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Characterization of the Gene Encoding Catechol 2,3-Dioxygenase from *Achromobacter xylosoxidans* KF701

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Catechol 2,3-dioxygenase (C23O) catalyzes a *meta* cleavage of the aromatic ring in catechol to form 2-hydroxymuconic semialdehyde. A C23O gene was cloned from chromosomal DNA of *A. xylosoxidans* KF701, a soil bacterium degrading biphenyl, and expressed in *E. coli* HB101. In substrate specificity to catechol and its analogs, the C23O exhibited the highest aromatic ring-fission activity to catechol, and its relative activity to other dihydroxylated aromatics was 4-chlorocatechol > 4-methylcatechol > 3-methylcatechol ≫ 2,3-dihydroxybiphenyl. Aromatic ring-fission activity of the C23O to catechol was about 40-fold higher than that to 2,3-dihydroxybiphenyl. Nucleotide sequence analysis of the C23O gene from *A. xylosoxidans* KF701 revealed an open reading frame consisting of 924 base pairs, and identified a putative ribosome-binding sequence (AGGTGA) at about 10 nucleotides upstream from the initiation codon. The open reading frame can encode a polypeptide chain with molecular weight of 34 kDa containing 307 amino acid residues. The deduced amino acid sequence of the C23O exhibited the highest homology with that of C23O from *Pseudomonas* sp. IC with 96% identity, and the least homology with that of C23O from *P. putida* F1 with 22% identity among reported C23O sequences. Furthermore, comparison of the C23O sequence with other extradiol dioxygenases has led to identification of evolutionally conserved amino acid residues whose possible catalytic and structural roles are proposed.

Thermal and chemical stability of aromatic compounds has led them to be persistent and thus accumulated to be environmental pollutants. One of attractive means to remove them from the environment is microbial degradation. Several soil microorganisms have been isolated to degrade a variety of natural and synthetic aromatic compounds. In the microbial degradation of aromatic compounds, monocyclic compounds are converted to catechol intermediates and polycyclic compounds to dihydroxyaromatics with two hydroxy substituents to adjacent aromatic carbons in one of the aromatic rings [1]. Aromatic ring-fissions of catechol intermediates and the dihydroxyaromatics are catalyzed by dioxygenases which incorporate both atoms of dioxygen into the substrates. The dioxygenases are broadly classified as intradiol or extradiol dioxygenase according to the site of ring cleavage relative to the dihydroxy groups of the substrate. Intradiol dioxygenase opens the aromatic ring by cleavage between hydroxylated two carbons, and extradiol dioxygenase cleaves between the hydroxylated carbon and adjacent nonhydroxylated carbon in aromatic ring [2]. Catechol 2,3-dioxygenase (C23O), one of the extradiol dioxygenases, catalyzes the conversion of catechol to 2-hydroxymuconic semialdehyde (Fig. 1), which plays important roles in dissimilation of catechol intermediates in the microbial degradation of monocyclic and polycyclic compounds.

Achromobacter xylosoxidans KF701 was isolated from soil as a degrader of biphenyl [3]. Since firstly reported in 1966 on environmental contamination of chlorinated biphenyls, a number of microorganisms have been isolated to utilize the chlorinated biphenyls as the sole carbon and energy source. Catabolic pathways of chlorinated biphenyls by microorganisms are initiated to convert them to corresponding benzoate by sequential reactions of biphenyl dioxygenase, dihydrodiol dehydrogenase, 2,3-dihydroxybiphenyl dioxygenase, and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase. Several species of the microorganisms

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Abbreviations used: C23O, catechol 2,3-dioxygenase; ORF, open reading frame; SDS, sodium dodecyl sulfate; PEG, polyethylene glycol; Ap, ampicillin; Tc, tetracycline.

