dbfB* gene product plays a role in this acquired catabolic activity is, however, not known.

Nevertheless, there are several characteristics of 2,2′,3-THB dioxygenase which distinguish it from the DHB dioxygenases. First, THB dioxygenase is much more oxygen labile than DHB dioxygenases, and this lability cannot be stabilized by organic solvents. Second, 2,2′,3-THB dioxygenase is monomeric. Although another monomeric extradiol dioxygenase has been reported (5), extradiol dioxygenases are generally either tetrameric, as in the case of catechol 2,3-dioxygenases (26, 35), or octameric, as in the case of 2,3-DHB 1,2-dioxygenases (8, 9, 34). The significance of these differences in quaternary structure is not clear.

In the absence of direct evidence of the identity of the amino acid residues that function as endogenous ligands to the iron in the extradiol dioxygenases, sequence homologies can provide a useful hint as to which residues may fulfill this role. Of the 14 strictly conserved residues among the 13 related dioxygenases, 6 residues (4 histidines, 1 tyrosine, and 1 glutamate) have been shown to be involved in iron coordination in other metalloproteins: e.g., the ferrous iron of isopenicillin *N*-synthase has been shown to be coordinated to three histidinyl residues and one aspartyl residue (23). Recent studies have suggested that the aspartyl ligand is displaced upon binding of the substrate. Magnetic circular dichroism measurements on catechol 2,3-dioxygenase (22) supported by earlier electron paramagnetic resonance measurements on protocatechuate 4,5-dioxygenase (2) have led to the proposal of an exchangeable coordination position at the ferrous center in these enzymes, which is activated upon substrate binding. It could be that glutamate functions in extradiol dioxygenases in the same manner as aspartate in isopenicillin *N*-synthase and that the iron coordination of these enzymes in the resting state is very similar.

The 3,4-DHB cleaving activity of 2,2′,3-THB dioxygenase is interesting. While the catabolism of biphenyl and its chlorinated derivatives (PCBs) is initiated by hydroxylation at positions 2 and 3 in most characterized aerobic biphenyl pathways, *Pseudomonas* sp. strain LB400 has been shown to hydroxylate some PCB congeners in positions 3 and 4 (24). Since the

2,3-DHB dioxygenase of LB400 cannot cleave 3,4-DHB, the PCB-degrading potential of LB400 might increase if a 3,4-DHB cleaving activity were acquired. While the 3,4-DHB cleaving activity of 2,2′,3-THB dioxygenase is not very high, various strategies might be employed to improve this activity. A limiting factor in the rational modification of this enzyme is the lack of three-dimensional structure information, which is currently a problem for all extradiol cleavage enzymes. The relatively small native size of the 2,2′,3-THB dioxygenase (32 kDa) presents the possibility of obtaining such information from nuclear magnetic resonance studies.

The availability of more detailed structure-function information on this enzyme would not only facilitate the manipulation of its catalytic properties but also increase greatly our understanding of extradiol dioxygenases in general.

REFERENCES

1. **Adams, R. H., C.-M. Huang, F. K. Higson, V. Brenner, and D. D. Focht.** 1992. Construction of a 3-chlorobiphenyl-utilizing recombinant from an intergeneric mating. Appl. Environ. Microbiol. **58:**647–654.
2. **Arciero, D. M., and J. D. Lipscomb.** 1986. Binding of ^{17}O-labeled substrate and inhibitors to protocatechuate 4,5-dioxygenase-nitrosyl complex. J. Biol. Chem. **261:**2170–2178.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley & Sons, New York.
4. **Bartilson, M., and V. Shingler.** 1989. Nucleotide sequence and expression of the catechol 2,3-dioxygenase-encoding gene of phenol-catabolizing *Pseudomonas* CF600. Gene **85:**233–238.
5. **Block, D. W., and F. Lingens.** 1992. Purification and properties of 1*H*-3-hydroxy-4-oxoquinoline oxygenase, a new extradiol cleavage enzyme from *Pseudomonas putida* strain 33/1. Biol. Chem. Hoppe-Seyler **373:**343–349.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Bünz, P.** 1992. Ph.D. thesis. University of Hamburg, Hamburg, Germany.
8. **Eltis, L., B. Hofmann, H.-J. Hecht, H. Lünsdorf, and K. N. Timmis.** 1993. Purification and crystallisation of 2,3-dihydroxybiphenyl 1,2-dioxygenase. J. Biol. Chem. **268:**2727–2732.
9. **Furukawa, K., and N. Arimura.** 1987. Purification and properties of 2,3-dihydroxybiphenyl dioxygenase from polychlorinated biphenyl-degrading strain *Pseudomonas pseudoalcaligenes* and *Pseudomonas aeruginosa* carrying the cloned *bphC* gene. J. Bacteriol. **169:**924–927.
10. **Furukawa, K., N. Arimura, and T. Miyazaki.** 1987. Nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene from *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **169:**427–429.
11. **Harayama, S., M. Kok, and E. Neidle.** 1992. Functional and evolutionary relationships among diverse dioxygenases. Annu. Rev. Microbiol. **46:**565–601.
12. **Harayama, S., and M. Rekik.** 1989. Bacterial aromatic ring-cleavage enzymes are classified in two different gene families. J. Biol. Chem. **264:**15328–15333.
13. **Harayama, S., M. Rekik, A. Wasserfallen, and A. Bairoch.** 1987.

