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Cloning and Overexpression of Methylcatechol 2,3-Dioxygenase Gene from Toluene-Degrading *Pseudomonas putida* mt-2 (pWWO)

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Abstract □ Methylcatechol 2,3-dioxygenase encoded in pWWO megaplasmid of *Pseudomonas putida* mt-2 has been cloned and overexpressed in *Escherichia coli*. This enzyme gene has been localized inside 2.3-kb *Xho*I fragment derived from the pWWO megaplasmid. Analysis of enzyme activity and SDS-PAGE showed that the cloned methylcatechol 2,3-dioxygenase gene in *E. coli* was about 100 fold overexpressed compared with the parental gene in *P. putida* mt-2 (pWWO). The cloned enzyme exhibited higher ring-fission activity to catechol than catechol derivatives including 3-methylcatechol, 4-methylcatechol, and 4-chlorocatechol.

Keywords □ Methylcatechol 2,3-dioxygenase, pWWO, gene cloning, overexpression.

Aromatic hydrocarbons released to the environment are catabolized to catechol or its derivatives, and then further oxidized to intermediates of TCA cycle by soil and water microorganisms. This microbial oxidation has played an important role for reutilization of hydrocarbons in the aromatic pollutants.

Toluene, an aromatic hydrocarbon, can be completely degraded by several microorganisms including *Pseudomonas putida* mt-2 (pWWO), *P. cepacia* G4, *P. picketti* PKO1 and *P. putida* F1[1-4]. Microbial degradation of toluene is initially proceeded by hydroxylation or dioxygenation. Toluene is hydroxylated at its methyl group to form benzylalcohol by *P. putida* mt-2 (pWWO), and at its aromatic ring to form *O*-cresol by *P. cepacia* G4 and *m*-cresol by *P. picketti* PKO1. Toluene is initially dioxygenated at its aromatic ring to form (+)-*cis*-1(S), 2(R)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) by *P. putida* F1. The benzylalcohol, *O*-cresol, *m*-cresol, and *cis*-toluene dihydrodiol are catabolically converged to 3-methylcatechol by sequential enzyme activities in each strain. The catabolic enzymes are: xylene oxygenase, benzylalcohol dehydrogenase, benzylaldehyde dehydrogenase, toluate 1,2-dioxygenase, and dihydrodihydroxybenzoate dehydrogenase in *P. putida* mt-2 (pWWO); toluene monoxygenases in *P. cepacia* G4 and *P. picketti* PKO1; toluene dioxygenase and *cis*-toluene dihydrodiol dehydrogenase in *P. putida* F1. The 3-methylcatechol is opened its benzene ring, and then fruther degraded through α-ketoadipate pathway to form intermediates of TCA cycle. The benzene-ring fission in toluene catabolism is catalyzed by methylcatechol 2,3-dioxygenase which converts 3-methylcatechol to 2-hydroxy-6-methylmuconic semialdehyde.

In this study, we have cloned and overexpressed methylcatechol 2,3-dioxygenase encoded in pWWO megaplasmid of *P. putida* mt-2. The cloned methylcatechol 2,3-dioxygenase has been identified as an yellow band in polyacrylamide gel by electrophoresis coupled with activity staining. The methylcatechol 2,3-dioxygenase was about 100 fold overexpressed in *E. coli* compared with that in the pare-

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ntal *P. putida* (pWWO).

EXPERIMENTAL METHODS

Materials

Aromatic hydrocarbons were purchased from Sigma (benzoate, catechol, 4-methylcatechol, and xylene), Tokyo Kasei (3-methlcatechol and 4-chlorocatechol), and Wako (*m*-toluate, 3-chlorobenzoate, and 4-chlorobenzoate). Other chemicals were obtained from BRL (agarose), IBI (acrylamide), and GIBCO (trypton, yeast extract, and agar). DNA modifying enzymes were purchased from BM and Pharmacia.

Strains and culture

P. putida mt-2 (pWWO) was supplied from P.A. Williams at University of North Wales, United Kingdom. The *P. putida* mt-2 (pWWO) was grown in MMO medium containing *m*-toluate (0.1%) as the sole carbon and energy source. *E. coli* haboring any recombinant plasmid was grown in LB medium supplimented with ampicillin (50 μg/ml).

DNA manipulations and electrophoresis

Total DNA was isolated and purified from *P. putida* mt-2 (pWWO) by proteinase K-SDS lysis followed by CsCl-ethidium bromide ultracentrifugation [5]. Plasmids were isolated by alkali lysis[6]. DNA restrictions were carried out according to conditions recommended by the enzyme suppliers. Other manipulations including ligation and transformation were performed as described in elsewhere[7]. DNA fragments were resolved on 0.7%-agarose gel with TAE buffer, and visualized by staining with ethidium bromide. Proteins were resolved on native 7.5 %-polyacrylamide gel with Tris-Glycine buffer, and then soaked with 0.5 M catechol or 3-methylcatechol for activity staining specific to methylcatechol 2,3-dioxygenase[8].

