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Research article

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

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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.
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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

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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

determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Construction of a catechol dioxygenase phylogenetic tree

The relevant amino acid sequences of different catechol dioxygenases were downloaded from the Protein Database of National Center for Biotechnology Information. Sequence alignments were generated by using the Clustal method [13]. Phylogenetic analysis of catechol dioxygenase amino acid sequences generated in this study was performed using MEGA 4.1 software [14].

2.6. Analysis of catechol dioxygenase enzyme activity and kinetics

C12O and 2,3-dioxygenase enzyme activities were assayed spectrophotometrically at 25°C, following the formation of *cis,cis*-muconic acid and 2-hydroxymuconic semialdehyde, by measuring absorbance at 260-nm and 390-nm wavelengths, respectively [4,15]. One unit of enzyme activity (U) was defined as the amount of enzyme required to form 1 μmol of product (*cis,cis*-muconic acid or 2-hydroxymuconic semialdehyde) per min. Specific activity was defined as units per milligramme of protein (U/mg). Protein concentrations were determined by the Bradford method, using bovine serum albumin as a standard [16].

The Michaelis–Menten (K_m) and maximal velocity (V_{max}) kinetic constants of C12O were calculated according to the Michaelis–Menten equation at different concentrations of catechol, ranging from 0 to 30 μmol/L at 25°C. All independent measurements were performed in triplicate for each substrate concentration.

2.7. Effect of pH and temperature on C12O and 2,3-dioxygenase activities

To determine the optimum pH, enzyme activity assays were conducted across a pH range of 5–10 in a sodium acetate buffer system (100 mmol/L) for 10 min. To evaluate pH stability, the purified enzymes were mixed with a series of buffers at different pH values (5.8–9.6) for 1 h. Catechol dioxygenase (both C12O and C23O) activity was then determined under standard assay conditions (see above). The highest activity was defined as 100%. Unless specifically stated, enzyme activity was measured at 30°C using the spectrophotometric assay described above. All experiments were performed in triplicate.

The optimum temperatures for C12O and C23O were determined by enzyme activity assays at different temperatures (25–60°C). For the thermal stability experiments, purified enzymes were mixed with 100 mmol/L buffer systems (pH 7.2) and maintained at 35°C, 40°C, 45°C, 50°C, 55°C or 60°C for 10 min, and catalytic activity was subsequently measured by using the method described above.

2.8. Effect of metal ions on C12O activity

The effect of various metal ions on the activity of C12O was investigated by adding 0.5 mmol/L of different metal ions ($FeCl_3 \cdot 6H_2O$, $MnSO_4 \cdot H_2O$, $CuCl_2 \cdot 2H_2O$, $ZnCl_2$, $CaCl_2 \cdot 2H_2O$, $BaCl_2 \cdot 2H_2O$, $AlCl_3 \cdot 6H_2O$ and $NiSO_4 \cdot 6H_2O$) to the standard assay conditions described above. The resulting values were recorded as percentages of enzyme activity at 0% concentration of metal ions. Reported values are the averages from triplicate tests.

2.9. RNA isolation and real-time quantitative RT-PCR (qRT-PCR)

P. putida ND6 was cultivated under agitation (180 rpm) at 30°C in MMB containing 0.5% glucose (w/v) in the presence or absence of salicylate (200 mg/L) for 36 h. RNA from *P. putida* ND6 was isolated using the TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Dalian, China) and reverse transcribed into cDNA using a PrimeScript ™ II First Strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's instructions. Transcript levels were determined by qRT-PCR using SYBR® Fast qPCR Mix (Takara, Dalian, China). The primers used are presented in Table S2. The expression levels of the different genes were normalised with regard to those of 16S rRNA.

3. Results and discussion

3.1. The arrangement of P. putida ND6 genes encoding catechol dioxygenase

Catechol dioxygenase enzymes, which play key roles in the cleavage of aromatic rings, include C12O (EC 1.13.11.1) and C23O (EC 1.13.11.2) [17]. Based on the analysis of the *P. putida* ND6 complete genome sequence, three diverse C12O-encoding sequences ($catA_I$, $catA_{II}$ and $catA_{III}$) were identified in the genome, and another C12O-encoding sequence (*catA*) was found in the naphthalene-degrading plasmid pND6-1 [8,9]. The genes $catA_I$ and $catA_{II}$ form operons with $catB_I$ and $catC_I$ as well as $catB_{II}$ and $catC_{II}$, respectively. The C23O-encoding gene *nahH* was clustered with other naphthalene-degrading genes and formed lower operon for naphthalene degradation in the pND6-1 plasmid (Fig. 1).

To examine the phylogenetic relationships among the four C12O genes in the ND6 strain, a phylogenetic tree was constructed using deduced amino acid sequences of C12O enzymes identified by a BLASTp search of the GenBank database (Fig. 2). The resulting phylogenetic neighbour-joining tree was clearly divided into four clades, where the four C12O enzymes from the ND6 strain each formed unique branches, together with related proteins from other organisms (Fig. 2). $CatA_I$ exhibited more similarity with $CatA_{III}$ than with $CatA_{II}$ or CatA. Many C12O sequences from the GenBank database showed similarity with $CatA_I$ or $CatA_{III}$, whereas fewer sequences were homologous with $CatA_{II}$ or CatA, thereby indicating an increased diversification of the $CatA_I$ and $CatA_{III}$ isozymes. The three C12O enzymes encoded in the genome of *P. putida* ND6 ($CatA_I$, $CatA_{II}$ and $CatA_{III}$) exhibited the highest similarity with C12O proteins from other strains of *P. putida*, whereas the CatA amino acid sequence encoded in the naphthalene-degrading plasmid pND6-1 showed similarity with CatA from *P. putida* DOT-T1E and *Stenotrophomonas rhizophila*.

