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Cloning and Nucleotide Sequence of Catechol 2,3-Dioxygenase Gene from the Naphthalene-Degrading *Pseudomonas putida* NA3

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ABSTRACT

Pseudomonas putida strain NA3 was found to degrade naphthalene very efficiently and use it as a main carbon and energy source to support its growth. Naphthalene was found to be metabolized via the extradiol *meta* ring-cleavage pathway. Catechol 2,3-dioxygenase was found to be the responsible key enzyme for the dearomatization of naphthalene. Catechol 2,3-dioxygenase gene from the naphthalene-degrading *P. putida* strain NA3 was cloned in *Escherichia coil* JM109. The plasmid, pGEM-T Easy, was isolated from the transformant containing catechol 2,3-dioxygenase encoding base sequences. The nucleotide base sequence of a 900 bp segment encoding the catechol 2,3-dioxygenase (C23DO) was determined. This segment showed an open reading frame (ORF), which encodes a polypeptide of 306 amino acids. The nucleotide base sequence of ***nahB*** gene as well as its corresponding amino acid sequence had a best matching with ***phe*B** gene coding for the same dioxygenase from the phenol-degrading *Pseudomonas aeruginosa* JI104.

Key Words: Catechol 2,3-dioxygenase; Naphthalene; Biodegradation; *Pseudomonas putida;* Sequencing

INTRODUCTION

Polycyclic aromatic compounds (PAHs) are ubiquitous environmental pollutants that have been found to have toxic, mutagenic, and carcinogenic properties (International Agency for Research on Cancer [IARC] (Ahn *et al*., 1999). Interest in the biodegradation mechanisms and environmental fate of PAHs is prompted by their ubiquitous distribution and their potentially deleterious effects on human health (Kanaly & Harayama, 2000).

Soil microorganisms such as *Pseudomonas putida* have the ability to aerobically catabolize a wide range of aromatic hydrocarbons via suites of specialized catabolic enzymes. Operons encoding these catabolic enzymes are frequently found on plasmids or, if located on the chromosome, are often carried on transposons or flanked by insertion sequences, ensuring a degree of transferability (Williams & Sayer, 1994).

The metabolism of naphthalene has been studied more extensively than that of any other Polycyclic Aromatic Hydrocarbons (PAH). *Pseudomonas* spp. metabolize naphthalene via naphthalene cis-1,2-dihydrodiol, 1,2-dihydroxynaphthalene, 2-hydroxychromene-2-carboxylic acid (HCCA), trans-O-hydroxybenzylidenepyruvic acid (tHBPA), salicylaldehyde, salicylic acid, and either catechol or gentisic acid (Utkin *et al*., 1990; Eaton & Chapman, 1992).

Catechol 2,3-dioxygenase transforms catechol by *meta*-cleavage to 2-hydroxymuconate semialdehyde. In *Pseudomonas* sp., *P. testosteroni*, and *P. stutzeri*, Catechol 2,3-dioxygenase was found to be induced by growth on naphthalene (Garcia-Valdes *et al*., 1988).

The *meta*-cleavage pathway is also believed to function in the degradation of other compounds like phenol. *Pseudomonas* sp. strain CF600 could grow efficiently with phenol as a sole carbon and energy source (Shingler *et al*., 1989). The ring-cleavage enzyme catechol 2,3-dioxygenase is encoded by *dmp*B (Powlowski & Shingler, 1994) and catalyzes the conversion of catechol to 2-hydroxymuconate semialdehyde.

The aim of this work is to gain an understanding of the distribution of the potential degradative enzyme, catechol 2,3-dioxygenase, in the environment, through cloning and determining the nucleotide base sequence of such enzyme which is well known for its involvement in phenol catabolism from the naphthalene-degrading bacterium *Pseudomonas putida* strain NA3.

