Electronic Journal of Biotechnology 34 (2018) 83–90

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Cloning and characterisation of four *catA* genes located on the chromosome and large plasmid of *Pseudomonas putida* ND6

Shanshan Li [a], Kun Qin [b], Huaying Li [b], Jin Guo [b], Dejin Li [b], Fang Liu [b], Zhilei Tan [b,c], Wei Yan [a], Shuling Qu [d], Huabing Zhao [b,c,*]

[a] Department of Environmental Science & Engineering, Xi'an Jiaotong University, Xi'an, Shaanxi 710049, China
[b] Tianjin Key Laboratory for Prevention and Control of Occupational and Environmental Hazards, Logistics University of Chinese People's Armed Police Forces, Tianjin 300309, China
[c] Tianjin Key Lab of Industrial Microbiology, College of Biotechnology, Tianjin University of Science and Technology, Tianjing 300457, China
[d] Tuanbowa Development Company of Dagang Oilfield Corporation, Tianjin 300000, China

ARTICLE INFO

Article history:
Received 15 January 2018
Accepted 4 June 2018
Available online 09 June 2018

Keywords:
Biodegradation pathways
Bioremediation
C12O
catA genes redundancy
Catechol 1,2-dioxygenase genes
Catechol dioxygenases
Gene redundancy
Isozymes
Microbial degradation
Pseudomonas putida ND6

ABSTRACT

Background: Although the functional redundancy of catechol 1,2-dioxygenase (C12O) genes has been reported in several microorganisms, limited enzymes were characterised, let alone the advantage of the coexistence of the multiple copies of C12O genes.
Results: In this study, four novel C12O genes, designated *catA*, $catA_I$, $catA_{II}$ and $catA_{III}$, in the naphthalene-degrading strain *Pseudomonas putida* ND6, were cloned and characterised. Phylogenetic analysis of their deduced amino acid sequences revealed that the four C12O isozymes each formed independent subtrees, together with homologues from other organisms. All four enzymes exhibited maximum activity at pH 7.4 and higher activity in alkaline than in acidic conditions. Furthermore, CatA, $CatA_I$ and $CatA_{III}$ were maximally active at a temperature of 45°C, whereas a higher optimum temperature was observed for $CatA_{II}$ at a temperature of 50°C. $CatA_I$ exhibited superior temperature stability compared with the other three C12O isozymes, and kinetic analysis indicated similar enzyme activities for CatA, $CatA_I$ and $CatA_{II}$, whereas that of $CatA_{III}$ was lower. Significantly, among metal ions tested, only Cu^{2+} substantially inhibited the activity of these C12O isozymes, thus indicating that they have potential to facilitate bioremediation in environments polluted with aromatics in the presence of metals. Moreover, gene expression analysis at the mRNA level and determination of enzyme activity clearly indicated that the redundancy of the *catA* genes has increased the levels of C12O.
Conclusion: The results clearly imply that the redundancy of *catA* genes increases the available amount of C12O in *P. putida* ND6, which would be beneficial for survival in challenging environments.
How to cite: Li S, Qin K, Li H, et al. Cloning and characterisation of four *catA* genes located on the chromosome and large plasmid of *Pseudomonas putida* ND6. Electron J Biotechnol 2018;34.

1. Introduction

Catechol dioxygenases play an essential role in the microbial degradation of aromatic compounds; they are responsible for the cleavage of the aromatic ring of catechol, which is a convergent point of a number of aerobic biodegradation pathways such as benzoate, aniline, phenol, naphthalene and pyrene [1,2,3]. Cleavage of the aromatic ring of catechol can occur through the *ortho*- or the *meta*-cleavage pathways, which are catalysed by catechol 1,2-dioxygenase (C12O, EC 1.13.1.1) and catechol 2,3-dioxygenase (C23O, EC 1.13.1.2), respectively. C12O enzymes use non-haeme ferric ions as a cofactor and catalyse the intradiol addition of molecular oxygen at the 1,2-(*ortho*-) position, thereby resulting in the conversion of catechol to *cis*,*cis*-muconate [4]. The existence of more than one C12O-encoding genes has been reported in several environmental microorganisms, including *Pseudomonas putida* KT2440 [3], *Acinetobacter lwoffii* K24 [5], *Acinetobacter radioresistens* [6] and *P. putida* DOT-T1E [7]. Different enzyme properties and induction patterns of C12O isozymes have been reported in these organisms, thus suggesting that they may be responsible for the metabolism of different substrates [6]. Jose et al. analysed the function of the second copy of the *catA* gene (coding for C12O) on the chromosome of *P. putida* mt-2 and confirmed the role of CatA2 as important for survival

* Corresponding author.
E-mail address: 13820664530@163.com (H. Zhao).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2018.06.001

under conditions of excess catechol, along with the stable co-existence of genes encoding the *meta* and *ortho* pathways [3].

P. putida ND6 is well characterised owing to its effective metabolism of naphthalene, which is considered a model aromatic compound pollutant [8,9,10,11,12]. Naphthalene is first converted to salicylate by the enzymes encoded by the upper pathway operon (*nahAaAbAcAdBFCED*) on plasmid pND6-1, and subsequently, the salicylate is oxidised to produce catechol, which can be further degraded through either a *meta*-cleavage pathway, catalysed by the catechol-2,3-dioxygenase NahH (C23O), or *ortho*-cleavage pathway by the CatA enzyme [9,11]. Functional gene analysis of *P. putida* ND6 DNA sequences indicates that there are three *catA* genes on its chromosome and one on the naphthalene-degrading plasmid pND6-1. Bioinformatic analysis revealed that the deduced amino acid sequences encoded by the four *catA* genes in the ND6 strain shared 74–81% identity with one another; however, the physiological function of the C12O isozymes in ND6, and whether they exhibit redundancy, remains unclear. As the lower operon on the plasmid pND6-1 encodes all enzymes necessary for the catabolism of catechol, roles for the other C12O proteins in the survival of the ND6 strain have not been demonstrated clearly.

