Function and Molecular Ecology Significance of Two Catechol-Degrading Gene Clusters in *Pseudomonas putida* ND6

Sanyuan Shi^{1†}, Liu Yang^{1†}, Chen Yang¹, Shanshan Li², Hong Zhao³, Lu Ren¹, Xiaokang Wang¹, Fuping Lu¹, Ying Li^{4*}, and Huabing Zhao^{1*}

¹Tianjin Key Laboratory of Industrial Microbiology, College of Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, P.R. China

²Department of Environmental Science and Engineering, Xi'an Jiaotong University, Xi'an 710048, P.R. China

³Tianjin Entry-Exit Inspection and Quarantine Bureau, Tianjin 300457, P.R. China

⁴China Animal Disease Control Center, Beijing 100000, P.R. China

Many bacteria metabolize aromatic compounds via catechol as a catabolic intermediate, and possess multiple genes or clusters encoding catechol-cleavage enzymes. The presence of multiple isozyme-encoding genes is a widespread phenomenon that seems to give the carrying strains a selective advantage in the natural environment over those with only a single copy. In the naphthalene-degrading strain *Pseudomonas putida* ND6, catechol can be converted into intermediates of the tricarboxylic acid cycle via either the *ortho*- or *meta*-cleavage pathways. In this study, we demonstrated that the catechol *ortho*-cleavage pathway genes (*catB_IC_IA_I* and *catB_{II}C_{II}A_{II}*) on the chromosome play an important role. The *cat_I* and *cat_{II}* operons are co-transcribed, whereas *catA_I* and *catA_{II}* are under independent transcriptional regulation. We examined the binding of regulatory proteins to promoters. In the presence of *cis*-*cis*-muconate, a well-studied inducer of the *cat* gene cluster, CatR_I and CatR_{II} occupy an additional downstream site, designated as the activation binding site. Notably, CatR_I binds to both the *cat_I* and *cat_{II}* promoters with high affinity, while CatR_{II} binds weakly. This is likely caused by a T to G mutation in the G/T-N₁₁-A motif. Specifically, we found that CatR_I and CatR_{II} regulate *catB_IC_IA_I* and *catB_{II}C_{II}A_{II}* in a cooperative manner, which provides new insights into naphthalene degradation.

Keywords: *Pseudomonas putida* ND6, catechol *ortho*-cleavage pathway, CatR_I and CatR_{II}, *catB_IC_IA_I* and *catB_{II}C_{II}A_{II}* operons, cross-regulation, evolution of catabolic pathways

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*Corresponding authors

H. Zhao
 Phone: +86-22-60601958
 Fax: +86-22-60602298
 E-mail: zhaohuabing@tust.edu.cn
 Y. Li
 Phone: +86-10-59198969
 Fax: +86-10-59198969
 E-mail: liyinglucky2001@aliyun.com

[†]These authors contributed equally to this work.

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Introduction

A number of aerobic biodegradation pathways of aromatic compounds like benzoate, aniline, phenol, pyrene and naphthalene converge at the catechol ring cleavage reaction, and eventually generate intermediates of the tricarboxylic acid cycle through two different routes [1-3]. The catechol *meta*-cleavage pathway is usually encoded by the *nah* and *xyl* genes on plasmids, while the catechol *ortho*-cleavage pathway is usually determined by the chromosomal *cat* and *ben* genes [4-9]. *Ortho*-cleavage is followed by further steps of the β -ketoadipate pathway which is catalyzed by the three enzymes catechol 1,2-dioxygenase (C12O, E.C. 1.13.11.1) (*catA*), *cis*,*cis*-muconate-lactonizing enzyme I (*catB*), and muconolactone isomerase (*catC*). The expression of this operon is regulated by CatR, and is induced by *cis*,*cis*-muconate (CCM) [9-15].

Several research groups have found that bacteria possess multiple catechol *ortho*-cleavage genes or clusters, which might indicate the importance of keeping the intracellular concentration of catechol and its derivatives low [6, 7, 16-20]. Notably, the strains with multiple catechol dioxygenase genes can usually grow on many more aromatic compounds than strains with one dioxygenase gene [14, 21]. Different enzymatic properties and induction patterns of C12O isozymes may be responsible for the metabolism of different substrates [22]. For example, three different C12O isozymes encoded by *catA_I*, *catA_{II}* and *catA_{III}* in the naphthalene-degrading strain *Pseudomonas putida* ND6 were found to have different enzymatic properties [17]. *Burkholderia* sp. strain TH2, a 2-chlorobenzoate (2CB)-degrading bacterium, metabolizes benzoate (BA) and 2CB via two catechol *ortho*-cleavage pathways (*cat₁* and *cat₂*). Interestingly, the inducer of *catA₁* was found to be BA, and not 2CB. It was also found that CCM or its metabolite acts as an inducer for *catA₂*. These results suggest that although *cat₂* genes are not indispensable for growth of TH2 on 2CB, they are advantageous [20]. Murakami *et al.* reported that the aniline-assimilating bacterium *Frateruia* sp. ANA-18 has two *cat* clusters, which are respectively located on the

chromosome and the large plasmid [19]. Two CatR proteins that differ in their binding affinity for the *catB* promoters control the expression of the two *cat* gene clusters, which is affected by the concentration of CCM. The *catB_I* promoter was shown to be active in the presence of CCM, while the *catB_{II}* promoter was activated only at low concentrations of CCM [23]. Hence, the presence of multiple catechol *ortho*-cleavage genes and pathways in the same cell seems to be a widespread phenomenon that gives the host strains a selective advantage in the natural environment. However, the research on function, regulation and evolutionary significance of these genes and clusters should be further intensified.

P. putida strain ND6, which was isolated by our group from industrial wastewater in Tianjin, China, was capable of growth on naphthalene as the sole carbon and energy source, and successfully degraded 98% of 2 g/l naphthalene in mineral medium in 48 h. Similar to other described strains [24–26], ND6 possesses an integrated naphthalene-degrading pathway, and catabolizes catechol through the *meta*-cleavage pathway. However, a unique feature of ND6 exists in the presence of two catechol *ortho*-cleavage pathways, encoded by *catR_IB_IC_IA_I* and *catR_{II}B_{II}C_{II}A_{II}* on the chromosome, in addition to the typical catechol *meta*-cleavage pathway on the plasmid. We investigated the cellular responses of *P. putida* ND6 grown in medium with 2 (normal concentration) and 4 g/l naphthalene (high concentration) using a quantitative proteomics-based approach. Comparative analysis of the proteomic data indicated that the expression levels of CatA_I, CatB_I, and CatB_{II}, which are involved in the catechol *ortho*-cleavage pathway, were upregulated [27]. This demonstrated that *P. putida* ND6 is able to survive in the presence of high naphthalene concentrations mainly because of the duplicated catechol *ortho*-cleavage operons. The two *ortho*-cleavage operons are regulated by the LysR-type regulatory proteins CatR_I and CatR_{II}, respectively. The distance between the two clusters was about 754 kb, and their sequence identity was 73.57%. Based on the previous work in our laboratory, we hypothesized that these two gene clusters have different functions and evolutionary origins, and might regulate each other via genetic cross-talk. In this paper, we compared the function, regulation and evolutionary significance of the two catechol *ortho*-cleavage gene clusters in *P. putida* strain ND6.