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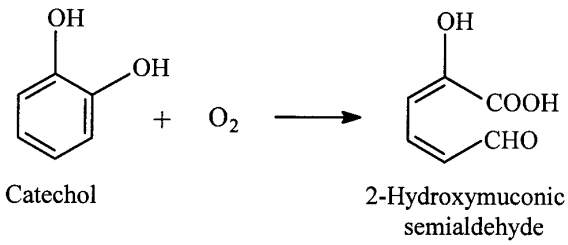

FIG. 1. The chemical reaction catalyzed by C23O.

are extensively studied for biochemical and genetic properties of enzymes involving in catabolic pathways of chlorinated biphenyls. However, *A. xylosoxidan* KF701 has not been characterized on the catabolism of aromatic compounds at molecular level.

In this study, a C23O gene has been cloned from chromosomal DNA of *A. xylosoxidans* KF701, expressed in *E. coli* HB101, and its nucleotide sequence has been determined.

MATERIALS AND METHODS

Strains and plasmids. Bacterial strains and plasmids used and prepared in this study are listed and described in Table 1. *A. xylosoxidans* KF701 is a soil bacterium which can grow in biphenyl as the sole carbon and energy source [3]. The strain was grown in LB medium or MMO medium containing 0.1% biphenyl. *E. coli* HB101 and *E. coli* JM101 were used as the recipient strains of recombinant plamsids and grown in LB medium or 2 × YT medium. For antibiotic selections, ampicillin or tetracycline was supplemented to the medium with 50 μg/ml or 15 μg/ml as a final concentration [4]. Plasmids of pBR322 and pUC18 were used as clonging vectors, and M13mp18 and M13mp19 as sequencing vectors.

Recombinant DNA techniques and sequencing. Plasmid was isolated by the alkali lysis method, and chromosomal DNA was isolated from *A. xylosoxidans* KF701 by the SDS-proteinase K lysis method [5,6]. DNA cleavage with restriction endonuclease and ligation of DNA fragments with T_4 DNA ligase were performed under standard conditions recommended by the supplier (Boehringer Mannheim). DNA was resolved on 0.7% or 1% agarose gel with TAE buffer by electrophoresis, stained with ethidium bromide, and visualized by UV irradiation [4]. Transformation was accomplished by the calcium chloride method [4]. For nucleotide sequencing, single-stranded DNA with M13mp18 or M13mp19 was subjected to dideoxy chain termination with T_7 DNA polymerase and [^{35}S]dATP. This sequencing mixture was resolved on 50% urea-6% polyacrylamide gel with TBE buffer by electrophoresis [4]. This gel was washed with 5% acetic acid-20% methanol, dried by heating under vacuum, and exposed to X-ray film for autoradiography. Nucleotide sequences obtained were analyzed by using several softwares of DNASIS, PROSIS, and Clustal V.

Assay of C23O activity. *E. coli* HB101 harboring each of recombinant plasmids was grown in LB medium supplemented with ampicillin or tetracycline to a log phase, harvested by centrifugation at 6,500 × g for 10 min, and washed once with 50 mM phosphate buffer (pH 7.5). The bacterial pellet was resuspended in the same buffer, and then sonicated with a cell disruptor followed by centrifugation at 12,300 × g for 1 hr to obtain supernatant as an enzyme source. C23O activity was spectrophotometrically measured in 50 mM phosphate buffer (pH 7.5) containing 0.5 mM catechol or dihydroxyaromatics as the substrate. One unit of the enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate to *meta* cleavage compound per minute. The molar extinction coefficient (ϵ) of 2-hydroxymuconic semialdehyde formed from catechol was taken as 33,000 M^{-1} cm^{-1} at a maximal wavelength (λ_{max}) of 375 nm [7]. Ring-fission activity of C23O to substrate analogs was determined by using extinction coefficient of respective *meta* cleavage compound fromed from each of following substrates: 3-methylcatechol (ϵ = 13,400 M^{-1} cm^{-1} at λ_{max} = 388 nm), 4-methylcatechol (ϵ = 28,000 M^{-1} cm^{-1} at λ_{max} = 382 nm), 4-chlorocatechol (ϵ = 39,600 M^{-1} cm^{-1} at λ_{max} = 379 nm), and 2,3-dihydroxybiphenyl (ϵ = 22,000 M^{-1} cm^{-1} at λ_{max} = 434 nm) [7]. Specific activity of the enzyme was defined as unit(s) per

