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

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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, catA, and catB, catA) on the chromosome play an important role. The cat, and cat, operons are co-transcribed, whereas catA, and catA, 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, and CatR, occupy an additional downstream site, designated as the activation binding site. Notably, CatR binds to both the cat, and cat, promoters with high affinity, while CatR, binds weakly. This is likely caused by a T to G mutation in the G/T-N 11- A motif. Specifically, we found that CatR, and CatR, regulate catB, catA, and catB, catA, in a cooperative manner, which provides new insights into naphthalene degradation.

Keywords: Pseudomonas putida ND6, catechol ortho-cleavage pathway, CatR, and CatR, catB, catA, and catB, catA, operons, cross-regulation, evolution of catabolic pathways

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 1 (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, catA, and catA, 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 (cat, and cat). Interestingly, the inducer of catA, was found to be BA, and not 2CB. It was also found that CCM or its metabolite acts as an inducer for catA. These results suggest that although cat, genes are not indispensable for growth of TH2 on 2CB, they are advantageous [20]. Murakami et al. reported that the aniline-assimilating bacterium Fraterulia sp. ANA-18 has two cat clusters, which are respectively located on the
Table 1. Strains and plasmids used in this study.

| Strains/plasmids | Genotype and description | Source/ reference |
|------------------|--------------------------|-------------------|
| E. coli          | JM109 Cloning strain     | Novagen           |
|                  | S17-1 recA pro hsdR RP4-2-Tc::Mu-Km::Tn7; donor strain for conjugation; | [28]              |
|                  | BL21 Expression strain   |                   |
| P. putida        | ND6 Ch'                   | [16]              |
|                  | ND6-ΔcatRI              | This study        |
|                  | ND6-ΔcatRII             | This study        |
|                  | ND6-ΔcatA               | This study        |
|                  | ND6-ΔcatAnahH           | This study        |
|                  | NC ND6 containing plasmid pDN19lacΩ; Ch', Sp', Sm' | This study        |
|                  | NC1 ND6 containing plasmid pDB1; Ch', Sp', Sm' | This study        |
|                  | ND1P1 ND6-ΔcatRI containing plasmid pDB2; Ch', Kan', Sp', Sm' | This study        |
|                  | ND1P2 ND6-ΔcatRII containing plasmid pDB2; Ch', Kan', Sp', Sm' | This study        |
|                  | ND2P1 ND6-ΔcatRI containing plasmid pDB1; Ch', Kan', Sp', Sm' | This study        |
|                  | ND2P2 ND6-ΔcatRII containing plasmid pDB2; Ch', Kan', Sp', Sm' | This study        |
| Plasmid          | pUC18-T simple Cloning vector; Amp' | TransGen          |
|                  | pEX18Tc Gene replacement; Tet', oriT' sacB' | [29]              |
|                  | pDN19lacΩ Broad host range shuttle vector; Sp', Sm' | [30]              |
|                  | pEASY-Blunt Source of kan gene; Amp', Kan' | TransGen          |
|                  | pXM191 Source of cat gene; Amp', Cm' | TransGen          |
|                  | pEX18Tc-R-Km pEX18Tc with ΔcatRI; Kan'; Tet' | This study        |
|                  | pEX18Tc-R-Km pEX18Tc with ΔcatRIII; Kan'; Tet' | This study        |
|                  | pEX18Tc-A-Km pEX18Tc with ΔcatRII; Kan'; Tet' | This study        |
|                  | pEX18Tc-H-Cm pEX18Tc with ΔnahH::Cm'; Tet' | This study        |
|                  | pEXS-catRI pEXS-catRI protein expression vector; Kan' | This study        |
|                  | pEXS-catRII pEXS-catRII protein expression vector; Kan' | This study        |
|                  | pUC19c-catB pUC19c with catB promoter; Amp' | This study        |
|                  | pUC19c-catB2 pUC19c with catB2 promoter; Amp' | This study        |
|                  | pDB1 cat promoter inserted between the EcoRl and BamHI sites of pDN19lacΩ; Sp', Sm' | This study        |
|                  | pDB2 cat promoter inserted between the EcoRl and BamHI sites of pDN19lacΩ; Sp', Sm' | This study        |