Materials and Methods

Bacterial Strains, Culture Conditions, Plasmids, Primers and DNA Manipulations

P. putida strain ND6 and mutants were cultured at 30°C and 180 rpm in Luria-Bertani (LB), or in mineral medium (MMB) with 0.2% naphthalene or 0.2% glucose as the carbon source. *Escherichia coli* strains were grown

Table 1. Strains and plasmids used in this study.

Strains/plasmids		Genotype and description	Source/reference
<i>E. coli</i>	JM109	Cloning strain	Novagen
	S17-1	<i>recA pro hsdR</i> RP4-2-Tc::Mu-Km::Tn7; donor strain for conjugation;	[28]
	BL21	Expression strain	Novagen
<i>P. putida</i>	ND6	Cb ^r	[16]
	ND6-Δ <i>catR_I</i>	<i>catR_I</i> mutant of ND6; Cb ^r , Kan ^r	This study
	ND6-Δ <i>catR_{II}</i>	<i>catR_{II}</i> mutant of ND6; Cb ^r , Kan ^r	This study
	ND6-Δ <i>catA</i>	<i>catA</i> mutant of ND6; Cb ^r , Kan ^r	This study
	ND6-Δ <i>catAnahH</i>	<i>catA</i> and <i>nahH</i> mutant of ND6; Cb ^r , Kan ^r , Cm ^r	This study
	NC	ND6 containing plasmid pDN19 <i>lac</i> Ω; Cb ^r , Sp ^r , Sm ^r	This study
	NP1	ND6 containing plasmid pDB1; Cb ^r , Sp ^r , Sm ^r	This study
	NP2	ND6 containing plasmid pDB2; Cb ^r , Sp ^r , Sm ^r	This study
	ND1P1	ND6-Δ <i>catR_I</i> containing plasmid pDB1; Cb ^r , Kan ^r , Sp ^r , Sm ^r	This study
	ND1P2	ND6-Δ <i>catR_I</i> containing plasmid pDB2; Cb ^r , Kan ^r , Sp ^r , Sm ^r	This study
	ND2P1	ND6-Δ <i>catR_{II}</i> containing plasmid pDB1; Cb ^r , Kan ^r , Sp ^r , Sm ^r	This study
	ND2P2	ND6-Δ <i>catR_{II}</i> containing plasmid pDB2; Cb ^r , Kan ^r , Sp ^r , Sm ^r	This study
	Plasmid pUC18-T simple	Cloning vector; Amp ^r	TransGen
	pEX18Tc	Gene replacement; Tet ^r , ori ^T <i>sacB</i> ⁺	[29]
Plasmid	pDN19 <i>lac</i> Ω	Broad host range shuttle vector; Sp ^r , Sm ^r	[30]
	pEASY-Blunt	Source of <i>kan</i> gene; Amp ^r , Kan ^r	TransGen
	pXMJ19	Source of <i>cat</i> gene; Amp ^r , Cm ^r	TransGen
	pEX18Tc-R _I Km	pEX18Tc with Δ <i>catR_I</i> ::Kan ^r ; Tet ^r	This study
	pEX18Tc-R _{II} Km	pEX18Tc with Δ <i>catR_{II}</i> ::Kan ^r ; Tet ^r	This study
	pEX18Tc-AKm	pEX18Tc with Δ <i>catA</i> ::Kan ^r ; Tet ^r	This study
	pEX18Tc-HCm	pEX18Tc with Δ <i>nahH</i> ::Cm ^r ; Tet ^r	This study
	pEX5-catR _I	CatR _I protein expression vector; Kan ^r	This study
	pEX5-catR _{II}	CatR _{II} protein expression vector; Kan ^r	This study
	pUC19c-catB _I	pUC19c with <i>catB_I</i> promoter; Amp ^r	This study
	pUC19c-catB _{II}	pUC19c with <i>catB_{II}</i> promoter; Amp ^r	This study
	pDB _I	<i>cat_I</i> promoter inserted between the EcoRI and BamHI sites of pDN19 <i>lac</i> Ω; Sp ^r , Sm ^r	This study
	pDB _{II}	<i>cat_{II}</i> promoter inserted between the EcoRI and BamHI sites of pDN19 <i>lac</i> Ω; Sp ^r , Sm ^r	This study

*Cb^r, carbenicillin resistant; Kan^r, kanamycin resistant; Cm^r, Chloramphenicol resistant; Sp^r, spectinomycin resistant; Sm^r, streptomycin resistant; Amp^r, ampicillin resistant; Tet^r, tetracycline resistant; Gm^r, gentamicin resistant.

in LB at 37°C and 180 rpm. Where appropriate, spectinomycin (Sp; 50 mg/l), streptomycin (Sm; 50 mg/l), chloramphenicol (Cm; 25 mg/l), ampicillin (Amp; 50 mg/l), kanamycin (Kan; 50 mg/l), carbenicillin (Cb; 75 mg/l), tetracycline (Tet; 10 mg/l), or gentamicin (Gm; 10 mg/l) was added for selection.

The bacterial strains, plasmids, and primers used in the present study are listed in Tables 1 and 2. All DNA manipulations were performed according to standard procedures [31]. Restriction enzymes, DNA polymerase and T4 DNA ligase were used in accordance with the manufacturers' specifications.

Table 2. Primers used in this study.

