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Molecular Cloning and M13 Subcloning of Genes Encoding Catechol Dioxygenases

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Abstract □ *Achromobacter xylosoxidans* KF701 and *Pseudomonas putida* (NAH7) were significantly different in degradative capability of aromatic compounds including benzoates, biphenyls, and naphthalene. However, both of the bacterial strains can grown on catechol as the sole carbon and energy source. Catechol 2,3-dioxygenase gene for naphthalene oxidation or biphenyl oxidation was cloned into *Escherichia coli* HB101 from NAH7 megaplasmid of *P. putida* or chromosomal DNA of *A. xylosoxidans* KF701. A *E. coli* HB101 clone containing catechol 2,3-dioxygenase gene from *P. putida* (NAH7) contains a recombinant plasmid with 3.6-kb pBR322 and 6-kb insert DNA. Another *E. coli* HB101 clone containing catechol 2,3-dioxygenase gene from *A. xylosoxidans* KF701 has a recombinant plasmid with 4.4-kb pBR322 and 10-kb insert DNA. Physical maps of the recombinant plasmids were constructed, and catechol 2,3-dioxygenase gene in the recombinant plasmid was further localized and subcloned into M13. The cloned-catechol 2,3-dioxygenase gene products were identified as yellow bands on nondenaturaing polyacrylamide gel after electrophoresis followed by activity staining with catechol solution.

Keywords □ *Aromatic pollutant, naphthalene, biphenyl, catechol dioxygenase, gene cloning.*

Man-made aromatic chemicals have been released into the biospher on a large scale. Many of the aromatic chemicals have potential toxicity which causes considerable environmental pollution and human health problems. However many soil and water microorganisms can use aromatic hydrocarbons as source of carbon and energy. This plays important roles in carbon cycle of ecosystem in addition to cleaning of environmental pollution. Microbial degradation of aromatic hydrocarbons such as benzene, benzoates, biphenyls, naphthalene, phenanthrene, phenol, and toluene is initially proceeded to form catechol or protocatechuate through special reaction sequences[1-3]. These dihydroxy group-substituted benzene and benzoate are completely degraded to intermediates of TCA cycle through α- or β-ketoadipate pathway[4,5]. The first enzyme in α-ketoadipate pathway of catechol is catechol 2,3-dioxygenase (catechol:oxygen 2,3-oxidoreductase, EC 1.13.11.2) which catalyzes *meta* cleavage of a benzene ring in catechol to form 2-hydroxymuconic semialdehyde.

To study genetic basis of the α-ketoadipate pathway, we have cloned two catechol 2,3-dioxygenases encoded in NAH7 megaplasmid of *Pseudomonas putida* and chromosomal DNA of *Achromobacter xyloxosidans* KF701. The cloned-catechol 2,3-dioxygenase gene products are involved in catabolism of naphthalene in *P. putida* and of biphenyl in *A. xyloxosidans* KF701.

EXPERIMENTAL METHODS

General chemicals and enzymes

General chemicals were obtained from GIBCO (trypton, yeast extract, and agar), Sigma (tetracycline, ampicillin, and RNase A), and Clontech (ultrapure phenol). DNA modifying enzymes were obtained from Boehringer Mannheim and BRL (restriction enzymes), and Pharmacia (T_4 DNA ligase).

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Bacterial strains and culture

P. putida (NAH7) and *A. xylosoxidans* KF701 were obtained from M.A. Schell (Univ. of Georgia, USA) and K. Furukawa (Kyushu Univ., JAPAN), respectively. These bacteria were grown in M9 salt medium containing 0.1% naphthalene for *P. putida* (NAH7) or 0.1% biphenyl for *A. xylosoxidans* KF701 as the sole carbon and energy source. *Escherichia coli* HB 101 was grown in LB medium or LB medium supplimented with ampicillin (50 μg/m*l*) or tetracycline (15 μ/m*l*). *E. coli* JM103 was used as a host for subcloning of catechol 2,3-dioxygenase gene into M 13mp18. This strain was grown in YT medium or YT medium supplimented with ampicillin (50 μg/m*l*).

DNA isolation and modification

Chromosomal DNA was isolated from *A. xylosoxidans* F701 by SDS-proteinase K lysis[6]. Plasmid DNA was isolated by alkali lysis[7]. NAH7 megaplasmid was isolated from *P. putida* by Sarkosyl lysis & PEG 6,000 precipitation[8]. DNA digestion with restriction enzymes was carried out according to conditions recommended by the enzyme suppliers.

