JOURNAL OF BACTERIOLOGY, Feb. 2000, p. 789–795 Vol. 182, No. 3
0021-9193/00/$04.00+0

Identification of an Extradiol Dioxygenase Involved in Tetralin Biodegradation: Gene Sequence Analysis and Purification and Characterization of the Gene Product

ELOÍSA ANDÚJAR,[1] MARÍA JOSÉ HERNÁEZ,[1] STEFAN R. KASCHABEK,[2] WALTER REINEKE,[2] AND EDUARDO SANTERO[1]*

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, 41080 Seville, Spain,[1] and Chemische Mikrobiologie, Bergische Universität-Gesamthochschule Wuppertal, D-42097 Wuppertal, Germany[2]

Received 8 September 1999/Accepted 9 November 1999

A genomic region involved in tetralin biodegradation was recently identified in *Sphingomonas* strain TFA. We have cloned and sequenced from this region a gene designated *thnC*, which codes for an extradiol dioxygenase required for tetralin utilization. Comparison to similar sequences allowed us to define a subfamily of 1,2-dihydroxynaphthalene extradiol dioxygenases, which comprises two clearly different groups, and to show that ThnC clusters within group 2 of this subfamily. 1,2-Dihydroxy-5,6,7,8-tetrahydronaphthalene was found to be the metabolite accumulated by a *thnC* insertion mutant. The ring cleavage product of this metabolite exhibited behavior typical of a hydroxymuconic semialdehyde toward pH-dependent changes and derivatization with ammonium to give a quinoline derivative. The gene product has been purified, and its biochemical properties have been studied. The enzyme is a decamer which requires Fe(II) for activity and shows high activity toward its substrate (V_{max}, 40.5 U mg^{-1}; K_m, 18.6 μM). The enzyme shows even higher activity with 1,2-dihydroxynaphthalene and also significant activity toward 1,2-dihydroxybiphenyl or methylated catechols. The broad substrate specificity of ThnC is consistent with that exhibited by other extradiol dioxygenases of the same group within the subfamily of 1,2-dihydroxynaphthalene dioxygenases.

Tetralin (1,2,3,4-tetrahydronaphthalene) is an organic solvent widely used as a degreasing agent and solvent for fats, resins, and waxes; as a substitute for turpentine in paints, lacquers, and shoe polishes; and also in the petrochemical industry in connection with coal liquefaction (12). Tetralin is very toxic to bacteria because of its accumulation in the cell membranes, which leads to changes in structure and function (39, 40), and also because of the formation of highly toxic hydroperoxides (11).

Tetralin is a bicyclic molecule composed of an aromatic moiety and an alicyclic moiety sharing two carbon atoms. Just a few bacterial strains able to grow on tetralin as the only carbon and energy source have been reported (37), and very little is known about the utilization of this molecule by bacteria. Identification of accumulated intermediates during growth on tetralin suggests that some bacteria, such as *Pseudomonas stutzeri* AS39, initially hydroxylate and further oxidize the alicyclic ring (35), while others, such as *Corynebacterium* sp. strain C125, initially dioxygenate the aromatic ring, which is subsequently cleaved in the extradiol position (38). In spite of previous reports showing modification and utilization of tetralin (35, 38, 41, 42), a complete biodegradation pathway has not yet been elucidated.

Key enzymes in the pathways of aromatic compounds are the metal-dependent ring cleavage dioxygenases, which act on the corresponding catechol-type derivatives, cleaving them at the intradiol position (*ortho* cleavage) or the extradiol position (*meta* cleavage) (18). While intradiol dioxygenases typically depend on Fe(III), most extradiol dioxygenases depend on Fe(II), although one magnesium-dependent (13) and several manganese-dependent extradiol dioxygenases (reference 5 and references therein) have also been described. Several phylogenetic analyses performed with over 30 extradiol dioxygenase sequences showed that the two-domain enzymes can be separated into two broad groups of enzymes (17) which show preferences for monocyclic or bicyclic compounds, respectively, and which may each be broken into five subfamilies (10).

A strain designated TFA, which is able to grow using tetralin as the only carbon and energy source, was recently isolated and tentatively assigned to *Sphingomonas macrogoltabidus*. Genetic analysis of insertion mutants unable to use tetralin allowed the identification of a genomic region comprising two divergent operons involved in tetralin biodegradation (21). In this paper, we describe (i) the identification and sequencing of a gene encoding a new extradiol dioxygenase (ii) and the purification and characterization of its product. The enzyme, whose sequence shows high similarity to those of 1,2-dihydroxynaphthalene (1,2-DHN) dioxygenases (1,2-DHNDOX), has a high affinity for its substrate, the catechol-type derivative of tetralin, and it also exhibits broad substrate specificity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* DH5α [F$^-$ φ80d *lacZΔM15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gypA relA1*] (16) was used for cloning and isolation of DNA for sequencing. *E. coli* strains were routinely grown in Luria-Bertani medium. Strain TFA and its mutant derivative K4 (21) were grown in mineral medium (9) with tetralin in the vapor phase and β-hydroxybutyrate (1 g liter^{-1}) as the carbon and energy source.

A 118-bp *Xba*I-*Bam*HI fragment from pET-16b (Novagen) was cloned between the *Xba*I and *Bam*HI sites of the multiple cloning sequence of pBluescript II KS (+) (Stratagene) to yield pIZ578. pIZ590 was constructed by cloning a 1,247-bp *Xma*I-*Hpa*I fragment from pIZ612 (21) into pIZ578 linearized with *Xma*I and *Eco*RV. The same *Xma*I-*Hpa*I fragment was cloned into pBluescript II KS (+) and commercially sequenced (Boehringer Mannheim). To construct pIZ591, an 89-bp deletion was constructed in pIZ590 by site-directed oligonucleotide mutagenesis, as previously described (26), using the mutagenic oligonucleotide 5′GAAGGTCGTGACAAGGC3′. The deletion was confirmed by se-

* Corresponding author. Mailing address: Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Ap. 1095, 41080 Sevilla, Spain. Phone: 34-95-4557106. Fax: 34-95-4557104. E-mail: esantero@cica.es.

quencing. The deletion of 89 bp was designed to fuse the initiation codon of *thnC* to the frame coding for the His tag located upstream.

