This article was downloaded by: [Universitat Politècnica de València]
On: 25 October 2014, At: 11:14
Publisher: Taylor & Francis
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Catechol 2,3-Dioxygenase from Pseudomonas sp. Strain ND6: Gene Sequence and Enzyme Characterization

Yun JIANG[a], Xianglong YANG[b], Bin LIU[c], Huabing ZHAO[a], Qiuxiang CHENG[a] & Baoli CAI[a]

[a] Department of Microbiology, Nankai University

[b] Department of Laboratory Science, Tianjin Medical University

[c] Genomics Institute, Chinese Academy of Sciences

Published online: 22 May 2014.

To cite this article: Yun JIANG, Xianglong YANG, Bin LIU, Huabing ZHAO, Qiuxiang CHENG & Baoli CAI (2004) Catechol 2,3-Dioxygenase from Pseudomonas sp. Strain ND6: Gene Sequence and Enzyme Characterization, Bioscience, Biotechnology, and Biochemistry, 68:8, 1798-1800, DOI: 10.1271/bbb.68.1798

To link to this article: http://dx.doi.org/10.1271/bbb.68.1798

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis makes every effort to ensure the accuracy of all the information (the "Content") contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden. Terms & Conditions of access and use can be found at http://www.tandfonline.com/page/terms-and-conditions

Biosci. Biotechnol. Biochem., **68** (8), 1798–1800, 2004

Note

Catechol 2,3-Dioxygenase from *Pseudomonas* sp. Strain ND6: Gene Sequence and Enzyme Characterization

Yun JIANG,[1] Xianglong YANG,[2] Bin LIU,[3] Huabing ZHAO,[1] Qiuxiang CHENG,[1] and Baoli CAI[1,†]

[1]*Department of Microbiology, Nankai University, Tianjin 300071, China*
[2]*Department of Laboratory Science, Tianjin Medical University, Tianjin 300020, China*
[3]*Genomics Institute, Chinese Academy of Sciences, Beijing 101300, China*

Received April 14, 2004; Accepted May 27, 2004

Downloaded by [Universitat Politècnica de València] at 11:14 25 October 2014

The catechol 2,3-dioxygenase (C23O) gene in naphthalene catabolic plasmid pND6-1 of *Pseudomonas* sp. ND6 was cloned and sequenced. The C23O gene was consisted of 924 nucleotides and encoded a polypeptide of molecular weight 36 kDa containing 307 amino acid residues. The C23O of *Pseudomonas* sp. ND6 exhibited 93% and 89% identities in amino acid sequence with C23Os encoded by naphthalene catabolic plasmid NAH7 from *Pseudomonas putida* G7 and the chromosome of *Pseudomonas stutzeri* AN10 respectively. The *Pseudomonas* sp. ND6 C23O gene was overexpressed in *Escherichia coli* DH 5α using the *lac* promoter of pUC18, and its gene product was purified by DEAE-Sephacel and Phenyl-Sepharose CL-4B chromatography. The enzymology experiments indicated that the specific activity and thermostability of C23O from *Pseudomonas* sp. ND6 were better than those of C23O from *Pseudomonas putida* G7.

Key words: *Pseudomonas* sp. ND6; catechol 2,3-dioxygenase; gene sequence; enzyme characterization

Many microorganisms are able to utilize xenobiotic aromatic compounds as the sole carbon and energe source for growth. Various efforts have been made to improve their capabilities for environmental pollutant degradation and bioremediation.[1)] The aromatic compounds, including biphenyl, naphthalene, toluene, phenol, and benzoates can be converged to catechol as a catabolic intermediate by microbial degradation. The catechol formed as an intermediate is subjected to its ring fission by catechol 1,2-dioxygenase or catechol 2,3-dioxygenase. The catechol 1,2-dioxygenase is an intradiol-type enzyme which cleaves the C–C bond at the 1,2-(*ortho*) position of catechol to make *cis*,*cis*-muconic acid, which is further degraded by the β-ketoadipate pathway. The catechol 2,3-dioxygenase (C23O), an extradiol-ring fission enzyme, catalyzes the conversion of catechol to 2-hydroxymuconic semialdehyde by breaking the C–C bond at the 2,3-(*meta*) position, which is part of the α-ketoadipate pathway.[2)] Many research works have been devoted to different aspects of C23O for its potential theoretical and applied values in environmental protection and other areas.[3)] At present, over 30 extradiol catechol dioxygenases has been reported[4)] and the nucleotide sequences of some catechol 2,3-dioxygenase genes has been determined.[2,5–8)]

Previously we isolated a naphthalene-degrading bacterium, strain ND6. The naphthalene catabolic genes of this strain were localized on the 102 kb plasmid, pND6-1, in a DNA hybridization experiment.[9)] In this study, we determined and analyzed the 16S rRNA gene sequence of strain ND6, cloned, expressed, and sequenced the C23O gene of strain ND6, and purified and characterized the catechol 2,3-dioxygenase expressed.

