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Molecular Cloning and Characterization of Catechol 2,3-Dioxygenase Gene from Aniline-Degrading, *Pseudomonas acidovorans*

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Catechol 2,3-dioxygenase(C23O) catalyses the oxidative ring cleavage of catechol to 2-hydroxymuconic semialdehyde. This is one of the key reactions in the metabolism of the widespread pollutant aniline. We have cloned a gene encoding C23O from cells of the aniline degrading bacteria, *Pseudomonas acidovorance* KCTC2494 strain and expressed in *E. coli*. A 11.3-kilobase *Sau*3A partial digested DNA fragment from KCTC2494 was cloned into phagemid vector pBluescript and designated as pLP201. The C23O gene was mapped to a 2.8-kb region, and the direction of transcription was determined. The cloned C23O gene contains its own promoter which can be recognized and employed by *E. coli* transcriptional apparatus. C23O activities of subclones were identified by enzyme assay and activity staining. The T7 RNA promoter/polymerase system and maxicell analysis showed that a polypeptide with Mw of 35 kDa is the C23O gene product.

KEY WORDS □ ***Peudomonas acidovorans*, catechol 2,3-dioxygenase, T7 RNA promoter/polymerase, maxicell, activity staining**

The necessity of studying microorganisms that utilize aniline as the sole source of carbon and nitrogen is due to the fact that many widely used herbicides and pesticides are aniline derivatives. The process of microbiological transformation of the herbicides is usually accompanied by the accumulation of toxic halosubstituted aniline in the surrounding media. The development of efficient biodegradation process for the destruction of aniline will require organisms with superior oxidative ability. These may be either naturally occuring strains isolated by enrichment culture or those created through laboratory selection or recombinant technology. An excellent candidate for use in which studies is *P. acidovorans* (KCTC2494), an organism capable of oxidizing aniline (1, 4, 10, 11).

There are several reports on the microbiological oxidation of anilines (2, 3, 6, 15). Aniline is metabolized by oxidative deamination to catechol and the aromatic ring is then cleaved via either the ortho- or meta-cleavage pathway (8). Aniline metabolism by KCTC2494 involves at least two different oxygenation process. One is the oxygenation by aniline dioxygenase, the other is the oxygenation by catechol 2,3-dioxygenase. This is the enzyme of the pathway leading to hydroxymuconic semialdehyde from catechol.

In this communication, we report the molecular cloning of 11.3-kb *Sau*3A partial digested DNA fragment from *P.acidovorans* which expresses catechol 2,3-dioxygenase activity in *E. coli*. The data on identification of the gene product and characterization of recombinant cell's C23O activity are included.

MATERIALS AND METHODS

(a) Bacteria, plasmid and media

The strains and plasmids used in this study are listed in Table 1. *E. coli* strain grown Luria broth medium at 37°C. Antibiotics ampicillin (50 μg/ml, w/v) and kanamycin (50 μg/ml, w/v) were used if needed. X-Gal and IPTG were used at final concentrations of 0.002%(w/v). The strain KCTC 2494 was grown at 30°C on aniline minimal medium (16) containing 0.1% aniline as a source of carbon and nitrogen.

(b) DNA techniques

P. acidovorans chromosomal DNA was prepared by a modification of Silhavy *et al*(13). The genomic DNA was partially digested with *Sau*3A and recovered 10-20 kb DNA fragment in range by electroelution and ligated with *Bam*H1-digested pBluescript vector. Plasmid DNA was prepared by the alkaline-sodium dodecyl sulfate procedure

Table 1. *Bacterial strains and plasmids*

Bacterial strains		
P. acidovorans KCTC2494	*Ani*$^+$	Cho (1988)
E. coli XL-1 blue	*rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi* Δ(*lac-pro*AB) F'[*tra*D36*pro*AB$^+$*lac*1 & *lac*ZΔM15	Stratagene Co.
Plasmids		
pBluescript SK(+)	Ap^r	Stratagene Co.
pTZ18R, pTZ19R	Ap^r	Tabor and Richardson (1984)
pGP1-2	Km^r	Tabor and Richardson (1984)
pLP201	pBluescript SK(+) carrying 11.3-kb fragment of chromosomal DNA from KCTC2494	This work
pLP202	Deleted 7.1-kb *Eco*RI fragment of pLP201	This work
pLP203	Deleted 4.9-kb *Eco*RI fragment of pLP201	This work
pLP204	7.0-kb *Sal*I-*Xba*I	This work
pLP205	2.8-kb *Sal*I-*Eco*R1(pTZ18R)	This work
pLP206	2.8-kb *Sal*I-*Eco*R1(pTZ19R)	This work
pLP206	4.3-kb *Xba*I-*Sal*I	This work

(9). DNA cleavage with restriction enzymes and ligation with T4 DNA ligase were performed as recommended by the supplier (Boehringer Mannheim GmbH, New England Biolabs), and cleavage product were visualized by agarose gel electrophoresis. *E. coli* was transformed by the calcium chlorid procedure (9).