Overexpression, purification, and electrophoretic conditions. For overexpression of *thnC*, *E. coli* NCM631/pIZ227 (14) was transformed with pIZ590 or pIZ591. The resulting transformants were grown in Luria-Bertani liquid medium at 26°C to an optical density at 600 nm of 0.7. They were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight (10 to 12 h). Cells were harvested by centrifugation, frozen in liquid nitrogen, broken with aluminum oxide 90 (Merck), and suspended in 0.5 volume of 20 mM Tris-HCl (pH 8.0)–100 mM NaCl. The purification was performed by affinity chromatography with Co^{2+}-bound resins, following the instructions of the TALON Metal Affinity Resin User Manual (Clontech Laboratories, Inc.). Imidazole (80 mM) was used to elute the protein. Sample preparation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed essentially as previously described (27). Gels were stained with GELCODE Blue stain reagent (Pierce).

Activity assays. One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate per min. Extradiol dioxygenase activity using 1,2-DHN as the substrate was assayed in 50 mM acetate buffer (pH 5.5) by measuring the substrate amount consumed as previously described (25). The extinction coefficient (ε) of 1,2-DHN (λ_{max} = 331 nm) was 2.60 mM^{-1} cm^{-1} (25). Extradiol dioxygenase activity toward other substrates was assayed by measuring the formation of the corresponding ring fission products in 50 mM Na-K phosphate buffer (pH 6.8). The extinction coefficients for the ring fission product of 5,6-dihydroxytetralin (DHT) were calculated by estimating the amount of DHT consumed by high-pressure liquid chromatography (HPLC) and the absorbance of the product at the λ_{max} using purified His-tagged protein. The change in absorbance as a function of pH was subsequently calculated by addition of diluted HCl or NaOH. The extinction coefficients used for the ring fission products of the following substrates were as follows: DHT, λ_{max} = 336 nm, ε = 12.26 mM^{-1} cm^{-1} (see Fig. 4A); catechol, λ_{max} = 375 nm, ε = 36 mM^{-1} cm^{-1}; 3-methylcatechol, λ_{max} = 388 nm, ε = 13.8 mM^{-1} cm^{-1}; 4-methylcatechol, λ_{max} = 382 nm, ε = 28.1 mM^{-1} cm^{-1}; 2,3-dihydroxybiphenyl (2,3-DHBP), λ_{max} = 434 nm, ε = 13.2 mM^{-1} cm^{-1} (20). Protein concentration was determined by the method of Bradford (6) with bovine serum albumin as the standard. All assays were quantified using a Beckman DU 640 spectrophotometer.

To perform enzymatic assays at different pHs, the following buffers were used at a concentration of 50 mM: citric acid, pH 5.0; MES [2-(*N*-morpholino)ethanesulfonic acid], pH 5.5 to 6.0; PIPES [piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)], pH 6.5 to 7.0; TRIZMA [Tris(hydroxymethyl)aminomethane], pH 7.5 to 9.

Molecular weight determination. The relative molecular weight of the native enzyme was determined by gel filtration through an Amersham-Pharmacia Biotech Sephacryl S-300 HR column (15-ml bed volume) calibrated with horse pancreas ferritin (M_r, 440,000), bovine liver catalase (M_r, 232,000), rabbit muscle aldolase (M_r, 158,000), and ovalbumin (M_r, 45,000) as reference proteins. Crude extracts of NCM631/pIZ227/pIZ590 were precipitated by serial addition of powdered ammonium sulfate, followed by continuous stirring for 40 min, and by centrifugation at 17,400 × *g* for 20 min. Dioxygenase activity precipitated between 40 and 50% ammonium sulfate. The precipitate was resuspended in one-sixth volume of column buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl). A 50-μl volume of the suspension was mixed with an equal volume of column buffer containing 10% sucrose and loaded onto the column. Protein elution from the column was in the same buffer at a flow rate of 0.4 ml min^{-1}. Fractions of 100 μl (single-drop fractions) were collected and assayed for activity.

Chemical synthesis. DHT was prepared with a 75% yield by demethylation of 5,6-dimethoxytetralin (34) with anhydrous aluminum chloride using chlorobenzene as the solvent in accordance with a general procedure (7). Synthesized DHT turns brown after incubation, and HPLC analysis showed that a new compound, presumably the autooxidation product, appeared while the peak of DHT was reduced. The half-life of DHT was estimated to be 5 h 15 min under standard conditions for activity assay. Under growth conditions, the half-life of DHT is 3 h.

Identification of intermediates. DHT and its ring fission product were analyzed by HPLC (HP 1100 Series; Hewlett-Packard, Waldbronn, Germany) with an apparatus equipped with a diode array detector and a reversed-phase column (ODS Hypersil [5 μm, 250 by 2 mm]; Hewlett-Packard).

To identify the ring fission product of the reaction, induced whole cells of NCM631/pIZ227/pIZ591 were used to produce large amounts of the compound. Cells were removed by centrifugation, and the supernatant containing the product was transformed into the picolinic acid derivative by overnight incubation at room temperature with 1.2 M NH_4Cl, an usual procedure used to characterize extradiol cleavage products (3, 8, 28, 31). The resulting product was analyzed by gas chromatography-mass spectrometry (GC-LC type HP 5890 S2 with a CPSIL [8 μm, 30 m by 0.25 μm by 0.25 μm] column; Hewlett Packard). The flame ionization temperature was 300°C. The column oven was programmed to go from a 99°C initial temperature to 250°C at a rate of 5°C/min.

Sequence analysis comparison. The resulting sequence of 1,247 bp was initially compared to those in the databases using the BLASTp and tBLASTn programs (2). Sequences which showed high similarity to that of strain TFA were aligned using the CLUSTAL W program (44) with default parameters. A distance matrix and a phylogenetic tree were constructed using the neighbor-joining method (33) and visualized with the NJPLOT program.

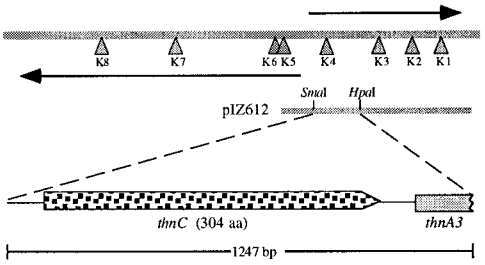

FIG. 1. Schematic representation of the genomic region of strain TFA involved in tetralin biodegradation and the sequenced gene. Arrows represent divergent operons. Triangles represent locations of KIXX insertions in mutants unable to grow on tetralin as the only carbon source.

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AF157565.