The primer pair 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (CGGYTACCTTGTTACGACTT)[10)] was used for PCR amplification of 16S rRNA gene. The PCR product was cloned into the pMD 18-T vector (Takara Biotechnology Co.) following the manufacturer's instructions. Primers targeted at the T7 and Sp6 promoters and a universal primer 533F (GTGCCAGCMGCCGCGGTAA)[10)] were used for sequencing to cover the entire length of the 16S rRNA gene. The final sequence of 1499 bp was submitted to the NCBI GenBank under accession no. AY589689. The BLAST search of this sequence indicated that it was matched at 99% to 33 strains of *Pseudomonas*. Hence strain ND6 was identified as a *Pseudomonas* sp.

Plasmid DNA of *Pseudomonas* sp. ND6 was isolated by the SDS-alkali lysis method[11)] and purified by CsCl-ethidium bromide ultracentrifugation.[12)] To construct a plasmid shotgun library, the purified plasmid DNA was broken by sonication and DNA fragments of 1∼3 kb in size were isolated by agarose gel electrophoresis. The DNA ends were filled in, ligated into plasmid pUC18, and transformed into *E. coli* DH 5α by electroporation.[12)] White clones containing inserts were randomly selected. DNA templates from white clones were

† To whom correspondence should be addressed. Tel: +86-22-23503617; Fax: +86-22-23508800; E-mail: caibaoli@nankai.edu.cn
Abbreviations: C23O, catechol 2,3-dioxygenase; IPTG, isopropylthio-β-D-galactoside; PAGE, polyacrylamide gel electrophoresis

Downloaded by [Universitat Politècnica de València] at 11:14 25 October 2014

prepared from 1 ml overnight cultures using the alkaline lysis method.[12] Each of cloned fragments was sequenced from both ends by using an ABI model 377A DNA sequencer (Applied Biosystems, Foster city, California, USA). The DNA sequences in the plasmid shotgun library were submitted to a BLAST search of the NCBI GenBank database to identify naphthalene degradation genes. Computational analysis indicated that 23 genes were predicted to be involved in naphthalene catabolism. The complete nucleotide sequence of pND6-1 from strain ND6 has been deposited in GenBank under accession no. AY208917 (Wei Li and Baoli Cai, unpublished).

In the sequence library of pND6-1, we found that the cloning fragment of 1551 bp (no. 0842) included a coding sequence of C23O. The recombinant plasmid containing the C23O gene was designated pNDN. The C23O gene was consisted of 924 nucleotides and encoded a polypeptide of molecular weight 36 kDa containing 307 amino acid residues. The sequence of catechol 2,3-dioxygenase of *Pseudomonas* sp. ND6 exhibited 95% homology at the nucleotide level and 93% homology at the amino acid level with the corresponding enzyme from *Pseudomonas putida* G7[5] (Fig. 1). Ishida *et al.* suggested that C23O from *Pseudomonas putida* mt-2 has five conserved active-site residues, His153, His199, His214, Tyr255, and Glu265.[4] These conserved active-site residues were not changed in the C23O of strain ND6. This study indicated that globally distributed catechol 2,3-dioxygenase genes are highly conserved among different bacterial species.

