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A Manganese-Dependent Dioxygenase from *Arthrobacter globiformis* CM-2 Belongs to the Major Extradiol Dioxygenase Family

YVONNE R. BOLDT,[1] MICHAEL J. SADOWSKY,[1,2] LYNDA B. M. ELLIS,[3] LAWRENCE QUE, JR.,[4] AND LAWRENCE P. WACKETT[1,5,6]*

Departments of Microbiology,[1] Soil Science,[2] and Biochemistry[5] and Institute for Advanced Studies in Biological Process Technology,[6] University of Minnesota, St. Paul, Minnesota 55108, and Departments of Laboratory Medicine and Pathology[3] and Chemistry,[4] University of Minnesota, Minneapolis, Minnesota 55455

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Almost all bacterial ring cleavage dioxygenases contain iron as the catalytic metal center. We report here the first available sequence for a manganese-dependent 3,4-dihydroxyphenylacetate (3,4-DHPA) 2,3-dioxygenase and its further characterization. This manganese-dependent extradiol dioxygenase from *Arthrobacter globiformis* CM-2, unlike iron-dependent extradiol dioxygenases, is not inactivated by hydrogen peroxide. Also, ferrous ions, which activate iron extradiol dioxygenases, inhibit 3,4-DHPA 2,3-dioxygenase. The gene encoding 3,4-DHPA 2,3-dioxygenase, *mndD*, was identified from an *A. globiformis* CM-2 cosmid library. *mndD* was subcloned as a 2.0-kb *Sma*I fragment in pUC18, from which manganese-dependent extradiol dioxygenase activity was expressed at high levels in Escherichia coli. The *mndD* open reading frame was identified by comparison with the known N-terminal amino acid sequence of purified manganese-dependent 3,4-DHPA 2,3-dioxygenase. Fourteen of 18 amino acids conserved in members of the iron-dependent extradiol dioxygenase family are also conserved in the manganese-dependent 3,4-DHPA 2,3-dioxygenase (MndD). Thus, MndD belongs to the extradiol family of dioxygenases and may share a common ancestry with the iron-dependent extradiol dioxygenases. We propose the revised consensus primary sequence (G,T,N,R)X(H,A)XXXXXXX(L,I,V,M,F)YXX(D,E,T,N,A)PX(G,P)X{2,3}E for this family. (Numbers in brackets indicate a gap of two or three residues at this point in the sequence.) The suggested common ancestry is also supported by sequence obtained from genes flanking *mndD*, which share significant sequence identity with *xylJ* and *xylG* from *Pseudomonas putida*.

Bacterial ring cleavage dioxygenases play a key role in degrading aromatic compounds for reentry into the carbon cycle (13). Aromatic compounds containing a benzene ring are particularly stable and prevalent in nature. They occur naturally in plant lignins, the amino acids tyrosine and phenylalanine, and man-made compounds such as herbicides and pesticides (4). Monocyclic and polycyclic substituted aromatic compounds are biodegraded to a limited number of intermediates which are the targets of ring cleavage dioxygenases. While the majority of bacterial ring cleavage dioxygenases that have been characterized contain iron as a catalytic metal center, there are three known bacterial dioxygenases that contain manganese: two from the genus *Arthrobacter* (reference 39 and this paper) and one from *Bacillus brevis* (40). Manganese dependence of the 3,4-dihydroxyphenylacetate (3,4-DHPA) 2,3-dioxygenase activities from *B. brevis* (40), and *Arthrobacter* sp. strain Mn-1 (39) have been demonstrated.

Two types of ring cleavage dioxygenases which act on catecholic substrates are distinguished by the position of ring cleavage (20). Intradiol dioxygenases cleave between the two hydroxyl groups (ortho cleavage), while extradiol dioxygenases cleave adjacent to one of the hydroxyl groups (meta cleavage). The manganese-dependent 3,4-DHPA 2,3-dioxygenase from *Arthrobacter globiformis* CM-2 catalyzes extradiol cleavage of 3,4-dihydroxyphenylacetic acid (Fig. 1). Both intradiol and extradiol ring cleavage dioxygenases operate in degradation pathways that convert aromatic compounds such as tyrosine and 4-hydroxyphenylacetate to tricarboxcylic acid cycle intermediates (3, 7, 43, 44). DNA and amino acid sequence analyses have shown that the intradiol and extradiol dioxygenases are evolutionarily distinct (15, 20, 23). There now appear to be at least three phylogenetically distinct families of extradiol dioxygenases (2).

Aromatic ring cleavage dioxygenases are widespread in both gram-positive and gram-negative bacteria, although most studies have focused on those isolated from gram-negative bacteria, especially *Pseudomonas* sp. strains (4, 20, 22, 23, 25, 30, 31, 49). Aromatic degradation pathways in gram-positive bacteria have been known since the 1970s (7, 43) and have been found in *Arthrobacter* spp. (7, 34), *Rhodococcus* spp. (2, 10), *Micrococcus* spp. (6), and *Bacillus* spp. (14). These gram-positive organisms have been shown to contain degradative pathways capable of catabolizing a wide variety of substituted aromatic compounds, including aromatic acids, chlorocatechols, and chlorinated biphenyls. Some of these compounds are the targets of bioremediation. Because the microorganisms most often useful for bioremediation are those naturally present in the soil and *Arthrobacter* spp. are the most numerous bacteria in many soils (37), it is important to better understand the metabolic potential of this genus.