Primer name	Sequence (5'–3')	Description
R ₁ L-F	GTGATGTGGCCGAATGCCTC	Used in the creation of ND6- Δ catR ₁ mutant
R ₁ L-R	CGGCATGAGCATTCGTGTC	Used in the creation of ND6- Δ catR ₁ mutant
R ₁ R-F	GGCCGAAGCCAATGTCTGA	Used in the creation of ND6- Δ catR ₁ mutant
R ₁ R-R	GCTTTCGATGCCGACTTG	Used in the creation of ND6- Δ catR ₁ mutant
R ₁₁ L-F	GACGGCCAAGTCGATGGTG	Used in the creation of ND6- Δ catR ₁₁ mutant
R ₁₁ L-R	TAGGCCAGCCGATATCGCT	Used in the creation of ND6- Δ catR ₁₁ mutant
R ₁₁ R-F	CCTGATAGATAGCCGCGTCTG	Used in the creation of ND6- Δ catR ₁₁ mutant
R ₁₁ R-R	AGCAAGCGAAGAATGACCAAGG	Used in the creation of ND6- Δ catR ₁₁ mutant
AL-F	GGGCCGCTTGATCAACGTCGT	Used in the creation of ND6- Δ AH mutant
AL-R	ATCTCAGGCAGGTTGAAATAG	Used in the creation of ND6- Δ AH mutant
AR-F	CAGTCCGAATACAACCTGCGC	Used in the creation of ND6- Δ AH mutant
AR-R	CCGAAGGCGACAAAGCTGGAG	Used in the creation of ND6- Δ AH mutant
HL-F	GCGCCGCTGGACGTGACAATGC	Used in the creation of ND6- Δ AH mutant
HL-R	TCCAGTACACGCAGTTGCACG	Used in the creation of ND6- Δ AH mutant
HR-F	TTATTTCTTCGACCCGTCCGG	Used in the creation of ND6- Δ AH mutant
HR-R	GGCCGGTCCGACTTTCAGGT	Used in the creation of ND6- Δ AH mutant
kan-F	GGGCGGTTTATGGACAGC	Used in the creation of ND6- Δ catR ₁ , ND6- Δ catR ₁₁ , ND6- Δ AH mutant
kan-R	CGGTGCTCAACGGGAATC	Used in the creation of ND6- Δ catR ₁ , ND6- Δ catR ₁₁ , ND6- Δ AH mutant
cat-F	CTTAAAAAATTTACGCCCCGCG	Used in the creation of ND6- Δ AH mutant
cat-R	TGATCGGCACGTAAGAGGTTTC	Used in the creation of ND6- Δ AH mutant
catB ₁ -P-F	AGCACGGTGCAGGTCTGTTC	Used for construction catB ₁ -lacZ fusions
catB ₁ -P-R	GCCCGAGTGATGCGTTTAC	Used for construction catB ₁ -lacZ fusions
catB ₁₁ -P-F	CAAGCTGTTTGAGCAAGGTGTC	Used for construction catB ₁₁ -lacZ fusions
catB ₁₁ -P-R	TAAGGCATCAATACCCATCG	Used for construction catB ₁₁ -lacZ fusions
CatR ₁ -F	ACTTGATTGTTGAAGGATT	Used for CatR ₁ protein expression
CatR ₁ -R	TCAGAGTGCCTGTTGCTC	Used for CatR ₁ protein expression
CatR ₁₁ -F	GTGGAGCTCAGGCAT	Used for CatR ₁₁ protein expression
CatR ₁₁ -R	TTATCGATTGATGGTCG	Used for CatR ₁₁ protein expression
q16S-F	CGGATCGCAGTCTGCAACTC	16S gene for RT-qPCR
q16S-R	ACACCGTGGTAACCGTCCTCC	16S gene for RT-qPCR
qcatA ₁ -F	AGGAATGCCTGGACCTGCTCG	catA ₁ gene amplification for RT-qPCR
qcatA ₁ -R	CGAATGACAACCTCGGCAAAGCG	catA ₁ gene amplification for RT-qPCR
qcatB ₁ -F	CTGGCATTTGCCTTGTACGG	catB ₁ gene amplification for RT-qPCR
qcatB ₁ -R	AGGCGCTGTTTCGTCCAATG	catB ₁ gene amplification for RT-qPCR
qcatC ₁ -F	ACATGGACCCGGCCAAAGG	catC ₁ gene amplification for RT-qPCR
qcatC ₁ -R	TCAGCGATCGTCGCTGTG	catC ₁ gene amplification for RT-qPCR
qcatA ₁₁ -F	AAGGGCTAATCGGCGAAGTG	catA ₁₁ gene amplification for RT-qPCR
qcatA ₁₁ -R	GCTGTTCCGGCAGTCTCAACC	catA ₁₁ gene amplification for RT-qPCR
qcatB ₁₁ -F	GGTGCGGCTCAATCAACG	catB ₁₁ gene amplification for RT-qPCR
qcatB ₁₁ -R	GCCTGGGCTATCTGGGCAG	catB ₁₁ gene amplification for RT-qPCR
qcatC ₁₁ -F	CAAGTGGCTGCCCGCTTG	catC ₁₁ gene amplification for RT-qPCR
qcatC ₁₁ -R	AGCGCTTCGACACTGTCCACA	catC ₁₁ gene amplification for RT-qPCR
catB ₁ -C ₁ -F	GGGCCTGACATTGGACGAAC	The region of catB ₁ gene and catC ₁ gene; RT-PCR
catB ₁ -C ₁ -R	ACACAGGCCGTCGACCTCA	The region of catB ₁ gene and catC ₁ gene; RT-PCR
catC ₁ -A ₁ -F	CGTACATGGACATTGAGGTCTGA	The region of catC ₁ gene and catA ₁ gene; RT-PCR
catC ₁ -A ₁ -R	AGGTGCGAGGAAGTGCTCGATG	The region of catC ₁ gene and catA ₁ gene; RT-PCR
catB ₁₁ -C ₁₁ -F	GATCAGCTGCAATGGCACAC	The region of catB ₁₁ gene and catC ₁₁ gene; RT-PCR
catB ₁₁ -C ₁₁ -R	AGCGCTTCGACACTGTCC	The region of catB ₁₁ gene and catC ₁₁ gene; RT-PCR
catC ₁₁ -A ₁₁ -F	CAAGTGGCTGCCCGCTT	The region of catC ₁₁ gene and catA ₁₁ gene; RT-PCR
catC ₁₁ -A ₁₁ -R	CGATAAACAACGTGGTGGAAC	The region of catC ₁₁ gene and catA ₁₁ gene; RT-PCR
M13F-11	CGCCAGGGTTTCCCAGTCACGAC	TaqMan primer used for catB ₁ or catB ₁₁ EMSA and DNase I footprinting template
M13R-12	AGCGGATAACAATTTACACAGGA	TaqMan primer used for catB ₁ or catB ₁₁ EMSA and DNase I footprinting template
catB ₁ -pe	TGTGTTCAATCAGCGCGCTTGTC	CatB ₁ gene-specific primer, Primer extension
catB ₁₁ -pe	GCTGCTGTCATTTACAGTTCCATC	CatB ₁₁ gene-specific primer, Primer extension