MATERIALS AND METHODS

Bacterial strain, plasmid and culture conditions. *Pseudomonas putida* NA3 that was previously isolated from wastes of tar plant and identified in previous study (GenBank accession number AB109013 (El-Sayed *et al*., 2003) was used in this study. Strain NA3 was cultured aerobically at 30ºC in mineral salt medium (Farrell & Quilty, 1999) with naphthalene as the sole carbon source. Growth was measured by following the optical density at 600 nm (OD_{600}). The harvested cells were stored at –20ºC. Competent cells of *Escherichia coli* JM109 obtained from Takara Shuzo Co., Ltd, Japan were used to produce recombinant plasmid DNA. The plasmid pGEM T-Easy (Promega Corp., Madison, USA) was used as a vector for *E. coli* JM109. *E. coli* cells carrying recombinant derivatives

of pGEM T-Easy were grown at 37ºC on Luria-Bertani (LB) medium (Sambrook & Russell, 2001) with ampicillin (100 mg L^{-1}).

Absorption spectra and catechol 2,3-dioxygenase assay. Cells were grown overnight at 30ºC with shacking in mineral salt medium with naphthalene in screw-caped bottles. catechol was fed at late exponential phase to induce catechol 2,3-dioxygenase expression. Cells were assayed for catechol 2,3-dioxygenase (Kaschabek *et al.,* 1998). The activity was measured by following the formation of 2-hydroxy-muconate semialdehyde (2-HMS), the *meta*-cleavage product of catechol monitored by the increase in absorbance at 375 nm. Spectra were recorded on a Shimadzu Recording Spectrophotometer.

PCR amplification of catechol 2,3-dioxygenase gene. Extraction of genomic DNA, gel electrophoresis and PCR were performed by standard procedure (Ausubel *et al*., 1999; Sambrook & Russell, 2001).

Based on the N-terminal amino acid sequences of the active protein fraction determined (unpublished data), degenerate primers ***nah*B F** and ***nah*B R** were designed. The degenerate sense primer (***nah*B F**) had the sequence (5`-GTCGTGATGAAAAAAGGAGAGTT-3`). The antisense primer (NahB R) was determined from the conserved region of ***phe*B** gene from *Pseudomonas aeruginosa* (GenBank accession number JQ0182) and had the sequence (5`-GGGCTTTCAGGTCAGCACGGTCA-3`).

Template DNA concentration was adjusted to 100 µg mL^{-1}. The reaction mixture was prepared to a final volume of 20 µL and thermal cycler temperature controller program was set as 95ºC for 5 min, 30 s, 52ºC for 30 s, 72ºC for 1 min, 10 min, and 4ºC forever. After 30 cycles the reaction was terminated and PCR product was detected by 1% agarose gel electrophoresis. The fragment was eluted by GenElute Minus EtBr spin column and purified by ethanol precipitation.

Cloning and DNA sequencing. The purified PCR product was ligated to pGEM-T Easy vector and used to transform *E. coli* JM109 cells. White colonies on LB plates containing 50 µg ml^{-1} ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were picked, and recombinant DNA was extracted and purified by Wizard plus plasmid DNA purification kit (Mezei & Storts, 1994). The nucleotide sequence analysis of the selected clones was determined by automated florescent dye terminator sequencing (Sanger *et al*., 1977) with a model ABI 310 sequencer (Applied Biosystems, CA, USA). T7 and M13 primers were selected to cover up the range of 900 bp. Related sequences and alignments were obtained from the GenBank database (National Center for Biotechnology Information, National Library of Medicine) using BLAST search program.

RESULTS AND DISCUSSION

Naphthalene biodegradation. Catechol 2,3-dioxygenase catalyzes the oxidative cleavage of catechol intermediates from phenol in a number of bacterial pathways (Harayama *et al*., 1992). UV/VIS spectrophotometric analysis showed that *P. putida* NA3 metabolizes naphthalene via extradiol *meta*-ring cleavage pathway. Fig. 1 shows the time-dependent change of the supernatant of an incubation containing naphthalene and cells of *P. putida* NA3. The respective increase and decrease in absorption spectrum indicates the formation and disappearance of intermediate compounds as a result of naphthalene degradation. It was found that *P. putida* NA3 starts naphthalene degradation by its hydroxylation to 1,2-dihydroxynaphthalene. Further metabolism of this compound results in the formation of salicylic acid and catechol, which undergoes *meta* ring-cleavage producing the chromophoric product 2HMS. When catechol was fed to the growing cells, an increase in