Toward this goal, this study was conducted to further characterize the 3,4-DHPA 2,3-dioxygenase from *A. globiformis* CM-2 and to define the relationship of this manganese-dependent extradiol dioxygenase to the iron-dependent extradiol dioxygenases. We report here that the manganese-dependent 3,4-DHPA 2,3-dioxygenase from *A. globiformis* CM-2 belongs to the major extradiol dioxygenase family. Portions of two genes flanking *mndD*, *mndC* and *mndE*, were also sequenced and showed 37 and 46% amino acid sequence identity to XylJ and XylG, respectively, from the TOL plasmid of *Pseudomonas putida*. These data suggest that the *Arthrobacter* genes are

* Corresponding author. Mailing address: Department of Biochemistry, University of Minnesota, 140 Gortner Lab., 1479 Gortner Ave., St. Paul, MN 55108. Phone: (612) 625-3785. Fax: (612) 625-5780. Electronic mail address: wackett@molbio.cbs.umn.edu.

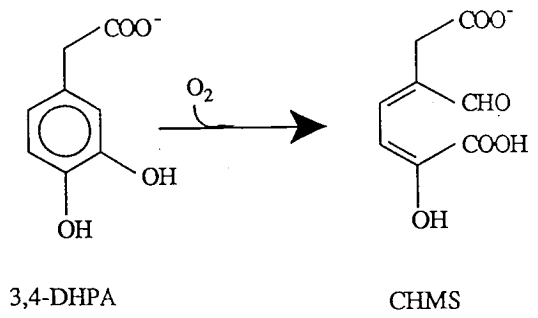

FIG. 1. Reaction catalyzed by the manganese-dependent 3,4-DHPA 2,3-dioxygenase from *A. globiformis* CM-2. CHMS, 5-carboxymethyl-2-hydroxymuconic semialdhyde.

evolutionarily related to the corresponding TOL plasmid genes and that MndD, which shares 21% amino acid identity with the TOL plasmid catechol 2,3-dioxygenase, XylE, is the most divergent of the three.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. globiformis* CM-2 cells (34) were grown on minimal media as described previously (40). *Escherichia coli* DH5α cells were grown on Luria-Bertani medium (28) or M9 minimal medium (28). Tetracycline (30 μg/ml) and kanamycin (30 μg/ml) were added as required.

Enzyme assay and product identification. 3,4-DHPA 2,3-dioxygenase activity was assayed by measuring the rate of formation of 5-carboxymethyl-2-hydroxymuconic semialdehyde at 380 nm (40). The reaction mixture (250 μl) contained 140 μM 3,4-DHPA in 50 mM phosphate buffer (pH 8.0). The reaction was started by adding 0.1 to 0.4 mg of crude cell extract protein from *E. coli* DH5α(pYB2). The specific activity was calculated with an extinction coefficient of 36 mM^{-1} cm^{-1} (40). For inhibitor studies, $FeSO_4$ (1 mM) was added immediately prior to initiation of the enzyme assay, KCN (1 mM) was preincubated with crude extract for 10 min, or H_2O_2 (1 mM) was preincubated with crude extracts for 20 min. All preincubations were at 4°C. The identities of the ring cleavage products from 4-methylcatechol and 3,4-DHPA were confirmed by their spectral properties as described previously (4, 35).

Genomic library construction. *A. globiformis* CM-2 genomic DNA was isolated by the method of Dabbs and Sole (12), except that 10% sodium lauroyl sarcosinate was substituted for 10% sodium dodecyl sulfate (SDS). The DNA was purified on a CsCl gradient (28). Genomic DNA was partially digested with *Hin*dIII and size selected on a sucrose gradient (28). Fragments of 18 to 23 kb were ligated to *Hin*dIII-digested cosmid vector pVK102 (27). Ligated DNA was packaged in vitro with the Packagene DNA packaging system (Promega, Madison, Wis.). *E. coli* DH5α cells were transfected with the packaging mix, and colonies were selected on Luria-Bertani medium containing tetracycline. Clones containing genomic inserts were identified by kanamycin sensitivity. The final library contained 1,942 clones.

Library screening. The entire library was replica plated onto Luria-Bertani medium with tetracycline and onto M9 minimal medium containing 0.1% (wt/vol) 4-hydroxyphenylacetic acid, 30 μg of tetracycline per ml, and 55 μM $MnSO_4$. Both replicates of the library were sprayed with a 2% solution of 4-methylcatechol in ethyl ether. Colonies expressing 3,4-DHPA 2,3-dioxygenase activity turned yellow because of the accumulation of 2-hydroxy-5-methylmuconic semialdehyde.

DNA manipulations. Subcloning, Southern blotting, and probing were done according to standard procedures (28). Transformation of *E. coli* DH5α was performed according to the method of Hanahan (18).

Western blot (immunoblot) analysis. SDS-polyacrylamide gel electrophoresis and Western blotting were performed with the Pharmacia PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden). Primary and secondary antibody hybridizations were performed as previously described (34).

DNA sequencing. DNA sequencing was done by the dideoxy chain termination method of Sanger et al. (42) with double-stranded DNA template and the Sequenase version 2.0 sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio).

Sequence analysis. The Molecular Biology Computer Center in the College of Biological Sciences, University of Minnesota was the computational resource. The GCG sequence analysis software package (Genetics Computer Group, Inc., Madison, Wis.) was used for all DNA and protein sequence comparisons. DNA sequences were scanned for promoters by the method of Staden (45).