C12O is indispensable enzymes for processing various aromatic compounds and is widely distributed among soil, water and sewage microorganisms. Some catechol-degrading bacteria are reported to possess several C12O proteins. It has been proposed that two isozymes of C12O can be induced in numerous aromatic-degrading bacterial strains including *P. putida* KT2440 [18], *P. putida* F1 [19], *P. putida* LS46 [20] and *A. lwoffii* K24 [5]. Additionally, it has been proposed that these two isozymes can be distinguished from each other on the basis of their different chemical and catalytic characteristics, regulation mechanisms and physiological significance. Although four isozymes of C12O (CD-I, II, III-1 and III-2) were isolated from *Arthrobacter* sp. BA-5-17, analysis of their NH_2-terminal amino acid sequences indicated that they were encoded by the same genes and that the presence of the four isozymes was probably due to post-translational modifications [21]. To our knowledge, there are only two bacterial strains reported to harbour four diverse C12O-encoding genes. One is *P. putida* DOT-T1E [7], where the four C12O genes (*catA*) are all located on the chromosome, despite this strain possessing a self-transmissible large plasmid, pGRT1. The other, *P. putida* ND6, is described here, and it is the first bacterial strain reported to have four *catA* genes separately distributed on both the chromosome and a large degrading-plasmid with diverse patterns of organisation. The four CatA proteins of *P. putida* ND6 shared extensive similarity with the corresponding enzymes in *P. putida* DOT-T1E (approximately 99%). Among them, two of the *catA* genes were organised with other salicylate-degrading genes in two *catBCA* clusters (located at 1910696–1913716 bp and 5,994,152–5,996,572 bp in

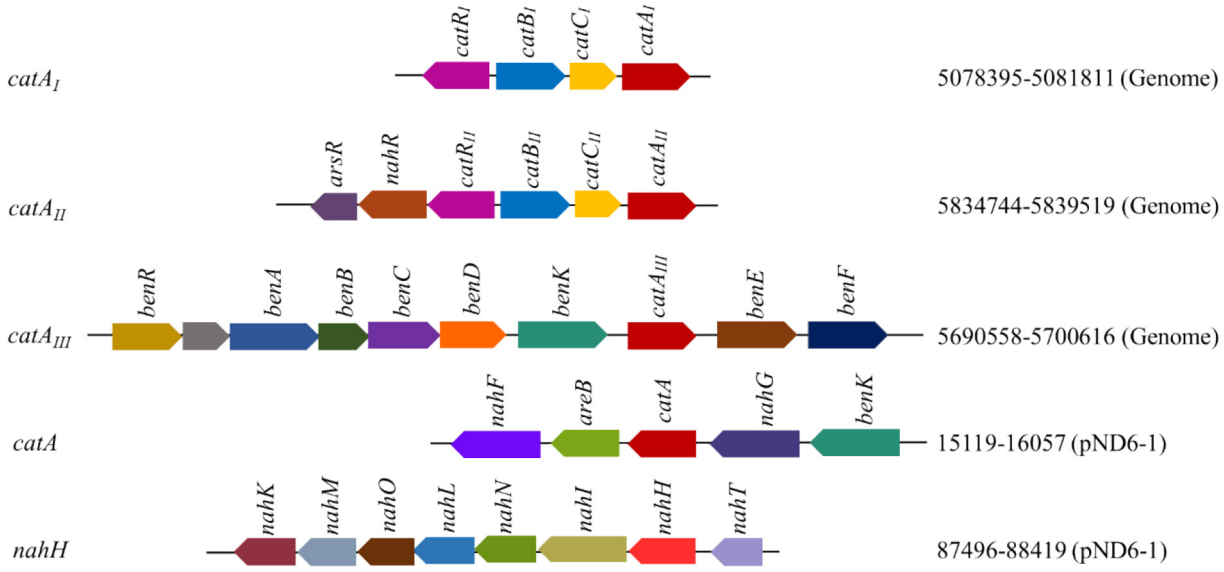

Fig. 1. Location of genes encoding catechol dioxygenase in *P. putida* ND6. The four *catA* gene clusters (three in the genome and one in plasmid pND6-1) and one *nahH* cluster in plasmid pND6-1 are shown. Gene homologues are indicated by the same colour. *catA*: catechol 1,2-dioxygenase genes; *catB*: muconate and chloromuconate cycloisomerase gene; *catC*: muconolactone delta-isomerase gene; *catR*: transcriptional regulator gene; *benR*: AraC family transcriptional regulator gene; *benA*: benzoate dioxygenase gene, alpha subunit; *benB*: benzoate 1,2-dioxygenase gene, beta subunit; *benC*: benzoate 1,2-dioxygenase electron transfer component gene; *benD*: 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase gene; *benK*: benzoate transporter gene; *benE*: putative benzoate transporter gene; *benF*: methyltransferase gene; *nahF*: salicylaldehyde dehydrogenase gene; *areB*: benzyl alcohol dehydrogenase gene; *nahG*: salicylate hydroxylase gene; *nahT*: chloroplast ferredoxin gene; *nahH*: catechol 2,3-dioxygenase gene; *nahI*: hydroxymuconic semialdehyde dehydrogenase gene; *nahN*: hydroxymuconic semialdehyde hydrolase gene; *nahL*: 2-oxopent-4-enoate hydratase gene; *nahM*: 4-hydroxy-2-ketovalerate aldolase gene; *nahK*: 4-oxalcrotonate decarboxylase gene.

