APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2004, p. 1804–1810 Vol. 70, No. 3
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.3.1804–1810.2004

Construction of Chimeric Catechol 2,3-Dioxygenase Exhibiting Improved Activity against the Suicide Inhibitor 4-Methylcatechol

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Received 7 July 2003/Accepted 16 December 2003

Catechol 2,3-dioxygenase (C23O; EC 1.3.11.2), exemplified by XylE and NahH, catalyzes the ring cleavage of catechol and some substituted catechols. C23O is inactivated at an appreciable rate during the ring cleavage of 4-methylcatechol due to the oxidation of the Fe(II) cofactor to Fe(III). In this study, a C23O exhibiting improved activity against 4-methylcatechol was isolated. To isolate this C23O, diverse C23O gene sequences were PCR amplified from DNA which had been isolated from mixed cultures of phenol-degrading bacteria and subcloned in the middle of a known C23O gene sequence (*xylE* or *nahH*) to construct a library of chimeric C23O genes. These chimeric C23O genes were then introduced into *Pseudomonas putida* possessing some of the toluene catabolic genes (*xylXYZLGFJQKJI*). When a C23O gene (e.g., *xylE*) is introduced into this strain, the transformants cannot generally grow on *p*-toluate because 4-methylcatechol, a metabolite of *p*-toluate, is a substrate as well as a suicide inhibitor of C23O. However, a transformant of this strain capable of growing on *p*-toluate was isolated, and a chimeric C23O (named NY8) in this transformant was characterized. The rate of enzyme inactivation by 4-methylcatechol was lower in NY8 than in XylE. Furthermore, the rate of the reactivation of inactive C23O in a solution containing Fe(II) and ascorbic acid was higher in NY8 than in XylE. These properties of NY8 might allow the efficient metabolism of 4-methylcatechol and thus allow host cells to grow on *p*-toluate.

Catechol 2,3-dioxygenase (C23O; EC 1.3.11.2), belonging to the extradiol dioxygenase family, catalyzes ring cleavage of catechol and chloro-, methyl-, and ethyl-substituted catechols in a meta fashion (9, 17). A wide variety of C23Os have been reported from different bacteria. While C23O basically catalyzes catechol more effectively than substituted catechols, the reactivity of C23O isolated from *Pseudomonas putida* UCC2 to 3-methylcatechol is higher than the reactivity of this C23O to catechol (42). Most C23Os are rather unstable and become inactivated during the catalysis of substituted catechols because the Fe(II) at the active sites is oxidized to Fe(III) (6). 4-Methylcatechol, which is an intermediate of the degradation of certain alkyl benzenes, such as *p*-xylene, *p*-methylbenzyl alcohol, and *p*-toluate, is one of the most effective inactivation compounds. Bacteria possess the repair system, which regenerates the oxidized Fe(III) to the reduced Fe(II) by a novel [2Fe-2S] ferredoxin (19) and makes it possible for them to grow even on *p*-xylene, *p*-methylbenzyl alcohol, or *p*-toluate (36).

DNA shuffling is a recently developed technique that allows accelerated and directed protein evolution in vitro (35, 38). This technique is called family shuffling when it is carried out with a set of related genes. Several applications of family shuffling have been demonstrated (8, 7, 29), and we have used this technique to improve C23O (6, 22). C23Os encoded by *xylE* and *nahH* in *P. putida* share 84% identity in their amino acid sequences. A set of *nahH-xylE* hybrid genes has been constructed by in vivo homologous recombination, and enzymes with altered substrate specificity have been obtained (6). Furthermore, some of the hybrid enzymes thus obtained were much more stable than the natural enzymes at a high temperature (J. Inoue, M. Kikuchi, and S. Harayama, unpublished data). The *xylE* and *nahH* genes have recently been applied to in vitro family shuffling, and hybrid enzymes with much greater stability than natural enzymes at a high temperature were isolated (22).

Although cloned genes are generally used as the starting materials for family shuffling, we have developed a PCR method called cassette PCR that allows a variety of chimeric genes to be isolated without using cloned genes (Fig. 1, 32). In this study, we used this technique to isolate a C23O exhibiting improved activity against 4-methylcatechol.

MATERIALS AND METHODS

Plasmid construction. The *Xba*I-*Hin*dIII fragment containing the entire operon encoding the *meta* cleavage enzymes of pWW0 (*xylXYZLTEGFJQKIH*), its regulation gene (*xylS*), and the 5′ end of *xylR* was isolated from pPL392 (11) and subcloned into the T7 expression vector, pT7-7, to produce pMETA21. A deletion extending between *xylT* and *xylE* was introduced into pMETA21 by the method outlined in Fig. 2A. First, four primers were designed. FxylL (5′-GCG CCA TCG TAA ATG TTT CCT-3′) corresponds to the middle sequence of *xylL*. The underlined sequence in RxylET (5′-ACC GGA CCA TCA **ATT CAT CCC AGA** TAC ACT AAA-3′) is complementary to the underlined sequence in FxylTE, while the rest of the sequence is complementary to the initiation codon region of *xylT*. The sequence in bold letters in FxylTE (5′-**TCT GGG ATG AAT** TGA TGG TCC GGT ACG ACT TAT-3′) is complementary to the sequence in bold letters in RxylET, while the rest of the sequence corresponds to the termination codon region of *xylE*. RxylG (5′-GGC GAC GAA CGC GTC GAA GAT-3′) is complementary to the middle sequence of *xylG*. In Fig. 2A, the boldface and underlined sequences in FxylTE and RxylET are indicated. With pPL392 as a template, a DNA segment upstream of *xylT* covering the 3′ half of