*Cbr, carbenicillin resistant; Kan', kanamycin resistant; Cm', Chloramphenicol resistant; Sp', spectinomycin resistant; Sm', streptomycin resistant; Amp', ampicillin resistant; Tet', tetracycline resistant; Gm', 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 |
|-------------|------------------|-------------|
| catRI-F     | GTGATATGTCGCGAGATGCCTC | Used in the creation of ND6-ΔcatRI mutant |
| catRI-R     | GCCAGACATTGTCGCTGC | Used in the creation of ND6-ΔcatRI mutant |
| CatRI-R     | GCTTTCGATGCCGGACTTG | Used in the creation of ND6-ΔcatR II mutant |
| CatRII-R    | AGCAAGCGAAGAATGACCAAGG | Used in the creation of ND6-ΔcatR II mutant |
| CatRII-F    | AGGCGAGGAGTATCGCTG | Used in the creation of ND6-ΔcatR II mutant |
| AL-F        | GGGCCGCTTGATCAACGTCGT | Used in the creation of ND6-Δmutant |
| AL-R        | ATCTCAGGCAGGTTGGAAATAG | Used in the creation of ND6-Δmutant |
| AR-F        | CAGTCGCGATACAACCTGCACG | Used in the creation of ND6-Δmutant |
| AR-R        | CCGAGGGCGGAGAACCTGCTGAG | Used in the creation of ND6-Δmutant |
| HL-F        | GCCGCCGCTTGACGTCG | Used in the creation of ND6-Δmutant |
| HL-R        | TTATTTCTTTCGACCCGTCCGG | Used in the creation of ND6-Δmutant |
| HR-F        | CAATGGCAGATACCCGAGAG | Used in the creation of ND6-Δmutant |
| HR-R        | GCTTGTTTGAGCAAGGTGTC | Used in the creation of ND6-Δmutant |
| kan-F       | GGGCGGTTTTATGGACAGC | Used in the creation of ND6-Δmutant |
| kan-R       | GCGGCAGTTTTATGGACACAG | Used in the creation of ND6-Δmutant |
| cat-R       | TGATCGGCACGTAAGAGGTTC | Used in the creation of ND6-Δmutant |
| M13F-11     | CGCCAGGGTTTTCCCAGTCACGAC | TaqMan primer used for footprinting template |

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Construction of *P. putida* ND6 Mutants

The suicide vector pEX18Tc was used for gene replacement in *Pseudomonas*. The catR\(_2\) replacement vector pEX18Tc-R\(_2\)Km was constructed by inserting the Kan\(^{\text{r}}\) cassette into the catR\(_2\) gene in the pEX18Tc vector to generate a partial deletion from 226 to 440 bp. Similarly, the catR\(_3\) replacement vector pEX18Tc-R\(_3\)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\(_2\)-Km, pEX18Tc-R\(_3\)-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\(_2\), catR\(_3\), and catA mutants. The resulting mutant strains were named *P. putida* ND6-\(\Delta\text{catR}\_2\), ND6-\(\Delta\text{catR}\_3\), and ND6-\(\Delta\text{catA}\), respectively.

The nahH replacement vector pEX18Tc-HCm was constructed by inserting the Cm\(^{\text{r}}\) 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-\(\Delta\text{catAnah}\)H was obtained.

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

The wild-type ND6, as well as the mutants ND6-\(\Delta\text{catR}\_2\), ND6-\(\Delta\text{catR}\_3\), and ND6-\(\Delta\text{catAnah}\)H, 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 mutants ND6-\(\Delta\text{catR}\_2\), ND6-\(\Delta\text{catR}\_3\), and ND6-\(\Delta\text{catAnah}\)H, were individually 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 RNXpreP Pure Bacteria Kit (Tiangen Biotech, China) and reverse-transcribed into cDNA using the FastKing RT Kit (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 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 transcript copy number. 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\(_2\) and CatR\(_3\)