TABLE 1

Bacterial Strains and Plasmids Used and Prepared in This Study: Antibiotic Resistance to Ampicillin (Ap^r) or Tetracycline (Tc^r)

Strain or plasmid	Description
Strains	
E. coli HB101	*supE*44 *hsdS*58 ($r_B^-m_B^-$) *rec*A13 *ara*-14 *proA2 lacY*1 *galK2 rpsL*20 *xyl*-5 *mtl*-1
E. coli JM101	*supE thi D* (*lac-proAB*) F′ (*traD*36 *proAB*$^+$*lacIq lacZ*ΔM15)
Achromobacter xylosoxidans KF701	A soil bacterium which can grow in biphenyl as the sole carbon source
Plasmids	
pBR322	Cloning vector, Ap^r and Tc^r
pUC18	Cloning vector, Ap^r
pCNU201	Plasmid clone selected from a genomic library of *Achromobacter xylosoxidans* KF701, a 10-kb *Bam*HI fragment of the KF701 inserted into the *Bam*HI site of pBR322, Ap^r
pCNU202	A 10-kb *Bam*HI fragment of pCNU201 inserted into the *Bam*HI site of pUC18, Ap^r
pCNU203	A 3.1-kb *Kpn*I fragment of pCNU202 inserted into *Kpn*I site of pUC18, Ap^r
pCNU204	A 6.9-kb *Bam*HI-*Kpn*I fragment of pCNU202 inserted into the *Bam*HI and *Kpn*I sites of pUC18, Ap^r
pCNU205	An 1.8-kb *Bam*HI-*Xho*I fragment of pCNU202 inserted into the *Bam*HI and *Sal*I sites of pUC18, Ap^r
pCNU207	An 1.1-kb *Pst*I-*Stu*I fragment of pCNU205 inserted into the *Pst*I and *Sma*I sites of pUC18, Ap^r
pCNU218	A 4.7-kb *Eco*RI fragment of pCNU202 inserted into the *Eco*RI site of pBR322, Ap^r and Tc^r
pCNU252	An 8.7-kb *Bam*HI fragment, a deletion derivative of pCNU218 lacking a *Bam*HI fragment, Ap^r

mg of proteins, where protein concentration was determined by the Lowry method [8].

RESULTS AND DISCUSSION

A. xylosoxidans KF701 was previously known to grow in biphenyl, 4-methylbiphenyl, 2-hydroxybiphenyl, benzoate or salicylate but not in 4-chlorobiphenyl, 2-bromobiphenyl, 2-nitrobiphenyl, diphenylmethane or *m*-toluate as the sole carbon and energy source [3]. However, biochemical and genetic properties of the catabolic enzymes have not been studied at all. In this study a C23O gene was cloned from chromosomal DNA of *A. xylosoxidans* KF701, expressed in *E. coli* HB101, and its nucleotide sequence was determined.

Cloning and location of the C23O gene. A genomic library of *A. xylosoxidans* KF701 was constructed by ligation of partially *Bam*HI-digested chromosomal DNA into the same endonuclease site of pBR322 vector followed by transformation into *E. coli* HB101. The genomic library was screened for the ability to be yellow colored by formation of 2-hydroxymuconic semialdehyde from colorless catechol, as indicative of C23O activity. A positive clone exhibiting ampicillin resistance and C23O activity by catechol spray test was selected. From the positive clone, a recombinant plasmid with a 10-kb *Bam*HI fragment of *A. xylosoxidans* KF701 inserted into the same endonuclease site of pBR322 vector was extracted and designated as pCNU201. A detailed physical map of pCNU201 was constructed by conventional single and multiple digestions, and is shown in Fig. 2. The 10-kb fragment from *A. xylosoxidans* KF701 was cut by *Bam*HI, *Eco*RI, *Kpn*I, *Pst*I, *Stu*I, and *Xho*I. To study the C23O gene in pCNU201 at molecular level, several subclones were constructed as described in Table 1, and their physical maps are shown in Fig. 2. The C23O gene was localized at an 1.8-kb *Bam*HI-*Xho*I fragment found in pCNU205, and its precise location was identified by comparison of nucleotide sequences. *E. coli* harboring pCNU207 did not exhibit C23O activity, and thus a *Stu*I site is located within the structural gene of C23O.