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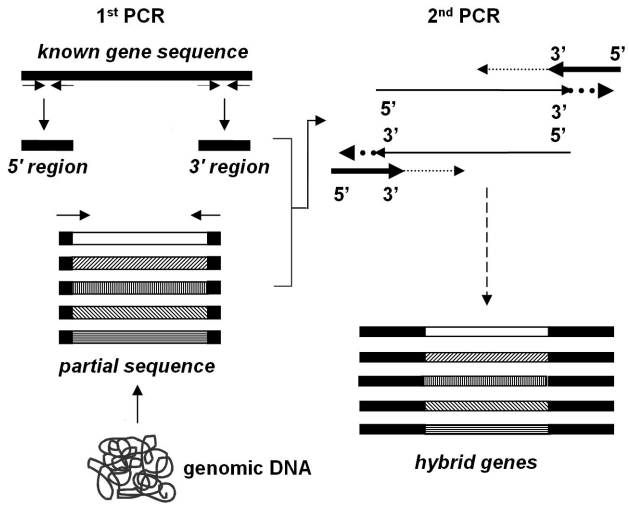

FIG. 1. Outline of cassette PCR.

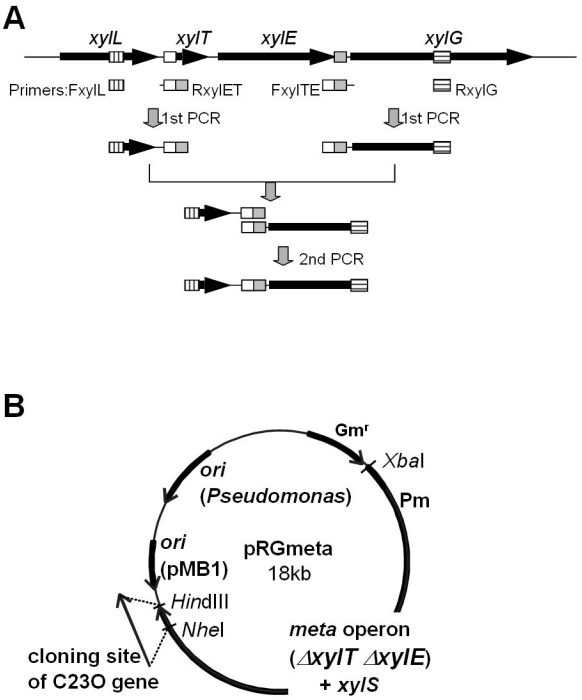

FIG. 2. Plasmid vector pRGmeta. (A) Construction of a deletion extending between the *xylT* and *xylE* genes. Genes on TOL plasmid pWW0 (*xylLTEG*) are indicated by arrows. The open and gray-shaded boxes on primers FxylTE and RxylET are complementary to each other. The products of the first PCR were mixed and subjected to the second PCR with the FxylL and RxylG primers. Details are described in Materials and Methods. (B) The pRGmeta plasmid contains two origins of replication, one functional in *Pseudomonas* and the other functional in *E. coli* (pMB1). In addition, the genes of the *meta* cleavage operon (except *xylT* and *xylE*) and its promoter (*Pm*) on TOL plasmid pWW0 were cloned in the plasmid as the *Xba*I-*Hin*dIII fragments.

xylL (′*xylL*) was amplified with the primer pair FxylL and RxylET, with the structure of the amplified sequence being 5′-(′*xylL*)-**TCT GGG ATG AAT** TGA TGG TCC GGT. Another DNA segment downstream of *xylE* corresponding to the 5′ half of *xylG* (*xylG*′) was amplified with the primer pair FxylTE and RxylG, again with pPL392 as a template. The structure of the amplified sequence was 5′-**TCT GGG ATG AAT** TGA TGG TCC GGT-(*xylG*′)-3′. The two DNA segments thus amplified were subsequently mixed together and subjected to a second PCR with the primers FxylL and RxylG. In this reaction, the 3′ end of 5′-(′*xylL*)-**TCT GGG ATG AAT** TGA TGG TCC GGT-3′ and the 5′ end of 5′-**TCT GGG ATG AAT** TGA TGG TCC GGT-(*xylG*′)-3′ were annealed to each other, and the DNA segment with the structure 5′-(′*xylL*)-**TCT GGG ATG AAT** TGA TGG TCC GGT-(*xylG*′)-3′ was amplified. The product was excised from agarose gel, digested with *Bam*HI (cleavage within *xylL*) and *Mlu*I (cleavage within *xylG*), and introduced into pMETA21 at the corresponding restriction enzyme sites to create pMETA213. The absence of any nucleotide substitutions and the successful introduction of the *xylT-xylE* deletion in this plasmid were confirmed by nucleotide sequencing.

The pRG vector was constructed based on the broad-host-range pRO1614 vector, possessing two origins of replication, one derived from pMB1 and the other derived from an endogenous plasmid in *Pseudomonas aeruginosa* (34). The pRG vector had these two origins of replication, a gentamicin resistance gene, and the *meta* operon promoter *Pm*. The *Xba*I-*Hin*dIII fragment of pMETA213, which contained all of the *meta* cleavage pathway genes except *xylT* and *xylE*, the regulatory gene, *xylS*, and the 5′ part of *xylR*, was subcloned into the same sites of pRG. The resultant plasmid, pRGmeta, was used for cloning C23O genes into the *Nhe*I and *Hin*dIII sites (Fig. 2B).