(c) Assay for C23O activity

Functional expression of gene was detected by spray on the antibiotic selection plates with an aqueous solution of 0.5 M catechol. Colonies of cells that express the gene become yellow due to the conversion by C23O of catechol to 2-hydroxymuconic semialdehyde as shown Fig. 1. For measuring C23O activity, cell cultures were grown overnight at 37°C in 10 ml LB. Cells were washed with 20 mM phosphate buffer(pH 7.5) and broken by sonication for 3 minutes. Crude extract were centrifuged at 12000 rpm for 15 minutes to remove cellular debris. The reaction initiated by addition of 0.1 M catechol, then C23O specific activity was determined spectrophotometrically by measuring the increase in optical density at 375-nm due to the formation of the reaction product hydroxymuconic semialdehyde (16). One miliunit corresponds to the formation of 1nmol 2-hydroxymuconic semialdehyde per minute at 30 °C. Protein concentrations were measured by the method of Lowry *et al.* (7).

(d)Analysis of C23O protein

C23O was partially purified from *E. coli* strain carrying hybrid plasmids pLP201, pLP202, pLP 203, pLP204, pLP205 and pLP206 by acetone precipitation. These protein fractions were

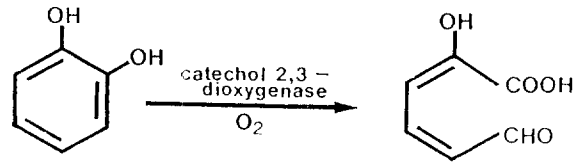

Fig. 1. *Diagramatic representation of chemical reaction catalyzed by catechol 2,3-dioxygenase. Catechol is converted to 2-hydroxymuconic semialdehyde by meta-cleavage of a benzene ring.*

analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and resolved on nondenaturing 8% polyacrylamide gel and then soaked with 0.5 M catechol solution for specific staining of C23O protein.

(e) Gene product analysis

Identification of plasmid-encoded proteins was accomplished by the maxicell method as described by Sancar(12). Alternatively, insert-specific gene products from plasmid containing a T7 promoter (e.g., pTZ18 R) were identified by a modification of the Tabor and Richardson T7 RNA polymerase procedure(14). Cells containing a plasmid with a T7 promoter were transformed with pGP1-2. The pGP1-2 plasmid contains the rifampin-resistant T7 RNA polymerase gene under the control of temperature-sensitive cI repressor. However, even without temperature induction, a sufficient amount of T7 RNA polymerase is made to transcribe the T7 promoter

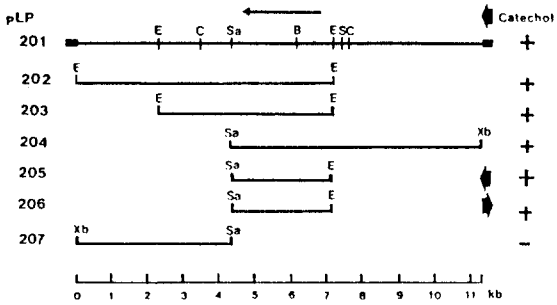

Fig. 2. *Physical map and subclones of the recombinant plasmid pLP201. Expression of C 23O in the subclones was identified by color development yellow(+) or colorless(−) after catechol spray. The direction of transcription of the lac promoter is indicated as thick arrow and the direction of transcription of C23O is indicated as thin arrow.*
Abbreviation: E, *Eco*R1: C, *Cla*1: Sa, *Sal*1: B, *BamH*1: S, *Sma*1: Xb, *Xba*1