*E. coli* BL21 carrying the CatR\(_2\) and CatR\(_3\) expression plasmids pEX5-CatR\(_2\) and pEX5-CatR\(_3\) 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\(_2\) and CatR\(_3\), cell pellets obtained from 1 liter cultures were collected at 13,000 ×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, and pUC19c-catB\(_2\) 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 μl of 50 mM Tris-HCl [pH 8.0], 100 mM KCl, 2.5 mM MgCl\(_2\), 0.2 mM diithiothreitol (DTT), with 2 μg 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×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 μl 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 catBI-lacZ and catBII-lacZ Fusions and β-Galactosidase Activity Assays**

A 309 bp DNA fragment corresponding to the catBI-initiation codon (from -347 to -39 relative to the catBI initiation codon) and a 330 bp DNA fragment corresponding to the catBII-initiation codon (from -358 to -29 relative to the catBII initiation codon) were amplified by PCR to construct catB promoter-lacZ fusion and catII promoter-lacZ fusion, respectively. Two amplified fragments of catI and catII promoter were digested with EcoRI and BamHI, respectively, and inserted into pDN19lacΩ that had been previously cut with the same enzymes. The resulting plasmids, pDBI and pDBII, were transformed into E. coli S17-1 by chemical transformation. The complementation was performed through the conjugation between E. coli S17-1 with plasmid pDBI and pDBII and P. putida ND6, P. putida ND6-ΔcatRI, and ND6-ΔcatRII. 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 (catRIBICIAI and catRIIBIICIIAII) (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-ΔcatAnahH 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 catI and catII clusters in catechol metabolism, we disrupted the catRI and catRII regulatory genes and examined the properties of the resulting strains. Interestingly, inactivation of catRI or catRII 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 catBICIAI and catBIICIIAII operons. The results indicated that either the catBICIAI or catBIICIIAII cluster can be co-transcribed
However, the results of RT-qPCR revealed that the transcription of the downstream genes \( \text{catC}_1 \) and \( \text{catA}_1 \) was higher than that of \( \text{catB}_1 \) (Fig. 5A), suggesting that \( \text{catC}_1 \) and \( \text{catA}_1 \) were independently transcribed, while the \( \text{catB}_1 \text{C}_1 \text{A}_1 \) cluster was co-transcribed. A similar phenomenon was also observed in the cluster \( \text{catB}_2 \text{C}_2 \text{A}_2 \) (Fig. 5A).

In the naphthalene medium, the activity of transcription (Fig. 5A) and expression levels (Fig. 2, small figure) of three \( \text{catA} \) genes and \( \text{C12O} \) were much higher than \( \text{nahH} \) gene and \( \text{C23O} \). Therefore, the naphthalene metabolism of \( \text{P. putida} \) 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 (\( \text{catA}_1 \), \( \text{catB}_1 \text{C}_1 \text{A}_1 \), \( \text{catA}_2 \), \( \text{catB}_2 \text{C}_2 \text{A}_2 \)) were all increased in naphthalene medium, while that of meta-cleavage pathways (\( \text{nahH} \)) 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 \( \text{catI} \) and \( \text{catII} \) in the mutant strains ND6-\( \Delta \text{catRI} \) and ND6-\( \Delta \text{catRII} \) following cultivation in the presence of glucose or naphthalene. For the ND6-\( \Delta \text{catRI} \) strain (only \( \text{catRI} \) is functional), the transcriptional levels of \( \text{catB}_1 \) were significantly decreased, while the \( \text{catB}_2 \) gene cluster could still be induced (Fig. 5B). For the ND6-\( \Delta \text{catRII} \) strain (only \( \text{catRII} \) is functional), the transcriptional levels of \( \text{catB}_2 \) were also significantly decreased, while the \( \text{catB}_1 \) gene cluster could still be induced (Fig. 5B).
strain (only catRI is functional), the transcriptional level of catBII was also decreased while the catBI gene cluster could still be induced (Fig. 5C). This result demonstrated that CatRI and CatRII are the native regulatory proteins of the catI cluster and the catII cluster, respectively.