Cassette PCR. DNA was isolated from phenol-acclimatized activated sludge harboring a variety of phenol-degrading bacteria (43). The central parts of the C23O genes were PCR amplified from DNA by using the primers CF and CR. Their respective sequences, 5′-**GCC GAT GAG CCA GGT ATG GA**(TC) TT(TC) ATG GG(TCAG) TT(TC) AA-3′ and 5′-**ACT TCG TTG CGG TTA CCG GA** (TCAG) TC(AG) AA(AG) AA(AG) TA(TCA) AT(TCAG) GT-3′, were based on the conserved sequences of known C23O genes (9). This primer pair amplified the C23O gene sequence corresponding to that of the 582-bp-long *xylE* at positions 4940 to 5522 of GenBank accession number M64747 (16). Boldface letters in the sequences of primers CF and CR were complementary to boldface letters in sequences of primers 5R and 3F, respectively, as shown below. Two primer pairs, 5F plus 5R and 3F plus 3R, were used for PCR amplification of the DNA segments corresponding to the 5′ and 3′ ends, respectively, of *nahH* and *xylE*. The sequences of these four primers were as follows: 5F, 5′-GGC GGC CGC TGA AGA GGT GAC GTC ATG AA-3′; 5R, 5′-**CAT ACC TGG CTC ATC GGC** TT-3′; 3R, 5′-GGG AAT TCT AAG TCG TAC CGG ACC ATC-3′; and 3F, 5′-**TCC GGT AAC CGC AAC GAA GT**-3′. Underlined sequences correspond to the recognition sites for *Not*I and *Eco*RI, respectively. The *xylE* segment was amplified from genomic DNA prepared from *P. putida* strain KT2440 containing TOL plasmid pWW0 (12), while the *nahH* segment was from pGSH2939 (13). Three PCR-amplified DNA segments, the 5′ end of *xylE* or *nahH*, the central parts of the C23O genes, and the 3′-end of *xylE* or *nahH*, were subsequently mixed together and subjected to a second PCR with the primers 5F and 3R. This PCR operation allowed the amplification of the hybrid genes to full length in the structure 5′-(*xylE* or *nahH* segment)-(central C23O gene segment)-(*xylE* or *nahH* segment)-3′. A full description of this method, called cassette PCR (Fig. 1), has previously been published (32).

Screening of C23Os with improved activity against 4-methylcatechol. The 1-kb final products of cassette PCR were separated by agarose gel electrophoresis, excised from agarose gel, and digested with *Not*I and *Eco*RI. The resulting fragment was ligated to pZErO-2 (Invitrogen) and introduced into *Escherichia coli* top10F′. Transformants were cultivated on Luria-Bertani plates each containing 20 μg of kanamycin/ml and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Colonies that appeared on the plates were collected, and plasmids were prepared en masse from the collected cells. These plasmids were then digested with *Xba*I and *Hin*dIII and ligated with pRGmeta that had been digested with *Nhe*I and *Hin*dIII (Fig. 2B). The resulting plasmids were introduced into *P. putida* PaW94 (25) by electroporation (10), and the transformants were cultivated at 30°C on minimal M9 plates containing 10 μg of gentamicin/ml and 5 mM *p*-toluate. The transformants generally could not grow on the plates, since 4-methylcatechol, an intermediate formed from *p*-toluate, cannot be efficiently metabolized by C23O. However, colonies appeared on the plates at a low frequency, and they were replicated on fresh plates of the same type. After two replications, about 2,000 colonies remained. All of these colonies were spread for single-colony isolation on plates of the same composition except for a lower concentration of *p*-toluate (0.5 mM). After purification twice, 11 clones remained. These 11 clones were cultivated at 30°C with shaking on a minimal M9 medium containing 5 mM *p*-toluate. One clone which exhibited reproducible growth on the medium was retained for further analysis, and the plasmid in this clone was named pRGmeta-NY8.

Purification of C23Os. The C23O gene on pRGmeta-NY8 was subcloned into the pTrc99A vector (1) and introduced into *E. coli* BL21. The resulting transformant was grown in 200 ml of Luria-Bertani medium containing 50 μg of ampicillin/ml to an A_{600} of 0.5 to 0.8. C23O was then induced by adding 1 mM IPTG, and the cells were subsequently cultivated for 5 h at 30°C. C23O encoded by pRGmeta-NY8 (hereinafter called NY8) was purified by a method described previously (32). XylE was similarly purified from *P. putida* PaW94 harboring TOL plasmid pWW0 (5).

Determination of the kinetic parameters of C23Os. Assays for C23O activity under the standard conditions were carried out with a 100 mM potassium phosphate buffer (pH 7.5) at 25°C with 330 μM catechol as a substrate, and the amount of the ring cleavage product of catechol (2-hydroxymuconic semialdehyde; $\varepsilon = 33\ mM^{-1}\ cm^{-1}$) was determined spectrophotometrically at 375 nm (5). The k_{cat} and K_m values of C23O for catechol, 3-methylcatechol, and 4-methylcatechol were determined under the standard conditions, except that the concentration of each substrate was varied in the range of 0.5 to 330 μM. The ring cleavage products of 3-methylcatechol (2-hydroxy-6-oxohepta-2,4-dinoate; $\varepsilon = 19.4\ mM^{-1}\ cm^{-1}$) and 4-methylcatechol (2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate; $\varepsilon = 28.1\ mM^{-1}\ cm^{-1}$) were detected at 388 and 382 nm, respectively.