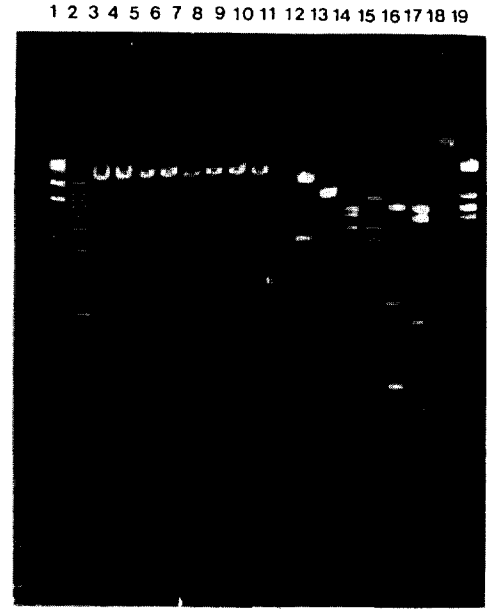

Fig. 3. *Restriction patterns of recombinant plasmid pLP201.*
(A) lane 1, λ-*Hind*III : 2, 1 kb ladder: 3-10, *Bam*H1, *Hind*III, *Xho*I, *Xba*I, *Bgl*II, *Hpa*I, *Sac*I, *Not*I: 12, *Sma*I: 13, *Sal*I: 14, *Eco*RI: 15, *Cla*I: 16, *Ava*I: 17, *Pst*I: 19, λ-*Eco*RI.

plasmsid.

The labeling procedure was as follows. An overnight inoculate minimal glucose medium supplemented with 0.3% casamino acid and the appropriate antibiotics for plasmid maintenance (5×10^6 cells/m*l*). Each 5 m*l* culture was then grown to an OD_{600} of 0.35 at which time the culture was split. Rifampin was added to half of the portions at a final concentration of 240μg/m*l*. Thirty minutes after, the addition of rifampin, the cells were pulse labeled for 5 min with 10μCi of [^{35}S] methionine per m*l*. Cells were harvested by centrifugation, washed with an equal volume of ice-cold M9 salt solution.

The pellet was finally suspended in 100μ*l* of sodium dodecyl sulfate (SDS) lysis buffer for SDS-PAGE analysis.

RESULTS AND DISCUSSION

(a) Cloning of the C23O gene

The cloning strategy is based on coloration of transformant that immediately turned yellow upon spraying with catechol solution indicating the conversion of catechol to the meta-cleavage compound hydroxymuconic semialdehyde. Result of such screening, one colony with this activity was isolated. This colony harbored the plasmid pLP201 (14.3 kb), that contained a 11.3-kb DNA insert in the unique *Bam*HI site of pBluescript. Plasmid pLP201 was mapped with restriction endonucleases, and the result is shown in Fig. 2.

(b) Subcloning of catechol 2,3-dioxygenase gene

The region of the 11.3 kb DNA fragment essential for C23O activity was determined by deletion mapping. Various regions of the DNA fragment were cloned individually into the *E. coli* expression vector pTZ18 and pTZ19 and the resulting strain were assayed for C23O activity. It was observed from a strain carrying recombinant plasmid pLP202, pLP203, pLP204, pLP205 and pLP206 but not from the strain carrying pLP207.(Fig. 3). Among the five plasmids having C23O activity, pLP205 and pLP206 carry the smallest insert, a 2.8-kb fragment defined by the recognition sites of *Eco*RI and *Sal*I. The C23O genes are therefore located between these two sites on the 11.3 kb fragment.

Since, in the presence of IPTG in medium,there was no rise in the rate of conversion of catechol to hydroxymuconic semialdehyde, the conclusion was made that *lac* promoter of the vector does not participate in transcription of the C23O gene in *E. coli* cells. The direction of the transcription was also determined(Fig. 2). The recombinant plasmid pLP206 containing the C23O gene in opposite orientation to the *lac* promoter of vector pTZ19R was constructed and shown to enable *E. coli* cells to convert catechol independence upon the presence or absence of *lac* promoter inducer IPTG in cultivation medium. Thus it seems that the cloned C23O gene contains its own promoter

Table 2. *Activity of catechol 2,3-dioxygenase gene in E. coli (XL-1-blue) catechol2,3-dioxygenase activities were measured spectrophotometrically at 375 nm. One unit of the enzyme activity was expressed as 1 μmole 2-hydroxymuconic semialdehyde formation per minute.*

Plasmid	Specific Activity (units/mg of protein)
None	0
pLP 201	231
pLP 202	223
pLP 203	290
pLP 204	177
pLP 205	369
pLP 206	392

signal which can be recognized and employed by *E. coli* transcription apparatus.