Expression of the C23O gene in E. coli HB101. C23O activity was identified in *E. coli* HB101 harboring pCNU201, pCNU202, pCNU203, pCNU205 or pCNU218, but not in *E. coli* HB101 harboring pCNU204, pCNU207 or pCNU252 (Fig. 2). Of course, *E. coli* HB101 harboring pBR322 or pUC18 used as cloning vectors did not exhibit C23O activity (Table 2). pCNU218 contained a 4.7-kb *Bam*HI-*Eco*RI fragment from *A. xylosoxidans* KF701 at downstream of P1 promoter in pBR322 vector, and pCNU252 did the same fragment at upstream of P2 promoter in pBR322 vector. C23O activity was identified in *E. coli* HB101 harboring pCNU218, but not in that harboring pCNU252.

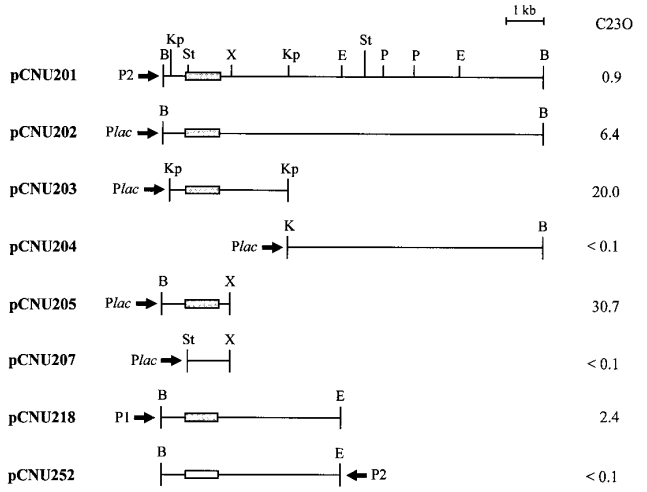

FIG. 2. Physical maps of pCNU201 and its subclones. The insert DNAs originated from chromosomal DNA of *A. xylosoxidans* KF701 are only shown. Restriction endonucleases are *Bam*HI (B), *Eco*RI (E), *Kpn*I (Kp), *Pst*I (P), *Stu*I (St), and *Xho*I (X). Orientations of the P1 or P2 promoter in pBR322 vector and the *lac* promoter (P*lac*) in pUC18 vector are indicated by an arrow. The C23O gene is located within a box. Enzyme activity of C23O is specific activity described in Materials and Methods.

This means that the 4.7-kb *Bam*HI-*Eco*RI fragment from *A. xylosoxidans* KF701 did not contain its own promoter or this promoter was not recognized by *E. coli* RNA polymerase.

Expression of C23O gene in pCNU201 or pCNU218 is under P2 or P1 promoter of pBR322 vector and that in pCNU202, pCNU203 or pCNU205 under *lac* promoter of pUC18 vector. Crude lysates of *E. coli* HB101 harboring pCNU201 or pCNU218 exhibited specific C23O activities with 0.9 unit to 2.4 units per mg of proteins. Crude lysates of *E. coli* HB101 harboring pCNU202, pCNU203 or pCNU205 exhibited specific C23O activities with 6.4 units to 30.7 units per mg of proteins. Therefore, *lac* promoter in pUC18 vector was much stronger in the expression of C23O gene from *A. xylosoxidans* KF701 in *E. coli* HB101 than P1 or P2 promoter in pBR322 vector. Specific C23O activities of clones under the *lac* promoter were pCNU205 $>$ pCNU203 $\gg$ pCNU202, which means the smaller insert DNA containing functional C23O gene expressed the higher amount of the enzyme.