Determination of the kinetic constants for the inactivation of C23Os. The method used for determining the rate constants for the inactivation of C23O during catalysis (suicide inhibition or mechanism-based inactivation) was that of Cerdan et al. (5). Briefly, purified C23O (50 to 100 ng) was added to 1 ml of 100 mM potassium phosphate buffer (pH 7.5) containing catechol, 3-methylcatechol, or 4-methylcatechol at various concentrations, and the formation of the ring cleavage products was measured by the absorbance change at 375, 388, or 382 nm, respectively. After the initiation of the enzyme reaction, the absorbance increased. The rate of the absorbance change decreased as the enzyme became inactivated. The slope of the absorbance change, $dA(t)/dt$, was calculated at each point. When the logarithms of the $dA(t)/dt$ values were plotted against t, a straight line was obtained. The slope represented the $k_{inact}\gamma$ value. In this formula, k_{inact} and γ are the rate constant for enzyme inactivation and a constant, respectively (5, 6). The γ value is constant if the concentrations of the substrates are constant. The $k_{inact}\gamma$ values were calculated at different substrate concentrations. When the concentrations of substrates, catechol or substituted catechols, and molecular oxygen are significantly higher than their respective K_m values, the γ value becomes 1. Curve fitting was carried out by using GraphPad Prism, version 3.03, for Windows (GraphPad Software) with nonlinear regression based on a one-site binding equation.

In vitro inactivation and reactivation of C23Os. Purified NY8 or XylE was diluted to 100 μM (14.8 μg/ml) in a 25 mM 3-(*N*-morpholino) propane sulfonic acid (MOPS) buffer (pH 7.5) containing 1% (vol/vol) isopropanol, and the solution was incubated with freshly prepared 200 μM 4-methylcatechol for 30 min at room temperature. As a control, an equivalent amount of each of these enzymes was similarly treated without 4-methylcatechol. After extensive dialysis twice against 1,000 volumes of a 25 mM MOPS buffer (pH 7.5) containing 1% (vol/vol) isopropanol, each enzyme sample was made anaerobic and kept under argon for 30 min at 4°C. The C23Os were then chemically reactivated in an anaerobic chamber in the presence of 1 mM $FeSO_4$ and 1 mM ascorbate (31). Samples were taken at different times, and their C23O activities were measured under the standard conditions.

Nucleotide sequence accession number. The nucleotide sequence data reported were deposited in the nucleotide sequence databases DDBJ, EMBL, GenBank, and GSDB under accession number AB074512.

RESULTS

Plasmid construction. PaW94 is a *P. putida* mutant defective in benzoate 1,2-dioxygenase. This strain cannot, therefore, grow on benzoate (25). The pRGmeta plasmid (Fig. 2B) was constructed as described in Materials and Methods. This plasmid contained all of the genes for the *meta* cleavage pathway enzymes except *xylT* and *xylE*. When this plasmid was introduced into *P. putida* PaW94 by electroporation, the transformant could grow on benzoate but not on *m*-toluate or *p*-toluate. This strain could grow on benzoate because benzoate was transformed to catechol by toluate 1,2-dioxygenase (XylXYZ) and *cis*-1,2-dihydroxycyclohexa-3,5-diene dehydrogenase (XylL) encoded by pRGmeta, which was further metabolized by *ortho* cleavage pathway enzymes encoded by genes on the *P. putida* PaW94 chromosome. This strain could not grow on *m*-toluate or *p*-toluate because 3-methylcatechol and 4-methylcatechol that had been formed from the respective substrates could not be metabolized by the *ortho* pathway enzymes.

TABLE 1. Growth of Paw94 transformants

Plasmid	Cell growth on substrate[a]		
	Benzoate	*m*-Toluate	*p*-Toluate
pRGmeta	+	−	−
pRGmeta-XylE	+	+	−
pRGmeta-XylTE	+	+	+
pRGmeta-NY8	+	+	+

[a] Paw94 cells transformed with plasmid were streaked on M9 medium with the substrate and incubated at 30°C for 2 days. The substrate concentration was 5 mM. +, growth; −, no growth.

The *xylE* or *xylT-xylE* gene sequence was amplified by PCR and subcloned at the *Nhe*I and *Hin*dIII sites of pRGmeta. The cloned genes in these constructs were under the control of the constitutively expressed *Pr* promoter (13, 25, 26). When pRGmeta carrying *xylE* (pRGmeta-XylE) was introduced into PaW94, the transformant could grow on benzoate and *m*-toluate but not on *p*-toluate as the sole carbon source (Table 1). This growth phenotype of PaW94 harboring pRGmeta-XylE was exactly the same as the phenotype of *P. putida* harboring pWW0 defective in *xylT* (36). *p*-Toluate was not a growth substrate for these strains because 4-methylcatechol, a metabolite of *p*-toluate, is a suicide inhibitor of C23O; during the catalysis of 4-methylcatechol, catalytic Fe(II) of C23O is oxidized to Fe(III), and the enzyme is inactivated (2). When pRGmeta carrying both *xylT* and *xylE* (pRGmeta-XylTE) was introduced into PaW94, the transformant could grow on benzoate, *m*-toluate, and *p*-toluate like *P. putida* harboring wild-type pWW0 (Table 1). *P. putida* possessing *xylT* could grow on *p*-toluate because the gene product of *xylT*, a plant-type [2Fe-2S] ferredoxin, regenerates C23O inactivated by 4-methylcatechol (19, 39).