Construction of *P. putida* ND6 Mutants

The suicide vector pEX18Tc was used for gene replacement in *Pseudomonas*. The *catR_I* replacement vector pEX18Tc-R_IKm was constructed by inserting the Kan^r cassette into the *catR_I* gene in the pEX18Tc vector to generate a partial deletion from 226 to 440 bp. Similarly, the *catR_{II}* replacement vector pEX18Tc-R_{II}Km was constructed by replacing 335 to 407 bp, and the *catA* replacement vector pEX18Tc-AKm by replacing between 10 to 514 bp. The knockout plasmids pEX18Tc-R_IKm, pEX18Tc-R_{II}Km, and pEX18Tc-AKm were transferred from *E. coli* S17-1 into *P. putida* ND6 by intergeneric conjugation, followed by selection on LB + Cb + Kan medium with 20% sucrose to generate the *catR_I*, *catR_{II}* and *catA* mutants. The resulting mutant strains were named *P. putida* ND6- Δ *catR_I*, ND6- Δ *catR_{II}*, and ND6- Δ *catA*, respectively.

The *nahH* replacement vector pEX18Tc-HCm was constructed by inserting the Cm^r cassette into the internally deleted *nahH* gene in the pEX18Tc vector, replacing the sequence between 204 to 742 bp. Finally, the *P. putida* strain ND6- Δ *catAnahH* was obtained.

Time-Courses of Growth, Naphthalene Degradation, and Catechol Dioxygenase Activity

The wild-type ND6, as well as the mutants ND6- Δ *catR_I*, ND6- Δ *catR_{II}* and ND6- Δ *catAnahH*, were individually grown in MMB medium with 0.2% naphthalene. Bacterial growth was monitored by measuring the optical density at 600 nm. Naphthalene in the culture broth was extracted twice with an equal volume of dichloromethane, and its concentration was measured according to a published method [32]. The activity of catechol dioxygenases (C12O and C23O) was assayed as described previously [17].

Quantitative Real-Time PCR (RT-qPCR)

The wild-type ND6, as well as the ND6- Δ *catR_I* and ND6- Δ *catR_{II}* mutants were cultured at 30°C in MMB with glucose or naphthalene. Cells were harvested at 25 h (end of the exponential growth phase) and washed with TE buffer. RNA was extracted using the RNeasy Pure Bacteria Kit (Qiagen Biotech, China) and reverse-transcribed into cDNA using the FastKing RT Kit (with gDNase) (Tiangen Biotech). The RT-qPCR was performed following the instructions of the Green Premix Ex Taq II Kit. All RT-qPCR reactions were conducted in three biological and three technical replicates.

Plasmid standards were used for absolute quantification of the corresponding gene fragments. The plasmid copy number was determined according to the molar mass derived from the plasmid and amplicon sequences [33]. For each standard sample, the RT-qPCR system was used to measure the cycle threshold values, which were used to draw a standard curve for each isoform by plotting the cycle threshold values versus the log value of the transcript copy number. Regression equations generated by the system software were used to calculate the transcript copy number for each isoform in each test sample, which was normalized to the value of the 16S rRNA and expressed as the absolute copy number per 1000 copies of 16S transcript. The 95% confidence interval was used to assess the significance of copy number differences, and analysis of variance (ANOVA) was used to assess the significance of the threshold cycle values.

Expression and Purification of CatR_I and CatR_{II}

E. coli BL21 carrying the CatR_I and CatR_{II} expression plasmids pEX5-CatR_I and pEX5-CatR_{II} were grown overnight in LB medium with kanamycin. The expression was induced overnight at 16°C by adding a final concentration of 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG). To purify CatR_I and CatR_{II}, cell pellets obtained from 1 liter cultures were collected at 13,000 $\times$ g for 3 min at 4°C and resuspended in 40 ml of binding buffer (50 mM sodium phosphate, 0.3 M NaCl, pH 7.4), and sonicated. The clarified lysate was applied to a nickel-nitrilotriacetic acid (NTA) affinity column and allowed to bind for 1 h, followed by washing with binding buffer containing 10 mM, 30 mM and 60 mM imidazole, and eluting with binding buffer containing 500 mM imidazole. The collected fractions were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The desalination column Sephadex G-25 (GE, USA) was used to remove the imidazole, and yielded the purified protein. The protein was stored in glycerol at -20°C.

Primer-Extension Assay

The primer extension assay was carried out according to the protocol of Fekete *et al.*, as published previously [34].

Electrophoretic Mobility Shift Assay (EMSA)

The fluorescent 6-fluorescein amidite (FAM)-labeled probes were prepared by amplifying the promoter regions of pUC19c-catB_I and pUC19c-catB_{II} by PCR using the primers M13F-11 and M13R-12 (Table 2). The Wizard SV Gel and PCR Clean-Up System (Promega) was used to purify the resulting FAM-labeled probes, which were then quantified using a NanoDrop 2000C instrument (Thermo Fisher Scientific, USA). The EMSA samples comprised 20 μ l of 50 mM Tris-HCl [pH 8.0], 100 mM KCl, 2.5 mM MgCl₂, 0.2 mM dithiothreitol (DTT), with 2 μ g salmon sperm DNA and 10% glycerol, as well as 40 ng of the probe and the indicated DNA-binding proteins. After incubation for 30 min at 30°C, the mixture was loaded onto a 10% PAGE gel buffered with 0.5 $\times$ Tris-borate-EDTA (TBE).

DNase I Footprinting

The fluorescent FAM-labeled probes were prepared as described above. The DNase I footprinting assays were conducted as published by Wang *et al.* [35]. For each assay, 400 ng of the probe in a total volume of 40 μ l was mixed with different amounts of the indicated DNA-binding protein. The preparation of the DNA ladder,

electrophoresis and data analysis were the same as described before [35], except that the GeneScan-LIZ500 size standard (Applied Biosystems) was used.