To analyze substrate specificity of the C23O, *meta* cleavage activities to dihydroxyaromatics of catechol, 3-methylcatechol, 4-methylcatechol, 4-chlorocatechol, and 2,3-dihydroxybiphenyl were determined with crude lysate of *E. coli* HB101 harboring pCNU205 (Table 2). The C23O exhibited the highest aromatic ring-fission activity to catechol as the substrate, and its relative activity to other compounds was 4-chlorocatechol $>$ 4-methylcatechol $>$ 3-methylcatechol $\gg$ 2,3-dihy-

TABLE 2

Aromatic Ring-Fission Activity of the C23O from *A. xylosoxidans* KF701

Substrate	Specific activity (unit/mg)		
	pUC18	pBR322	pCNU205
Catechol	<0.1	<0.1	30.7
3-Methylcatechol	n.d.	n.d.	13.9
4-Methylcatechol	n.d.	n.d.	21.3
4-Chlorocatechol	n.d.	n.d.	25.6
2,3-Dihydroxybiphenyl	<0.1	<0.1	0.8

Note. One unit of the enzyme activity was defined as formation of 1 μmol of *meta* cleavage compound per minute and specific activity as unit(s) per mg of proteins, where "n.d." means "not determined."

droxybiphenyl. Aromatic ring-fission activity of the C23O to catechol was about 40-fold higher than that to 2,3-dihydroxybiphenyl.

Nucleotide sequence of the C23O gene. An open reading frame (ORF) corresponding to C23O gene was identified within the 1.8-kb fragment in pCNU205. The determined nucleotide sequence and deduced amino acid sequence of C23O are shown in Fig. 3. The ORF corresponding to C23O gene was consisted of 924 nucleotides initiating at ATG codon and terminating at TGA codon. The C23O gene exhibited 58% of G + C content, and its codon usage preferred G or C in the wobble position. A putative ribosome-binding sequence, AGGTGA, was identified at about 10 nucleotides upstream from the initiation codon. This ribosome-binding sequence is the same as those of C23Os encoded in NAH7 plasmid of *P. putida* PpG7 and chromosomal DNA of *Pseudomonas* sp. CF600 [9,10]. The ORF corresponding to C23O gene from *A. xylosoxidans* KF701 predicts a polypeptide chain with molecular weight of 34 kDa containing 307 amino acid residues. Another ORF corresponding to 2-hydroxymuconic semialdehyde dehydrogenase gene initiating at ATG codon of position 958 was also identified at downstream of the C23O gene.

Sequence comparison of the C23O with other extradiol dioxygenases. C23O from *A. xylosoxidans* KF701 exhibited significant sequence homology with other reported C23Os at nucleotide and amino acid levels (Table 3). The highest sequence homology with C23O from *A. xylosoxidans* KF701 was identified with corresponding enzyme from *Pseudomonas* sp. IC exhibiting 95% identity at nucleotide sequence and 96% indentity at amino acid sequence [11]. Amino acid sequence of the C23O from *A. xylosoxidans* KF701 exhibited more than 80% identity with those of nine C23Os, and less than 50% identity with those of eight C23Os. The C23O from *A. xylosoxidans* KF701 exhibited the least homology at amino acid sequence with that from *P. putida* F1, sequence of which, among published sequences of C23Os, shows the highest homology with those of 2,3-dihydroxybiphenyl dioxygenases [12]. The C23O from *A. xylosoxidans* KF701 exhibited about 20% sequence identity with 2,3-dihydroxybiphenyl dioxygenases at amino acid level [13].

Even though low sequence homology among C23Os and 2,3-dihydroxybiphenyl dioxygenases was shown, amino acid sequence of the C23O from *A. xylosoxidans*