Screening of C23O with improved activity against 4-methylcatechol. Genomic DNA was isolated from phenol-acclimatized activated sludge. This sludge sample contained a variety of phenol-degrading bacteria, many of which were likely to harbor C23O genes (28). The central parts of the C23O genes were prepared by PCR with this DNA sample, while the 5′ and 3′ ends of the C23O genes were prepared by PCR with *xylE* of the pWW0 plasmid (12) or with *nahH* of the NAH7 plasmid (13) as a template. Primers for the amplification of the central parts of C23O genes were designed based on amino acid sequences that are highly conserved among extradiol dioxygenases (9, 32). The cassette PCR products were subcloned into pRGmeta and then introduced into *P. putida* PaW94 by electroporation (25), and the transformants capable of growing on *p*-toluate were screened. Although many transformants grew on M9 minimal agar plates containing 5 mM *p*-toluate as a sole carbon source, almost all colonies became brownish. This development indicated that the intermediate, 4-methylcatechol, was accumulated in the transformants without being utilized. When these brownish colonies were replicated on *p*-toluate at the lower concentration (0.5 mM), they did not grow and then

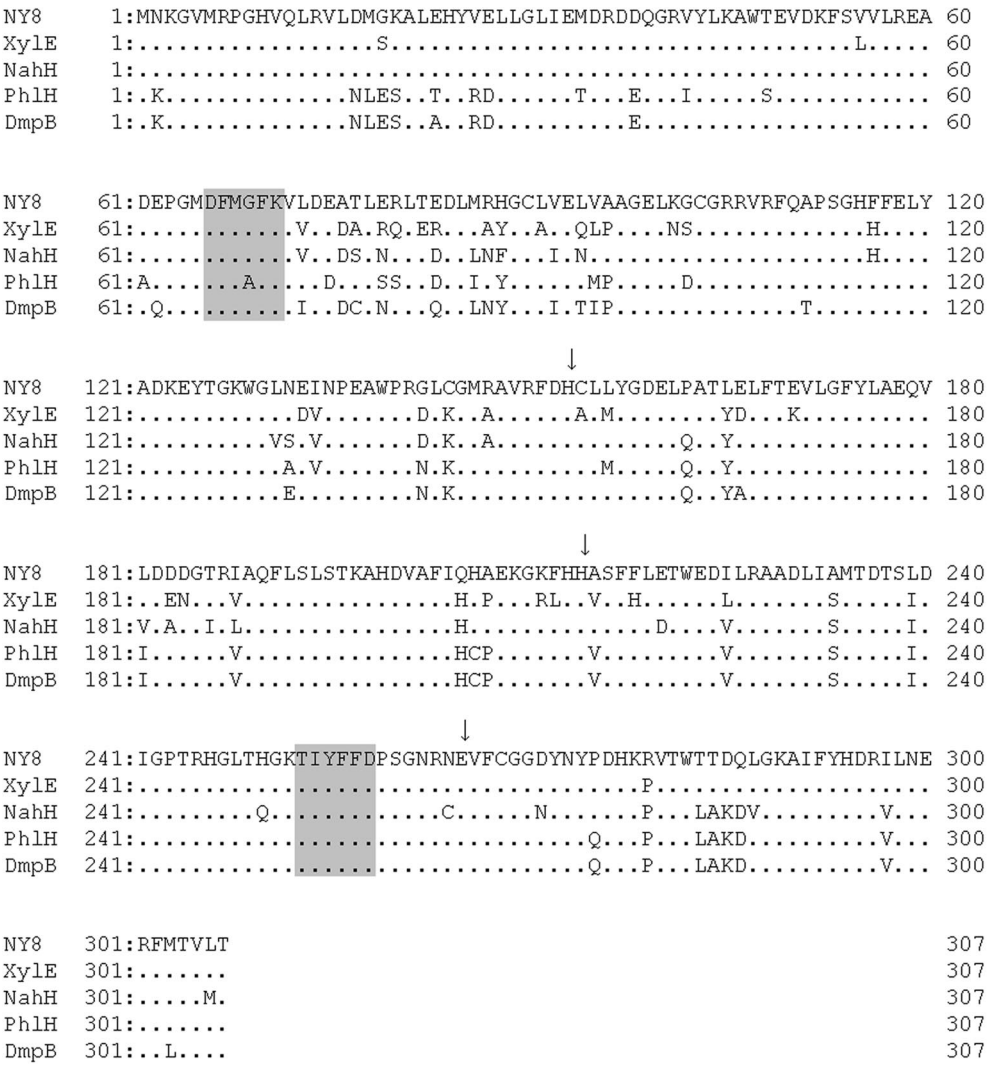

FIG. 3. Alignment of the deduced amino acid sequences of C23Os. Dots indicate residues identical to those in NY8, and arrows indicate iron ligand residues. Gray-shaded boxes represent the PCR primer regions used for cassette PCR. The sequences are as follows: NY8, hybrid C23O obtained in this study; XylE, C23O of *P. putida* mt-2 (28); NahH, C23O of *P. putida* PpG7 (45); PhlH, C23O of *P. putida* strain H (18); DmpB, C23O of *Pseudomonas* sp. strain CF600 (3).