Construction of *catB_I-lacZ* and *catB_{II}-lacZ* Fusions and β -Galactosidase Activity Assays

A 309 bp DNA fragment corresponding to the *catR_I-catB_I* intergenic region (from -347 to -39 relative to the *catB_I* initiation codon) and a 330 bp DNA fragment corresponding to the *catR_{II}-catB_{II}* intergenic region (from -358 to -29 relative to the *catB_{II}* initiation codon) were amplified by PCR to construct *cat_I* promoter-*lacZ* fusion and *cat_{II}* promoter-*lacZ* fusion, respectively. Two amplified fragments of *cat_I* and *cat_{II}* promoter were digested with EcoRI and BamHI, respectively, and inserted into pDN19lac Ω that had been previously cut with the same enzymes. The resulting plasmids, pDB_I and pDB_{II}, were transformed into *E. coli* S17-1 by chemical transformation. The complementation was performed through the conjugation between *E. coli* S17-1 with plasmid pDB_I and pDB_{II} and *P. putida* ND6, *P. putida* ND6- Δ *catR_I*, and ND6- Δ *catR_{II}*. Recombinant *P. putida* was selected on MMB plate containing 0.2% salicylate (w/v %) with Cb, Sp and Sm, and subcultured several times to ensure plasmid stabilization. The β -galactosidase activity was measured according to the method of Green [31].

Phylogenetic Tree and Analysis of Conserved Sequences

Database searches were performed using BLAST at the National Center for Biotechnology Information (NCBI) website. Multiple sequence alignments were performed using Clustal W. Then, a neighbor-joining (NJ) phylogenetic tree was constructed using MEGA software version 5.0, and the branching reliability was tested using bootstrap re-sampling (1,000 pseudo-replicates). Analysis of conserved motifs based on known sequences was conducted using WebLogo to generate LOGO diagrams.

Results

Functional Analysis of the Two Catechol-Degrading Gene Clusters

As mentioned above, the genome of *P. putida* ND6 encodes two catechol-degrading gene clusters (*catR_IB_IC_IA_I* and *catR_{II}B_{II}C_{II}A_{II}*) (Fig. 1). To investigate the possible physiological role of these two orthologous clusters, *catA* (encoding C12O) and *nahH* (encoding C23O) from the large plasmid pND6-1, which are responsible for catechol ring cleavage [16], were knocked out. The obtained double-mutant strain ND6- Δ *catA**nahH* was still able to grow on naphthalene by employing the catechol-degrading gene clusters in the genome as a backup, although the C12O activity dropped from 74.60 to 43.02 U/mg (Fig. 2). Furthermore, to examine the relative roles of the *cat_I* and *cat_{II}* clusters in catechol metabolism, we disrupted the *catR_I* and *catR_{II}* regulatory genes and examined the properties of the resulting strains. The ability of these strains to use naphthalene as the sole carbon source was tested in growth and degradation experiments. Interestingly, inactivation of *catR_I* or *catR_{II}* had no obvious effect on the strains' ability to utilize naphthalene (Fig. 3).

Transcriptional Analysis of the Two Catechol-Degrading Gene Clusters

The cDNA of *P. putida* ND6 was used as template to amplify the intergenic regions of the *catB_IC_IA_I* and *catB_{II}C_{II}A_{II}* operons. The results indicated that either the *catB_IC_IA_I* or *catB_{II}C_{II}A_{II}* cluster can be co-transcribed

Fig. 1. Genetic, regulatory and biochemical connections of naphthalene catabolism in *P. putida* ND6. The figure sketches the pathways involved in the naphthalene catabolism of *P. putida* ND6: the upstream operon (*nahABCDE*) for the conversion of naphthalene into salicylate; the downstream operon for the conversion of salicylate into catechol and eventually into intermediates of the central carbon metabolism. Catechol metabolism can proceed via either the *ortho*- or the *meta*-cleavage pathway. Both pathways converge towards catechol and diverge at that point, as this compound can be cleaved between positions 1 and 2 by *catA*, whereas *nahH* cleaves between positions 2 and 3. The three *catA* genes are located on the chromosome and large plasmid, respectively.

Fig. 2. Naphthalene-degradation phenotype and catechol dioxygenase enzyme activities of *P. putida* ND6 and the ND6-Δ*catAnahH* mutant. Naphthalene (0.2%, w/v %) was provided as the sole carbon source. The small figure shows the C12O and C23O enzyme activity of *P. putida* ND6 and ND6-Δ*catAnahH*.

(Fig. 4). However, the results of RT-qPCR revealed that the transcription of the downstream genes *catC_I* and *catA_I* was higher than that of *catB_I* (Fig. 5A), suggesting that *catC_I* and *catA_I* were independently transcribed, while the *catB_IC_IA_I* cluster was co-transcribed. A similar phenomenon was also observed in the cluster *catB_{II}C_{II}A_{II}* (Fig. 5A). In the naphthalene medium, the activity of transcription (Fig. 5A) and expression levels (Fig. 2, small figure) of three *catA* genes and C12O were much higher than *nahH* gene and C23O. Therefore, the naphthalene metabolism of *P. putida* ND6 proceeds via catechol cleavage in both the *ortho* and *meta* positions, whereby the *ortho*-cleavage pathway is the main cleavage pathway. The transcriptional levels of the *ortho*-cleavage pathways (*catA_I*, *catB_I*, *catC_I*, *catA_{II}*, *catB_{II}*, *catC_{II}*, *catA*) were all increased in naphthalene medium, while that of *meta*-cleavage pathways (*nahH*) was not (Fig. 5A). It indicates that the *ortho*-cleavage pathways can be induced by naphthalene or its metabolites but the *meta*-cleavage pathway apparently cannot. Furthermore, we measured the transcriptional levels of *cat_I* and *cat_{II}* in the mutant strains ND6-Δ*catR_I* and ND6-Δ*catR_{II}*, following cultivation in the presence of glucose or naphthalene. For the ND6-Δ*catR_I* strain (only *catR_{II}* is functional), the transcriptional levels of *catB_I* were significantly decreased, while the *catB_{II}* gene cluster could still be induced (Fig. 5B). For the ND6-Δ*catR_{II}*

Fig. 3. Growth and naphthalene-degradation curves of *P. putida* ND6 wild type, as well as the mutants ND6-Δ*catR_I* and ND6-Δ*catR_{II}*. Naphthalene (0.2%, w/v) was provided as the sole carbon source.

Fig. 4. Agarose gel plots were used to determine the transcription patterns of the two *cat* gene clusters. M1: Tiangen DNA Marker I; M2: Tiangen DNA Marker II; Lane 1: Band of the *catB_I-catC_I* intergenic region DNA fragment; Lane 2: Band of the *catC_I-catA_I* intergenic region DNA fragment; Lane 3: Band of the *catB_I-catA_I* intergenic region DNA fragment; Lane 4: Band of the *catB_{II}-catC_{II}* intergenic region DNA fragment; Lane 5: Band of the *catC_{II}-catA_{II}* intergenic region DNA fragment; Lane 6: Band of the *catB_{II}-catA_{II}* intergenic region DNA fragment; Lane 7: negative control.

strain (only *catR_I* is functional), the transcriptional level of *catB_{II}* was also decreased while the *catB_I* gene cluster could still be induced (Fig. 5C). This result demonstrated that *CatR_I* and *CatR_{II}* are the native regulatory proteins of the *cat_I* cluster and the *cat_{II}* cluster, respectively.