RESULTS

Properties of 3,4-DHPA 2,3-dioxygenase from *A. globiformis* CM-2. We previously reported an immunologically unique 3,4-DHPA 2,3-dioxygenase from *Arthrobacter* sp. strain Mn-1 (34). Qi (39) demonstrated that the activity of the 3,4-DHPA 2,3-dioxygenase is manganese dependent. Polyclonal antibodies raised against this manganese-dependent dioxygenase from *Arthrobacter* sp. strain Mn-1 cross-react with a protein from *A. globiformis* CM-2 (34). Immunoprecipitation experiments with these antibodies resulted in a loss of >95% of the 3,4-DHPA 2,3-dioxygenase activity from *A. globiformis* CM-2 crude extracts (34). To determine the extent of similarity between the 3,4-DHPA 2,3-dioxygenase from *A. globiformis* CM-2 and the manganese-dependent dioxygenase from *Arthrobacter* sp. strain Mn-1, we determined the kinetic properties for the dioxygenase from *A. globiformis* CM-2. The apparent K_m of 3,4-DHPA 2,3-dioxygenase from *A. globiformis* CM-2 of 19 μM is similar to that for the purified manganese-dependent dioxygenase from *Arthrobacter* sp. strain Mn-1 (14 μM) (39). The oxidizing reagent H_2O_2 (1 mM) inhibited 3,4-DHPA 2,3-dioxygenase activity by 49%. The metal binding reagent CN^- (1 mM) inhibited activity by 38%, and ferrous ions (1 mM), which activate iron-dependent extradiol dioxygenases, inhibited activity by 40%. These properties are similar to the behaviors of two other manganese-dependent dioxygenases (Table 1) and are consistent with the dependence of the *A. globiformis* CM-2 3,4-DHPA 2,3-dioxygenase catalytic activity on manganese, which has recently been demonstrated with purified enzyme (46).

Cloning of the 3,4-DHPA 2,3-dioxygenase from *A. globiformis* CM-2. We cloned and sequenced the manganese-depen-

TABLE 1. Effectors of extradiol dioxygenase enzyme activity

Enzyme and source	Catalytic metal	Fe^{2+} activation[a]	H_2O_2 inactivation[b]	Reference
3,4-DHPA 2,3-dioxygenase				
Arthrobacter globiformis CM-2[c]	Mn^{2+}	No	No	This paper
Arthrobacter sp. strain MN-1	Mn^{2+}	No	No	39
Bacillus brevis	Mn^{2+}	No	No	40
Protocatechuate 2,3-dioxygenase, *Bacillus macerans*	Fe^{2+}	Yes	Yes	48
3,4-DHPA 2,3-dioxygenase, *Pseudomonas ovalis*	Fe^{2+}	Yes	Yes	26, 35
Catechol 2,3-dioxygenase, *Pseudomonas arvilla*	Fe^{2+}	Yes	Yes	33

[a] The *Arthrobacter* dioxygenases exhibited >40% inhibition with 0.1 mM Fe^{2+}.
[b] The *Arthrobacter* dioxygenases exhibited <50% inhibition with 1 mM hydrogen peroxide. The *Bacillus* dioxygenase exhibited no inhibition with 10 mM hydrogen peroxide. The iron-dependent dioxygenases were inactivated by 1 mM hydrogen peroxide.
[c] Experiments were performed with MndD from *E. coli* DH5α(pYB2).

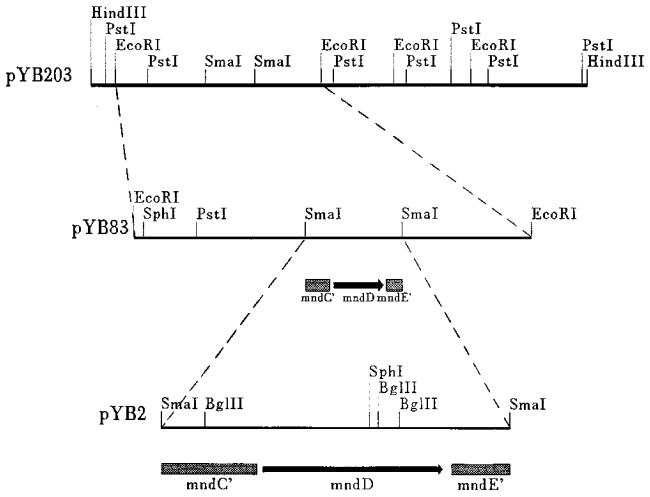

FIG. 2. Physical relationship of clones expressing manganese-dependent 3,4-DHPA 2,3-dioxygenase activity. Cosmid pYB203 is a 20.3-kb *Hin*dIII fragment in pVK102. Plasmid pYB83 is an 8.3-kb *Eco*RI fragment from pYB203 cloned into pUC18. Plasmid pYB2 is a 2.0-kb *Sma*I fragment from pYB83 cloned into pUC18. All three clones express MndD activity in *E. coli* DH5α.

dent extradiol dioxygenase from *A. globiformis* CM-2 in order to determine its relationship with the far more common iron-dependent extradiol dioxygenases. Our cloning strategy was based on the ability to identify 2,3-dioxygenase activity in *A. globiformis* CM-2 by the accumulation of a yellow ring fission product (verified below) after spraying colonies with 4-methylcatechol. Similar screening procedures have been used to clone other extradiol dioxygenases (17, 20, 25, 31).

To construct an *A. globiformis* CM-2 genomic library, *A. globiformis* CM-2 total genomic DNA was partially digested with *Hin*dIII, ligated to the cosmid vector pVK102, packaged in vitro, and used to transfect *E. coli* DH5α. The completed library contained 1,942 Tet^r Kan^s clones. To identify 3,4-DHPA 2,3-dioxygenase-expressing clones, the library was sprayed with 4-methylcatechol. Fourteen yellow colonies were identified. All 14 clones contained the same 20.3-kb *Hin*dIII fragment, and one of these cosmid clones, pYB203, was used for subsequent analysis (Fig. 2).