RESULTS

Sequence analysis of ThnC and other 1,2-DHNDOX. Among the nonpolar KIXX insertion mutants unable to grow on tetralin which were previously constructed (21), mutant K4 excreted a brownish pigment when grown in the presence of β-hydroxybutyrate plus tetralin, suggesting the accumulation of an intermediate of the tetralin biodegradation pathway. A 1,247-bp DNA fragment encompassing the insertion site in the mutant K4 was subcloned from pIZ612 (21) and sequenced (Fig. 1).

Translation of the nucleotide sequence in all possible reading frames revealed the existence of a complete open reading frame (ORF) of 304 amino acids followed by an ORF for which only the sequence corresponding to the first 51 amino acids was obtained. The initiation codons of both ORFs were preceded by putative Shine-Dalgarno sequences, suggesting that they were translated. Comparison of the deduced amino acid sequence of the complete ORF to those in the databases revealed that it was highly similar to extradiol dioxygenases which preferentially cleave bicyclic substrates. Similarity was highest to BphC encoded in the recently sequenced plasmid pNL1 of *S. aromaticivoras* F199 (32), with which it showed 77% similarity. The incomplete ORF showed high similarity to the ferredoxin component of hydroxylating dioxygenases. Therefore, the identified ORFs were designated *thnC* and *thnA3*, respectively (Fig. 1). Restriction analysis of the sequence showed that the KIXX cassette was inserted at codon 104 of *thnC* in the mutant K4.

Figure 2 shows a dendrogram resulting from comparison of two-domain extradiol dioxygenases of the family I.3, which preferentially cleave bicyclic substrates. Five subfamilies were previously defined within this family (10), which are also clearly observed in Fig. 2. The arrangement of the nodes leading to branches of subfamilies I.3.C and I.3.D is different from that previously described. The DbfD SPRW1 sequence has been recently updated and significantly modified. For instance, the highly conserved Phe-187 and Ala-198 residues, which were not conserved in the older sequence, are present in the updated sequence. This and the use of additional sequences in our alignment may be responsible for these differences. In any case, the bootstrap values of these nodes are low and therefore their arrangement is uncertain. In our analysis, many more representatives of 1,2-DHNDOX of different species have been used. Interestingly, all of them cluster within the subfamily I.3.E. The higher number of sequences used in this analysis also allowed the identification of two clearly defined groups

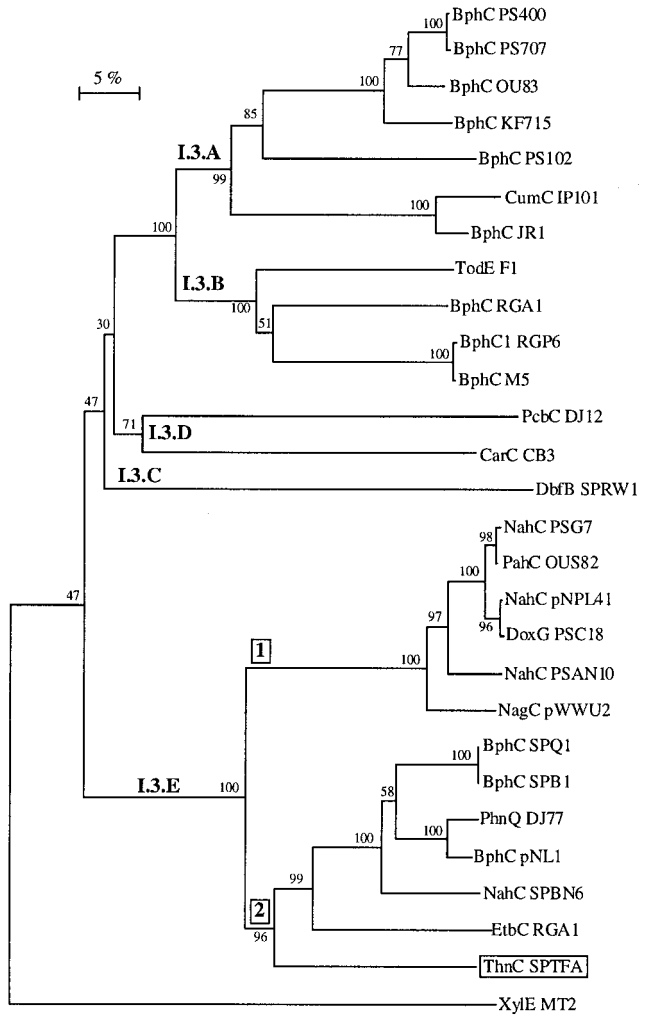

FIG. 2. Dendrogram showing the best tree obtained by the neighbor-joining method from the alignment of 26 sequences showing high similarity to ThnC. XylE MT2 was used as an outgroup. The scale represents distance expressed as percent divergence. The numbers at nodes are bootstrap values expressed as percentages. The ThnC sequence is boxed. The other sequences and their GenBank accession numbers are as follows: BphC PS400, *Burkholderia cepacia* LB400 2,3-DHBDOX (X66122); BphC PS707, *P. pseudoalcaligenes* KF707 2,3-DHBDOX (M83673); BphC OU83, *P. putida* OU83 2,3-DHBDOX (X91876); BphC PS715, *P. putida* KF715 2,3-DHBDOX (M33813); BphC PS102, *Pseudomonas* sp. strain KKS702 2,3-DHBDOX (M26443); CumC IP101, *P. fluorescens* IP101 catechol 2,3-dioxygenase (D37828); BphC JR1, *Pseudomonas* sp. strain JR1 2,3-DHBDOX (U53507); TodE PSF1, *P. putida* F1 3-methylcatechol 2,3-dioxygenase (JO4996); BphC RGA1, *Rhodococcus* sp. strain RHA1 2,3-DHBDOX (D32142); BphC1 RGP6, *Rhodococcus globerulus* P6 2,3-DHBDOX I (X75633); BphC M5, *Rhodococcus* sp. strain M5 2,3-DHBDOX (U27591); PcbC DJ12, *Pseudomonas* sp. strain DJ12 2,3-DHBDOX (D44550); CarC CB3, *Sphingomonas* sp. strain CB3 extradiol dioxygenase involved in carbazole degradation (AF060489); DbfB SPRW1, *S. paucimobilis* RW1 2,2′,3-trihydroxybiphenyl dioxygenase (X72850); NahC PSG7, *P. putida* PpG7 1,2-DHNDOX (J04994); PahC OUS82, *P. putida* OUS82 polycyclic aromatic hydrocarbon extradiol dioxygenase (D16629); NahC pNPL41, *P. putida* 1,2-DHNDOX (Y14173); DoxG PSC18, *Pseudomonas* sp. strain C18 1,2-DHNDOX (M60405); NahC PSA10, *P. stutzeri* AN10 1,2-DHNDOX (AF039533); NagC pWWU2, *Pseudomonas* sp. strain U2 1,2-DHNDOX in plasmid pWWU2 (AF036940); BphC SPQ1, *S. yanoikuyae* Q1 2,3-DHBDOX (M20640); BphC SPB1, *S. yanoikuyae* B1 2,3-DHBDOX (U23374); PhnQ DJ77, *Pseudomonas* sp. DJ77 2,3-DHBDOX (AF061802); BphC pNL1, *S. aromaticivorans* F199 2,3-DHBDOX in plasmid pNL1 (AF079317); NahC SPBN6, *Sphingomonas* sp. strain BN6 1,2-DHNDOX (U65001 [This sequence corresponds to the enzyme purified as described in reference 25); EtbC RGA1, *Rhodococcus* sp. strain RHA1 alternative 2,3-DHB DOX (D78322); XylE MT2, *P. putida* mt-2 catechol 2,3-dioxygenase (V01161).