DOT-T1E as well as 5,079,403–5,081,811 bp and 5,837,099–5,839,519 bp in ND6), and this organisation was also observed in other *P. putida* and *Pseudomonas monteilii* strains [22,23]. Of the two other *catA* genes identified in the ND6 and DOT-T1E strains, one ($catA_{III}$) exhibited 98% identity with the $catA_{II}$ gene of KT2440 and was clustered with the *benRABCDK* genes, which encode proteins involved in benzoate degradation. This organisation was also identified in other *P. Putida* strains including GB1, F1, LS46 and TRO1, which also have clusters containing *ben* and *cat* genes. This observation indicates that the ND6 and DOT-T1E strain $catA_{III}$ genes may have been acquired through horizontal gene transfer, independently of other *catA* genes. Of particular interest, the fourth *catA* gene was found only in the ND6 and DOT-T1E strains. Analysis of the amino acid sequences of the CatA proteins from the two strains indicated that they were homologues and the encoding genes both clustered with genes encoding salicylate-degrading proteins (Fig. 2); however, the *catA* gene of *P. putida* DOT-T1E was located on the chromosome, whereas *catA* of *P. putida* ND6 was located on the large plasmid pND6-1, which harbours the operons encoding naphthalene-degrading enzymes. Further DNA-based comparative analysis revealed that a large segment of the ND6 strain pND6-1 plasmid DNA sequence shared extensive similarity to part of the genome of *P. putida* DOT-T1E, thus indicating that this fragment was acquired through horizontal gene transfer. The presence of different C12O gene arrangements and coding sequences can likely be ascribed to bacterial evolution and environmental adaption.

3.2. Effect of pH on P. putida ND6 C12O enzyme activity and stability

To determine the effect of pH on the activity of the four C12O enzymes, we detected their activities at pH values ranging from 5.0 to 9.6 using catechol as a substrate. All four C12O enzymes exhibited maximum activity at pH 7.0–7.4 and higher activity under alkaline than under acidic conditions (Fig. 3a); however, the decrease in the rate of $CatA_{III}$ activity under increasing acidity was much more pronounced than that of the other C12O enzymes. The activity of $CatA_{III}$ was close to zero at pH 5.8, whereas the residual activities of CatA, $CatA_I$ and $CatA_{II}$ were 62%, 60% and 78%, respectively, under the same conditions. Moreover, tests of pH stability (Fig. 3b) demonstrated similar trends among the four C12O enzymes, with all exhibiting greater stability under alkaline than under acidic conditions. All four C12O isozymes from the ND6 strain were stable in a pH range of 7.4–9.6, with only an 18% reduction in activity after 1-h incubation at pH 9.6 compared to almost 100% of activity lost after 1 h at pH 5.8.

A similar phenomenon was observed in *Candida tropicalis* JH8 [15], as recombinant C12O from this strain was unstable below pH 5.0 and stable from pH 7.0 to 9.0 and had an optimum pH value of 7.5. By contrast, purified C12O from the phenol-degrading *Acinetobacter* sp. Y64 was more stable under acidic conditions and lost only 18% of its activity after 6-h incubation at pH 5, whereas at pH 4, approximately 50% activity was lost after 6 h [4].

3.3. Effect of temperature on P. putida ND6 C12O enzyme activity and stability

The effect of reaction temperatures of 25–60°C on C12O activity was determined under standard assay conditions using catechol as a substrate. CatA, $CatA_I$ and $CatA_{III}$ exhibited maximum activities at 45°C, whereas a higher optimum temperature (50°C) was observed for $CatA_{II}$ (Fig. 4a). The results of temperature stability experiments suggested similar trends for the four C12O enzymes, which were all sensitive to temperature change. The residual activities of all four C12O enzymes from the ND6 strain decreased rapidly with increasing temperature (Fig. 4b), with the purified enzymes losing 25–35% of their activities when incubated at 40°C for 1 h. $CatA_I$ lost all of its enzyme activity after exposure to 45°C for 1 h, whereas the activities of the other three C12O isozymes decreased to zero at 50°C.

In general, the catalytic activities of enzymes are highly influenced by reaction conditions, particularly pH and temperature. As reported previously, the majority of C12O isolated from wild bacteria such as *Acinetobacter* sp. Y64 [4], *C. tropicalis* JH8 [15] and *Corynebacterium glutamicum* [24] exhibit optimum temperatures of 30–37°C, similar to