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-42  CGT TGG CGG AAA CAA ACC TGA CAA CAG AGA AAA GGT GAC GTC
                                                 RBS
  1  ATG AAC AAA GGT GTA ATG CGC CCC GGC CAT GTG CAG CTG CGT
  1  Met Asn Lys Gly Val Met Arg Pro Gly His Val Gln Leu Arg
 43  GTA CTG GAC ATG AGC AAG GCC TTG GAA CAC TAC GTC GAG TTG
 15  Val Leu Asp Met Ser Lys Ala Leu Glu His Tyr Val Glu Leu
 85  CTG GGC CTA ATC GAG ATG GAC CGT GAC GAT CAG GGC CGT GTC
 29  Leu Gly Leu Ile Glu Met Asp Arg Asp Asp Gln Gly Arg Val
127  TAT CTC AAG GCT TGG ACC GAA GTG GAC AAG TTT TCC CTG GTG
 43  Tyr Leu Lys Ala Trp Thr Glu Val Asp Lys Phe Ser Leu Val
169  CTG CGT GAA GCG GAT GAG CCG GGC ATG GAT TTC ATG GGC CCC
 57  Leu Arg Glu Ala Asp Glu Pro Gly Met Asp Phe Met Gly Pro
211  AAG GTG ATC GAT GAT GAG TGC CTG GTC CGT CTG ACC CAG GAC
 71  Lys Val Ile Asp Asp Glu Cys Leu Val Arg Leu Thr Gln Asp
253  CTG ATC GAC TAC GGC TGC CTG ATC GAG ACC ATT CCC GCC GGA
 85  Leu Ile Asp Tyr Gly Cys Leu Ile Glu Thr Ile Pro Ala Gly
295  GAA CTC AGG GGC TGT GGC CGT CGC GTG CGC TTC CAG GCA TCC
 99  Glu Leu Arg Gly Cys Gly Arg Arg Val Arg Phe Gln Ala Ser
337  TCC GGG CAT CAC TTC GAG TTG TAT GCA GAC AAG GAA TAT ACT
113  Ser Gly His His Phe Glu Leu Tyr Ala Asp Lys Glu Tyr Thr
379  GGA AAG TGG GGT GTG AAT GAG GTC AAT CCC GAG GCA TGG CCG
127  Gly Lys Trp Gly Val Asn Glu Val Asn Pro Glu Ala Trp Pro
421  CGC GAT TTG AAA GGT ATG GCG GCT GTG CGT TTC GAC CAC GCC
141  Arg Asp Leu Lys Gly Met Ala Ala Val Arg Phe Asp His Ala
463  CTC ATG TAT GGC GAC CAA TTG CCG GCG ACT TAT GAC CTG TTC
155  Leu Met Tyr Gly Asp Gln Leu Pro Ala Thr Tyr Asp Leu Phe
505  ACC AAG GTG CTC GGC TTC TAT CTG GCC GAA CAG GTG CTG GAC
169  Thr Lys Val Leu Gly Phe Tyr Leu Ala Glu Gln Val Leu Asp
547  GAA AAT GGC ACG CGC GTC GCC CAG TTC CTC AGC CTG TCG ACC
183  Glu Asn Gly Thr Arg Val Ala Gln Phe Leu Ser Leu Ser Thr
589  AAG GCC CAC GAC GTG CCT TTC ATT CAC CAT CCG GAA AAA GGC
197  Lys Ala His Asp Val Pro Phe Ile His His Pro Glu Lys Gly
631  CGC CTC CAT CAT GTG TCC TTC CAC CTC GAA ACC TGG GAA GAC
211  Arg Leu His His Val Ser Phe His Leu Glu Thr Trp Glu Asp
673  GTG CTT CGC GCC GCC GAC CTG ATC TCC ATG ACC GAC ACC TCG
225  Val Leu Arg Ala Ala Asp Leu Ile Ser Met Thr Asp Thr Ser
715  ATC GAC ATC GGC CCA ACC CGC CAC GGC CTC ACT CAC GGC AAG
239  Ile Asp Ile Gly Pro Thr Arg His Gly Leu Thr His Gly Lys
757  ACC ATC TAC TTC TTC GAC CCG TCC GGT AAC CGC AAC GAA GTG
253  Thr Ile Tyr Phe Phe Asp Pro Ser Gly Asn Arg Asn Glu Val
799  TTC TGC GGG GGA GAT TAC AAC TAC CCG GAC CAC AAA CCG GTG
267  Phe Cys Gly Gly Asp Tyr Asn Tyr Pro Asp His Lys Pro Val
841  ACC TGG ACC ACT GAC CAG CTG GGC AAG GCG ATC TTT TAC CAC
281  Thr Trp Thr Thr Asp Gln Leu Gly Lys Ala Ile Phe Tyr His
883  GAC CGC ATT CTC AAC GAA CGA TTC ATG ACC GTG CTG ACG TGA
295  Asp Arg Ile Leu Asn Glu Arg Phe Met Thr Val Leu Thr ***
925  AGG CCC GGT TCG ACT TAT TGC AGA GAT TGC GAG ATG AAA GAA
  1                                          Met Lys Glu
967  ATC AAG CAT TTC ATT AAC GGT GCC TTC GTC GGT TCG GGC AGC
  4  Ile Lys His Phe Ile Asn Gly Ala Phe Val Gly Ser Gly Ser
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FIG. 3. Nucleotide sequence and deduced amino acid sequence of the C23O from *A. xylosoxidans* KF701. An open reading frame corresponding to the C23O gene at positions 1 to 924 and another open reading frame corresponding to 2-hydroxymuconic semialdehyde dehydrogenase gene from position 958 are shown. A putative ribosome-binding sequence (RBS) is underlined, and the termination codon of the C23O gene is indicated by ***.