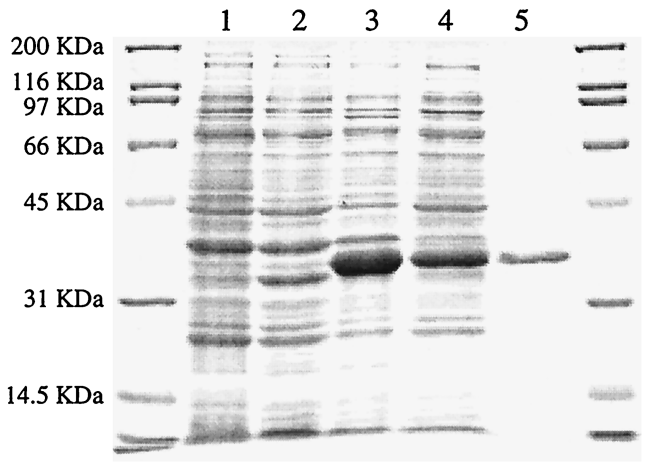

FIG. 3. Polyacrylamide gel showing overproduction and purification of native or His-tagged ThnC. The lanes contained boiled whole induced cells of NCM631/pIZ227 bearing pIZ578 (lane 1, vector), pIZ590 (lane 2, native ThnC), or pIZ591 (lane 3, His-tagged ThnC); crude extracts of NCM631/pIZ227 bearing pIZ591 after breakage and centrifugation (lane 4); and purified His-tagged ThnC after affinity chromatography (lane 5). For induction conditions, see Materials and Methods.

within this subfamily. ThnC clustered within group 2 of this subfamily (Fig. 2).

Alignment of extradiol dioxygenases confirmed the conservation of the nine residues which were previously identified as universally conserved among extradiol dioxygenases and other highly conserved residues in this type of enzyme (10). In addition to this, 39 positions have been identified which have residues highly conserved in extradiol dioxygenases which preferentially cleave bicyclic substrates (family I.3) and are characteristic of this family. Another 61 positions have highly conserved residues characteristic of the members of the I.3.E subfamily. Seventeen of these positions are also highly conserved in the other members of the I.3 family, although they contain a different residue, while 46 of these positions are not. An additional 23 positions allowed distinction between the two groups of the subfamily I.3.E. Residues in these positions are highly conserved in the members of each group but are different in the two groups. Seven of these positions are also highly conserved in the other members of the I.3 family. Finally, ThnC has specific substitutions in 13 of the 86 positions which are highly conserved within the I.3.E subfamily or are discriminatory between the two groups of this subfamily.

Overproduction and purification of the enzyme. The *thnC* gene was cloned under control of the T7 promoter, and the resulting plasmid, pIZ590, was used to transform *E. coli* strain NCM631/pIZ227 (14) in order to overproduce the native gene product. To purify the enzyme in a single step, an 89-bp deletion was created by site-directed mutagenesis in pIZ590 to fuse the initiation codon of *thnC* to an upstream ORF coding for an His_{10} tag and a signal sequence for protease factor Xa. When introduced into strain NCM631/pIZ227, the resulting plasmid, pIZ591, should drive production of an His-tagged extradiol dioxygenase containing 20 extra amino acids in its N terminus.

The overproducing strain NCM631/pIZ227 bearing pIZ590 accumulated a protein with an apparent molecular mass of 36.9 kDa (Fig. 3, lane 2), slightly higher than the molecular mass (33,827 Da) deduced from its coding sequence. When bearing plasmid pIZ591, the overproducing strain accumulated significantly higher amounts of a product of 40.7 kDa (Fig. 3, lane 3), consistent with the predicted molecular mass of the His-

tagged protein (36,080 Da). Most of the His-tagged protein was soluble (Fig. 3, lane 4) and could be purified in an active form by affinity chromatography with cobalt-bound resin (Fig. 3, lane 5). Attempts to remove the N-terminal tail of the purified His-tagged protein with factor Xa were unsuccessful, suggesting that its signal sequence is occluded in the native conformation of this protein. Therefore, the purified His-tagged protein was used for subsequent studies.

Identification of the substrate and product of the reaction. Since the mutant K4 has a nonpolar insertion in the *thnC* gene, it was expected that this mutant accumulated the substrate of the extradiol dioxygenase. To identify the intermediate accumulated in this mutant, the putative substrate of the reaction, DHT, was chemically synthesized and used as an internal standard in HPLC analysis. Mutant K4 accumulated a single intermediate which eluted at exactly the same time. Chromatography of the product with the standard showed the same result. Both the product and the standard had the same absorption spectrum (not shown), indicating that DHT is an intermediate of the tetralin pathway and the real substrate of the extradiol dioxygenase.

Addition of DHT to resting cells of strain NCM631/pIZ227 bearing pIZ590 or pIZ591, which had been grown under inducing conditions, resulted in rapid production of a yellow compound with two absorption peaks at 336 and 417 nm, which shifted depending on the pH, suggesting keto-enol tautomerism, which is typical of ring fission products of extradiol dioxygenases. Production of the yellow compound was dependent on the overproducing plasmids and was maximal under the inducing conditions (data not shown). The same yellow product was obtained using the purified enzyme. The extinction coefficients at the two absorption maxima were calculated by estimating the substrate amount consumed and are shown as a function of pH in Fig. 4A. The yellow ring cleavage product was transformed with ammonium and analyzed by gas chromatography-mass spectrometry. The mass spectrum (Fig. 4B), which fully matched that of 5,6,7,8-tetrahydroquinoline, was dominated by a fragment at $m/z = 133$, indicating loss of CO_2 from M^+, which is characteristic of pyridine-2-carboxylic acids (29, 31). This indicates that the extradiol cleavage is proximal to the alicyclic ring, yielding 4-(2-oxocyclohexyl)-2-hydroxy-buta-2,4-dienoic acid (Fig. 5).