Evolutionary relationships between catabolic pathways for aromatics: conservation of gene order and nucleotide sequence of catechol oxidation genes of pWWO and NAH7 plasmids. Mol. Gen. Genet. **210**:241–247.

14. **Hayase, N., K. Taira, and K. Furukawa.** 1990. *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and chlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria. J. Bacteriol. **172**:1160–1164.

15. **Hofer, B., L. D. Eltis, D. N. Dowling, and K. N. Timmis.** 1993. Genetic analysis of a *Pseudomonas* locus encoding a pathway for biphenyl/polychlorinated biphenyl degradation. Gene **130**:47–55.

16. **Kabisch, M., and P. Fortnagel.** 1990. Nucleotide sequence of metapyrocatechase I (catechol 2,3-oxygenase I) gene *mpcI* from *Alcaligenes eutrophus* JMP222. Nucleic Acids Res. **18**:3405–3406.

17. **Kimbara, K., H. Toshiyuki, M. Fukuda, T. Koana, M. Takagi, M. Oishi, and K. Yano.** 1989. Cloning and sequencing of two tandem genes involved in degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS102. J. Bacteriol. **171**:2740–2747.

18. **Kurnit, D. A.** 1989. *Escherichia coli recA* deletion strains that are highly competent for transformation and for in vivo packaging. Gene **82**:313–315.

19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

21. **Manoil, C., and J. Beckwith.** 1985. Tn5 *phoA*, a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**:8129–8133.

22. **Marbrouk, P. A., A. M. Orville, J. D. Lipscomb, and E. I. Solomon.** 1991. Variable-temperature variable-field magnetic circular dichroism studies of the Fe(II) active site in metapyrocatechase: implications for the molecular mechanism of extradiol-dioxygenases. J. Am. Chem. Soc. **113**:4053–4061.

23. **Ming, L.-J., and L. J. Que.** 1991. NMR studies of the active site of isopenicillin N synthase, a non-heme iron (II) enzyme. Biochemistry **30**:11653–11659.

24. **Nadim, L., M. J. Schocken, F. J. Higson, D. T. Gibson, D. L. Bedard, L. H. Bopp, and F. J. Mondello.** 1987. Bacterial oxidation of polychlorinated biphenyls, p. 395–402. *In* Proceedings of the 13th Annual Research Symposium on Land Disposal, Remediation Action, Incineration and Treatment of Hazardous Wastes. EPA/600/015. U.S. Environmental Protection Agency, Cincinnati.

25. **Nakai, C., K. Hori, H. Kagamiyama, T. Nakazawa, and M. Nozaki.** 1983. Purification, subunit structure, and partial amino acid sequence of metapyrocatechase. J. Biol. Chem. **258**:2916–2922.

26. **Nakai, C., H. Kagamiyama, M. Nozaki, T. Nakazawa, S. Inouye, Y. Ebina, and A. Nakazawa.** 1983. Complete nucleotide sequence of the metapyrocatechase gene on the TOL plasmid of *Pseudomonas putida* mt-2. J. Biol. Chem. **258**:2923–2928.

27. **Ramos, J. L., A. Wasserfallen, K. Rose, and K. N. Timmis.** 1987. Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. Science **235**:593–596.

28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.

29. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilisation system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1**:783–791.

30. **Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinae*. J. Bacteriol. **169**:5789–5794.

31. **Strubel, V., K.-H. Engesser, P. Fischer, and H.-J. Knackmuss.** 1991. 3-(2-Hydroxyphenyl)catechol as substrate for proximal *meta* ring cleavage in dibenzofuran degradation by *Brevibacterium* sp. strain DPO 1361. J. Bacteriol. **173**:1932–1937.

32. **Studier, W. F., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.

33. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.

34. **Taira, K., N. Hayase, N. Arimura, S. Yamashita, T. Miyazaki, and K. Furukawa.** 1988. Cloning and nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene from the PCB-degrading strain of *Pseudomonas paucimobilis* Q1. Biochemistry **27**:3990–3996.

35. **Wallis, M. G., and S. K. Chapman.** 1990. Isolation and partial characterization of an extradiol non-heme iron dioxygenase which preferentially cleaves 3-methylcatechol. Biochem. J. **266**:605–609.

36. **Wittich, R.-M., H. Wilkes, V. Sinnwell, W. Francke, and P. Fortnagel.** 1992. Metabolism of dibenzo-*p*-dioxin by *Sphingomonas* sp. strain RW1. Appl. Environ. Microbiol. **58**:1005–1010.

37. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the todC1C2BADE genes and their expression in *E. coli*. J. Biol. Chem. **264**:14940–14946.