TABLE 3

Sequence Identity of the C23O from *A. xylosoxidans* KF701 with Other Reported C23Os

Strain	Sequence identity (%)		GenBank accession number
	Nucleotide	Amino acid	
Pseudomonas sp. IC	95	96	U01825
P. putida mt-2 (TOL)	92	93	V01161
P. aeruginosa JI104	88	89	X60740
P. putida PpG7 (NAH7)	82	85	X06412
P. putida CF600 (pVI150)	81	85	M33263
P. putida HS1 (pDK1)	83	83	M65205
P. putida H (pPGH1)	79	83	X80765
P. putida P35X	81	82	X77856
Alcaligenes sp. KF711	80	81	S77084
Pseudomonas sp. HV3	58	50	L10655
S. yanoikuyae B1	58	49	U23375
P. putida MT15	46	43	U01826
P. putida UCC2	52	41	X59790
R. picketti PKO1	52	39	U20258
R. rhodochrous CTM (pTC1)	51	32	X69504
B. Stearothermophilus FDTP-3	45	25	X67860
P. Putida F1	47	22	J04996

KF701 was well aligned with those of 2,3-dihydroxybiphenyl dioxygenases from *P. cepacia* LB400 and *Pseudomonas* sp. KKS102 whose three dimensional structures are known [14,15]. Among five extradiol dioxygenases aligned, fourty two amino acids are shown as conserved residues (Fig. 4). Six (four His, one Tyr, and one Glu) of them have shown to be involved in coordination of active site ferrous ion in extradiol dioxygenases. Histidyl residues seems to be particularly critical ligands for coordination of the ferrous ion, which has been documented by site-specific mutagenesis of 2,3-dihydroxybiphenyl dioxygenase from *P. pseudoalcaligenes* KF707 and chemical modification of the C23O from *R. rhodochrous* CTM as well as by recent analysis of crystal structures of 2,3-dihydroxybiphenyl dioxygenases from *P. cepacia* LB400 and *Pseudomonas* sp. KKS102 [14-17]. The catalytic residues were well conserved in His-153, His-199, His-214, His-246, Tyr-255, Glu-265 of the C23O from *A. xylosoxinas* KF701.