Subcloning of *mndD*. To more precisely determine the region of pYB203 encoding the manganese-dependent dioxygenase activity, pYB203 was digested with *Eco*RI and the mixture was ligated into *Eco*RI-digested pUC18 (32). A 3,4-DHPA

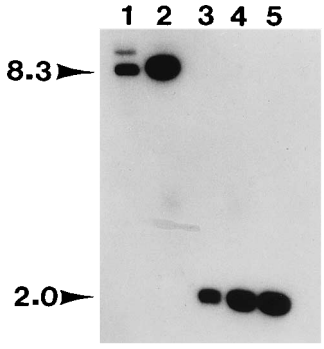

FIG. 3. Southern hybridization analysis of *A. globiformis* CM-2 genomic DNA and *mndD*-containing clones with the 2.0-kb *Sma*I fragment of pYB2 as probe. Lanes: 1, *Eco*RI genomic digest; 2, *Eco*RI digest of pYB83; 3, *Sma*I genomic digest; 4, *Sma*I digest of pYB83; 5, *Sma*I digest of pYB2.

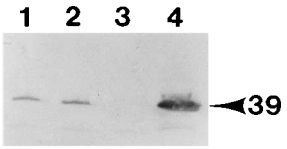

FIG. 4. Western blot analysis of *E. coli* DH5α clones expressing *mndD* by using anti-manganese-dependent dioxygenase polyclonal antibodies raised against purified protein from *Arthrobacter* sp. strain Mn-1. Lanes: 1, crude extract from *E. coli* DH5α(pYB2); 2, crude extract from *E. coli* DH5α(pYB83); 3, crude extract from *E. coli* DH5α(pUC18); 4, purified manganese-dependent 3,4-DHPA 2,3-dioxygenase.

2,3-dioxygenase-expressing subclone was identified by spraying recombinant colonies with 4-methylcatechol. This subclone, pYB83 (Fig. 2), contained an 8.3-kb *Eco*RI fragment. Plasmid pYB83 was further subcloned by digestion with *Sma*I, followed by ligation into *Sma*I-digested pUC18. Again, 3,4-DHPA 2,3-dioxygenase-expressing subclones were identified by spraying recombinant clones with 4-methylcatechol. This strategy led to the isolation of pYB2 (Fig. 2), which contained a 2.0-kb *Sma*I fragment in pUC18. Colonies containing pYB2 became bright yellow in less than 30 s after being sprayed with 4-methylcatechol. This high level of 3,4-DHPA 2,3-dioxygenase activity is due to gene expression from the pUC18 *lacZ* promoter. Plasmid pYB22, which contained the 2.0-kb *Sma*I fragment in the reverse orientation, did not express 3,4-DHPA 2,3-dioxygenase activity (data not shown). The 2.0-kb *Sma*I fragment of pYB2 was used as a hybridization probe to *Sma*I-digested and *Eco*RI-digested *A. globiformis* CM-2 genomic DNA. The probe hybridized to a 2.0-kb *Sma*I genomic DNA fragment and an 8.3-kb *Eco*RI genomic DNA fragment (Fig. 3), indicating that the cloned 2.0-kb *Sma*I fragment came from *A. globiformis* CM-2.

Verification of 3,4-DHPA 2,3-dioxygenase expression in *E. coli* DH5α. Anti-manganese-dependent dioxygenase polyclonal antibodies (34) were used in Western blot analysis of crude

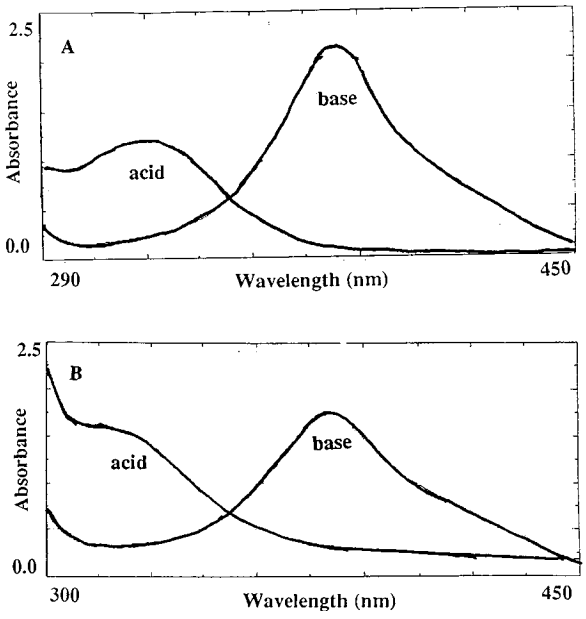

FIG. 5. Absorption spectra of products resulting from extradiol ring cleavage of 3,4-DHPA (A) and 4-methylcatechol (B) in crude extracts of *E. coli* DH5α(pYB2).