Subunit composition. To estimate the size of ThnC, we decided to use the native protein rather than the purified His-tagged protein, since the His tag could potentially alter subunit interactions. Crude extracts of NCM631/pIZ227 bearing pIZ590 were prepared after induction and fractionated by ammonium sulfate precipitation. Most of the extradiol dioxygenase activity precipitated between 40 and 50% ammonium sulfate. The size of active ThnC in this fraction, as determined by gel filtration, was estimated to be 342.4 ± 8.5 kDa in three different runs, which indicates that active ThnC consists of 10 subunits.

Temperature and pH optima. The optimum reaction temperature was shown to be between 25 and 30°C. Activity slowly decayed with increasing temperature above 30°C (data not shown).

Activity was tested at a pH range of 5 to 9. The reaction rate exhibited a quite sharp peak of activity with a pH optimum at 7. A very drastic decrease in activity was observed at higher pH values (data not shown). Therefore, subsequent reactions were routinely performed at pH 6.8.

Metal dependence. Different preparations of the purified enzyme showed different specific activities, indicating that the enzyme was inactivated to different extents during the rapid-purification step. However, incubation on ice of different enzyme preparations with Fe^{2+}, but not with Mn^{2+} or Fe^{3+}, resulted in similar and significantly higher specific activities.

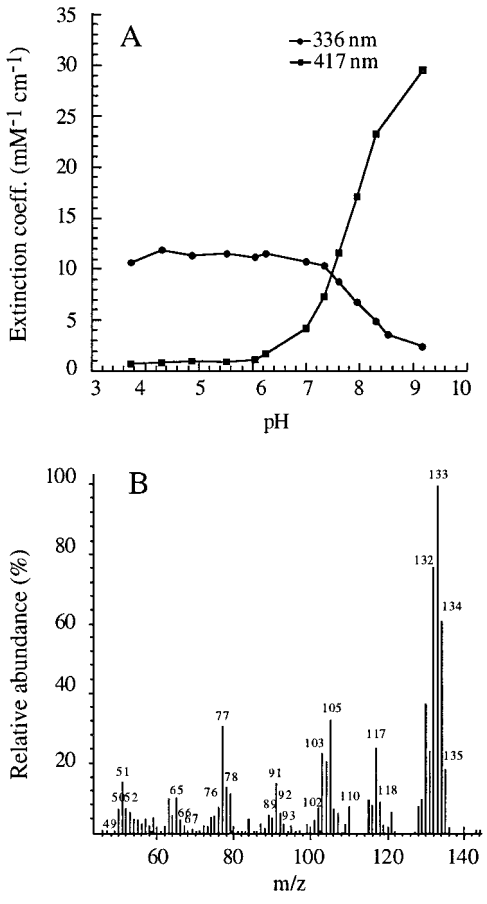

FIG. 4. (A) Variation of the extinction coefficients of 4-(2-oxocyclohexyl)-2-hydroxy-buta-2,4-dienoic acid at the two absorption maxima as a function of pH. (B) Mass spectrum of the product resulting from the incubation of 4-(2-oxocyclohexyl)-2-hydroxy-buta-2,4-dienoic acid with ammonium chloride.

To confirm the dependence on Fe^{2+}, the purified enzyme was incubated on ice in the presence or absence of Mn^{2+}. As shown in Fig. 6A, mere incubation on ice led to a clear decay in activity and the inactivation rate increased in the presence of Mn^{2+}. After most of the enzyme was inactivated on ice, activity was fully restored 20 min after the addition of Fe^{2+}. In the presence of Mn^{2+}, reactivation of the enzyme was also evident but much less efficient. Attempts to reactivate the enzyme with Fe^{3+} were unsuccessful, and in fact, addition of Fe^{3+} reduced efficiency of reactivation by Fe^{2+} (data not shown).

Kinetic parameters. Activity of the enzyme was assayed at different concentrations of DHT. A Lineweaver-Burk representation is shown in Fig. 6B, and it indicates evident inhibition of activity at substrate concentrations higher than 25 μM. This phenomenon has been previously observed with different extradiol dioxygenases (1, 4, 22) and is described by the polynomial expression $[S]/V = K_m/V_{max} + (1/V_{max})\,[S] + (1/K_{ss} \cdot V_{max})\,[S]^2$. Our data fit this expression well (inset in Fig. 6B; $R^2 = 0.998$) and gave the following theoretical kinetic parameters: $V_{max} = 40.5$ U mg of protein^{-1} $K_m = 18.6$ μM, and $K_{ss} = 40.3$ μM. The low inhibition constant indicates that the enzyme is highly sensitive to substrate inhibition, and because of this, the V_{obs} at the optimal substrate concentration (25 μM) is 21.9 U mg of protein^{-1}, just 54% of the theoretical V_{max}.

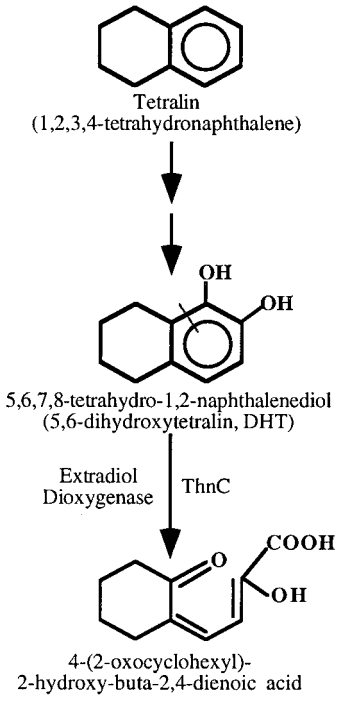

FIG. 5. Reaction catalyzed by ThnC in the tetralin biodegradation pathway of *S. macrogoltabidus* TFA.