To investigate their possible regulatory roles, the impacts of *catR_I* and *catR_{II}* were further analyzed by determining the transcriptional levels of all six catechol-degradation genes in wild-type ND6 and the mutants when grown with naphthalene as the sole carbon source. When either *catR_I* or *catR_{II}* was knocked out, the transcription of the *cat_I* or *cat_{II}* cluster could still be detected (Fig. 5D). It indicates that *CatR_I* and *CatR_{II}* might

Fig. 5. Copy number determination by absolute RT-qPCR using plasmid standards. Transcript copy number of genes per 1000 mRNA transcript copies of the 16S rRNA in ND6 (A), ND6- Δ *catR_I* (B), and ND6- Δ *catR_{II}* (C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between cells grown on naphthalene and on glucose. (D) Transcript copy number of two *cat* gene clusters per 1000 mRNA transcript copies of the 16S rRNA in ND6, ND6- Δ *catR_I* and ND6- Δ *catR_{II}*. Different superscript letters indicate statistically significant differences among experimental groups ($p < 0.05$; Duncan's multiple range test). Either naphthalene (N-) or glucose (G-) was provided as the sole carbon source. Bars show the standard errors of the means.

Fig. 6. Results of the primer-extension assay. Characterization of the *cat_I* transcription start site (TSS) using the primer extension assay. The primer extension experiments used the primer *catB_I-pe* (Table 2), which was designed to bind downstream of the *catB_I* initiation codon. Comparison of the sequencing results of the cDNA (above) and the genomic DNA (below) to determine the TSS (marked with a red asterisk).

cross-regulate the transcription of each other's target genes, and explains that the naphthalene degradation curves of ND6-Δ*catR_I* and ND6-Δ*catR_{II}* did not exhibit significant changes (Fig. 3).

Promoter Structure of the Two Catechol-Degrading Gene Clusters

The primer-extension assay indicated that the transcription start site (TSS) of *cat_I* is located at a G nucleotide (Fig. 6). Surprisingly, the primer extension assay of *cat_{II}* failed three times based on reliable methods, probably because the transcript abundance of the *cat_{II}* cluster is particularly low.

The CatR_I and CatR_{II} binding regions were also studied using DNase I footprinting (Fig. 7). Purified CatR_I was

Fig. 7. DNase I footprinting analysis of CatR_I (A, B) binding to the *catB_I* promoter and CatR_{II} (C, D) binding to the *catB_{II}* promoter with or without CCM. Based on these results and earlier reports on bacterial promoter prediction [39], we identified the promoter structure of the two catechol-degrading gene clusters (Fig. 8). The putative TSS of *cat_I* was consistent with the primer-extension experiment, and the putative TSS of *cat_{II}* was located 67 bp upstream of the *catB_{II}* initiation codon. Other motifs of the *cat_I* and *cat_{II}* promoters were identified by comparing to the reported conserved sequences recognized by RNA polymerase [11, 13, 19, 23, 40, 41], including the -35 element (positions -35 to -30, if the transcriptional start site is denoted as +1), the -10 element (positions -12 to -7) and the discriminator element (Dis; -6 to -4) (Fig. 8).

Fig. 8. Structure of the *catB_I* and *catB_{II}* promoter regions. (A) The promoter region of the *cat_I* gene cluster. (B) The promoter region of the *cat_{II}* gene cluster. The transcription start site is shown as +1. The predicted discriminator element (Dis), initiation codon (arrows, the direction represents the direction of transcription), and -10 and -35 regions of the promoter were also designated above the sequence. The promoter regions of *cat_I* and *cat_{II}* protected by CatR_I or CatR_{II} from DNase I digestion without CCM, called the repression binding site (RBS), are marked with black solid lines; the extended protected region in the presence of CCM, called the activation binding site (ABS), is marked with the black dotted line.

incubated with a 408 bp fragment labeled with FAM, with a sequence corresponding to the promoter region of *cat_I*, and the resulting complexes were treated with DNase I, followed by DNA fragment analysis by capillary electrophoresis. The binding sequence of the CatR_{II}-*catB_{II}* complex was assessed using the same method, and a 403 bp FAM-labeled DNA fragment was incubated with purified CatR_{II}. In the absence of CCM, CatR_I protected a continuous 26 bp region that had also been determined to be located from -137 to -112 relative to the *catB_I* codon (Fig. 7A). CatR_{II} protected a continuous 25 bp region that had also been determined to be located from -137 to -113 relative to the *catB_I* initiation codon (Fig. 7C). In the presence of CCM, CatR_I protected a continuous 48 bp region that was determined to be located from -137 to -90 relative to the *catB_I* initiation codon (Fig. 7B). CatR_{II} protected a continuous 41 bp region that was determined to be located from -137 to -97 relative to the *catB_I* initiation codon (Fig. 7D). These results demonstrated that CCM has a significant effect on the DNA binding mode of CatR. In the absence of CCM, CatR bound the repression binding site (RBS), which might be associated with the negative regulation of itself. In the presence of CCM, CatR occupied an adjacent downstream site (Fig. 8), designated as the activation binding site (ABS) [36, 37]. Like most LysR proteins, the DNA-binding sites of CatR_I and CatR_{II} invariably contain incomplete inverted repeats (G-N₁₁-A) or inverted repeats (T-N₁₁-A), respectively [38]. The results also indicated that the -35 and -10 regions of the *cat* promoter were important for promoter activity but not for CatR binding.