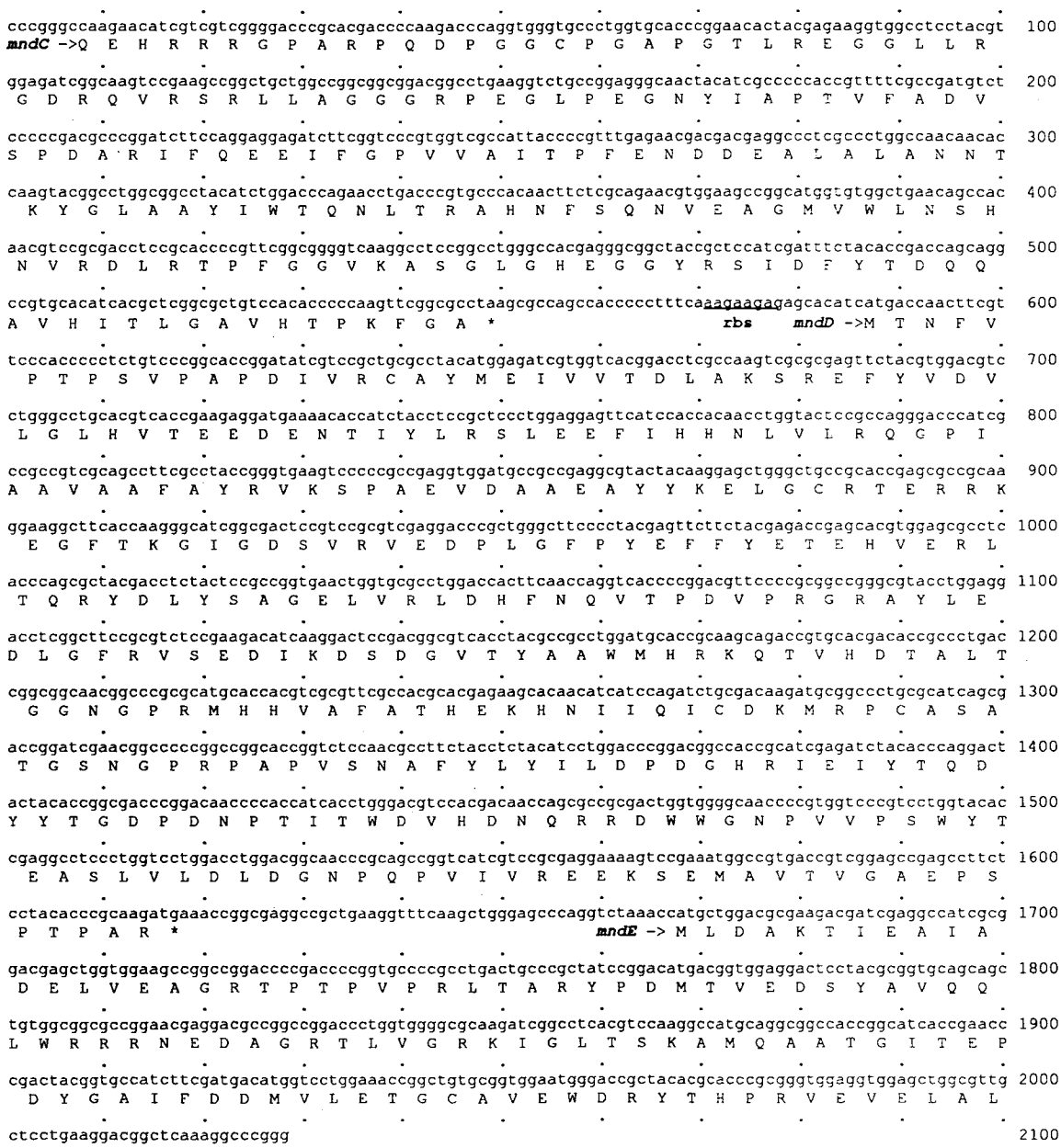

FIG. 6. DNA sequence of the 2.0-kb *Sma*I fragment carrying the manganese-dependent dioxygenase gene, *mndD*. A potential ribosome binding site (rbs) is underlined. The deduced amino acid sequence for MndD is shown. Partial DNA and deduced amino acid sequences are shown for *mndC'*, a putative semialdehyde dehydrogenase gene, and *mndE'*, a putative 2-hydroxy-pent-2,4-dienoate hydratase gene.

extract from *E. coli* DH5α clones containing pYB83, pYB2, or pUC18 (Fig. 4). The anti-manganese-dependent dioxygenase polyclonal antibodies reacted with a protein product in the pYB2- and pYB83-containing clones that was the same size (39 kDa) as the purified manganese-dependent dioxygenase.

The appearance of a yellow product when 3,4-DHPA or 4-methylcatechol is incubated with crude extracts from *E. coli* DH5α(pYB2) is consistent with the production of muconic semialdehydes via extradiol ring cleavage. The identity of these extradiol ring cleavage products was verified by their spectral properties (Fig. 5). The observed product with 3,4-DHPA had a λ_{max} at 379 nm at pH 8.0 and above and a λ_{max} at 321 nm below pH 4. This is consistent with what has been previously reported for 5-carboxymethyl-2-hydroxymuconic semialdehyde (1). The observed product with 4-methylcatechol had a λ_{max} at 382 nm at pH 8.0 and above and a λ_{max} at 320 nm below pH 4. This is consistent with what has been previously reported for 2-hydroxy-5-methylmuconic semialdehyde (4). Crude extract from *E. coli* DH5α containing only the vector pUC18 did not yield a yellow product in the presence of 3,4-DHPA or 4-methylcatechol (data not shown).