Gel electrophoresis

DNA fragments were resolved on 0.7% agarose gel with TAE buffer, and then visualized by staining with ethidium bromide[9]. Proteins in crude lysate were resolved on nondenaturing 7.5%-polyacrylamide gel with Tris-glycine buffer[10]. This gel was soaked with 0.5 M catechol for specific staining of catechol 2,3-dioxygenase.

RESULTS AND DISCUSSION

A. xylosoxidans KF701 and *P. putida* (NAH7) were compared their degradative properties of aromatic hydrocarbons including benzoates, biphenyls, catechol, and naphthalene (Table I). Both of the bacterial strains can grow on benzoate, 2-hydroxybenzoate, and catechol but not on 3-methylbenzoate as the sole carbon and energy source. *A. xylosoxidans* KF701 can grow on biphenyls and *P. putida* (NAH 7) can grow on naphthalene. However, *A. xylosoxidans* KF701 cannot use naphthalene and *P. putida* (NAH7) cannot use biphenyls as the sole carbon and energy source for growth. *A. xylosoxidans* KF701 contains biphenyls-degrading enzymes encoded in its chromosomal DNA[11]. *P. putida* (NAH7) contains naphthalene-degrading enzymes encoded in NAH7 megaplasmid[12,13].

Table I. Biodegradation of aromatic hydrocarbons by *A. xylosoxidans* KF701 and *P. putida* (NAH7)

Aromatic hydrocarbons	Bacterial strains	
	A. xylosoxidans KF701	*P. putida* (NAH7)
Benzoate	+	+
2-Hydroxybenzoate	+	+
3-Methylbenzoate	−	−
Biphenyl	+	−
2-Hydroxybiphenyl	+	−
4-Methylbiphenyl	+	−
Catechol	+	+
Naphthalene	−	+

Each bacterial strain can (+) or cannot (−) use the aromatic hydrocarbon as the sole carbon and energy source. Each bacterial strain was inoculated into M9 salt medium containing 0.1% of an aromatic hydrocarbon.

For molecular cloning of each catechol 2,3-dioxygenase for biphenyl oxidation in *A. xylosoxidans* KF 701 or naphthalene oxidation in *P. putida* (NAH7), the chromosomal DNA of *A. xylosoxidans* KF701 was digested with BamHI, and NAH7 megaplasmid of *P. putida* was digested with EcoRI plus PstI. The BamHI-digested chromosomal DNA of *A. xylosoxidans* KF701 was ligated to a unique BamHI site in pBR322, and NAH7 megaplasmid fragmented by EcoRI plus PstI was ligated to the same restiction enzyme sites in pBR322. Each ligation mixture was transformed to *E. coli* HB101, which does not have catechol 2,3-dioxygenase, to make each library. Each library was subjected to antibiotic and chromogenic screenings to identify clones(s) containing catechol 2,3-dioxygenase gene. *A. xylosoxidans* KF701 library was subjected to ampicillin resistance, and NAH7 library to tetracycline resistance. Transformants selected by the antibiotic resistance were further screened by yellow coloring after catechol spray. This chromogenic screening for catechl 2,3-dioxygenase is based on formation of yellow-colored 2-hydroxymuconic semialdehyde from colorless catechol by the enzyme. One yellow clone out of 1,200,000 transformants in *A. xylosoxidans* KF701 library was selected and designated as pCNU201.

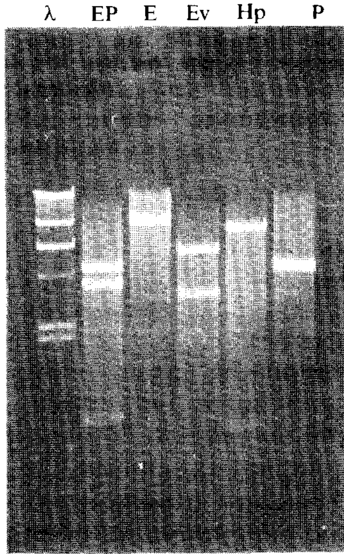

Fig. 1. Gel pattern of pCNU101.
Size marker is HindIII-digested λ DNA with 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.5 kb (lane λ). pCNU101 was digested with a variety of restriction endonucleases, EcoRI plus PstI (EP), EcoRI(E), EcoRV(Ev), HpaI(Hp), and PstI(P). The DNA fragments were resolved on 0.7% agarose gel by electrophoresis, and then visualized by staining with ethidium bromide.