Fig. 1. Absorption spectra of supernatant of cell suspension of *P. putida* NA3 with naphthalene showing the time dependent biotransformation of naphthalene via *meta*-cleavage pathway, catechol biotransformation to 2HMS as a result of *meta*-ring cleavage (inset figure).

a = 1,2-Dihydroxynaphthalene; b = Salicylic acid; c = Catechol; d = 2-Hydroxymuconate semialdehyde

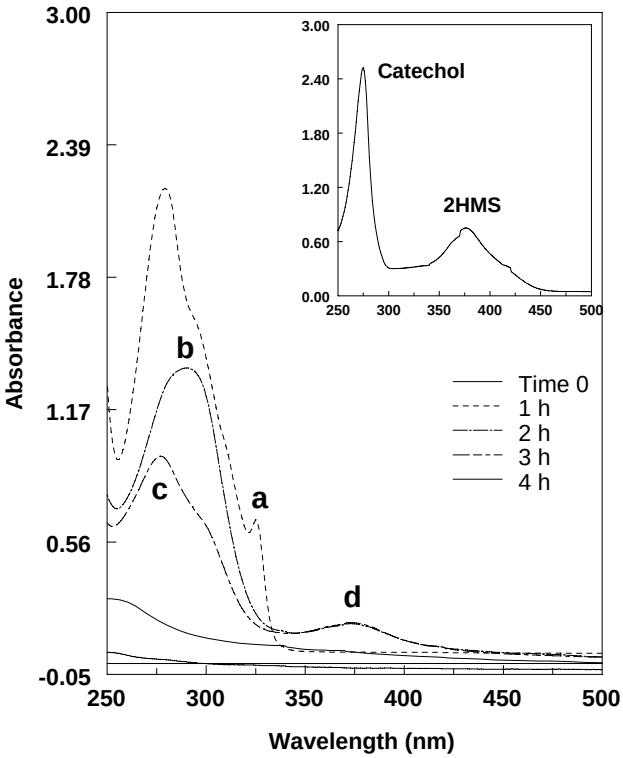
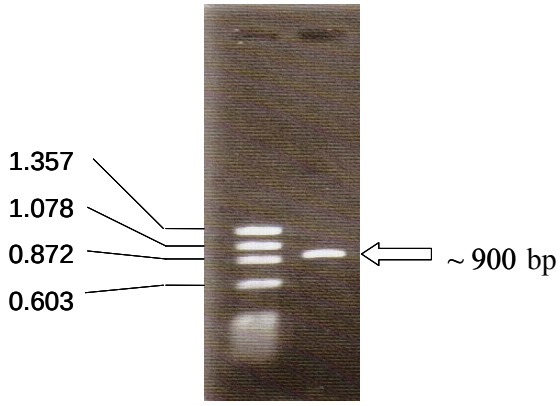


Fig. 2. PCR amplification of catechol 2,3-dioxygenase encoding gene n*ah*B from the naphthalene-degrading *Pseudomonas putida* NA3



cells suspension indicating 2HMS formation (Fig. 1, inset figure). Extradiol *meta*-ring cleavage pathway is controlled by the catechol 2,3-dioxygenase which cleaves the aromatic ring producing 2HMS. It was found that the extradiol *meta*-ring cleavage is the most crucial step in the biodegradation pathway as the compound of interest was found to lose its aromaticity due to the activity of catechol 2,3 dioxygenase enzyme and the produced aliphatic compound was found to be easily metabolized in most cases.