Sequence analysis of *mndD*. The DNA and deduced amino acid sequences of the 2.0-kb *Sma*I fragment are shown in Fig. 6. A single open reading frame of 1,032 bases for the 3,4-

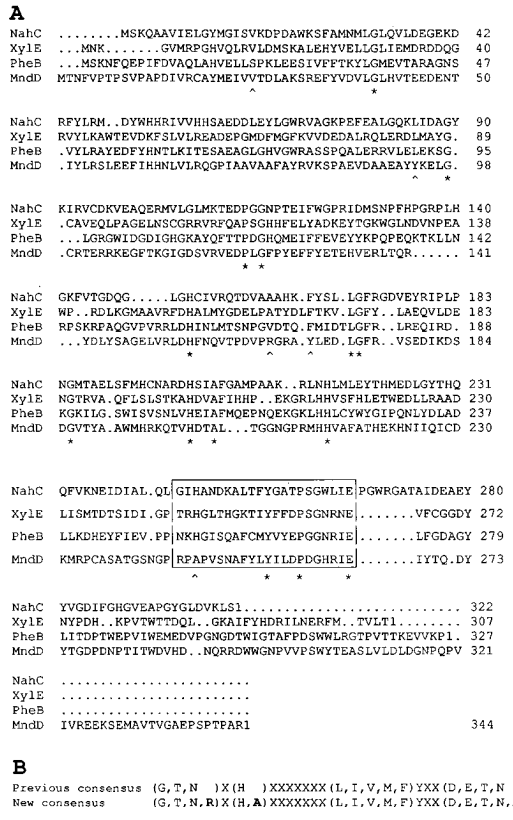

FIG. 7. (A) Sequence alignment of four extradiol dioxygenases. NahC, napthalene dioxygenase from *P. putida*; XylE, catechol 2,3-dioxygenase from *P. putida* mt-2; PheB, catechol 2,3-dioxygenase from *B. stearothermophilus*; MndD, manganese-dependent dioxygenase from *A. globiformis* CM-2. Asterisks indicate amino acids conserved in all 14 members of the extradiol dioxygenase family; ^ indicates amino acids conserved in all extradiol family members except PheB and/or MndD. The box indicates residues which form a consensus sequence. (B) Primary consensus sequence for extradiol dioxygenase family. The top sequence, designed by Harayama and Rekik (20), fits 12 original members of the extradiol dioxygenase family. The revised consensus sequence is shown at the bottom, with the additions given in boldface. Numbers in brackets indicate a gap of two or three residues at this point in the sequence.

DHPA 2,3-dioxygenase was identified by homology to the N-terminal amino acid sequence obtained from analysis of purified 3,4-DHPA 2,3-dioxygenase from *Arthrobacter* sp. strain Mn-1 (39). A potential ribosome binding site is present 10 bases upstream of the putative translation start site. The DNA sequence upstream of the putative translation start for *mndD* did not show similarities to previously identified *E. coli* promoter sequences by the method of Staden (45).

Amino acid sequence comparisons indicate that the manganese-dependent 3,4-DHPA 2,3-dioxygenase from *A. globiformis* CM-2, MndD, belongs to the major family of extradiol ring cleavage dioxygenases. MndD shares 78% (14 of 18) of the amino acid residues previously identified by Hofer et al. (23) as being conserved in this family (Fig. 7A). The dendrogram in Fig. 8 shows the relationship of MndD with other extradiol dioxygenases. This tree shows that MndD is more similar to the subfamily of extradiol dioxygenases that act on single-ring substrates than it is to those that act on multiring substrates and that catechol 2,3-dioxygenases from *Pseudomonas* spp. are more similar to MndD than they are to biphenyl extradiol dioxygenases from *Pseudomonas* spp. Furthermore, MndD is more similar to most of the dioxygenases shown than is McpII from *Alcaligenes eutrophus*, which had previously been assigned to this family (23).

MndD also contains identical residues in six of the eight positions of the primary consensus sequence for extradiol dioxygenases developed by Harayama and Rekik (21) (Fig. 7B). A revised primary consensus sequence pattern is proposed (Fig. 7B). This revised pattern is found in all 12 previously known members of the extradiol dioxygenase family (23), MndD from *A. globiformis* CM-2, the *Bacillus stearothermophilus* catechol 2,3-dioxygenase (accession no. S25157; PIR 40), and at least four more recently reported extradiol dioxygenases (2, 10, 19, 49). No other sequences in the 67,423-member PIR 40 database or the 36,000-member Swiss-Prot 28 database match this revised pattern, giving 100% sensitivity and specificity. MndD shares the greatest amino acid sequence identity with catechol 2,3-dioxygenases, with 27.6% identity to Cdo from *Rhodococcus rhodochrous* CTM (10), 25.4% identity to McpII from *A. eutrophus* (accession no. DAAL2E; PIR 40), 24.3% identity to PheB from *B. stearothermophilus*, and 22.3% identity to XylE from *P. putida* mt-2 (30). MndD does not show any significant sequence identity to HpcB, a 3,4-DHPA 2,3-dioxygenase from *E. coli* C that does not belong to this extradiol dioxygenase family (11).

Sequence analysis of *mndC* and *mndE*. Nucleotide sequence analysis suggested that there were two other putative genes located on pYB2. We have named these *mndC* and *mndE* (Fig. 6); these show very strong homology to semialdehyde dehydrogenases and hydratases, respectively. The deduced amino acid sequence for *mndE* begins with the ATG at position 1667, 50 bases downstream of the *mndD* TGA stop codon (Fig. 6), and it shows strong identity to 2-hydroxy-pent-2,4-dienoate hydratases (Table 2). The putative reading frame for *mndC* was identified by translating the nucleotide sequence upstream of the *mndD* putative start site in all three reading frames, followed by a homology search with each deduced amino acid sequence. The deduced amino acid sequence shown in Fig. 6, which ends 36 bases upstream of the *mndD* putative start codon, is the only reading frame that had strong homology to any known protein and, like the DNA sequence, was homologous to semialdehyde dehydrogenases. Both *mndC* and *mndE* represent enzymes that are utilized in an aromatic extradiol degradation pathway (7, 9, 41, 44). On the basis of the previously reported sizes of *xylG* and *xylJ* from the TOL plasmid (24), the available sequences for *mndC* and *mndE* comprise 44 and 54%, respectively, of the predicted amino acid sequence for these genes. The partial sequences for *mndC* and *mndE* share significantly higher levels of homology at both the DNA and protein levels with *xylG* and *xylJ*, respectively, than does *mndD* with the sequence for the TOL iron-dependent 2,3-dioxygenase *xylE* (Table 2).