(c) Expression of C23O gene in *E. coli*

Strains of *E. coli* harboring the recombinant plasmid pLP201, pLP202, pLP203, pLP204, pLP 205 and pLP206 were tested for their ability to convert catechol to hydroxymuconic semialdehyde (i.e., for expression of the C23O gene). The results showed that the recombinant plasmids had the C23O activity (Fig. 2).

To analyze more quantitatively the nature and magnitude of the expression of the cloned gene in *E. coli*, measurements of the specific activities of C23O gene were made in *E. coli* strains harboring the recombinant plasmid (Table 2).

As expected, C23O activity was detected in *E. coli* cells harboring recombinant DNA, where as none could be found in the parental *E. coli* cells.

(d) Identification of C23O

To identify whether C23O is produced by the cloned cells, partially purified cell-free extracts from each clone were resolved by polyacrylamide gel system allow rapid identification of C23O by spraying the gel with catechol solution after electrophoresis was terminated. As a result, yellow band appeared at coincident locations in the gel for all extract except *E. coli* strain haboring pBluscript only(Fig. 4).

In an additional experiment, protein fractions were resolved by 12% SDS-PAGE. In this case, to show the intact form of C23O protein, protein fractions were suspended with SDS-lysis buffer except β-mecaptoethanol and then resolved by 12% SDS-PAGE. As a result, one 35 kDa protein band appeared on the gel(Fig. 5).

(e) Polypetide analysis of the C23O gene

To determine the polypeptide responsible for the C23O activity, we analyzed plasmid-encoded polypeptides produced by maxicell and T7 RNA polymerase/promoter system harboring different plasmids. In maxicell system, proteins encoded

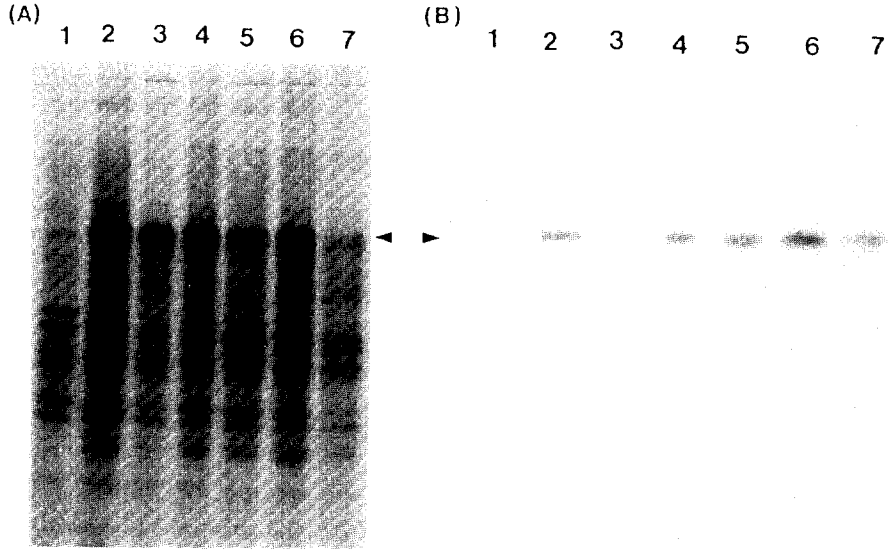

Fig. 4. *Activity staining of C23O from E. coli cells harboring hybrid plasmids. Protein fractions prepared from E. coli cells harboring pTZ18R (lane 1), pLP201(lane 2), pLP202(lane 3), pLP203(lane 4), pLP204(lane 5), pLP205 (lane 6) and pLP206(lane 7) were resolved on 8% nondenaturing polyacrylamide gel by electrophoresis. A gel was stained with comassie blue staining solution and B gel was stained with 0.5 M catechol solution after electrophorisis. Black arrowhead points to C23O protein.*