Nucleotide sequence of catechol 2,3-dioxygenase from strain NA3. To isolate the gene coding for catechol 2,3-dioxygenase, a PCR amplification procedure of the extracted plasmid DNA was performed with a sense primer deduced from the N-terminal amino acid sequence determined in a former study (El-Sayed *et al*., 2003) and an antisense primer deduced from the sequence of amino acids determined previously (Bartilson & Shingler, 1989). An amplified DNA fragment of ~ 900 bp was cloned in pGEM-

Fig. 3. Nucleotide base sequence and deduced amino acid sequence of catechol 2,3-dioxygenase encoding gene *nah*B showing an ORF that starts with the start codon ATG

```
[GENETYX-WIN : Translation of Nucleotides into Amino Acids
Filename         : CDO-NA3
Sequence Size    : 924
Sequence Position: 1 - 924
Translation Position: 1 -  924
Genetic Code        : Universal

         10        20        30        40        50        60        70        80
  ATGAAAAAAGGAGTTATGCGCCCCGGTCACGTCCAGCTTCGCGTACTGAACCTGGAGAGCGCTCTGGCCCACTACCGCGA
   M  K  K  G  V  M  R  P  G  H  V  Q  L  R  V  L  N  L  E  S  A  L  A  H  Y  R  D

         90       100       110       120       130       140       150       160
  CCTGCTCGGTCTGATCGAAATGGACCGTGACGAGCAAGGGCGCGTCTACCTGAAGGCCTGGaccGAGGTCGACAAATTCT
    L  L  G  L  I  E  M  D  R  D  E  Q  G  R  V  Y  L  K  A  W  T  E  V  D  K  F

       170       180       190       200       210       220       230       240
  CCGTGGTGCTGCGCGAGGCCGATCAGCCGGGCATGGATTTcATGGGCTTCAAGGTGCTCGACGAGGACTACCTGAACCGC
  S  V  V  L  R  E  A  D  Q  P  G  M  D  F  M  G  F  K  V  L  D  E  D  Y  L  N  R

       250       260       270       280       290       300       310       320
  TCAcCGAGGACCTGCTCAACTATGGCTGTCTGGTCGAGAGTATCGCGCCGGCGAACTCAAGGGGTGTGGCCGACGGGTGC
   S  P  R  T  C  S  T  M  A  V  W  S  R  V  S  R  R  R  T  Q  G  V  W  P  T  G  A

       330       340       350       360       370       380       390       400
  GCTTCGGGCACCGTCGGGCACTTCTTCGAgCTCTATGCGGACAaCGAGTAACCGGCAAATGGGGcTTGGCCGAGGTCAAC
    L  R  A  P  S  G  T  S  S  S  S  M  R  T  T  S  N  R  Q  M  G  L  G  R  G  Q

       410       420       430       440       450       460       470       480
  CGGAGGCCTGGCCGGCAACTCAAcGATggTtAACCACTTTGAGTTTTACCGAGTGTCGGTTTTACTTGCATGAGCTGCAA
  P  E  A  W  P  A  T  Q  R  W  L  T  T  L  S  F  T  E  C  R  F  Y  L  H  E  L  Q

       490       500       510       520       530       540       550       560
  GCCACCTATGAGCTGTTCACCGAGGTGCTCGGTTTCTACCTGGCCGAGCAGGTGATCGACGACGACGGCAACCCGCTTCG
   A  T  Y  E  L  F  T  E  V  L  G  F  Y  L  A  E  Q  V  I  D  D  D  G  N  P  L  R

       570       580       590       600       610       620       630       640
  CGCAGTTCCTCAGCCTGTCGACCAAAGCGCACGACGTGCCTTCAATCCATTGCCCGGAGAAGGGCAAGTTCCACCATGTG
    A  V  P  Q  P  V  D  Q  S  A  R  R  A  F  N  P  L  P  G  E  G  Q  V  P  P  C

       650       660       670       680       690       700       710       720
  TCGTTCTTCCTGgAAACCTGGGAgGACGTGCTGCGCGCAgCCGACCTGATCTCCATGACCGATACCTCTATCGACATAGG
  V  V  L  P  G  N  L  G  G  R  A  A  R  S  R  P  D  L  H  D  R  Y  L  Y  R  H  R

       730       740       750       760       770       780       790       800
  CCCGACCCGACACGGCCTGACTCACGgcAAGACCATCTACTTCTTCGACCCTTCGGGCAACCGCAACGAGGTGTTCTGTG
   P  D  P  T  R  P  D  S  R  Q  D  H  L  L  L  R  P  F  G  Q  P  Q  R  G  V  L  W

       810       820       830       840       850       860       870       880
  GCGGCGATTACAACTACCAGGACCACAAACCCGTGACCTGGCTGGCCAAGGATCTGGGCAAGGCGATCTTCTACCACGAC
    R  R  L  Q  L  P  G  P  Q  T  R  D  L  A  G  Q  G  S  G  Q  G  D  L  L  P  R

       890       900       910       920       930
  CGTGTGCTCAACGAACGCTTCCTGACCGTGCTGACcCTGAAAGC
  P  C  A  Q  R  T  L  P  D  R  A  D  P  E  S
```