were discarded. In contrast, nonbrownish colonies could grow even on the minimal agar plates with 0.5 mM *p*-toluate. These clones were cultivated in the liquid minimal medium with 0.5 mM *p*-toluate several times, and one clone that reproducibly grew in the liquid medium, PaW94(pRGmeta-NY8), was thus isolated. When the C23O gene from pRGmeta-NY8 was recloned into pRGmeta at the same restriction sites and introduced into *P. putida* PaW94, the resulting transformant was capable of growing on M9 minimal agar plates with 0.5 mM *p*-toluate as before, indicating that the isolated C23O gene, indeed, conferred to PaW94 the ability to grow on *p*-toluate (Table 1). PaW94(pRGmeta-NY8) grew twofold slower than PaW94(pRGmeta-XylTE) on *p*-toluate.

The nucleotide sequence of the C23O gene on pRGmeta-NY8 was determined. The deduced amino acid sequence of NY8 showed NahH and XylE sequences in the N-and C-terminal regions, respectively (Fig. 3), and the central region showed 86 and 84% similarity to those of C23Os from *P. putida* strain H (PhlH) (18) and *Pseudomonas* sp. strain CF600 (DmpB) (3).

Kinetic parameters. Two C23Os, XylE and NY8, were purified, and their K_m and k_{cat} values for catechol, 3-methylcatechol, and 4-methylcatechol were determined (Table 2). The K_m and k_{cat} values of XylE for catechol, 3-methylcatechol, and 4-methylcatechol were almost identical to results obtained previously (6). The K_m values of NY8 for catechol, 3-methylcatechol, and 4-methylcatechol were the same as or even smaller than those of XylE. On the other hand, the k_{cat} values of NY8 for all of the substrates were much smaller than those of XylE. In our experience, the hybrid C23Os sometimes shows the lower catalytic activity, partly because the formation of a tetramer, which is absolutely necessary for enzyme activity, is insufficient (33).

During the catalysis of 3-methylcatechol and 4-methylcat-

TABLE 2. Michaelis-Menten kinetic parameters for NY8 and XylE[a]

Enzyme	K_m (μM) for substrate[b]			k_{cat} (s^{-1}) for substrate[b]		
	Catechol	3-Methyl-catechol	4-Methyl-catechol	Catechol	3-Methyl-catechol	4-Methyl-catechol
NY8	0.9 ± 0.2	1.1 ± 0.1	1.5 ± 0.5	121 ± 5	84 ± 2	291 ± 12
XylE	2.7 ± 1.5	3.4 ± 1.4	3.9 ± 1.3	979 ± 96	338 ± 27	1,142 ± 96

[a] Fifty to 1,000 ng of purified enzymes was used for the C23O assay.
[b] Mean ± standard deviation of at least three measurements.

echol, the catalytic speed of XylE and NY8 decreased with time as the enzymes were inactivated concomitantly by oxidation of the substrates. The rate of enzyme inactivation could be quantified by determining $k_{inact}\gamma$ at different substrate concentrations. Since the K_m values of both enzymes for 4-methylcatechol were around 2 μM (Table 2), the value of γ was supposed to be close to 1 at a concentration of more than 300 μM, and the value of $k_{inact}\gamma$ was taken as k_{inact}. The k_{inact} value for 4-methylcatechol was calculated by curve fitting (Fig. 4A) or with a double-reciprocal plot (Fig. 4B). The k_{inact} values of XylE and NY8 were not much different from each other ($6.8 \times 10^{-3} \pm 0.3 \times 10^{-3}$ and $6.5 \times 10^{-3} \pm 0.5 \times 10^{-3}$ s^{-1}, respectively). On the other hand, the concentrations of 4-methylcatechol required for the half-maximum k_{inact} values of XylE and NY8 were 9.7 ± 2.9 and 30.8 ± 8.9 μM, respectively (Fig. 4). The k_{inact} values of NY8 and XylE for 3-methylcatechol were 6.3×10^{-3} and 3.3×10^{-3} s^{-1}, respectively. The k_{inact} values for catechol of NY8 and XylE were not very different from each other (1.0×10^{-3} and 0.8×10^{-3} s^{-1}, respectively).

In vitro reactivation of C23O. XylE and NY8 were inactivated by exposure to 200 μM 4-methylcatechol. After this treatment, XylE and NY8 both showed 1 to 3% of their initial activities. After dialysis of the inactivated enzymes against the 25 mM MOPS buffer (pH 7.5) containing 1% (vol/vol) isopropanol, the C23Os were chemically reactivated under the anaerobic condition in the presence of 1 mM $FeSO_4$ and 1 mM ascorbate (31). Samples were taken at different times, and their C23O activities were determined under the standard conditions. Under these conditions, the time required for XylE to recover the half-maximum activity was 2.8 min, while that required for NY8 was 0.5 min, showing that NY8 could be reactivated much faster than XylE without XylT ferredoxin (Fig. 5).