Quantitative analysis of ring-fission compounds

Bacterial culture grown in LB medium supplimented with 0.1% benzoate, 4-chlorobenzoate, or *m*-toluate was centrifuged twice at 27,000×g for each 20 min. The supernatant was used for quantitative analysis of the ring-fission compound produced from each benzoate derivative. Each ring-fission product was spectrophotometrically quantitated by using extinction coefficients (ε) as follow; benzoate to 2-hydroxymuconic semialdehyde (ε=33,000 M^{-1} at 375 nm), 4-chlorobenzoate to 2-hydroxy-5-chloromuconic semialdehyde (ε=39,600 M^{-1} at 379 nm), and *m*-toluate to 2-hydroxy-6-methylmuconic semialdehyde (ε=28,100 M^{-1} at 382 nm).

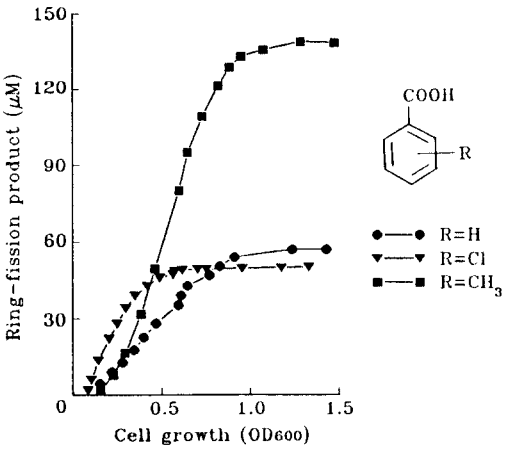

Fig. 1. Formation of ring-fission products from benzoate and its derivatives by pCNU701.

RESULTS AND DISCUSSION

P. putida mt-2 (pWWO) can grow in toluene or xylenes as the sole carbon and energy source. Toluene and xylenes are degraded by the same enzymes encoded in pWWO megaplasmid with two operons, upper and lower pathways[9,10]. The upper pathway enzymes catabolize toluene and xylenes to benzoate and toluates which are further degraded to pyruvate, acetaldehyde, and propanal by the lower pathway enzymes[11,12].

For molecular cloning of the methylcatechol 2,3-dioxygenase gene, pWWO megaplasmid was partially digested with *Bam*HI, and ligated to the same endonuclease site in pBR322 to make a libary. This library was subjected to selection of ampicillin-resistant and tetracycline-sensitive colonies followed by selection of chromogenic colonies by 3-methylcatechol spray. This chromogenic identification of methylcatechol 2,3-dioxygenase is based on yellow colored 2-hydroxy-6-methylmuconic semialdehyde produced from colorless 3-methylcatechol by the enzyme activity. One yellow colony was selected from the library and this clone is designated as pCNU701. The pCNU701 clone has shown to con-

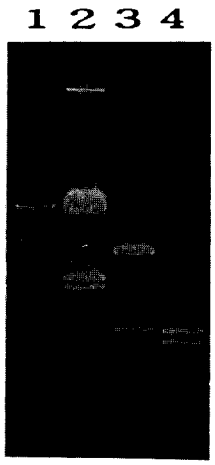

Fig. 2. Gel pattern of pCNU701, pCNU703 and pCNU 705.
The recombinant plasmids were resolved on 0.7 % agarose gel by electrophoresis. Lambda DNA-digested with *Hin*dIII is a size marker with 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb (lane 1). pCNU701 was digested with *Bam*HI (lane 2) and pCNU703 with *Eco*RI (lane 3). pCNU705 was digested with *Eco*RI and *Hin*dIII (lane 4).

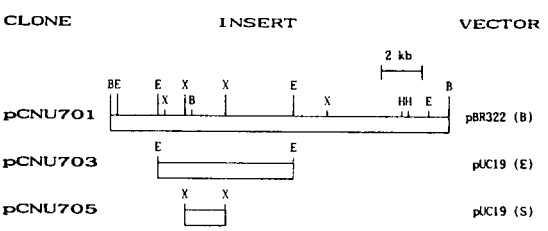

Fig. 3. Physical maps of pCNU701, pCNU703, pCNU 705.
Each insert DNA was cloned to the endonuclease site inside parenthesis of pBR322 or pUC 19. Endonuclease are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Sal*I (S) and *Xho*I (X).

tain toluate 1,2-dioxygenase, dihydrodihydroxybenzoate dehydrogenase in addition to the methylcatechol 2,3-dioxygenase derived from pWWO megaplasmid. The pCNU701 clone can degrade benzoate derivatives to corresponding ring-fission products. *m*-Toluate was most efficiently catabolized and then benzoate and 4-clorobenzoate by the pCNU701 clone as shown in Fig. 1.