Fig. 4. Amino acid alignments of CDO from *Pseudomonas putida* NA3 with other catechol dioxygenases

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cdona3 aa      1:MKKGVMRPGHVQLRVLNLESALAHYRDLLGLIEMDRDEQGRVYLKAWTEVDKFSVVLREA 60
CDO  db_xref   1:MKKGVMRPGHVHVRVLNLESALAHYCDLLGLIEMDRDEQGRVYLKAWTEVDKFSVVLREA 60
CDO  JI104     1:MKKGVMRPGHVHVRVLNLESALAHYRDLLGLIEMDRDEQGRVYLKAWTEVDKFSVVVREA 60
CDO  P35X      1:MKKGVMRPGHVQLRVLNLEAALTHYRDLLGLIEMDRDEQGRVYLKAWSEVDKFSVVLREA 60

cdona3 aa     61:DQPGMDFMGFKVLDEDYLNRSPR---TCSTMAVWSRVSRRRTQGVWPTGALRAPSGTSSS 117
CDO  db_xref  61:DQPGMDFMGFKVIDEDCLNRLTQDLLNYGCLIETIPAGELKGCGRRG--GFQAPSGHFFE 118
CDO  JI104    61:DQPGMDFMGFKVLDEDYLNRLTEDLLNYGCLVESMPAGELKGCGRRV--RFRAPSGHFFE 118
CDO  P35X     61:DQPGMDFMAFKVLDEDCLNRLTEDLLNYGCLVESIAAGELKGCGRRV--RFRAPSGHFFE 118

cdona3 aa    118:SMRTTSNRQM-GLGRGQPEAWPATQRWLTTLSFTECRFYLHELQATYELFTEVLGFYLAE 176
CDO  db_xref 119:LYADKEYTGKWGLEEINPEAWPRNLKGMRRVRFDHCLLYGDELQATYALFTEVLGFYLAE 178
CDO  JI104   119:LYADKEYTGKWGLAEVNPEAWPRNLKGMRAVRFDHCLLYGDELQATYELFTEVLGFYLAE 178
CDO  P35X    119:LYADKQYTGKWGVEEINPEAWPRDLKGMRAVRFDHCLMYGDELQATYELFTEVLGFYLAE 178

cdona3 aa    177:QVIDDDGNPLRAVPQ-PVDQSARR-AFNPLPGEGQVPPCVVLPGNLGGRAARSRPDLHDR 234
CDO  db_xref 179:QVVDDNGTRIQ-FLSLST-KA-HDVAFIQHTEKG------------------------- 209
CDO  JI104   179:QVIDDDGTRVAQFLSLST-KA-HDVAFIHCPEKG------------------------- 210
CDO  P35X    179:QVIDDNGTRMAQFLSLST-KA-HDVAFIHCPEKG------------------------- 210

cdona3 aa    235:YLYRHRPDPTRPDSRQDHLLLRPFGQPQRGVLWRRLQLPGPQTRDLAGQGSGQGDLLPRP 294
CDO  db_xref 210:------------------------------------------------------------ 210
CDO  JI104   211:------------------------------------------------------------ 211
CDO  P35X    211:------------------------------------------------------------ 211