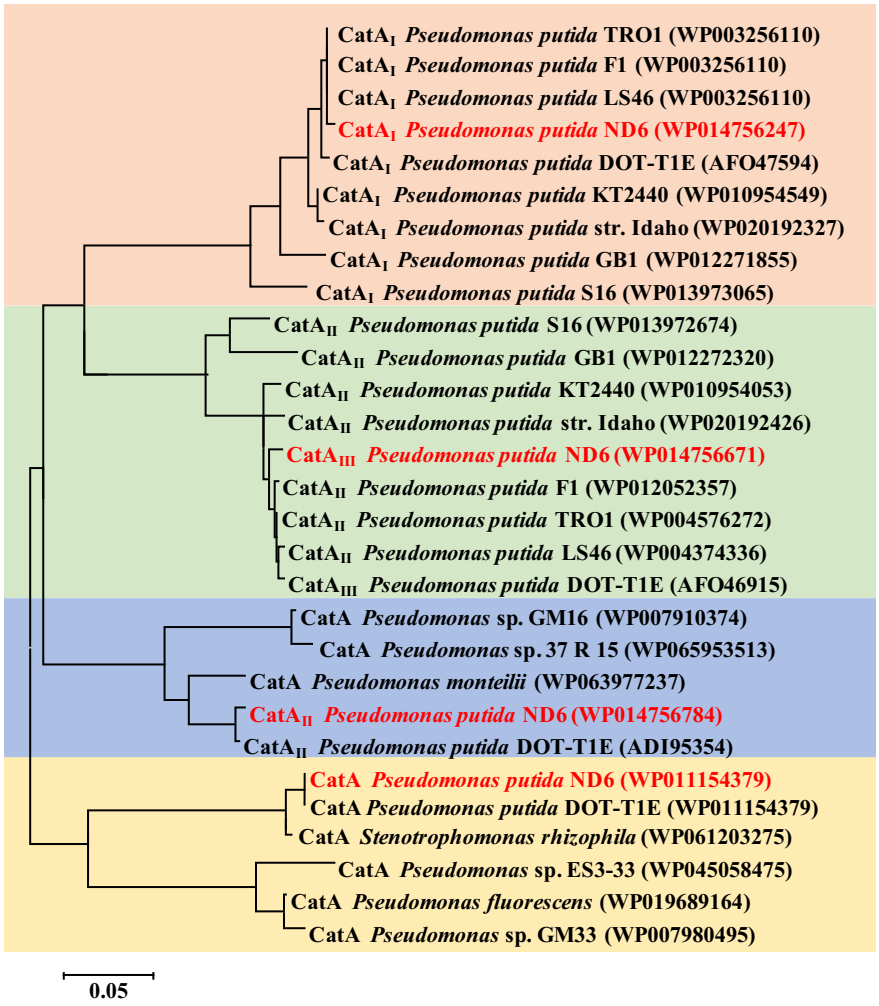

Fig. 2. Phylogenetic tree based on the four C12O sequences of *P. putida* ND6. The amino acid sequences of CatA enzymes from the ND6 strain are indicated in red. Different C12O enzyme sequences from the ND6 strain and related species formed unique clades, indicated by different background colours. The tree was constructed by using the neighbour-joining method, with1000 replicate bootstrap tests. Bar, 0.05 substitution amino acids per site.

the temperatures at which these species grow; however, the optimum reaction temperatures for the C12O enzymes from the ND6 strain were between 45°C and 50°C, which is higher than the optimum growth temperature for this strain (30°C). Furthermore, among the four C12O enzymes, the optimum reaction temperature of CatA$_{II}$ was slightly higher than that of the other three proteins. We propose that the diverse optimal reaction temperatures among the C12O isozymes may represent a beneficial adaptation of the ND6 strain to varying environmental conditions. There are a few other reports describing thermophilic C12O proteins. The C12O enzyme from *Streptomyces setonii* ATCC 39116 exhibits thermophilic enzyme activity, within a broad temperature range from 25°C up to 65°C (maximum activity at

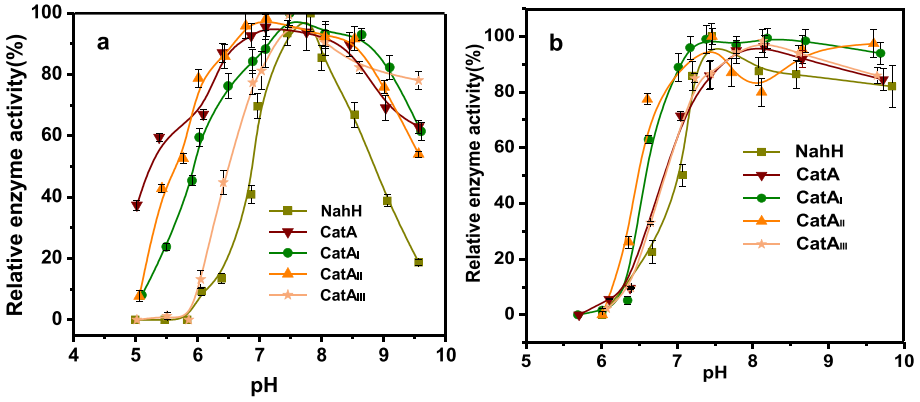

Fig. 3. The pH dependency of *P. putida* ND6 C12O enzymes. (a) Optimum pH, (b) pH stability. The values shown represent averages from triplicate experiments. The residual relative activity of each enzyme was determined by the standard assay and compared with the highest activity of the enzyme exhibited at different pH values.

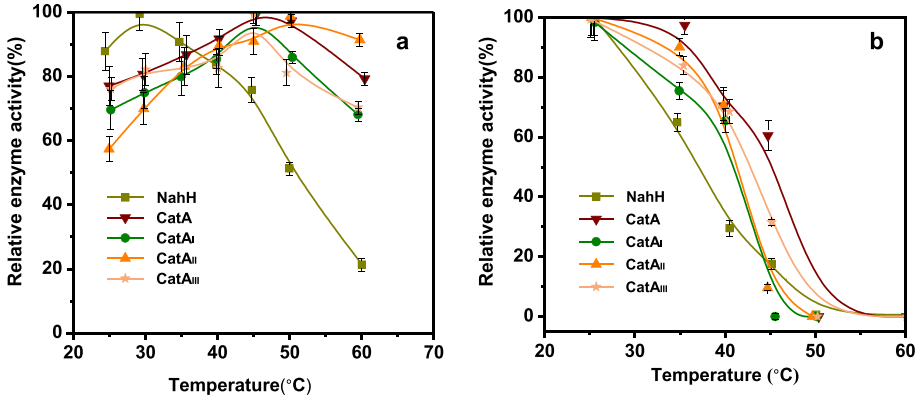

Fig. 4. Temperature dependency of *P. putida* ND6 C12O enzymes. (a) Optimum temperature, (b) Temperature stability. Data are presented as averages of triplicate experiments. Error bars represent the standard deviation. The residual relative activity of each enzyme was determined by the standard assay and compared with the highest activity of the enzyme exhibited at different temperatures.

45°C). Notably, the optimal temperature of C12O from *A. radioresistens* S13 shifted from 30°C for the free protein to 50°C for the immobilised protein [25].

3.4. Effect of metal ions on the activity of P. putida ND6 C12O enzymes

Metal ions are often important cofactors for the stabilisation and activation of enzymes. Some metal ions play roles within the active centres of enzymes such as transferring electrons during the reaction process, aiding in the formation of a stable transition state of the ternary complex during the reaction between enzyme and substrate or reducing the activation energy of the reaction. Moreover, other metal ions are not necessary for the enzyme reaction, but rather have regulatory roles.