DISCUSSION

MndD exhibits biochemical properties that distinguish it from the iron-dependent extradiol dioxygenases. Table 1 shows a comparison of three manganese-dependent extradiol dioxygenases with three iron-dependent extradiol dioxygenases. The difference in catalytic metal centers correlates with differences in kinetic properties. The manganese-dependent extradiol dioxygenases are only partially inhibited by the oxidizing agent H_2O_2, while iron-dependent extradiol dioxygenases are rapidly inactivated. Moreover, ferrous ions, which activate the iron-dependent extradiol dioxygenases, inhibit the manganese-dependent extradiol dioxygenases.

The use of manganese in the place of iron as a catalytic metal center and the corresponding differences in biochemical

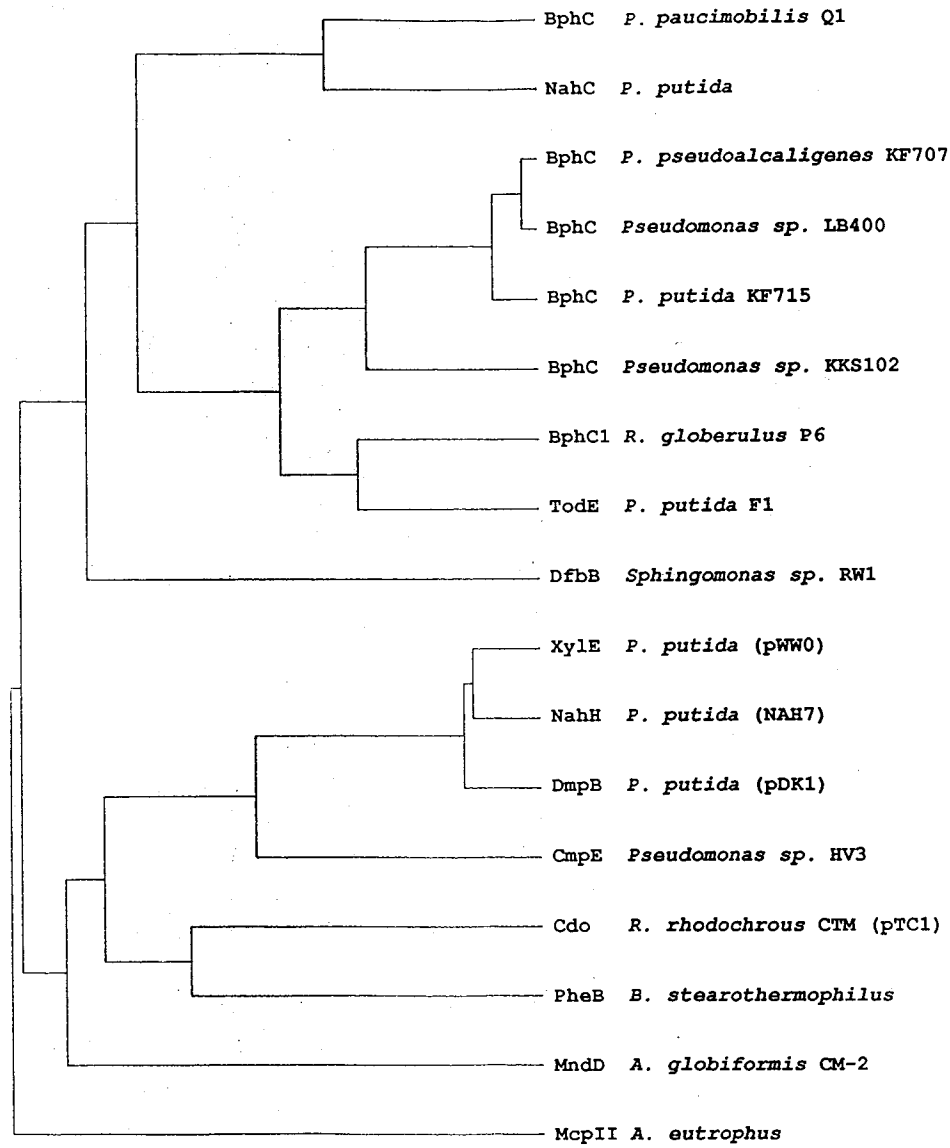

FIG. 8. Dendrogram based on pairwise alignments of 17 extradiol dioxygenases. Nonitalicized accession numbers (in parentheses) refer to the PIR protein database, and italicized accession numbers (in parentheses) refer to the GenBank and EMBL DNA databases: 2,3-dihydroxybiphenyl 1,2-dioxygenases, BphC from *P. paucimobilis* Q1 (A28718), *P. pseudoalcaligenes* KF707 (G42409), *Pseudomonas* sp. strain LB400 (JN0815), *P. putida* KF715 (B35124), and *Pseudomonas* sp. strain KKS102 (DAPSPC); BphC1, *R. globerulus* P6 (*X75633*); 1,2-dihydroxynaphthalene dioxygenase, NahC from *P. putida* (A34343); 3-methylcatechol dioxygenase, TodE from *P. putida* F1 (F36516); 2,2′,3-trihydroxybiphenyl dioxygenase, DfbB from *Sphingomonas* sp. strain RW1 (*X72850*); catechol 2,3-dioxygenase, XylE from *P. putida* pWW0 (A20852), NahH from *P. putida* NAH7 (A27389), DmpB from *P. putida* pDK1 (A42733), CmpE from *Pseudomonas* sp. HV3 (*L10655*), Cdo from *R. rhodochrous* CTM(pTC1) (*X69504*), PheB from *B. stearothermophilus* (S25157), MndD from *A. globiformis* CM-2 (U19817), and McpII from *A. eutrophus* (DAAL2E).