With the exception of pNDN containing the *Pseudomonas* sp. ND6 C23O gene, we also constructed a recombinant plasmid, pNDG, containing the C23O gene from *Pseudomonas putida* G7 using pUC18 as a vector.[9] *E. coli* DH 5α containing pNDN or pNDG were grown in LB medium supplemented with ampicillin for 12 h, then IPTG was added at a final concentration of 0.1 mM and growth continued for 4 h to induce gene expression. The cells were harvested by centrifuging 100 ml of culture and washed with 20 mM sodium phosphate buffer (pH7.5). Pelleted cells were resuspended in 8 ml of the same buffer, 2 ml of cold acetone was added, and then the mixture was subjected to sonication with a cell disruptor. The crude extract was obtained by centrifugation at 13,682 $\times$ *g* for 30 min at 4 °C. The supernatant was fractionated by 20%, 40%, and 60% $(NH_4)_2SO_4$ saturations. The precipitates of the 40% and 60% $(NH_4)_2SO_4$ saturations were dissolved in 2 ml of 20 mM sodium phosphate buffer, mixed, and dialyzed overnight against the same buffer. The dialysate was applied to a DEAE-Sephacel column (2 $\times$ 30 cm) preequilibrated with 20 mM sodium phosphate buffer. Proteins in the column were eluted with a linear NaCl gradient up to 0.7 M in 20 mM sodium phosphate buffer. Fractions with C23O activity were pooled and then applied to a Phenyl-Sepharose CL-4B column (1.5 $\times$ 20 cm) preequilibrated with 50 mM sodium phosphate buffer (pH7.5). Proteins were eluted with a linear gradient of 50 mM sodium phosphate buffer and distilled water. Fractions with C23O activity were pooled and concentrated by ultrafiltration. C23O activity was measured spectrophotometrically following the formation of 2-hydroxymuconic semialdehyde from catechol at 375 nm. Activity assays were performed in 50 mM sodium phosphate buffer containing 0.5 mM catechol as substrate. One unit of enzyme activity was defined as the

```
ND6 C230  MKKGVMRPGH VQLRVLDMGK ALEHYIELLG LIEMDRDDQG RVYLKAWSEV  50
G7  C230  MNKGVMRPGH VQLRVLDMGK ALEHYVELLG LIEMDRDDQG RVYLKAWTEV
ND6 C230  DKFSVVLREA DEPGMDFMGF KVVDEVSLQQ LEQDLQAHGC SVEQVPAGEL 100
G7  C230  DKFSVVLREA DEPGMDFMGF KVVDEDSLNR LTDDLLNFGC LIENVAAGEL
ND6 C230  NSCGRRVRFQ APSGHHFELY ADKEYTGKWG VNEVNPEAWP RDLKGMSAVR  150
G7  C230  KGCGRRVRFQ APSGHHFELY ADKEYTGKWG VSEVNPEAWP RDLKGMAAVR
ND6 C230  FDHCLLYGDE LQATYELFTE VLGFYLAEQV VDAEGIRLAQ FLSLSTKAHD  200
G7  C230  FDHCLLYGDE LQATYELFTE VLGFYLAEQV VDADGIRLAQ FLSLSTKAHD
ND6 C230  VAFIQHAEKG KFHHASFLLD TWEDVLRAAD LISMTDTSID IGPTRHGLTH  250
G7  C230  VAFIHHAEKG KFHHASFFLD TWEDVLRAAD LISMTDTSID IGPTRHGLTQ
ND6 C230  GKTIYFFDPS GNRCEVFCGG NYNYPDHKPV TWLAKDVGKA IFYHDRVLNE  300
G7  C230  GKTIYFFDPS GNRCEVFCGG NYNYPDHKPV TWLAKDVGKA IFYHDRVLNE
ND6 C230  RFMTVMT  307
G7  C230  RFMTVMT
```

Fig. 1. Comparison of Deduced Amino Acid Sequences of Catechol 2,3-Dioxygenases from *Pseudomonas* sp. Strain ND6 and *Pseudomonas putida* G7.

The different amino acid residues of the two enzymes are indicated by frames.

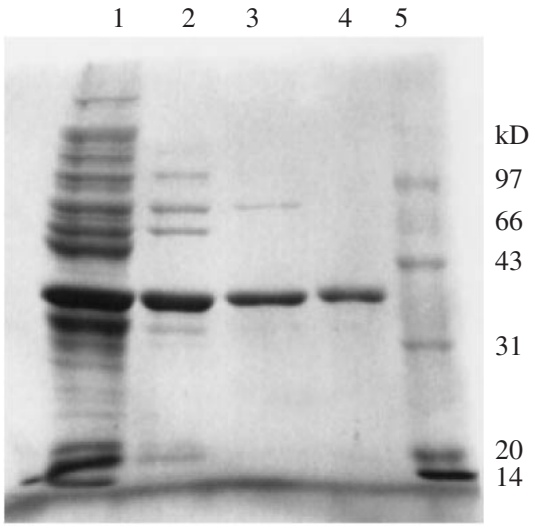

Fig. 2. SDS-PAGE of Catechol 2,3-Dioxygenases from *Pseudomonas* sp. Strain ND6.

(1) crude cell extract; (2) 40% and 60% ammonium sulfate fractions; (3) partially purified enzyme by DEAE- Sephacel chromatography; (4) purified enzyme by DEAE-Sephacel chromatography and Phenyl-Sepharose CL-4B chromatography; (5) Protein molecular weight markers.

amount of enzyme that converts 1 μmol of substrate per minute. The specific activity of the enzyme was defined in terms of units per mg of protein.[2)] Enzyme kinetic parameters were determined by double-reciprocal plots. The thermostability of C23O was determined by incubating an enzyme solution at 57 °C in 50 mM sodium phosphate buffer. C23O activity was determined every 5 min.

