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CHARACTERIZATION OF CATECHOL 2,3-DIOXYGENASES*

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SUMMARY : Three catechol 2,3-dioxygenases for biphenyl, naphthalene/salicylate, and toluene/xylene oxidation were cloned from *Achromobacter xylosoxidans* KF701, *Pseudomonas putida* (NAH7), and *Pseudomonas* sp. (pWWO). The cloned catechol 2,3-dioxygenases were identified by enzymatic activity assay in addition to yellow bands on polyacrylamide gel after electrophoresis and activity staining. All of the cloned catechol 2,3-dioxygenases exhibited their highest activities on catechol as a substrate compared with catechol derivatives including 4-chlorocatechol, 3-methylcatechol, and 4-methylcatechol. The cloned catechol 2,3-dioxygenases are not fused proteins but were significantly different from one another in their electrophoretic mobilities on nondenaturing 7.5%-polyacrylamide gel.

Many microorganisms have been identified to degrade aromatic pollutants to simple aliphatic compounds which can be recycled in ecosystem. The microbial catabolism of a variety of aromatic pollutants proceeeds through initial conversion to catechol or protocatechuate (1). The catechol or protocatechuate is further metabolized by *ortho* and/or *meta* cleavage pathway(s) (2).

The *meta* cleavage of a benzene ring in catechol is catalyzed by catechol 2,3-dioxygenase (catechol:oxygen 2,3-oxidoreductase, EC 1.13.11.2), an oxygenase which converts catechol to 2-hydroxymuconic semialdehyde with incorporation of molecular oxygen. Biochemical and genetic properties of the catechol 2,3-dioxygenase are known in detail about the enzyme encoded in *nah*H of NAH7 plasmid (3,4) or *xyl*E of pWWO plasmid (5,6). NAH7 plasmid or pWWO plasmid encodes a set of enzymes for naphthalene/salicylate oxidation (7-9) or toluene/xylene oxidation (10-12). However, little is known about catechol 2,3-dioxygenases involved in oxidation of other aromatic compounds.

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In this paper, a catechol 2,3-dioxygenase for biphenyl oxidation is cloned from chromosomal DNA of *Achromobacter xylosoxidans* KF701 and its enzymatic and physical properties are compared with those from NAH7 and pWWO plasmids.

MATERIALS AND METHODS

Chemicals and Bacterial Culture. Catechol and antibiotics were obtained from Sigma Chemical Co. Restriction enzymes and T_4 DNA ligase were obtained from Boehringer Mannheim. Trypton, yeast extract, and agar were purchased from GIBCO. *E. coli* HB101 was grown on LB medium or LB medium supplimented with ampicillin (50 μg/ml) or tetracycline (15 μg/ml). *A. xylosoxidans* KF701 was a generous gift from K. Furukawa at Kyushu University (JAPAN). This strain was grown on M9 salt medium containing 0.1% biphenyl as the sole carbon and energy source. *Pseudomonas putida* (NAH7) or *Pseudomonas* sp. (pWWO) was grown on M9 salt medium containing 0.1% salicylate or toluene as the sole carbon and energy source.

DNA Manipulations. Chromosomal DNA from *A. xylosoxidans* KF701 was isolated by SDS-proteinase K lysis (13). NAH7 and pWWO plasmids were prepared by Sarkosyl-DOC lysis & Cabowax 6000 precipitation (14), and other plasmids were obtained by alkali lysis (15). DNA digestion with restriction enzymes was carried out according to the recommended conditions by the suppliers. DNA fragments digested with restriction enzymes were ligated to pBR322, and then transformed to Ca^{++}-treated *E. coli* HB101 (16). Other manipulations were followed by the standard protocols for recombinant DNA technology.

Gel Electrophoresis. DNA was resolved on 0.7% agarose gel with TAE buffer and visualized by staining with ethidium bromide. Proteins were resolved on nondenaturing 7.5%-polyacrylamide gel and then soaked with 0.5 M catechol solution for specific staining of catechol 2,3-dioxygenase.

Assay for Catechol 2,3-Dioxygenase Activity. *E. coli* HB101 containing a catechol 2,3-dioxygenase gene from NAH7, pWWO, or chromosomal DNA of *A. xylosoxidans* KF701 was grown to log phase, and crude lysates were prepared from the each bacterial pellet by sonication followed by centrifugation. The crude lysates were subjected to heat treatment at 50 °C, and then used as samples for assay of catechol 2,3-dioxygenase activity. Catechol 2,3-dioxygenase activities were spectrophotometrically measured. This assay consisted of 0.5 mM catechol or its derivative, 10 μl crude lysate, and 50 mM phosphate buffer (pH 7.5) in 1-ml final volume. One unit of the enzyme activity is defined as formation of 1 μmol of the respective ring-fission product from catechol (375 nm, ϵ=33,000 M^{-1}), 4-chlorocatechol (379 nm, ϵ=39,600 M^{-1}), 3-methylcatechol (388 nm, ϵ=13,400 M^{-1}), or 4-methylcatechol (382 nm, ϵ=28,100 M^{-1}) per minute (17). Protein concentration was determined by the method of Lowry *et al.* (18).