Our results indicated minimal influence of metal ions on the different C12O isozymes from the ND6 strain, other than Fe^{2+} (Table 2). The activities of all four C12O isozymes were slightly inhibited by Zn^{2+}, Ba^{2+}, Ca^{2+}, Fe^{3+} and Al^{3+}, whereas they all lost 30–40% of their activity in the presence of Cu^{2+}. The activity of CatA was enhanced by Mn^{2+} and Al^{3+}, while that of $CatA_{II}$ increased with the addition of Ni^{2+} and Al^{3+}. Many studies have revealed that heavy metals such as Cu^{2+} and Hg^{2+} are extremely effective inhibitors of C12O enzyme activity. In *Rhodococcus* sp. NCIM 2891, the addition of Hg^{2+} or Cu^{2+} at a final concentration of 1 mM resulted in complete loss of C12O activity [26]. In addition, the C12O proteins in *Arthrobacter* sp. BA-517 lost 56–81% of their activity with addition of Cu^{2+} (0.1 mM) [21]. Of particular significance, our findings demonstrate that none of the metal ions tested, other than Cu^{2+}, exhibited significant inhibition of activity of these C12O isozymes, hence indicating that they could be useful for the bioremediation of pollution with aromatics in the presence of metals.

3.5. Determination of the kinetic parameters of P. putida ND6 C12O isozymes

The K_m values of the ND6 strain C12O isozymes involved in catechol *ortho*-cleavage were in the range of 1–2 μM with little difference except CatA (Table 3) [27]. The minimum K_m value, 0.019 μM, was that of CatA, which is significantly lower than that of C12Os from other strains such as *Stenotrophomonas maltophilia* KB2 [28], *Acinetobacter* sp. Y64 [4] and *Acinetobacter* sp. DS002 enzymes [26]. These results suggest that the ND6 C12O isozymes can catalyse the catechol-cleavage reaction and achieve their maximum rate at relatively low substrate concentrations.

Moreover, the enzyme activities of $CatA_I$ and $CatA_{II}$ were similar (9.11 and 8.28 U/mg), whereas those of CatA and $CatA_{III}$ were lower (1.434 and 2.17 U/mg). By contrast, the four C12O enzymes from *Arthrobacter* sp. BA-5-17 showed similar enzyme activities (between 35 and 41 U/mg) [29], and the activities of the three C12O isozymes from *Pseudomonas arvilla* C-l have also been reported as similar [30].

The increasing reports of the presence of multiple C12O forms in single microorganisms led us to consider the advantages and disadvantages to the host of possessing multiple isozymes. The four C12O proteins from *P. putida* ND6 exhibited very similar enzyme activities; however, their different physical and chemical properties such as pH and thermal stability may represent a physiological advantage, thus enabling them to regulate their metabolism in adaption to changes in the microenvironment. Furthermore, C12Os encoded by *catA*, $catA_I$, $catA_{II}$ and $catA_{III}$ are more adaptive to the change in pH and temperature than C23O encoded by *nahH*. The adaptability and stability of C23O to

Table 2
Effect of some metal ions on the enzyme activity of different C12Os.

Metal ions	CatA	$CatA_I$	$CatA_{II}$	$CatA_{III}$
Control	100	100	100	100
Mn^{2+}	106 ± 9	100 ± 8	100 ± 10	100 ± 6
Zn^{2+}	96 ± 6	95 ± 8	94 ± 4	99 ± 8
Cu^{2+}	62 ± 3	63 ± 6	66 ± 4	70 ± 6
Ba^{2+}	97 ± 5	95 ± 8	90 ± 8	92 ± 8
Ca^{2+}	100 ± 6	96 ± 5	90 ± 7	97 ± 6
Fe^{3+}	90 ± 7	93 ± 7	90 ± 6	90 ± 5
Fe^{2+}	143 ± 13	141 ± 11	139 ± 8	138 ± 12
Ni^{2+}	100 ± 11	100 ± 9	105 ± 12	100 ± 8
Al^{3+}	113 ± 8	100 ± 8	105 ± 9	100 ± 6

Data are expressed as mean ± standard deviation ($n = 3$).

Table 3
Kinetic parameters of different C12O isozymes.

Enzyme	K_m (μM)	V_{max} (U/mg)	Origin
CatA	0.019	1.434	[27]
$CatA_I$	1.72 ± 0.13	9.11 ± 0.71	This study
$CatA_{II}$	1.12 ± 0.18	8.28 ± 0.67	This study
$CatA_{III}$	2.07 ± 0.14	2.21 ± 0.19	This study
C12O from *Candida tropicalis* JH8	9.2	1.08	[15]
C12O from *Acinetobacter* sp. Y64	17.53	1.95	[4]
C12O from *P. putida* DSM 437	12.43	2.91	[28]
C12O from *Stenotrophomonas maltophilia* KB2	12.18	1218.8	[27]
C12O from *Acinetobacter* sp. DS002	1.58	2	[26]

Errors (standard deviations) were calculated on the basis of at least three independent experiments.

temperature and pH are both less than those of C12O (Fig. 3 and Fig. 4). Much effort should be focused on basic research to identify promoter regions and clarify the transcriptional mechanisms regulating expression of these enzymes.