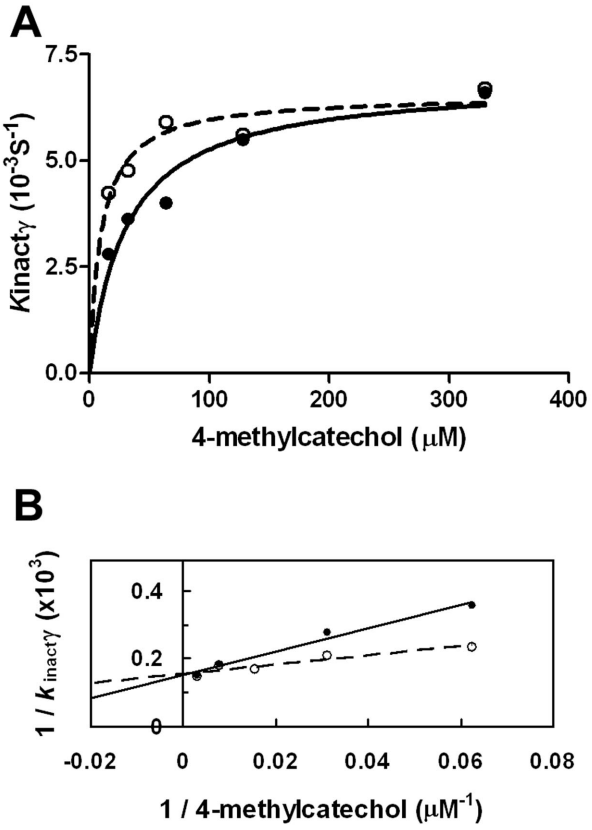

FIG. 4. $k_{inact}\gamma$ values for 4-methylcatechol. The $k_{inact}\gamma$ values for 4-methylcatechol at various concentrations were determined as described in Materials and Methods by curve fitting (A) and with a double-reciprocal plot (B). For XylE (open circles), the dotted curve was calculated assuming a k_{inact} value of $6.8 \times 10^{-3} \pm 0.3 \times 10^{-3}$ s^{-1} and the concentration of 4-methylcatechol required for the half-maximum k_{inact} value was 9.7 ± 2.9 μM. For NY8 (closed circles), the solid curve was calculated assuming a k_{inact} value of $6.5 \times 10^{-3} \pm 0.5 \times 10^{-3}$ s^{-1} and the concentration of 4-methylcatechol required for the half-maximum k_{inact} value was 30.8 ± 8.9 μM.

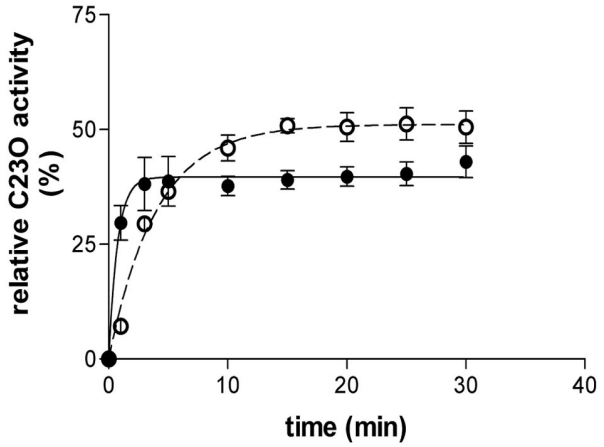

FIG. 5. In vitro reactivation of C23Os. XylE (open circles) or NY8 (closed circles) inactivated by 4-methylcatechol was incubated anaerobically in 25 mM MOPS buffer (pH 7.5) with 1 mM $FeSO_4$ and 1 buffer mM ascorbate, and the regenerated C23O activity was measured over an interval of 30 min. The C23O activity before inactivation was taken to be 100%. The averages of five measurements of C23O activity before inactivation were 208 units/mg (XylE) and 100 units/mg (NY8). The dotted (XylE) and the solid (NY8) curves were calculated assuming that the times to half-recovery were 2.8 min for XylE and 0.5 min for NY8.

DISCUSSION

TOL plasmid pWW0 of *P. putida* encodes the gene cluster responsible for growth on benzoate, *m*-toluate, and *p*-toluate (44). Benzoate, *m*-toluate, and *p*-toluate are transformed to catechol, 3-methylcatechol, and 4-methylcatechol (12), respectively, on this pathway. C23O catalyzes the ring cleavage of these catechols, and the ring cleavage products are further metabolized to precursors of TCA cycle intermediates. 4-Methylcatechol is a suicide inhibitor of C23O; during the ring cleavage of 4-methylcatechol, catalytic Fe(II) of C23O is oxidized to Fe(III) and inactivated to a high degree (2). Interestingly, TOL plasmid pWW0 encodes the mechanism that regenerates the inactivated C23O. The gene called *xylT* encodes a ferredoxin, and this XylT ferredoxin regenerates the inactivated C23O by reducing the oxidized catalytic iron, thus keeping the catalytic efficiency of C23O sufficiently high to support cell growth, even with 4-methylcatechol (19). In consequence, *P. putida* containing the wild-type TOL plasmid can grow on *p*-toluate, but *xylT* mutants defective in the regeneration system cannot grow on it (36).