RESULTS AND DISCUSSION

P. putida (NAH7) can grow on naphthalene or salicylate, and *Pseudomonas* sp. (pWWO) on toluene or xylene as the sole carbon and energy source. Degradative pathways of the aromatic compounds by enzymes encoded in NAH7 and pWWO plasmids are initially catabolized to catechol or methylcatechol. This catechol or

methylcatechol is first subjected to its benzene-ring fission by catechol 2,3-dioxygenase, and then further degraded to intermediates in common pathways (EM and TCA cycle) (7,11). *A. xylosoxidans* KF701 can grow on biphenyl, 4-methylbiphenyl, or 2-hydroxybiphenyl as the sole carbon and energy source (19). This strain catabolizes biphenyl to benzoate by sequential enzyme activities of biphenyl dioxygenase, 2,3-dihydroxy-4-phenylhexa-2,4-diene dehydrogenase, 2,3-dihydroxy biphenyl dioxygenase, and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase (19,20). However, downstream of the biphenyl catabolism including benzene-ring fission is not characterized well.

To study biochemical and genetic basis of a benzene-ring fission by catechol 2,3-dioxygenase, we have cloned and expressed catechol 2,3-dioxygenases encoded in NAH7, pWWO, and chromosomal DNA of *A. xylosoxidans* KF701 into *E. coli* HB101. NAH7-catechol 2,3-dioxygenase gene was cloned to EcoRI and PstI sites in pBR322, and this clone is designated as pCNU101. pWWO-catechol 2,3-dioxygenase gene was cloned to BamHI site in pBR322, and this clone is designated as pCNU701. For molecular cloning of catechol 2,3-dioxygenase for biphenyl oxidation, chromosomal DNA of *A. xylosoxidans* KF701 was partially digested with BamHI and then ligated to a unique BamHI site in the tetracycline locus of pBR322. This ligation mixture was transformed to *E. coli* HB101 to make a genomic library. This genomic library was screened by ampicillin resistance and tetracycline sensitivity, and then subjected to a chromogenic screening by catechol spray. This chromogenic screening is based on formation of yellow-colored 2-hydroxymuconic semialdehyde from colorless catechol by catechol 2,3-dioxygenase. One yellow clone was selected from 1,200,000 transformants in the genomic library, and this clone is designated as pCNU201.

Gel patterns of pCNU101, pCNU201, and pCNU701 are shown in Fig. 1. pCNU101 is a hybrid plasmid with 6-kb NAH7 insert and 3.6-kb pBR322, where the 6 kb insert is fragmented into 5 kb and 1 kb bands. pCNU201 is a hybrid plasmid with 10-kb insert from *A. xylosoxidans* KF701 and 4.4-kb pBR322. pCNU701 is a hybrid plasmid with 18-kb pWWO insert and 4.4-kb pBR322. Physical maps of the three clones were constructed and are shown in Fig. 2. The 6-kb insert in pCNU101 was cut by ClaI, EcoRI, EcoRV, HpaI, KpnI, and PstI. The 10-kb insert in pCNU201 was cut by BamHI, ClaI, HindIII, KpnI, PstI, SmaI, StuI, and XhoI but not by BclI. The 18-kb insert in pCNU701 was cut by BamHI, EcoRI, HindIII, NotI, and XhoI.

Crude lysates prepared from *E. coli* HB101 harboring pBR322, pCNU101, pCNU201, or pCNU701 were resolved on nondenaturing polyacrylamide gel by electrophoresis, and then this gel was soaked with catechol solution for specific staining of catechol 2,3-dioxygenase. *E. coli* HB101 harboring pCNU101, pCNU201, or pCNU701 expressed each catechol 2,3-dioxygenase but *E. coli* HB101 harboring pBR322 did not (Fig. 3). Catechol 2,3-dioxygenases expressed from pCNU101, pCNU201, and pCNU701 do exhibit significant differences in their electrophoretic mobilities one another. Each catechol 2,3-dioxygenase from the hybrid plasmids did