3.6. In vivo expression profile of P. putida ND6 strain genes encoding C12O proteins

To elucidate the *in vivo* transcription levels and regulation profiles of the four C12O isozymes in *P. putida* ND6, RT-PCR was performed using template mRNA from ND6 cells cultured with glucose in the presence or absence of the inducer salicylic acid. The results provided several interesting insights into the transcriptional regulation of multiple sets of catechol-dioxygenase genes. First, all five catechol-dioxygenase genes (the C12O-encoding-genes *catA*, $catA_I$, $catA_{II}$ and $catA_{III}$) and the C23O-encoding gene *nahH* were expressed at low levels in the absence of salicylic acid. When cells were cultured with glucose as the sole energy and carbon source, mRNA expression levels of all five genes were low in *P. putida* ND6 (Fig. 5). Second, the expression of the four C12O-encoding genes in ND6 strain could be induced. Once salicylic acid was added into the ND6 culture, the expression level of the four C12O-encoding genes increased significantly. In particular, the mRNA expression levels of $catA_{II}$ and $catA_{III}$ increased by 1.3 and 2.7 times, respectively, on addition of salicylate. Similar results have been reported for other related strains including *P. putida* KT2440 [3], *P. putida* P8 [31] and *Burkholderia* sp. strain TH2 [32]. The third interesting insight was that the expression of the C23O gene (*nahH*) was not induced in the presence of salicylate. This result was not consistent with previous observations of the other well-characterised naphthalene-degrading plasmids NAH7 and pDTG-1.

Investigation of the regulatory mechanisms of naphthalene-degrading genes demonstrated that salicylate bound to NahR could initiate the transcription of both the upper and lower operons (including *nahH*) by interacting with DNA enhancer sequences upstream of the promoter region. It is surprising to note that the transcription level of *nahH* in the ND6 strain did not exhibit any apparent difference in response to salicylate. In accordance with *nahH* induction analysis at the level of transcription, C23O enzyme activity also did not alter according to the presence or absence of salicylate (Fig. 6). These results strongly suggest that the

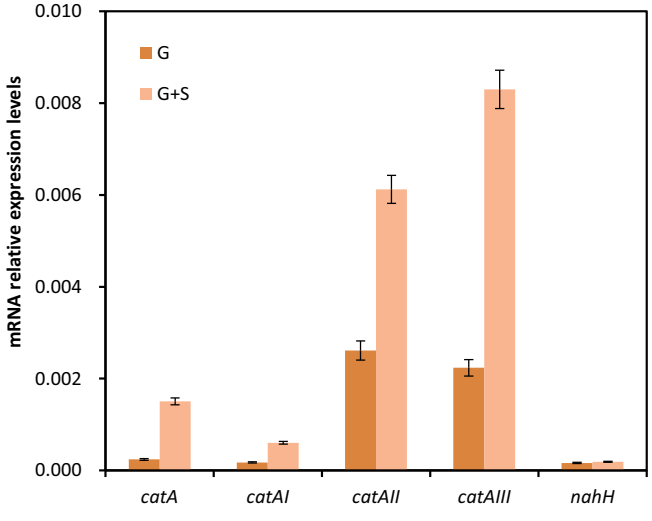

Fig. 5. The relative mRNA expression levels of the C12O- and C23O-encoding genes of glucose-grown *P. putida* ND6 in the presence (G + S) or absence (G) of salicylate. Levels of 16S rRNA in each sample were used as an internal reference to normalise the data, and mRNA expression levels are presented relative to their corresponding 16S rRNA values. Data are presented as the means of three replicate experiments, and error bars represent the standard deviation.

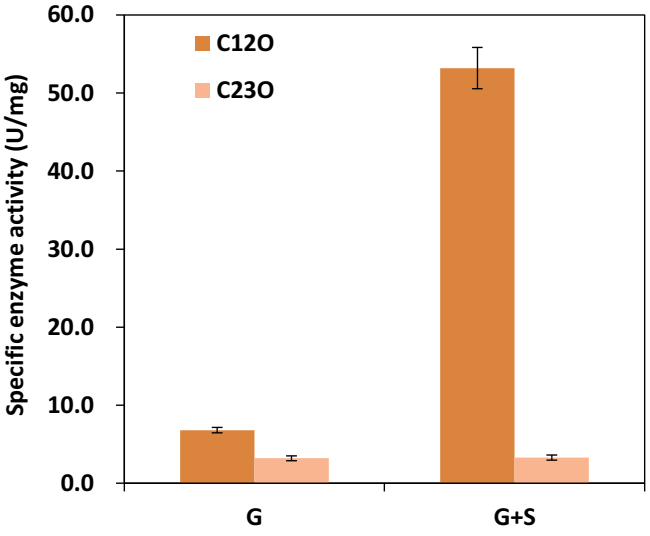

Fig. 6. Catechol dioxygenase activity analysis. The ND6 strain was cultivated with glucose in the presence (G + S) or absence (G) of salicylate. C12O and C23O activities were determined by using the methods described above. Data are presented as the means of three replicate experiments, and error bars represent the standard deviation.

C23O-encoding gene, *nahH*, from the lower operon cannot be induced by salicylate. These findings are consistent with observations in *S. maltophilia* KB2, which can produce different enzymes of the dioxygenase family. When cultivated with benzoate and catechol, strain KB2 exhibited only C12O activity, whereas C23O enzyme activity was detected after phenol induction [33]. The ability of microorganisms to induce and express different dioxygenases in response to diverse substrates or inducers under different conditions is likely beneficial to their environmental adaptability and will facilitate their use in the bioremediation of various contaminants.