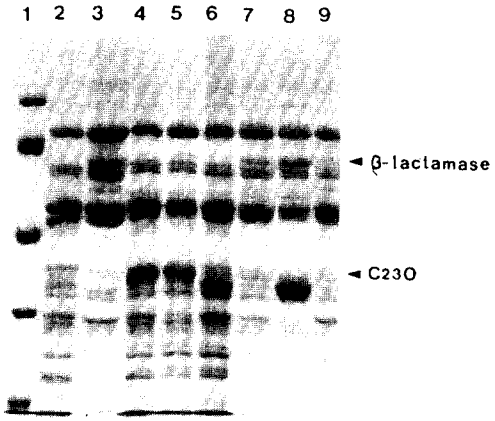

Fig. 5. *SDS-PAGE analysis of C23O products produced in E. coli cells carrying pLP201 through pLP205. Various fractions collected from acetone precipitation were analyzed by 12% SDS-PAGE. Total proteins of E. coli cell carrying no plasmid (lane 2), pBluescript(lane 3), pLP201(lane 4, 5), pLP202(lane 6), pLP203 (lane 7), pLP204(lane 8), and pLP205(lane 9) are shown for comparison. The molecular weight standards (lane 1) are soybean trypsin inhibitor(21 kDa), bovine carbonic anhydrase (31 kDa), hen egg white ovalbumin(45 kDa), bovine serum albumin(66 kDa), and rabbit muscle phosphorylase b(97 kDa).*

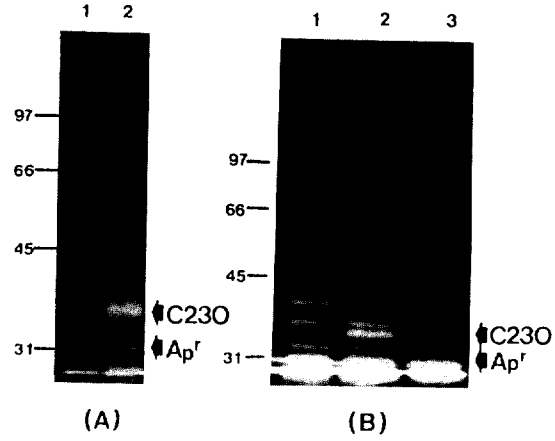

Fig. 6. *Radioautograph of [^{35}S]-methionine labeled proteins synthesized in E. coli cells carrying hybrid plasmid.*
(A) Identification of C23O in maxicells. Lane 1, pTZ18R; lane 2, pLP20 (B) Identfication of C23O by the *in vivo* T7 RNA polymerase protocol of Tabor and Richardson (14). Lane 1, Rifampin-treated cells containing pTZ18R; lane 2, pLP203; lane 3, pLP205. Size markers are shown on the left in kilodaltons. The molecular masses of the protein were calculated from unlabeled size standards run on the same gel.

by plasmid DNA are specifically labeled in UV irradiated cells of *E. coli* carrying *rec*R and *uvr*A mutations because extensive degradation of chromosomal DNA occurs concurrentry with amplification of plasmid DNA. In T7 RNA polymerase/promoter system, synthesis of large amounts of foreign gene products is directed by the phage T7 gene 10 promoter which uses T7 RNA polymerase. This polymerase transcribes the gene 10 promoter so efficiently that it uses up most of ribonucleotide triphosphates in the cell and drastically inhibits transcription of genes by the host polymerase. The plasmid-encoded polypeptides of harboring these hybrid plasmids are shown in Fig. 6. As expected, strain harboring plasmid that encode and express the DNA required for C23O activity expressed about 35 kDa polypeptide. It seems therefore reasonable to assume that the 35 kDa polypeptide is the product of the C23O gene.

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RFERENCE

1. **Alexander, M.**, 1981. Biodegradation of chemicals environmental concern. *Science*, **211**, 132-138.
2. **Anson, J.G. and G. mackinnon,** 1984. Novel *Pseudomonas plasmid* involved in aniline degradation. *Appl. & Environ. Microbiol.*, **48**, 868-869.
3. **Aoki, K., R. Shinke and H. Nishira**, 1983a. Metabolism of aniline by Rhodococcus erythropolis AN-13. *Agric. Biol. Chem.*, **47**, 1611-1616.
4. **Cho, K.Y., I.H. Ha, K.S. Bae and Y.H. Kho**. 1988. Isolation and characterization of aniline degrading bacteria. *Kor. J. Microbiol Bioeng.*, **16**, 421-426.
5. **Hallas, L.E. and M. Alexander**, 1983. Microbiol transformation of nitroaromatic compound in sewage effluent. *Appl. & Environ. Microbiol.*, **45**, **4**, 1234-1241.
6. **Loida, M., C. Hinteregger, G. Ditzelmuller, A. Ferschl and F. Streichsbier**, 1990. Degradation of aniline and monochlorinated anilines by