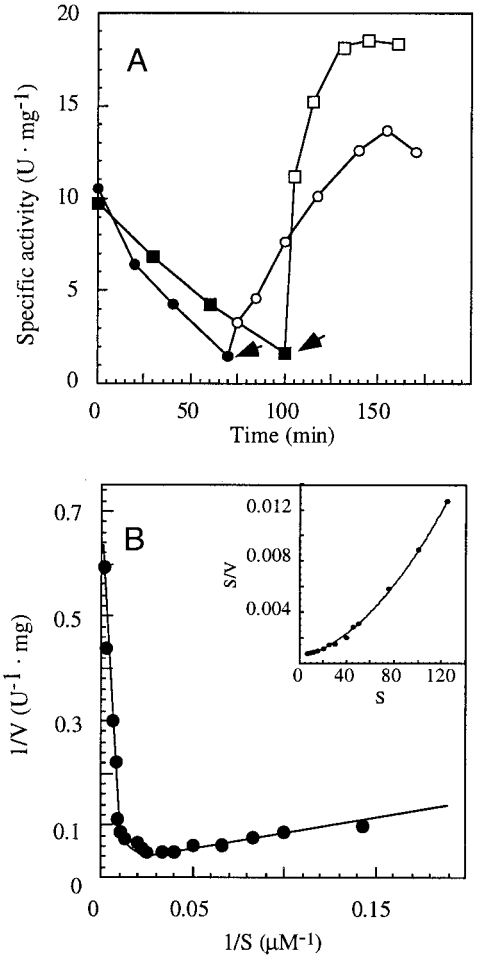

FIG. 6. (A) Inactivation of ThnC in elution buffer (■) or in elution buffer plus 0.1 mM $MnCl_2$ (●) after incubation on ice and reactivation of the enzyme by the addition of 2 mM ferrous ammonium sulfate (□, ○). Arrows show times of addition of Fe^{2+}. (B) Lineweaver-Burk plot of ThnC activity using DHT as the substrate. The inset represents the plot of $[S]/V$ versus $[S]$ and computer fitting of the data to the polynomial expression from which kinetic parameters were calculated.

Substrate specificity. For comparison to other extradiol dioxygenases of the same group, the activity of ThnC with different monocyclic or bicyclic catechol derivatives was determined. Since the activity of the enzyme showed strong substrate inhibition with DHT, we empirically estimated the optimal concentration of each substrate. For each substrate, concentrations above those shown in Table 1 resulted in lower specific activity; therefore, the enzyme exhibited inhibition by all substrates. Inhibitory concentrations varied depending on the substrate, but they were significantly higher than that for DHT. Significantly high activity of ThnC was observed toward 2,3-DHBP and also toward 3-methylcatechol or 4-methylcatechol, while the lowest activity observed was toward catechol. Interestingly, the specific activity of ThnC with 1,2-DHN was even higher than that with DHT. These data suggest that ThnC is a bona fide 1,2-DHNDOX which has broad substrate specificity.

DISCUSSION

A gene designated *thnC*, which potentially codes for a ring cleavage dioxygenase involved in tetralin utilization, has been identified by subcloning and sequencing of a DNA region in which the KIXX insertion of the mutant K4 (21) was located. Comparison to other sequences in the databases suggested that the gene product is an extradiol dioxygenase. Subcloning of *thnC* in overproducing plasmids resulted in accumulation of a polypeptide whose estimated size closely matched that predicted by its sequence (Fig. 3), showing that the gene is actually expressed. The function of ThnC in the degradation of tetralin was shown by the Thn^- phenotype of the mutant K4, by identification of DHT as the intermediate accumulated in the mutant K4, and by characterization of the product of the reaction catalyzed by ThnC (Fig. 4). Identification of the product of the reaction also confirmed the prediction based on sequence comparison, suggesting that ThnC is an extradiol dioxygenase involved in tetralin biodegradation. Taken together, these data clearly demonstrate that degradation of tetralin by strain TFA involves initial dihydroxylation of the aromatic ring, which is subsequently cleaved in the extradiol position.

Ring cleavage catalyzed by ThnC is in the position proximal to the nonhydroxylated ring, in a way similar to that of the

TABLE 1. Substrate specificity of purified ThnC

Substrate	Concn (μM)	V_{obs} (U mg^{-1})	Relative activity (%)
DHT	25	21.9	100
2,3-DHBP	200	2.1	10
3-Methylcatechol	400	1.4	7
4-Methylcatechol	800	1.2	5
Catechol	800	0.3	1
1,2-DHN[a]	100	17.3	166

[a] Activity toward 1,2-DHN was assayed at pH 5.5. At this pH, activity toward DHT was 10.4 U mg^{-1}.

reaction catalyzed by other 1,2-DHNDOX, the enzymes which are most similar to ThnC. However, an interesting feature of ThnC is the substrate on which it can act, which is significantly different from that of 1,2-DHN. Although both DHT and 1,2-DHN are bicyclic molecules sharing two carbon atoms, the nonhydroxylated ring of 1,2-DHN is also aromatic and therefore the molecule is constrained in a rigid planar structure while that of DHT is alicyclic and therefore nonplanar. Based on intermediates accumulated by *Corynebacterium* sp. strain C125 growing on tetralin, an activity similar to that of ThnC was reported (38), although neither the enzyme nor its gene has been characterized. Therefore, ThnC is the first characterized extradiol dioxygenase able to act on this type of molecule.

Estimation of the molecular mass of active ThnC by gel filtration consistently indicated that ThnC is a decamer. Different subunit structures, including the tetradecameric structure of the dioxygenase of *Pseudomonas* sp. strain NCIB9816 (30), have been reported for more distantly related extradiol dioxygenases. Nevertheless, this result is surprising because it is in contrast to the octameric structure frequently reported for extradiol dioxygenases, including those such as BphC SPQ1 (43), NahC SPBN6 (25), and EtbC RGA1 (19), which are very similar to ThnC (Fig. 2).