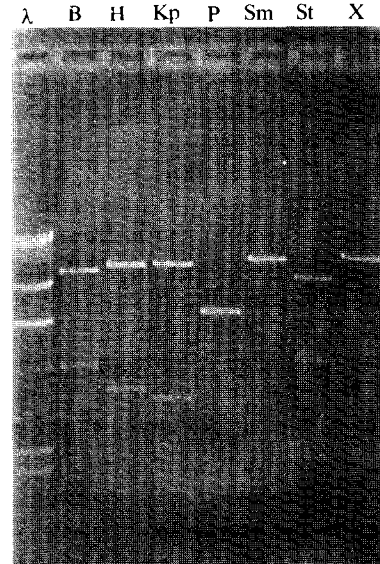

Fig. 2. Gel pattern of pCNU201.
Size marker is HindIII-digested λ DNA with 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb in size (lane λ). pCNU201 was digested with a variety of restriction endonucleases, BamHI(B), HindIII(H), KpnI(Kp), PstI(P), SmaI(Sm), StuI (St), and XhoI(X). The DNA fragments were resolved on 0.7% agarose gel by electrophoresis, and then visualized by staining with ethidium bromide.

Another yellow clone out of 600 transformants in NAH 7 library was selected and designated as pCNY101.

pCNU101 contains a recmbinant plasmid with 9.6 kb in size (Fig. 1). The 6-kb NAH7 insert and 3.6-kb pBR322 were regenerated from pCNU101 digested with EcoRI plus PstI, where the NAH7 insert was fragmented into two bands with 5 kb and 1 kb in size. pCNU201 contains a recombinant plasmid with 14.4 kb in size (Fig. 2). The 10-kb *A. xylosoxidans* insert and 4.4-kb pBR322 were regenerated from pCNU201 digested with BamHI. Physical maps of pCNU101 and pCNU201 plasmids were constructed and are shown in Fig. 3. NAH7-insert DNA in pCNU101 was cut by ClaI, EcoRI, EcoRV, HpaI, KpnI, and PstI but not by AvaI, BamHI, PvuII, and SalI. The entire catechol 2,3-dioxygenase gene in pCNU101 plasmid was further localized within 2.0-kb insert fragmented by HpaI and ClaI, and this DNA fragment was subcloned to M13mp

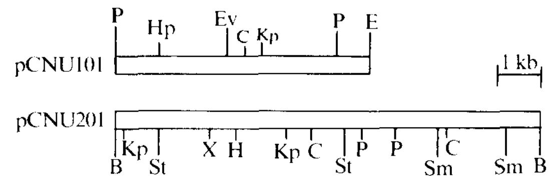

Fig. 3. Physical maps of inserts in pCNU101 and pCNU201.
pCNU101 is a recombinant DNA with 6-kb insert in PstI and EcoRI sites of pBR322. pCNU201 is a recombinant DNA with 10-kb insert in BamHI site of pBR322. Restriction endonucleases are BamHI(B), ClaI(C), EcoRI(E), EcoRV(Ev) HindIII(H), HpaI(Hp), KpnI(Kp), PstI(P), SmaI(Sm), StuI (St), and XhoI(X).

18. The *A. xylosoxidans* insert in pCNU201 was cut by BamHI, ClaI, HindIII, KpnI, PstI, SmaI, StuI, and XhoI but not by BclI.

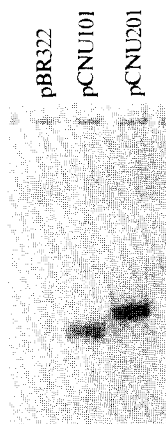

Fig. 4. Chromogenic identification of catechol 2,3-dioxygenases expressed from pCNU101 and pCNU201.
Catechol 2,3-dioxygenase in the crude lysate prepared from *E. coli* HB101 containing pCNU101 or pCNU201 was resolved on nondenaturing 7.5%-polyacrylamide gel by electrophoresis, and then identified as a yellow band by soaking with 0.5 M catechol.

Crude lysates were prepared from pCNU101 and pCNU201 clones, respectively. The catechol 2,3-dioxygenases in the crude lysates were resolved on nondenaturing polyacrylamide gel by electrophoresis, and then identified as yellow bands after soaking the gel with catechol solution (Fig. 4). The two catechol 2,3-dioxygenases exhibited significant difference in electrophoretic mobility on the gel.

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