Transcriptional Cross-Regulation between the Two Catechol-Degrading Gene Clusters

The regulatory proteins CatR_I and CatR_{II} were cloned into a vector to introduce six histidine residues at the C-terminus of the protein, and were expressed and purified from *E. coli* as described in the Materials and Methods section. The binding abilities of CatR_I and CatR_{II} to the upstream regulatory regions of *cat_I* and *cat_{II}* were studied

Fig. 9. EMSA of CatR_I and CatR_{II} binding to the *cat_I* and *cat_{II}* promoters, respectively. (A) EMSA of CatR_I binding to the *cat_I* promoter. (B) EMSA of CatR_{II} binding to the *cat_{II}* promoter. (C) EMSA of CatR_I binding to the *cat_{II}* promoter. (D) EMSA of CatR_{II} binding to the *cat_I* promoter. The first lane contains the free fragment, lanes 2-7 contain labeled probe and CatR_I or CatR_{II} protein, with 0, 0.1, 1.0, 10, 100, and 500 μM muconic acid, respectively. The concentration of the labeled probe was fixed at 40 ng.

by EMSA using a concentration gradient of CCM (Fig. 9). As expected, the addition of 60 ng CatR_I to 40 ng of the labeled probe caused a strong shift in the mobility of the *cat_I* promoter fragment (Fig. 9A). Notably, CatR_I could also strongly bind to the *cat_{II}* promoter region (Fig. 9C). By contrast, CatR_{II} was able to specifically bind the *cat_{II}* and *cat_I* promoter regions, but the binding stability of the CatR_{II}-*cat_{II}* and CatR_{II}-*cat_I* complexes was obviously reduced (Figs. 9B and 9D). These results support the hypothesis that CatR_I and CatR_I might cross-regulate the transcription of each other's target genes. In addition, CCM did not affect the binding of CatR_I to the corresponding target regions, which was in agreement with previous reports [10, 13]. However, the binding regions in the presence and absence of CCM might be different. As can be seen in Figs. 9B and 9D, adding CCM to the reaction mixture decreased the intensity of the DNA band, indicating that CatR_{II} recognizes and binds to the *cat_{II}* and *cat_I* promoters when CCM is at a lower concentration.

Promoter Activity Analysis of the Two Catechol-Degrading Gene Clusters

The primer extension assay indicated that there might be significant differences in the abundance of transcripts between the *cat_I* and *cat_{II}* clusters. To test this idea, the promoter activities of *cat_I* and *cat_{II}* (transcriptionally fused to the *lacZ* gene in pDN19lacΩ) were examined using the classical reporter β-galactosidase (Table 3).

As expected, in strain NP2 (ND6 wild type containing the *cat_{II}* promoter-*lacZ* fusion), the β-galactosidase activity was low in both glucose and naphthalene media. By contrast, the β-galactosidase activity of strain NP1 (ND6 wild type containing the *cat_I* promoter-*lacZ* fusion) was very high, indicating that the *cat_I* promoter is much stronger than the *cat_{II}* promoter. The activity of the *cat_{II}* promoter in cells grown on glucose or naphthalene showed little variation, probably because the transcript abundance was too low. The β-galactosidase activity of NP1 cells grown on glucose was approximately 75.9% of the activity of cells grown on naphthalene, indicating that naphthalene or its metabolites can slightly induce the *cat_I* promoter but are not necessary for its activity.

To confirm the function of CatR_I, we introduced the plasmid pDB_I (pDN19lacΩ + *cat_I* promoter) and plasmid pDB_{II} (pDN19lacΩ + *cat_{II}* promoter) into ND6-Δ*catR_I* and ND6-Δ*catR_{II}*, respectively, resulting in the strains ND1P1 and ND1P2. A drastic reduction of β-galactosidase activity was observed in ND1P1, whereby the promoter activity of *cat_I* was almost equal to that of the control strain NC (ND6 containing pDN19lacΩ) in glucose-grown cells, but the *cat_I* promoter could be still induced slightly in naphthalene-grown cells. This result suggested CatR_{II} might function as a backup regulator. Interestingly, the promoter activity of *cat_{II}* was also decreased in the CatR_I mutant (ND1P2) compared with NP2, suggesting that CatR_I might competitively bind the promoter region of *cat_{II}* and initiate transcription more effectively in the wild-type ND6. On the other hand, we found that the β-galactosidase activity of ND2P1 (ND6-Δ*catR_{II}* containing pDN19lacΩ + *cat_I* promoter) was obviously higher than that of NP1, both on glucose and on naphthalene. This result suggested that CatR_I could bind the *cat_{II}* promoter and initiate transcription more efficiently with or without the inducer when CatR_{II} is unavailable. All these results were in agreement with the EMSA results. Through in vitro and in vivo experiments, we have confirmed that two regulatory proteins CatR_I and CatR_{II} have interactive regulation on the *cat_I* and *cat_{II}* gene clusters, and CatR_I has a stronger regulatory and activation effect on the gene clusters than CatR_{II}.

Evolutionary Analysis of the Two Catechol-Degrading Gene Clusters and Their Promoter Regions

To analyze the diversity of the two catechol-degrading gene clusters in the ND6 genome, 24 sequences of different *cat* gene clusters were downloaded from NCBI to construct a phylogenetic tree (Fig. 10). Overall, the phylogenetic analysis showed that the *cat* gene clusters have significant phylogenetic diversity, and could be divided into two main groups depending on the genus. Most *Pseudomonas* spp. have two *cat* clusters, which constituted one group, including *cat_I* and *cat_{II}* of the ND6 strain. In the *Pseudomonas* spp. group, most second copies of the *cat* gene clusters, including *catR_{II}B_{II}C_{II}A_{II}* of *P. putida* ND6, formed a monophyletic clade. By contrast, most *cat_I* gene clusters, including *catR_IB_IC_IA_I* of *P. putida* ND6, formed a separate clade, indicating that *cat_I* and *cat_{II}* of ND6 have different evolutionary origins. Interestingly, in three *Burkholderia* sp. strains (GenBank: CP001052.1, GenBank: AB035483.1 and GenBank: CP026112.1), *catA* was found to be transcribed individually while *catR-catB-catC* was transcribed in the opposite direction, which is different from the common order *catB-catC/catA-catA/catC*. Therefore, the expression of C12O, encoded by *catA*, must be very adaptable to respond to the environment. However, gene clusters with different transcription sequences have a far-reaching relationship. Consistent with a previous study[38], analysis of an alignment of nine intergenic regions of *catB* downloaded from NCBI confirmed the presence of two types of binding motifs, with conserved sequences T-N₁₁-A and mutant sequences G-N₁₁-A (Fig. 11). In agreement with a report by Parsek *et al.* [37], the mutation of a T to a G resulted in an increase in the binding of CatR_I to both the *catB_I* and *catB_{II}* promoters (Fig. 9).

Table 3. Determination of relative promoter activities via β-galactosidase expression (measured in Miller units).

Strains	NC	NP1	NP2	ND1P1	ND1P2	ND2P1	ND2P2
MMB + glucose	8.37	151.30	15.89	8.09	9.45	196.37	8.19
MMB + naphthalene	8.30	199.31	11.52	11.87	9.94	297.38	11.54

*The β-galactosidase activity was determined in the following strains: NC (ND6 containing pDN19lacΩ); NP1 (ND6 containing pDN19lacΩ + *cat_I* promoter); NP2 (ND6 containing pDN19lacΩ + *cat_{II}* promoter); ND1P1 (ND6-Δ*catR_I* containing pDN19lacΩ + *cat_I* promoter); ND1P2 (ND6-Δ*catR_{II}* containing pDN19lacΩ + *cat_{II}* promoter); ND2P1 (ND6-Δ*catR_I* containing pDN19lacΩ + *cat_I* promoter); ND2P2 (ND6-Δ*catR_{II}* containing pDN19lacΩ + *cat_{II}* promoter).