soilborn *Pseudomonas acidovorans* strains. Arch. Microbiol., **155**, 56-61.
7. **Lowory, O.H. , N.J. Rosebrough , A.L. Farr and R. Randall**, 1951. Protein measurement with Folin phenol reagent . *J. Biol.-Chem.*, **193**, 265-273.
8. **Lyons, C.D., S. Katz and R. Bartha**, 1984. Mechanisms and pathways of aniline elimination from aquatic environments. *Appl. & Environ. Microbiol.*, **48**, 3, 491-496.
9. **Maniatis, T., E.F. Fritch and J. Sambrook**, 1990. Molecular cloning: a laboratory mannual. Cold Spring Garbor Laboratory, New York.
10. **Minard, R.D., S. Russel and J.M. Ballay**, 1977. Chemical transformation of 4-chloroaniline to a triazine in a bacterial culture medium. *J. Agric. Food. Chem.*, **25**, 841-844.
11. **Pemberbon, J.M.**, 1979. Pesticide degrading plasmid; A biological answer to environmental pollution by phenoxy herbicides. *Anbio.*, **8**, **5**, 202-205.
12. **Sancar, A., A.M. Hack, and W.D. Rupp**, 1979. Simple method for identification of plasmid-coded proteins. *J. Bacteriol.*, **137**, 692-693.
13. **Silhavy, T.J., M.L. Berman and L.W. Enquist**, 1984. Experiments with gene fusion. Cold spring Harbor Laboratory, New York.
14. **Taber, S. and C.C. Richardson**, 1985. A bacteriophage T7 RNA Polymerase promoter system for controlled exclusive expression of specific genes . *Proc.-Natl. Acad. Sci. USA.*, **82**, 1074-1078
15. **You, I.S. and R. Bartha**, 1982. Metabolism of dichloroaniline by *Pseudomonas putida*. *J. Agric. Food. Chem.*, **30**, 274-277.
16. **Zeyer, J., A.W. Wasserfallen and K.N. Timmis**, 1985. Microbiol mineralization of ring-substituted anilines through an ortho Cleavage pathway. *Appl. Environ. Microbiol.*, **50**, 447-453.
17. **Zukowski, M.A., O.F. Gaffney , D. Speck, M. Kauffmann, A. Coisecup and J.-P. Lewcq**, 1983. Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned *Pseudomonas gene*. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 1101-1105.

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초 록: Aniline을 분해하는 *Pseudomonas acidovorans*의 Catechol 2,3-Dioxygenase 유전자의 Cloning과 특성

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Catechol 2,3-dioxygenase(C23O)는 oxidative ring cleavage를 촉매하며 catechol을 2-hydroxymuconic semialdehyde로 분해한다. 이는 자연환경에 널리 분포한 제초제의 일종인 aniline의 분해 대사과정의 주요 반응중의 하나이다. 자연에서 분리한 *Pseudomonas acidovorans* KCTC2494 균주는 단일 탄소원과 질소원으로 aniline을 이용할 수 있는 분해능을 가진다. Aniline은 산화적 deamination 과정에 의해 catechol로 개열되고, 이러한 방향족 고리는 그 후 C23O에 의해서 *meta*-cleavage경로를 통하여 분해된다. 이 연구에서는 KCTC2494 균주의 chromosome DNA로부터 C23O 유전자의 클로닝을 수행하였다. KCTC2494로부터 분리한 chromosome DNA를 *Sau*3A로 부분절단하여 *Bam*H1으로 절단된 phagemid vector pBleuscript SK^+에 삽입하여 *E. coli* XL-1 blue균주로 형질전환하였으며, 이를 pLP201로 명명하였다. 11.3-kb 재조합 plasmid pLP201은 catechol을 노란색 대사산물인 hydroxymuconic semialdehyde로 전환시킬 수 있으며, cloned cell에서의 C23O의 실제적 발현정도는 KCTC2494 균주에서 보다 훨씬 높았다. Delectional subcloning실험에 의해 이 C23O 유전자는 2.8-kb정도로 제한되었으며, *E. coli*전사 기구에 의해서 인지되고 실행될 수 있는 자체의 promoter로 작동함과 전사방향도 알게되었다. SDS-PAGE, maxicell 그리고 T7 RNA promoter/polymerase system에 의해서 35 kDa정도의 polypeptide가 합성됨이 확인되었고, 이는 C23O유전자 산물로 여겨진다.