In this study, we focused on the physiological properties of the multiple C12O proteins in *P. putida* ND6. The four C12O-encoding genes were cloned and expressed in *Escherichia coli*, and the enzymatic properties of the purified C12O enzymes were characterised. Phylogenetic analysis based on deduced amino acid sequences revealed the evolutionary subfamilies to which each C12O protein belongs. Furthermore, expression levels of the mRNAs encoding the C12O proteins and their enzyme activities were investigated in the presence and absence of the inducer salicylate. As few reports have previously characterised four diverse C12O coding genes on a bacterial chromosome and plasmid, this study provides novel information in the field of environmental microbiology.

2. Materials and methods

2.1. Bacterial strains, media and plasmids

The strains and plasmids used in this study are listed in Table 1. *P. putida* ND6 can utilise naphthalene as its sole carbon and energy source. The genome of *P. putida* ND6 has previously been sequenced and characterised, and the data are available from the NCBI GenBank database (Accession number, CP003588) [8]. *P. putida* ND6 was cultivated under agitation (180 rpm) at 30°C in a mineral medium (MMB) containing either 0.5% glucose (w/v) or 2 g/L naphthalene as carbon sources [12]. When appropriate, the antibiotics ampicillin (30 μg/ml) and kanamycin (50 μg/ml) were added to the media. Cell growth was periodically monitored and determined on the basis of optical density of the culture at 600-nm wavelength (OD_{600}) using a Model iMarker Microplate Reader (Bio-Rad, USA).

2.2. DNA manipulation

The genomic DNA of ND6 strain was extracted with Ezup Column Bacteria Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. PCR was carried out using a PC-320 thermal cycler (Bio-Rad, CA, USA) with Takara PrimeSTAR HS DNA Polymerase (Takara Biotech, Dalian, China) in a 50-μL mixture. DNA agarose gel electrophoresis, plasmid DNA transformation and isolation were performed using standard procedures.

2.3. Construction of catechol dioxygenase expression vectors

To determine the characteristics of the five *P. putida* ND6 catechol dioxygenases (four C12O s and one C23O), specific expression vectors were constructed for each gene. The gene sequences encoding each catechol dioxygenase were amplified by PCR using the primers listed in Table S1. The resulting PCR products were digested with appropriate restriction endonucleases, purified and ligated into the pET21-b(−) or pET28-a(+) expression vectors. Nucleotide sequencing was performed by Sangon Biotech Co., Ltd. (Shanghai, China).

2.4. Expression and purification of catechol dioxygenase

The vectors for the expression of each of the catechol dioxygenase genes were transformed into *E. coli* BL21 (DE3) for expression analysis; *E. coli* was cultured in Luria-Bertani medium containing suitable antibiotics at 37°C to an OD_{600} of 0.6; then, the inducer isopropyl β-D-1-thiogalactopyranoside (IPTG, 100 μmol/L) was added to the medium. After cultivation at 18°C for 5 h, cells were harvested by centrifugation (8000 *g*, 4°C, 10 min) and washed twice in phosphate-buffered saline (PBS, pH 7.2). Bacterial cells were subsequently disrupted by sonication. Insoluble cell debris was removed by centrifugation (12,000 *g*, 4°C, 30 min), and the supernatant was applied to a column packed with nickel-nitrilotriacetic acid (Ni-NTA) for purification by metal-affinity chromatography under native conditions, according to the manufacturer's instructions (QIAGEN, Germany). Finally, the purity of the extracted proteins was

Table 1
Bacterial strains and plasmids.

Strain/plasmid		Genotype and description	Source/reference
Strains			
E. coli	DH5α	F^- ϕ80(*lacZ*) ΔM15 Δ (*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17* (*rK*$^-$, *mK*$^+$) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Novagen
	BL21(DE3)	*F*$^-$ *ompT hsdS* (*rB*$^-$ *mB*$^-$) *gal dcm*(*DE3*)	Novagen
	ECAT	*E. coli* BL21(DE3) containing plasmid pECAT	This study
	ECATI	*E. coli* BL21(DE3) containing plasmid pECATI	This study
	ECATII	*E. coli* BL21(DE3) containing plasmid pECATII	This study
	ECATIII	*E. coli* BL21(DE3) containing plasmid pECATIII	This study
	ENAH	*E. coli* BL21(DE3) containing plasmid pENAH	This study
P. putida	ND6	Cb^r	[8]
Plasmids			
pET21-b(−)		Ap^r; expression vector	Novagen
pET28-a(+)		Km^r; expression vector	Novagen
pECATA		*catA* gene inserted into *Nde*I and *Xho*I site of pET21-b(−), Ap^r	This study
pECATAI		$catA_I$ gene inserted into *Nde*I and *Xho*I site of pET21-b(−), Ap^r	This study
pECATAII		$catA_{II}$ gene inserted into *Nde*I and *Xho*I site of pET21-b(−), Ap^r	This study
pECATAIII		$catA_{III}$ gene inserted into *Nde*I and *Xho*I site of pET21-b(−), Ap^r	This study
pENAH		*nahH* gene inserted into *Nde*I and *Xho*I site of pET28-a(+), Km^r	This study