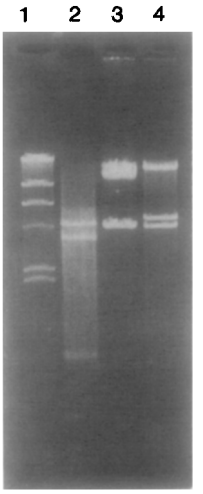

Fig. 1. Gel patterns of pCNU101, pCNU201, and pCNU701.
Recombinant plasmids were resolved on 0.7% agarose gel by electrophoresis. Size marker is HindIII-digested lambda DNA with 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb in size (lane 1). pCNU101 was digested with EcoRI plus PstI (lane 2), pCNU201 was digested with BamHI (lane 3). pCNU701 was digested with BamHI (lane 4).

exhibit the same electrophoretic mobility with its parental enzyme from *P. putida* (NAH7), *A. xylosoxidans* KF701, or *Pseudomonas* sp. (pWWO). pCNU101 expressed 10 to 50 fold higher catechol 2,3-dioxygenase activity than pCNU201 and pCNU701 (Table 1). All of the cloned catechol 2,3-dioxygenases exhibited their highest activities on catechol as a substrate compared with catechol derivatives including 4-

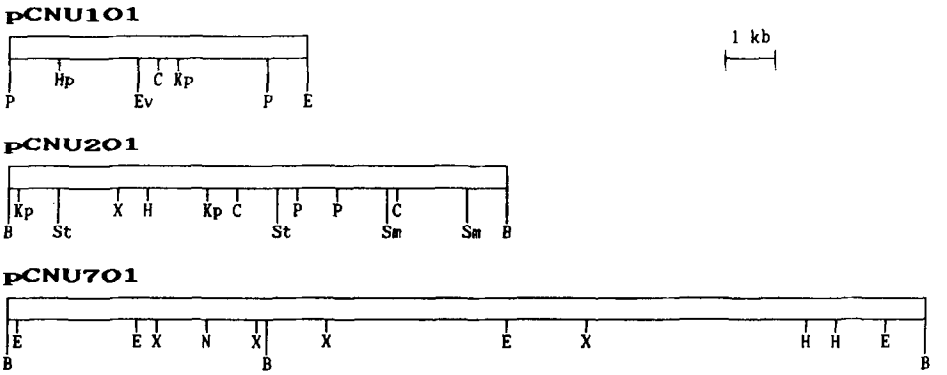

Fig. 2. Physical maps of inserts in pCNU101, pCNU201, and pCNU701.
Restriction enzymes are BamHI (B), ClaI (C), EcoRI (E), EcoRV (Ev), HindIII (H), HpaI (Hp), NotI (N), KpnI (Kp), PstI (P), SmaI (Sm), StuI (St), and XhoI (X).

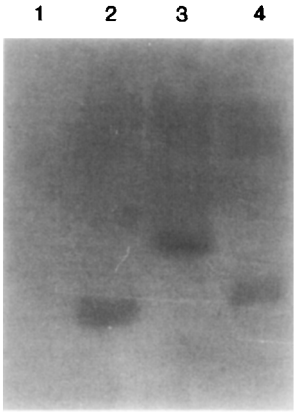

Fig. 3. Activity staining of catechol 2,3-dioxygenases from pCNU101, pCNU201, and pCNU701.

Crude lysates prepared from *E. coli* HB101 harboring pBR322 (lane 1), pCNU101 (lane 2), pCNU201 (lane 3), and pCNU701 (lane 4) were resolved on nondenaturing 7.5%-polyacrylamide gel by electrophoresis. This gel was soaked with 0.5 M catechol to stain catechol 2,3-dioxygenases as yellow bands.

chlorocatechol, 3-methylcatechol, and 4-methylcatechol. Catechol 2,3-dioxygenase from pCNU201 exhibited higher relative activities on 4-chlorocatechol as a substrate than those from pCNU101 and pCNU701. However, the enzyme from pCNU701

Table 1. Enzyme activity of catechol 2,3-dioxygenases expressed from pCNU101, pCNU201, and pCNU701

Plasmid	Substrate			
	Catechol	4-Cl-catechol	3-CH_3-catechol	4-CH_3-catechol
pBR322	(<0.001)*			
pCNU101	(21.466)*			
	100%	7%	22%	59%
pCNU201	(2.229)*			
	100%	87%	50%	74%
pCNU701	(0.420)*			
	100%	77%	61%	80%

The catechol 2,3-dioxygenase activities to catechol derivatives are shown in relative activities (%) to catechol as a substrate. One unit of the enzyme is defined as formation of 1-μmole ring fission product per minute. Specific activity of the enzyme is units per mg of protein. Protein content was determined by method of Lowry *et al.* with bovine serum albumin as a standard.

* Specific activity to catechol as a substrate.

exhibited higher relative activities on 3- or 4-methycatechol as a substrate than those from other hybrid plasmids. This suggests that the three catechol 2,3-dioxygenases may be isofunctional enzymes with different primary or high order structures.

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