The sequences which showed the highest percentage of similarity to ThnC correspond to extradiol dioxygenases belonging to the I.3 family, as defined by Eltis and Bolin (10). Comparison analysis of these sequences resulted in a dendrogram in which the five subfamilies previously defined were also evident (Fig. 2). However, in our analysis, the number of sequences which cluster within the subfamily I.3.E is much higher. This allowed a more detailed analysis of the cladistic relationships among members of this subfamily, which resulted in the establishment of two clearly defined groups (the bootstrap value of the node separating the two groups is 100%; Fig. 2). Group 1 is highly conserved and comprised of 1,2-DHNDOX from different naphthalene-degrading strains. Group 2 is more diverse in sequence, and enzymes of this group have shown activity with different substrates. Five enzymes of this group were previously characterized and reported to show significantly high activity toward 2,3-DHBP and methylated catechols. In fact, four of them were described as 2,3-DHBP dioxygenases (2,3-DHBDOX) (19, 23, 32, 43). However, all of the enzymes of this group whose activity toward 1,2-DHN was tested clearly showed higher activity toward this substrate (24, 25, 32). As shown in Table 1, ThnC, which belongs to group 2, also has significant activity using 2,3-DHBP, 3-methylcatechol, or 4-methylcatechol but its highest activity is toward 1,2-DHN. These data clearly indicate that enzymes from group 2 really are 1,2-DHNDOX that have broad substrate specificity. In turn, these data also allow definition of the subfamily I.3.E as the subfamily of 1,2-DHNDOX.

A common evolutionary origin of dioxygenases of group 2 is suggested not only by amino acid sequence conservation but also by their gene arrangement. Except for EtbC RGA1, the sequence downstream of the genes coding for these enzymes showed a coding potential for a Rieske-type ferredoxin. This conserved gene arrangement is unusual, since ferredoxin genes commonly cluster with the genes coding for the initial hydroxylating dioxygenase.

There are a number of highly conserved residues among 1,2-DHNDOX which are characteristics of the members of the I.3.E subfamily or of one of the groups within this subfamily. Conservation of some of these residues may just reflect common evolutionary origins, but conservation of others may be relevant for the ability of these enzymes to use 1,2-DHN as a substrate, since these residues contribute to the Fe^{2+} environment or form the substrate binding site in BphC LB400 (15) and BphC KKS102 (36), the two enzymes whose crystal structures are available. His-209 (LB400 numbering), which is highly conserved among extradiol dioxygenases (10), forms a hydrogen bond with the Fe ligand His-210, and also borders on the distal ring binding site, is substituted for Asn in all members of the I.3.E subfamily except PhnQ DJ77. Similarly, His-194, which forms a hydrogen bond with the active-site residue His-195 and is conserved in all other members of the I.3 family, is substituted for Asp in group 1 of the I.3.E subfamily or for Gln in all members of group 2, except in EtbC RGA1. His-194 also forms a hydrogen bond with Asp-171, which is also conserved in all other members of the I.3 family. Significantly, Asp-171 is substituted for Val in all members of the I.3.E subfamily. Met-246 and Ile-173, which are highly conserved among other members of the I.3 family and help to define the substrate binding site, are substituted for Ala and Tyr, respectively, in all members of the I.3.E subfamily. Other positions, such as 202 and 280, that also form the substrate binding site, are not particularly well conserved among 2,3-DHBDOX of the I.3 family, suggesting that they are not important for specific binding of biphenyl. However, these two positions are well conserved in the I.3.E subfamily and discriminate between the two groups of the subfamily. Met-202 is conserved in group 1 and Gly-202 is conserved in group 2. Gly-280 is conserved in group 1, and Arg-280 is conserved in all members of group 2 except ThnC, which has Ala.

Given the characteristics of these residues, it is tempting to speculate that some of them favor substrate binding to 1,2-DHNDOX in a way similar to mode A (15), which is more appropriate for binding of planar molecules such as 1,2-DHN, rather than in mode B, which is favored in 2,3-DHBDOX (15) and implies a torsion angle between the two rings of ~80°. However, involvement of these residues in activity or substrate specificity remains to be elucidated by mutational analysis.

ACKNOWLEDGMENTS

This work was supported by the Spanish Comisión Interministerial de Ciencia y Tecnología, grant BIO96-0908; by the European Union under the ENVIRONMENT Program, contract EV5V-CT92-0192; and by fellowships of the Spanish Ministerio de Educación to E.A. and M.J.H.

We thank H.-G. Schmalz, TU-Berlin, for providing 5,6-dimethoxytetralin for synthesis of DHT and Gabriel Gutierrez for his assistance in DNA sequence analysis.

REFERENCES

1. **Adams, R., C.-M. Huang, F. 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. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Asano, Y., Y. Yamamoto, and H. Yamada.** 1994. Catechol 2,3-dioxygenase-catalyzed synthesis of picolinic acids from catechols. Biosci. Biotech. Biochem. **58:**2054–2056.
4. **Asturias, J., and K. N. Timmis.** 1993. Three different 2,3-dihydroxybiphenyl 1,2-dioxygenase genes in the gram-positive polychlorobiphenyl-degrading bacterium *Rhodococcus globerulus* P6. J. Bacteriol. **175:**4631–4640.
5. **Boldt, Y., A. Whiting, M. Wagner, M. Sadowsky, L. Que, Jr., and L. Wackett.** 1997. Manganese(II) active site mutants of 3,4-dihydroxyphenylacetate 2,3-dioxygenase from *Arthrobacter globiformis* strain CM-2. Biochemistry **36:** 2147–2153.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–252.
7. **Corse, J., and L. L. Ingraham.** 1951. The monofluorocatechols. J. Org. Chem. **16:**1345–1348.
8. **Dagley, S., W. C. Evans, and D. W. Ribbons.** 1960. New pathways in the oxidative metabolism of aromatic compounds by microorganisms. Nature (London) **188:**560–566.