Functional gene redundancy is believed to contribute to the maintenance of genetic stability against mutations, thus generating proteins with novel biochemical functions and improving adaption to diverse environments. With the improvement of genome sequencing and analytical methods, observations of functional gene redundancy are increasingly reported in environmental bacteria, including diverse gene clusters involved in benzoate degradation in *Burkholderia xenovorans* LB400, various operons for benzoate and phthalate metabolism in *Rhodococcus jostii* RHA1, and the redundancy of C12O in the catechol-degrading pathways of several *Pseudomonas* species [34,35]. In *P. putida* mt-2, two C12O-encoding genes (*catA* and *catA2*) involved in the *ortho*-cleavage pathway were found in the genome, whereas the C23O gene associated with the *meta*-cleavage pathway is located on the plasmid pWW0 [3]. The expression of *catA2* was found to be enhanced after the pWW0 plasmid was eliminated, thus reducing the accumulation of toxic intermediates (catechol). Furthermore, if the *catA2* gene was knocked out, *P. putida* mt-2 would quickly lose the plasmid PWW0 when grown on toluene, thereby suggesting an indispensable role for *catA2* in maintaining the stability of pWW0. These results clearly demonstrate that *catA* gene redundancy helps to increase the levels of C12O and thus reduce excess levels of catechol, thereby facilitating the adaption of the organism to various growth conditions [3]. A similar phenomenon was found in *P. putida* ND6. The presence of four diverse *catA* genes led to an increase in the levels of active C12O, thus benefitting the survival of ND6 cells under conditions of excess catechol. Moreover, redundancy is recognised as a source of novel biochemical functions [36]. Hence, *catA* gene redundancy could be associated with adaptive interactions of the ND6 strain with its environment by increasing its phenotypic diversity.

4. Conclusions

In the present study, we cloned and characterised four *catA* genes from *P. putida* ND6 for the first time. The results illustrate that all four of the different genes encoded active C12O. Furthermore, the four recombinant C12O isozymes possessed varying enzymatic properties including optimum temperatures and pH values, thermal and pH stabilities as well as kinetic parameters. Together with mRNA expression levels and enzyme activity analysis, these results clearly imply that the redundancy of *catA* genes increases the available amount of C12O and will result in tangible benefits to facilitate survival of *P. putida* ND6 in challenging environments.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2018.06.001.