properties, along with the observation that the extradiol dioxygenases from numerous *Arthrobacter* species are immunologically distinct from other dioxygenases (34), suggested that MndD might represent a distinct family of bacterial extradiol dioxygenases. However, the overall amino acid identity between MndD and the other iron-dependent extradiol dioxygenases is as high as 28%, and pairwise amino acid sequence alignments place MndD within the single-ring substrate subfamily of extradiol dioxygenases (Fig. 8). Furthermore, the conservation of 78% of the amino acid residues previously identified as conserved in members of the major extradiol iron-dependent dioxygenase family and the presence of the primary consensus sequence for extradiol dioxygenases (discussed below) clearly place MndD in this family.

There are proposed to be three families of extradiol dioxygenases (2), and HpcB from *E. coli* C, which is not homologous to any other known extradiol dioxygenases (11), may represent a fourth. Three of these four families contain only one or two members. The majority of extradiol dioxygenases (>20) belong to the remaining family, which is identified by the consensus pattern originally proposed by Harayama and Rekik (21) and modified in this study. This pattern is located in a region of the enzyme that contains both a high concentration of completely conserved residues and minimal gaps or insertions in multiple sequence alignments and is thus expected to be functionally and/or structurally significant. Our revision of this pattern is less restrictive than the original pattern (Fig. 7B). It adds one amino acid to the first, second, and fifth pattern positions.

TABLE 2. Percentage of identity between *mndD*, *mndC′*, and *mndE′* with their corresponding genes from the TOL plasmid

Gene	Sequence compared	% of identity	
		DNA	Protein
mndD	Dioxygenase (*xylE*)	46	21
mndE′	Dehydrogenase (*xylG*)	60	46
mndC′	Hydratase (*xylJ*)	57	37

However, even with a decrease in constraint, the only additional sequences selected out of more than 67,000 are those of MndD and four recently reported extradiol dioxygenases (2, 11, 19, 49). The least restrictive pattern that retains 100% sensitivity and specificity is preferred; thus, our revised pattern is an improvement over the original extradiol dioxygenase pattern.

Our observation that the open reading frame comprising *mndD* has a ribosome binding site but no −10 or −35 promoter-like sequences upstream of *mndD* suggests that this gene may be part of an operon. The observation of the partial sequences *mndC* and *mndE*, which closely flank *mndD* and encode other enzymes expected in a 3,4-DHPA meta-cleavage degradation pathway, further supports this hypothesis. All three sequences, *mndCDE*, bear significant homology with their respective genes from the *P. putida* mt-2 TOL plasmid, pWW0. One possible explanation for this similarity is horizontal gene transfer of the entire 3,4-DHPA degradation pathway. Transfer of the TOL plasmid, which encodes transposon sequences and functions, is well known (9, 41), and gene transfer between gram-negative and gram-positive organisms has been observed (5, 29, 47).

Both the DNA and amino acid sequences of *mndC* and *mndE* exhibit a 20% higher level of identity with their respective counterparts on the TOL plasmid. *xylG* and *xylJ*, than does *mndD* to the TOL plasmid iron-dependent dioxygenase *xylE* (Table 1). This is not surprising given that the two dioxygenases use different metals for catalytic activity. Although it is difficult to statistically prove the evolutionary relationship between the manganese- and iron-dependent extradiol dioxygenases, the 78% identity with 18 residues conserved in many iron-dependent extradiol dioxygenases, the overall amino acid sequence similarity that places MndD within the single-ring substrate subfamily of extradiol dioxygenases (Fig. 8), the observation that MndD fits an extradiol dioxygenase consensus pattern, and the contiguous *mndCDE* DNA sequence lead us to propose a common ancestor for manganese- and iron-dependent extradiol dioxygenases. In our opinion, this is more likely than convergent evolution of a gene for a manganese-dependent extradiol dioxygenase, which for mechanistic or structural reasons contains the same residues conserved in the iron-dependent enzymes, and its insertion between *mndC* and *mndE*.

Recently, the first structure of an extradiol dioxygenase family member was determined for BphC from *Pseudomonas* sp. strain LB400 (8). Previously, sequence alignments had suggested six possible amino acids as potential metal ligands (2, 20, 23). It is of interest that the metal ligands identified for BphC, H-146, H-210, and E-260, align with amino acids H-155, H-214, and E-267 in MndD. Some or all of these corresponding amino acids may also coordinate manganese in MndD, because the same amino acids are responsible for ligating either manganese or iron in the superoxide dismutases (36).

We conclude that the manganese-dependent extradiol dioxygenase, MndD, belongs to the extradiol dioxygenase superfamily, propose a revised primary consensus sequence for this family, and propose that MndD shares a common ancestor with iron-dependent extradiol dioxygenases. We hypothesize that the amino acids H-155, H-200, H-214, Y-256, and E-267 of MndD are the most likely candidates for metal ligation. Future work will test this hypothesis.

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