cdona3 aa    295:CAQRTLPDRADPES---------------------------------------------- 308
CDO  db_xref 210:------------RFHHASFFLETWEDVLRARDLISMTDTSIDIGPTRHGLTHGKTIYFFE 257
CDO  JI104   211:------------KFHHVSFFLETWEDVLRAADLISMTDTSIDIGPTRHGLTHGKTIYFFD 258
CDO  P35X    211:------------KFHHVSFFLETWEDVLRARDLISMTDTSIDIGPTRHGLTHGKTIYFFD 258

cdona3 aa    309:-------------------------------------------------          309
```

Table I. Characterization of catechol 2,3-dioxygenase enzyme from *P. aeruginosa* NA3

Protein identity	Catechol 2,3-dioxygenase NA3
Sequence size	308
Sequence position	1 - 308
Average molecular weight	34867.10 D
Hydrophobic residues (152 , 49.67%)	No. %
Gly	26 8.44
Ile	2 0.65
Pro	27 8.77
Ala	19 6.17
Met	8 2.6
Val	19 6.17
Phe	9 2.92
Leu	36 11.69
Trp	6 1.96
Neutral residues (65, 21.1%)	
Ser	18 5.84
Cys	4 1.3
Thr	17 5.52
Asn	6 1.95
Gln	26 6.49
Hydrophilic residues (91, 29.55%)	
Asp	21 6.82
Arg	37 12.01
Glu	14 4.55
Tyr	8 2.6
Lys	5 1.62
His	6 1.95

T Easy vector and sequenced. The determined sequence and deduced amino acid sequence (Fig. 3). Analysis of the nucleotide sequence revealed an open reading frame (ORF) encoding 306 amino acids with a calculated molecular mass of 35.0 kDa (Table I).

Comparison of the deduced amino acid sequence of *nah*B gene product with the peptide sequence from other bacteria. Comparisons of the predicted ***nah*B** polypeptide sequences with sequences in the GenBank database revealed a strong homology to the catechol 2,3-dioxygenase encoded by ***phe*** operon of *Pseudomonas aeruginosa* JI104 (Kitayama *et al*., 1996). ***phe*B** coding catechol 2,3-dioxygenase is one of the genes coding for the *meta*-cleavage pathway and reside on the downstream part of the ***phe*** operon (*pheQBDCEFGHI*) (Powlowski & Shingler, 1994). Alignment of the encoded amino acid sequence of catechol 2,3-dioxygenase gene from strain NA3 with other catechol dioxygenases from different pseudomonads is shown in Fig. 4. The organization of the genes encoding these similar polypeptides was conserved among ***phe*** operons (Johnson & Olsen, 1995) and should be considered now in ***nah*** plasmid. The comparison revealed that most of the polypeptide chain was conserved as well as the active sites. However, some parts were found to be nonconserved. Noda *et al*. (1990) pointed out that small number of extradiol dioxygenases appear to show no similarity with the major enzyme family. Furthermore, three much smaller dioxygenases (21 kDa) have been identified in a naphthalene sulfonate-degrading strain P6 (Asturias *et*

al., 1994). The nonconservative parts of ***nah*B** gene could be attributed to the different ecological habitats from which the bacterial strain was isolated. From the molecular ecology point of view a degree of nonconservation should exist among similar genes. Therefore ***nah*B** gene coding for catechol 2,3-dioxygenase from the naphthalene-degrading isolate *P. putida* AN3 isolated from Egypt would have a unique base sequences when compared to similar catechol 2,3-dioxygenase encoding genes.

The gene coding for catechol 2,3-dioxygenase is over distributed in many bacterial strains. Originally it was found on the ***phe*** operon coding for phenol biodegradation and now is proved that it could be isolated from bacteria with a different biodegradation pathways like naphthalene catabolism. The distribution of catechol 2,3-dioxygenase encoding genes in bacterial strains with different biodegradation pathways is evident by the homology of the ***nah*B** gene from *P. putida* NA3 with other genes from ***phe*** operons coding for phenol biodegradation. Characterization of the genes, which encode degradative activities, may contribute to the evaluation of microbial populations optimal for biodegradation and bioremediation technologies.

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