References

[1] Singh SN, Kumari B, Upadhyay SK, et al. Bacterial degradation of pyrene in minimal salt medium mediated by catechol dioxygenases: Enzyme purification and molecular size determination. Bioresour Technol 2013;133:293–300. https://doi.org/10.1016/j.biortech.2013.01.068.
[2] Wackett LP. *Pseudomonas putida*-a versatile biocatalyst. Nat Biotechnol 2003;21(2):136–8. https://doi.org/10.1038/nbt0203-136.
[3] Jimenez JI, Perez-Pantoja D, Chavarria M, et al. A second chromosomal copy of the catA gene endows *Pseudomonas putida* mt-2 with an enzymatic safety valve for excess of catechol. Environ Microbiol 2014;16(6):1767–78. https://doi.org/10.1111/1462-2920.12361.
[4] Lin J, Milase RN. Purification and characterization of catechol 1,2-Dioxygenase from *Acinetobacter* sp. Y64 strain and *Escherichia coli* transformants. Protein J 2015;34(6):421–33. https://doi.org/10.1007/s10930-015-9637-7.
[5] Yoon YH, Yun SH, Park SH, et al. Characterization of a new catechol branch of the β-ketoadipate pathway induced for benzoate degradation in *Acinetobacter lwoffii* K24. Biochem Biophys Res Commun 2007;360(3):513–9. https://doi.org/10.1016/j.bbrc.2007.05.132.
[6] Pessione E, Giuffrida MG, Mazzoli R, et al. The catechol 1,2 dioxygenase system of *Acinetobacter radioresistens*: isoenzymes, inductors and gene localisation. Biol Chem 2001;382(8):1253–61. https://doi.org/10.1515/BC.2001.156.
[7] Udaondo Z, Molina L, Daniels C, et al. Metabolic potential of the organic-solvent tolerant *Pseudomonas putida* DOT-T1E deduced from its annotated genome. J Microbial Biotechnol 2013;6(5):598–611. https://doi.org/10.1111/1751-7915.12061.
[8] Li S, Zhao H, Li Y, et al. Complete genome sequence of the naphthalene-degrading *Pseudomonas putida* strain ND6. J Bacteriol 2012;194(18):5154–5. https://doi.org/10.1128/JB.01190-12.
[9] Li W, Shi J, Wang X, et al. Complete nucleotide sequence and organization of the naphthalene catabolic plasmid pND6-1 from *Pseudomonas* sp. strain ND6. Gene 2004;336(2):231–40. https://doi.org/10.1016/j.gene.2004.03.027.
[10] Li S, Zhao H, Li Y, et al. Complete nucleotide sequence of plasmid pND6-2 from *Pseudomonas putida* ND6 and characterization of conjugative genes. Gene 2013;512(1):148–56. https://doi.org/10.1016/j.gene.2012.09.065.
[11] Zhao H, Li Y, Chen W, et al. A novel salicylaldehyde dehydrogenase-NahV involved in catabolism of naphthalene from *Pseudomonas putida* ND6. Chin Sci Bull 2007;52(14):1942–8. https://doi.org/10.1007/s11434-007-0296-8.
[12] Li S, Li X, Zhao H, et al. Physiological role of the novel salicylaldehyde dehydrogenase NahV in mineralization of naphthalene by *Pseudomonas putida* ND6. Microbiol Res 2011;166(8):643–53. https://doi.org/10.1016/j.micres.2011.01.003.
[13] Larkin MA, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007;23(21):2947–8. https://doi.org/10.1093/bioinformatics/btm404.
[14] Kumar S, Nei M, Dudley J, et al. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 2008;9(4):299–306. https://doi.org/10.1093/bib/bbn017.
[15] Long Y, Yang S, Xie Z, et al. Cloning, expression, and characterization of catechol 1,2-dioxygenase from a phenol-degrading *Candida tropicalis* JH8 strain. Prep Biochem Biotechnol 2016;46(7):673–8. https://doi.org/10.1080/10826068.2015.1135449.
[16] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72(1–2):248–54. https://doi.org/10.1006/abio.1976.9999.
[17] Wang Y, Li J, Liu A. Oxygen activation by mononuclear nonheme iron dioxygenases involved in the degradation of aromatics. J Biol Inorg Chem 2017;22(2–3):395–405. https://doi.org/10.1007/s00775-017-1436-5.
[18] Kuepper J, Dickler J, Biggel M, et al. Metabolic engineering of *Pseudomonas putida* KT2440 to produce anthranilate from glucose. Front Microbiol 2015;6. https://doi.org/10.3389/fmicb.2015.01310.
[19] George KW, Hay A. Less is more: reduced catechol production permits *Pseudomonas putida* F1 to grow on styrene. Microbiology 2012;158:2781–8. https://doi.org/10.1099/mic.0.058230-0.
[20] Sharma PK, Fu J, Zhang XL, et al. Genome features of *Pseudomonas putida* LS46, a novel polyhydroxyalkanoate producer and its comparison with other *P. putida* strains. Amb Express 2014;4(37). https://doi.org/10.1186/s13568-014-0037-8.
[21] Murakami S, Kohsaka C, Okuno T, et al. Purification, characterization, and gene cloning of *cis,cis*-muconate cycloisomerase from benzamide-assimilating *Arthrobacter* sp BA-5-17. FEMS Microbiol Lett 2004;231(1):119–24. https://doi.org/10.1016/S0378-1097(03)00933-9.
[22] Weyens N, Beckers B, Schellingen K, et al. The potential of the Ni-resistant TCE-degrading *Pseudomonas putida* W619-TCE to reduce Phytotoxicity and improve phytoremediation efficiency of poplar cuttings on a Ni-TCE co-contamination. Int J Phytoremediation 2015;17(1–6):40–8. https://doi.org/10.1080/15226514.2013.828016.
[23] Guo W, Wang Y, Song C, et al. Complete genome of *Pseudomonas mendocina* NK-01, which synthesizes medium-chain-length Polyhydroxyalkanoates and alginate oligosaccharides. J Bacteriol 2011;193(13):3413–4. https://doi.org/10.1128/jb.05068-11.
[24] Shen XH, Liu ZP, Liu SJ. Functional identification of the gene locus (*ncg*12319 and characterization of catechol 1,2-dioxygenase in *Corynebacterium glutamicum*. Biotechnol Lett 2004;26(7):575–80. https://doi.org/10.1023/b:bile.0000021958.86258.08.
[25] Di Nardo G, Roggero C, Campolongo S, et al. Catalytic properties of catechol 1,2-dioxygenase from *Acinetobacter radioresistens* S13 immobilized on nanosponges. Dalton Trans 2009(33):6507–12. https://doi.org/10.1039/b903105g.
[26] Pandeeti EVP, Siddavattam D. Purification and characterization of catechol 1,2-dioxygenase from *Acinetobacter* sp DS002 and cloning, sequencing of partial *catA* gene. Indian J Microbiol 2011;51(3):312–8. https://doi.org/10.1007/s12088-011-0123-4.
[27] Zhao HB, Chen W, Cai BL. Cloning and expression of catA gene from pseudomonas putida ND6 and study on the catechol cleavage pathway. Wei Sheng Wu Xue Bao [In Chinese] 2007;47(3):387–91.
[28] Guzik U, Hupert-Kocurek K, Sitnik M, et al. High activity catechol 1,2-dioxygenase from *Stenotrophomonas maltophilia* strain KB2 as a useful tool in *cis,cis*-muconic acid production. Antonie Van Leeuwenhoek 2013;103(6):1297–307. https://doi.org/10.1007/s10482-013-9910-8.
[29] Murakami S, Wang CL, Naito A, et al. Purification and characterization of four catechol 1,2-dioxygenase isozymes from the benzamide-assimilating bacterium *Arthrobacter* species BA-5-17. Microbiol Res 1998;153(2):163–71. https://doi.org/10.1016/S0944-5013(98)80036-0.
[30] Nakai C, Horiike K, Kuramitsu S, et al. Three isozymes of catechol 1,2-dioxygenase (pyrocatechase), alpha alpha, alpha beta, and beta beta, from pseudomonas arvilla C-1. J Biol Chem 1990;265(2):660–5.
[31] Cao B, Geng A, Loh KC. Induction of ortho- and meta-cleavage pathways in *Pseudomonas* in biodegradation of high benzoate concentration: MS identification of catabolic enzymes. Appl Microbiol Biotechnol 2008;81(1):99–107. https://doi.org/10.1007/s00253-008-1728-3.
[32] Suzuki K, Ichimura A, Ogawa N, et al. Differential expression of two catechol 1,2-dioxygenases in *Burkholderia* sp strain TH2. J Bacteriol 2002;184(20):5714–22. https://doi.org/10.1128/Jb.184.20.5714-5722.2002.
[33] Wojcieszynska D, Guzik U, Gren I, et al. Induction of aromatic ring: cleavage dioxygenases in Stenotrophomonas maltophilia strain KB2 in cometabolic systems. World J Microbiol Biotechnol 2011;27(4):805–11. https://doi.org/10.1007/s11274-010-0520-6.
[34] Bratlie MS, Johansen J, Sherman BT, et al. Gene duplications in prokaryotes can be associated with environmental adaptation. BMC Genomics 2010;11(1):588. https://doi.org/10.1186/1471-2164-11-588.
[35] Gevers D, Vandepoele K, Simillion C, et al. Gene duplication and biased functional retention of paralogs in bacterial genomes. Trends Microbiol 2004;12(4):148–54. https://doi.org/10.1016/j.tim.2004.02.007.
[36] Treangen TJ, Rocha EP. Horizontal transfer, not duplication, drives the expansion of protein families in prokaryotes. PLoS Genet 2011;7(1):e1001284. https://doi.org/10.1371/journal.pgen.1001284.