The pCNU701 clone was subcloned to form

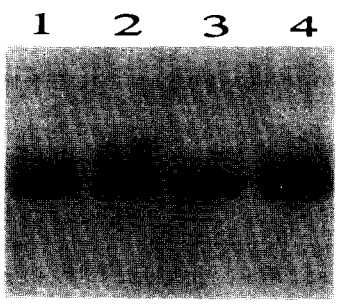

Fig. 4. Activity staining of methylcatechol 2,3-dioxygenase.
Catechol 2,3-dioxygenase expressed from pWWO (lane 1), pCNU701 (lane 2), pCNU703 (lane 3) or pCNU705 (lane 4) was resolved on native 7.5%-polyacrylamide gel by electrophoresis, and then soaked with 0.5 M 3-methylcatechol for specific staining of the enzyme as a yellow band.

pCNU703 and pCNU705. The pCNU701 plasmid was digested with *Eco*RI, and then ligated to the same endonuclease site of pUC19 to make pCNU 703. The pCNU703 was digested with *Xho*I, and then ligated to *Sal*I site of pUC19 to form pCUN 705. These three clones contain a functional methylcatechol 2,3-dioxygenase gene from pWWO of *P. putida* mt-2. All of the recombinant plasmids have been identified on agarose gel by electrophoresis (Fig. 2). The pCNU701 clone has a recombinant plasmid with an 18-kb insert in pBR322, where the insert was fragmented into 4.5-kb and 13.5-kb bands by *Bam*HI digestion. The pCNU703 clone has a recombinant plasmid with a 7.3-kb insert in pUC19. The pCUN705 clone has a recombinant plasmid with a 2.3-kb insert in pUC19. Physical maps of the recombinant plasmids have been constructed as shown in Fig. 3. The pCNU701 insert was cut by *Bam*HI, *Eco*RI, *Hin*dIII and *Xho*I. The pCNU703 insert was cut by *Bam*HI, *Eco*RI and *Xho*I but not by *Hin*dIII. The pCNU705 insert was cut by *Bam*HI but not by *Xho*I ligated with *Sal*I site in pUC19.

Methylcatechol 2,3-dioxygenases cloned in pCUN 701, pCUN703 and pCNU705 were identified as yellow bands on nondenaturing polyacrylamide gel by electrophoresis followed by activity staining (Fig. 4). All of the cloned methylcatechol 2,3-dioxygenases have exhibited the same electrophoretic mobility with the parental enzyme in *P. putida* (pWWO). Thus the cloned methylcatechol 2,3-dioxygenases

Table I. Enzyme activity of methylcatechol 2,3-dioxygenase expressed from each plasmid

Substrates	Plasmids					
	pWWO	pBR322	pUC19	pCNU701	pCNU703	pCNU705
Catechol	0.49	<0.01	<0.01	0.61	1.45	56.70
3-Methylcatechol						6.07
4-Methylcatechol						23.31
4-Chlorocatechol						35.51

One unit of the enzyme is defined as 1-μmol ring-fission product formation per minute.
Specific activity of the enzyme is unit per mg of proteins. Protein concentration was determined by dye method.

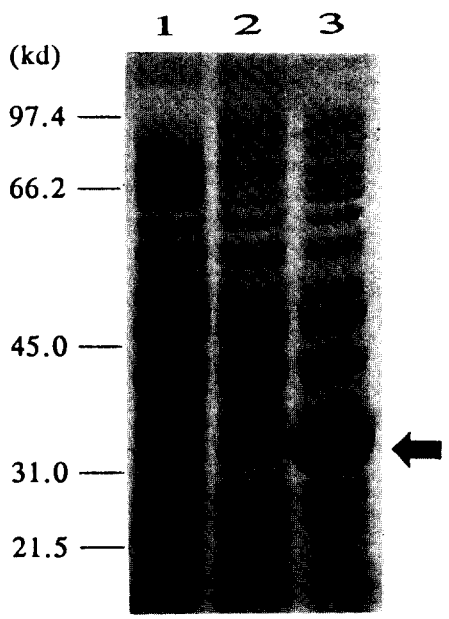

Fig. 5. Overexpression of methylcatechol 2,3-dioxygenase by pCNU705.
Crude lysate of pCNU701 (lane 1), pCNU703 (lane 2), or pCNU705 (lane 3) was resolved on SDS-polyacrylamide gel by electrophoresis and then stained with Coomassie brilliant blue R-250. Methylcatechol 2,3-dioxygenase is indicated as an arrow.

are not fused proteins. Furthermore *E. coli* haboring pCNU701, pCNU703 or pCNU705 did exhibit ring-fission activity but *E. coli* haboring pBR322 or pUC 19 did not show this activity (Table I). The methylcatechol 2,3-dioxygenase expressed from pCNU705 has exhibited about 115 fold higher specific ring-fission activity than that in the parental *P. putida* (pWWO). The methylcatechol 2,3-dioxygenase cloned in pCNU705 has shown the highest ring-fission activity to catechol among catechol, 3-methylcatechol, 4-methylcatechol and 4-chlorocatechol. The enzyme has exhibited higher ring-fission activity to 4-chlorocatechol than 3- and 4-methylcatchols. Overexpression of the methylcatechol 2,3-dioxygenase in pCNU705 is also demonstrated on SDS-PAGE as shown in Fig. 5, where the enzyme is 33 kd in size.

ACKNOWLEGEMENT

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