9. **Dorn, E., M. Hellwig, W. Reineke, and H.-J. Knackmuss.** 1974. Isolation and characterization of a 3-chlorobenzoate degrading degrading pseudomonad. Arch. Microbiol. **99:**61–70.
10. **Eltis, L., and J. Bolin.** 1996. Evolutionary relationships among extradiol dioxygenases. J. Bacteriol. **178:**5930–5937.
11. **Ferrante, A. A., J. Augliera, K. Lewis, and A. M. Klibanov.** 1995. Cloning of an organic solvent-resistance gene in *Escherichia coli*: the unexpected role of alkylhydroperoxide reductase. Proc. Natl. Acad. Sci. USA **92:**7617–7621.
12. **Gaydos, R. M.** 1981. Naphthalene, p. 698–719. *In* M. Grayson and D. Eckroth (ed.), Kirk-Othmer encyclopedia of chemical technology, 3rd ed. John Wiley & Sons, Inc., New York, N.Y.
13. **Gibello, A., E. Ferrer, M. Martin, and A. Garrido-Pertierra.** 1994. 3,4-Dihydroxyphenylacetate 2,3-dioxygenase from *Klebsiella pneumoniae*, a Mg^{2+}-containing dioxygenase involved in aromatic catabolism. Biochem. J. **301:**145–150.
14. **Govantes, F. J. A. Molina-López, and E. Santero.** 1996. Mechanism of coordinated synthesis of the antagonistic regulatory proteins NifL and NifA of *Klebsiella pneumoniae*. J. Bacteriol. **178:**6817–6823.
15. **Han, S., L. D. Eltis, K. N. Timmis, S. W. Muchmore, and J. T. Bolin.** 1995. Crystal structure of the biphenyl-cleaving extradiol dioxygenase from a PCB-degrading pseudomonad. Science **270:**976–980.
16. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
17. **Harayama, S., and M. Rekik.** 1989. Bacterial aromatic ring-cleavage enzymes are classified in two different gene families. J. Biol. Chem. **264:**15328–15333.
18. **Harayama, S., M. Kok, and E. L. Neidle.** 1992. Functional and evolutionary relationships among diverse dioxygenases. Annu. Rev. Microbiol. **46:**565–601.
19. **Hauschild, J. E., E. Masai, K. Sugiyama, T. Hatta, K. Kimbara, M. Fukuda, and K. Yano.** 1996. Identification of an alternative 2,3-dihydroxybiphenyl 1,2-dioxygenase in *Rhodococcus* sp. strain RHA1 and cloning of the gene. Appl. Environ. Microbiol. **62:**2940–2946.
20. **Heiss, G., A. Stolz, A. E. Kuhm, C. Müller, J. Klein, J. Altenbuchner, and H.-J. Knackmuss.** 1995. Characterization of a 2,3-dihydroxybiphenyl dioxygenase from the naphthalenesulfonate-degrading bacterium strain BN6. J. Bacteriol. **177:**5865–5871.
21. **Hernáez, M. J., W. Reineke, and E. Santero.** 1999. Genetic analysis of biodegradation of tetralin by a *Sphingomonas* strain. Appl. Environ. Microbiol. **65:**1806–1810.
22. **Higson, F. K., and D. D. Focht.** 1989. Bacterial metabolism of hydroxylated biphenyls. Appl. Environ. Microbiol. **55:**798–805.
23. **Kim, E., and G. Zylstra.** 1995. Molecular and biochemical characterization of two *meta*-cleavage dioxygenases involved in biphenyl and *m*-xylene degradation by *Beijerinckia* sp. strain B1. J. Bacteriol. **177:**3095–3103.
24. **Kuhm, A. E., A. Stolz, and H.-J. Knackmuss.** 1991. Metabolism of naphthalene by the biphenyl-degrading bacterium *Pseudomonas paucimobilis* Q1. Biodegradation **2:**115–120.
25. **Kuhm, A. E., A. Stolz, K.-N. Ngai, and H.-J. Knackmuss.** 1991. Purification and characterization of a 1,2-dihydroxynaphthalene dioxygenase from a bacterium that degrades naphthalenesulfonic acids. J. Bacteriol. **173:**3795–3802.
26. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
28. **Matthews, C., J. T. Rossiter, and D. W. Ribbons.** 1995. Production of pyridine synthons by biotransformations of benzene precursors and their cyclation with nitrogen nucleophiles. Biocatal. Biotransform. **12:**241–254.
29. **Moser, R. J., and E. V. Brown.** 1970. Mass spectra of some 5- and 6-substituted 2-pyridinecarboxylic acids. Nature of fragmentation step for loss of CO_2. Org. Mass Spectrom. **4:**555–561.
30. **Patel, T. R., and E. A. Barnsley.** 1980. Naphthalene metabolism by *Pseudomonas*: purification and properties of 1,2-dihydroxynaphthalene oxygenase. J. Bacteriol. **143:**668–673.
31. **Riegert, U., G. Heiss, P. Fischer, and A. Stolz.** 1998. Distal cleavage of 3-chlorocatechol by an extradiol dioxygenase to 3-chloro-2-hydroxymuconic semialdehyde. J. Bacteriol. **180:**2849–2853.
32. **Romine, M., L. Stillwell, K.-K. Wong, S. Thurston, E. Sisk, C. Sensen, T. Gaasterland, J. Fredrickson, and J. Saffer.** 1999. Complete sequence of a 184-kilobase catabolic plasmid from *Sphingomonas aromaticivorans* F199. J. Bacteriol. **181:**1585–1602.
33. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
34. **Schmalz, H.-G., M. Arnold, J. Hollander, and J. W. Bats.** 1994. Zur Totalsynthese von *cis*-7,8-Dihydroxy-11,12-dehydrocalamenen durch regio- und diastereoselektive Alkylierung chiraler η^6-Arentricarbonylchrom-Komplexe: ein unerwarterter Fall nucleophiler aromatischer *tele*-Substitution mit Methoxid als Fluchtgruppe. Angew. Chem. **106:**77–79.
35. **Schreiber, A. F., and U. K. Winkler.** 1983. Transformation of tetralin by whole cells of *Pseudomonas stutzeri* AS 39. Eur. J. Appl. Microbiol. Biotechnol. **18:**6–10.
36. **Senda, T., K. Sugiyama, H. Narita, T. Yamamoto, K. Kimbara, M. Fukuda, M. Sato, K. Yano, and Y. Mitsui.** 1996. Three-dimensional structures of free form and two substrate complexes of an extradiol ring-cleavage type dioxygenase, the BphC enzyme from *Pseudomonas* sp. strain KKS102. J. Mol. Biol. **255:**735–752.
37. **Sikkema, J., and J. A. M. de Bont.** 1991. Isolation and initial characterization of bacteria growing on tetralin. Biodegradation **2:**15–23.
38. **Sikkema, J., and J. A. M. de Bont.** 1993. Metabolism of tetralin (1,2,3,4-tetrahydronaphthalene) in *Corynebacterium* sp. strain C125. Appl. Environ. Microbiol. **59:**567–572.
39. **Sikkema, J., J. A. M. de Bont, and B. Poolman.** 1994. Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem. **269:**8022–8028.
40. **Sikkema, J., B. Poolman, W. N. Konings, and J. A. M. de Bont.** 1992. Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. J. Bacteriol. **174:**2986–2992.
41. **Soli, G., and E. M. Bens.** 1972. Bacteria which attack petroleum hydrocarbons in a saline medium. Biotechnol. Bioeng. **14:**319–330.
42. **Strawinsky, R. J., and R. W. Stone.** 1940. The utilization of hydrocarbons by bacteria. J. Bacteriol. **40:**461–462.
43. **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.
44. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.