The Phe-187 in 2,3-dihydroxybiphenyl dioxygenase from *P. cepacia* LB400 was known to interact with the dihydroxylated ring of the bicyclic substrate, and Ala-198 and Asn-243 were shown to serve to properly position the aromatic side chain of Phe-187 in the substrate-binding pocket [14]. The residue corresponding to Phe-187 was conserved in Phe-191 of the C23O from *A. xylosoxinas* KF701 but residues corresponding to Ala-198 and Asn-243 were replaced with Pro-202 and Leu-248 in the C23O. These amino acid differences between 2,3-dihydroxybiphenyl dioxygenase from *P. cepacia* LB400 and the C23O from *A. xylosoxinas* KF701 would make them be different in their substrate specificities, preferentially cleaving bicyclic substrates by the former extradiol dioxygenase and monocyclic substrates by the latter enzyme. The Leu-27Gly-28 and Leu-165Gly-166 in 2,3-dihydroxybiphenyl dioxygenase from *P. cepacia* LB400 were shown to play important roles in the formation of each α-helix in two $\beta\alpha\beta\beta\beta$ motifs, and Pro-109Gly-111 and Pro-254Gly-256 were within the turn linking second and third β-pleated sheets in the $\beta\alpha\beta\beta\beta$ motifs [13-15]. Structurally important residues corresponding to Leu-27, Gly-28, Gly-111, Leu-165, Gly-166, Pro-254, and Gly-256 in the 2,3-

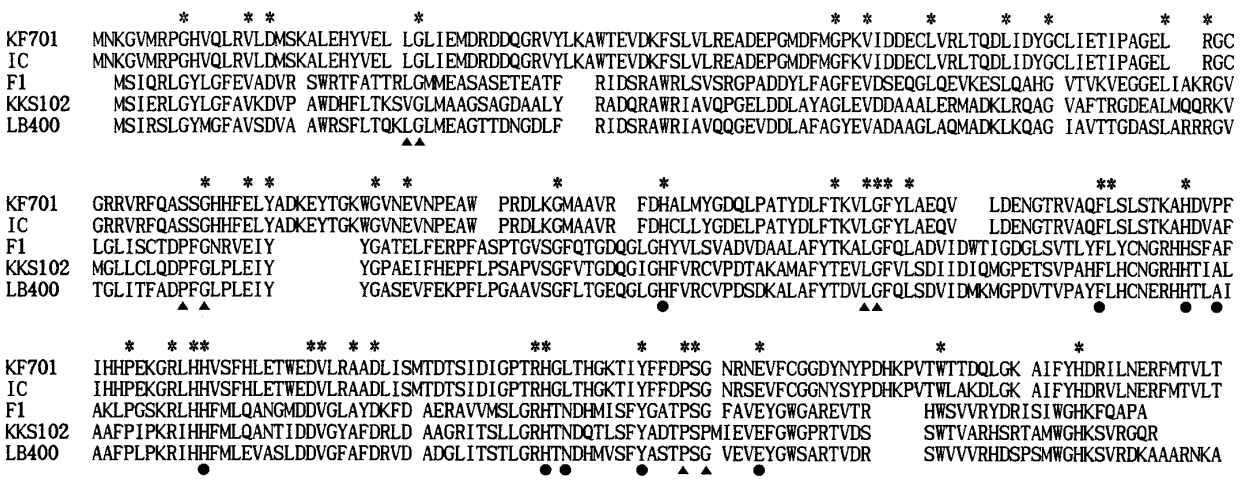

FIG. 4. Amino acid alignment of the extradiol dioxygenases. Enzymes are C23Os from *A. xylosoxidans* KF701 (KF701), *Pseudomonas* sp. IC (IC) and *P. putida* F1 (F1), and 2,3-dihydroxybiphenyl dioxygenases from *Pseudomonas* sp. KKS102 (KKS102) and *P. cepacia* LB400 (LB400). Indentical residues among aligned 5 extradiol dioxygenases are indicated by an asterisk, and catalytically or structurally important residues in the 2,3-dihydroxybiphenyl dioxygenases are indicated by a closed circle or triangle.

dihydroxybiphenyl dioxygenase were well conserved in the C23O from *A. xylosoxinas* KF701, but residue corresponding to Pro-109 was replaced with Ser-112 in the C23O.

In conclusion, C23O from *A. xylosoxinas* KF701 preferentially cleaves dihydroxylated monocyclic substrates, whose sequence comparison with other extradiol dioxygenases exhibited subtle differences in amino acid residues involving in binding to the subsrates and forming supersecondary motifs. Importance of the subtle amino acid differences will be elucidated by site-directed mutagenesis in a future study.

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