A number of XylT-like ferredoxins have been found to be encoded in the operons for the biodegradation of aromatic compounds like naphthalene (*nahT*) (45), phenol (*phhQ* [30] and *dmpQ* [37]), toluate (*xylT* [15] and *tbuW* [24]), and 3-chlorobenzene (*cbzT*) (27). The genes for ferredoxins are located just before the genes for C23Os in most operons. In the cases of *Sphingomonas yanoikuyae* B1 and *Novosphingobium aromaticivorans* F199, *xylT* genes are located about 5 kb downstream of *xylE* genes (23). Some of the XylT-like ferredoxins have been experimentally demonstrated to reactivate C23O (19, 20). The prevailing occurrence of XylT-like ferredoxins suggests that C23O may have inherent difficulty in metabolizing 4-methylcatechol and therefore that a ferredoxin has been evolutionarily recruited to expand the substrate specificity of C23O against 4-methylcatechol by creating a recycling system for inactivated C23O.

Using the recombinant DNA technique called cassette PCR, we were able to isolate a gene encoding C23O that can support the growth of host cells on *p*-toluate even in the absence of XylT. This method thus achieved an in vitro evolution of C23O that could not be accomplished by natural evolution. We characterized the recombinant C23O, named NY8. As expected, NY8 was more resistant to inactivation by 4-methylcatechol than XylE; the $k_{inact}\gamma$ value of NY8 was lower than that of XylE at low concentrations of 4-methylcatechol (Fig. 4). Based on the curve fitting, at around the K_m concentration of 4-methylcatechol (2 μM), the $k_{inact}\gamma$ values of XylE and NY8 were calculated as 1.2×10^{-3} and 0.4×10^{-3} s^{-1}, respectively. The $k_{inact}\gamma$ value of NY8 was one-third of the XylE value. These data show that NY8 was much more resistant to 4-methylcatechol inactivation than XylE was at the low substrate concentrations. Since the specific activities of the first and second enzymes of the *meta* cleavage pathway, namely, toluate 1,2-dioxygenase and *cis*-1,2-dihydroxycyclohexa-3,5-diene dehydrogenase, are much lower than that of C23O (25), a low intracellular concentration of 4-methylcatechol is generally assured even in the presence of 5 mM *p*-toluate in the medium.

The reactivation of NY8 that had been inactivated by 4-methylcatechol in a solution containing 1 mM $FeSO_4$ and 1 mM ascorbate was six times faster than that of XylE (Fig. 5). The reactivation mixture did not contain XylT. It can thus be expected that the reactivation of NY8 by reducing agents other than XylT might also occur in vivo and partly contribute to the efficient metabolism of 4-methylcatechol by NY8. Extradiol dioxygenases for bicyclic aromatic compounds do not have either XylT-like or Rieske-type ferredoxins (4, 21). The extradiol dioxygenase family can be divided into two large subfamilies comprising dioxygenases for monocyclic compounds and those for bicyclic aromatic compounds (9, 14). The latter dioxygenases are exemplified by 2,3-dihydroxybiphenyl dioxygenases (DHBDs). Like C23O, DHBD is also subject to suicide inhibition by the oxidation of the active site Fe(II) to the catalytically-incompetent Fe(III) (40). The partition ratios of XylE of *P. putida* mt-2 for catechol and DHBD of *P. putida* LB400 for 2,3-dihydroxybiphenyl are 1,400,000 and 84,900, respectively (5, 40), indicating that DHBD is much more susceptible than is C23O to suicide inhibition even by its preferred substrate. Despite the higher susceptibility of DHBD to suicide inhibition, none of the DHBDs have their cognate ferredoxins. The DHBD of *P. putida* LB400 is demonstrated to be inactivated by suicide inhibition of 3-chlorocatechol and reactivated by a nonspecific electron transfer system (41), and this process is slower than XylT-like-ferredoxin-mediated Fe(III) reduction to Fe(II). NY8 might use such nonspecific electron transfer protein to regenerate oxidized Fe(III) to the reduced form of Fe(II).

NY8 was isolated from mixed cultures of phenol-degrading bacteria as the hybrid form with the N-terminal NahH sequence of NAH7 and the C-terminal XylE sequence of pWW0. Although we have not yet specified the bacterium (strain X) which possesses the NY8 central sequence, the sequence is most similar to the C23Os identified in the phenol degraders *P. putida* strain H and *Pseudomonas* sp. strain CF600 (3, 18). This result was pretty much expected, since the NY8 central sequence was extracted from a mixed culture of phenol-degrading bacteria. By using this central sequence as a probe, we are planning to isolate strain X from the consortium of phenol-degrading bacteria. First, once strain X is isolated and the full length of the C23O gene is cloned, the suicide inhibition of C23Os of both strain X and NY8 by 4-methylcatechol will be compared. By this comparison, the effect of the flanking region on susceptibility to 4-methylcatechol will be clarified. Since most of the amino acid residues involved in substrate and Fe(II) binding reside in the central region (Fig. 3), the central region in NY8 might be enough to produce the resistance. Second, we could change the flanking region. XylE-NahH hybrid C23Os show different susceptibilities to 3-methylcatechol (6). The C23Os subjected to cassette PCR with NahH on both termini show higher specific activity against 3-chlorocatechol (32). We need to make three more different hybrid C23Os, NahH-NY8-NahH, XylE-NY8-XylE, and XylE-NY8-NahH hybrids, and the degree of the suicide inhibition of these hybrids by 4-methylcatechol will be determined. We might be able to obtain more resistant hybrid C23Os.

ACKNOWLEDGMENTS

We thank H. Itazawa for technical assistance.

This work was performed as part of a research and development project of the Industrial Science and Technology Frontier Program

supported by the New Energy and Industrial Technology Development Organization (NEDO).

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