Overexpression of the C23O gene of *Pseudomonas* sp. ND6 in *E. coli* DH 5α was demonstrated by determining the C23O activity and SDS-PAGE of the crude extract (Fig. 2). The purified C23O exhibited a single band of about 36 kDa on SDS-PAGE (Fig. 2). The enzyme kinetic experiments indicated that K_m and the specific activity of the C23O of strain ND6 for catechol were 11 μM and 11.96 U/mg of protein respectively, higher than those of the C23O from *Pseudomonas putida* G7 (4.5 μM and 7.43 U/mg of protein respectively). The thermostability of the C23O from strain ND6 was better than that of the C23O from *Pseudomonas putida* G7. After incubating at 57 °C for 10 min, the residual activity of ND6 C23O was 37%, while that of G7 C23O was 28% under the same conditions.

In conclusion, we have cloned and expressed a catechol 2,3-dioxygenase gene from naphthalene-degrading *Pseudomonas* sp. strain ND6. The gene product was purified by DEAE-Sephacel and Phenyl-Sepharose CL-4B chromatography. The specific activity and thermostability of the C23O from *Pseudomonas* sp. ND6 were better than those of C23O from *Pseudomonas putida* G7.

Acknowledgments

This work was supported by grant no. 30270274 from the National Natural Science Foundation of China.

References

1) Dua, M., Singh, A., Sethunathan, N., and Johri, A. K., Biotechnology and bioremediation: successes and limitations. *Appl. Microbiol. Biotechnol.*, **59**, 143–152 (2002).
2) Moon, J., Chang, H., Min, K. R., and Kim, Y., Cloning and sequencing of the catechol 2,3-dioxygenase gene of *Alcaligenes* sp. KF711. *Biochem. Biophys. Res. Commun.*, **208**, 943–949 (1995).
3) Jiang, T., Ji, C. N., Sheng, X. Y., Chen, M. Q., Xie, Y., Gong, W. M., and Mao, Y. M., Crystal structure of thermostable catechol 2,3-dioxygenase determined by multiwavelength anomalous dispersion method. *Chinese Science Bulletin*, **47**, 307–309 (2002).
4) Ishida, T., Kita, A., Miki, K., Nozaki, M., and Horiike, K., Structure and reaction mechanism of catechol 2,3-dioxygenase (metapyrocatechase). *International Congress Series*, **1233**, 213–220 (2002).
5) Ghosal, D., You, I. S., and Gunsalus, I. C., Nucleotide sequence and expression of gene *nahH* of plasmid NAH7 and homology with gene *xylE* of TOL plasmid pWWO. *Gene*, **55**, 19–28 (1987).
6) Bartilson, M., and Shingler, V., Nucleotide sequence and expression of the catechol 2,3-dioxygenase gene of phenol-degrading *Pseudomonas* CF600. *Gene*, **85**, 233–238 (1989).
7) He, Z. Q., Mao, Y. M., Sheng, Z. J., and Shen, R. Q., Complete nucleotide sequence of *pheB* gene encoding catechol 2,3-dioxygenase from *Bacillus stearothermophilus*. *Chin. Biochem.*, **11**, 114–116 (1995).
8) Oh, J. M., Kang, E., Min, K. R., Kim, C.-K., Kim, Y.-C., Lim, J.-Y., Lee, K.-S., Min, K.-H., and Kim, Y., Structure of catechol 2,3-dioxygenase gene encoded in TOM plasmid of *Pseudomonas cepacia* G4. *Biochem. Biophys. Res. Commun.*, **234**, 578–581 (1997).
9) Zhang, X., Yue, X., Huang, J., Niu, S., and Cai, B., Isolation and identification of naphthalene-degrading plasmid pND6. *Chin. J. Appl. Environ. Biol.*, **6**, 187–190 (2000).
10) Lane, D. J., 16S/23S rRNA sequencing. In "Nucleic Acid Techniques in Bacterial Systematics", eds. Stackebrandt, E., and Goodfellow, M., John Wiley & Sons, Chester, UK, pp. 371–375 (1991).
11) Cai, B., Gao, C., and Jiao, R., A simple method for the isolation of large plasmids in *Pseudomonas*. *Acta Scientiae Circumstantiae*, **4**, 291–295 (1984).
12) Sambrook, J., Fritsch, E. F., and Maniatis, T., Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

Downloaded by [Universitat Politècnica de València] at 11:14 25 October 2014