Fig. 10. Phylogenetic analysis of *cat* gene clusters. The phylogenetic tree was generated using MEGA 5.0 with maximum parsimony and 1000 bootstrap replicates. Reference DNA sequences were selected by BLAST searches in the NCBI database.

Fig. 11. LOGO diagrams of the binding sites occupied by CatR. The sequence was derived from the *catR-B* intergenic region of the reported *catRBCA* gene clusters, including *P. putida* PRS2000 [13], *P. putida* RB1 [11], *Pseudomonas resinovorans* strain CA10 [40], *Acinetobacter lwoffii* K24 [41], *Fratureia* sp. ANA-18 [19, 23], and *P. putida* ND6. The LOGO diagrams were built using all the predicted binding sites for the respective CatR in all studied genomes in all *catR-B* intergenic regions. The sequence between the two dashed lines is an inverted repeat (T-N₁₁-A) or an incomplete inverted repeat (G-N₁₁-A). The T/G and A residues of the T/G-N₁₁-A motif were marked in red and green, respectively.

Discussion

A number of aerobic biodegradation pathways of aromatic compounds like phenol, benzoate, or naphthalene converge into catechol ring cleavage [42]. Our previous study showed that each C12O isozyme in *P. putida* ND6, encoded by the separate *catA* genes on the chromosome and the large pND6-1 plasmid, belonged to independent branches of the phylogenetic tree and have different enzymatic properties [17]. The two *catA* genes located on the chromosome (Fig. 1) were found to significantly contribute to the fitness of the host strain that is adapted to high concentrations of naphthalene [27]. The phenotypic determination showed that the growth curve of *P. putida* ND6- Δ *catAnahH* decreased slightly compared to the wild type, indicating that the two catechol *ortho*-cleavage clusters in the genome play an important role but are not the only genes with this function. In nature, many bacteria grow and develop by catechol *ortho*- and *meta*-cleavage pathways to adapt to the presence of aromatics in the environment [3, 43]. The results of RT-qPCR and the catechol dioxygenase enzyme activity assay supported this idea. In addition to *P. putida* ND6, many other strains, especially of *Pseudomonas* and *Burkholderia* spp., possess multiple *cat* gene clusters. In *Burkholderia* sp. strain TH2, although *catA*₂ is not indispensable for the

growth on 2CB, the presence of the *cat*₂ gene cluster is beneficial [44]. Similar observations were made in *Acinetobacter lwoffii* K24, and *Frateriura* species ANA-18 [19]. Thus, the presence of multiple *cat* genes or clusters in the same cell is a widespread phenomenon [14] that may help the host adapt to changing environments by either reducing the intracellular concentration of catechol, which is a toxic intermediate of the degradation of various aromatic compounds, or by substituting for each other to circumvent harmful mutations [3].

It is interesting to note the different arrangement of *cat* genes in Fig. 10. Most of them are arranged in the order *catB-catC/catA-catA/catC*, while the *catR* gene is divergently transcribed from the operon. Furthermore, the *catA* gene is transcribed separately from *catR-catB-catC* as arranged in *Paraburkholderia terrae* strain DSM 17804, *Burkholderia* sp. strain TH2 and *Paraburkholderia phytofirmans* strain PsJN. At the same time, we found that *catA* is under independent transcriptional regulation in the ND6 strain, while the *catBCA* operon is co-transcribed. Consequently, we assumed that individual transcription of the *catA* gene might be important and not an accidental evolutionary event. Notably, *catA* encodes the rate-limited enzyme C12O in the catechol *ortho*-cleavage pathway, so the abundance of C12O should be regulated sensitively and quickly to adapt to the concentration of the substrate. Therefore, the independent transcription of *catA* might represent a positively selected genetic adaptation [3].

The intricate regulatory interplay of multiple *cat* gene clusters suggests an intimate mutual adaptation [45]. For instance, CatR is able to activate the *clcABD* promoter but ClcR cannot activate the *catBCA* promoter in *P. putida* PRS2000 [46]. As a result, transcription from the *clc* promoter is repressed by the TCA-cycle intermediates succinate, citrate and fumarate, while the presence of these organic acids does not affect the transcription from the *cat* promoter. This difference provides some flexibility to respond to different environmental signals in addition to the presence of the inducer [47]. Similarly, the question of how the two *catBCA* gene clusters are regulated in *P. putida* ND6 was investigated. We found that the core transcriptional activation mechanisms of the two *ortho*-cleavage operons are conserved. First, both can be induced by CCM. Second, CatR_I and CatR_{II} are the native regulatory proteins of the *cat_I* cluster and *cat_{II}* cluster, respectively. Third, in the absence of CCM, CatR_I and CatR_{II} bind to the RBS, which contains a T/G-N₁₁-A motif, presumably allowing CatR to negatively regulate its own expression, while in the presence of CCM, CatR_I and CatR_{II} occupy an adjacent downstream site, designated as the ABS. Based on these conserved characters, CatR_I and CatR_{II} share functional similarities which allow them to complement each other's mutants at the transcriptional level. Different from CatR and ClcR of *P. putida*, CatR_I and CatR_{II} can activate the promoters of the other, which provides another level of flexibility to respond to harmful mutations of each regulator. Additionally, EMSA demonstrated that CatR_I binds to both the *cat_I* and *cat_{II}* promoters with high affinity, while CatR_{II} binds weakly. These observations were confirmed by *lacZ* transcriptional-fusion expression experiments, which indicated that CatR_I might competitively bind the promoter region of *cat_{II}* and initiate transcription more effectively (Table 3). A mutation in the binding motif from T-N₁₁-A (*cat_{II}*) to G-N₁₁-A (*cat_I*) may explain the difference of binding characteristics. In conclusion, the two *cat* gene clusters in *P. putida* ND6 can cross-regulate each other as a result of similar evolutionary origins, but they diverged due to the accumulation of mutations that appear to be evolutionarily advantageous.

In this study, we found that the *catC_I/catC_{II}* and *catA_I/catA_{II}* genes are transcribed independently, in addition to the co-transcription of the *catBCA* operon. We are conducting further experiments to demonstrate why and how the regulation of *cat* genes in *P. putida* ND6 is so complex.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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