To investigate their possible regulatory roles, the impacts of catRI and catRII 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 catRI or catRII was knocked out, the transcription of the catI or catII cluster could still be detected (Fig. 5D). It indicates that CatRI and CatRII might

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; Lane1: Band of the catBI-catCI intergenic region DNA fragment; Lane 2: Band of the catCI-catAI intergenic region DNA fragment; Lane 3: Band of the catBI-catAI intergenic region DNA fragment; Lane 4: Band of the catBII-catCII intergenic region DNA fragment; Lane 5: Band of the catCII-catAII intergenic region DNA fragment; Lane 6: Band of the catBII-catAII intergenic region DNA fragment; Lane 7: negative control.

Fig. 5. Copy number determination by absolute RT-qPCT using plasmid standards. Transcript copy number of genes per 1000 mRNA transcript copies of the 16S rRNA in ND6 (A), ND6-ΔcatRI (B), and ND6-ΔcatRII (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-ΔcatRI, and ND6-ΔcatRII. 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.
cross-regulate the transcription of each other's target genes, and explains that the naphthalene degradation curves of ND6-ΔcatRI and ND6-ΔcatRII 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 catI is located at a G nucleotide (Fig. 6). Surprisingly, the primer extension assay of catII failed three times based on reliable methods, probably because the transcript abundance of the catII cluster is particularly low.

The CatRI and CatRII binding regions were also studied using DNase I footprinting (Fig. 7). 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 catI was consistent with the primer-extension experiment, and the putative TSS of catII was located 67 bp upstream of the catBII initiation codon. Other motifs of the catI and catII 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).
incubated with a 408 bp fragment labeled with FAM, with a sequence corresponding to the promoter region of catI, and the resulting complexes were treated with DNase I, followed by DNA fragment analysis by capillary electrophoresis. The binding sequence of the CatRI–catBII complex was assessed using the same method, and a 403 bp FAM-labeled DNA fragment was incubated with purified CatRII. In the absence of CCM, CatRI protected a continuous 26 bp region that had also been determined to be located from -137 to -112 relative to the catBII initiation codon (Fig. 7A). CatRII protected a continuous 25 bp region that had also been determined to be located from -137 to -113 relative to the catBII initiation codon (Fig. 7C). In the presence of CCM, CatRI protected a continuous 48 bp region that was determined to be located from -137 to -90 relative to the catBII initiation codon (Fig. 7B). CatRII protected a continuous 41 bp region that was determined to be located from -137 to -97 relative to the catBII 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 CatRI and CatRII 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 CatRI and CatRII 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 CatRI and CatRII to the upstream regulatory regions of catI and catII were studied.
by EMSA using a concentration gradient of CCM (Fig. 9). As expected, the addition of 60 ng CatR to 40 ng of the labeled probe caused a strong shift in the mobility of the cat promoter fragment (Fig. 9A). Notably, CatR could also strongly bind to the catI promoter region (Fig. 9C). By contrast, CatR was able to specifically bind the catI and catII promoter regions, but the binding stability of the CatR-catI and CatR-catII complexes was obviously reduced (Figs. 9B and 9D). These results support the hypothesis that CatR and CatR might cross-regulate the transcription of each other’s target genes. In addition, CCM did not affect the binding of CatR 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 recognizes and binds to the catI and catII 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 catI and catII clusters. To test this idea, the promoter activities of catI and catII (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 catI 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 catII promoter-lacZ fusion) was very high, indicating that the catII promoter is much stronger than the catI promoter. The activity of the catI 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 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 catI promoter but are not necessary for its activity.

To confirm the function of CatR, we introduced the plasmid pDBI (pDN19lacΩ + catI promoter) and plasmid pDBII (pDN19lacII + catII promoter) into ND6-ΔcatR and ND6-ΔcatRII respectively, resulting in the strains ND1P1 and ND1P2. A drastic reduction of β-galactosidase activity was observed in ND1P1, whereby the promoter activity of catI was almost equal to that of the control strain NC (ND6 containing pDN19lacΩ) in glucose-grown cells, but the catI promoter could be still induced slightly in naphthalene-grown cells. This result suggested CatR might function as a backup regulator. Interestingly, the promoter activity of catI was also decreased in the CatR VII mutant (ND1P2) compared with NP2, suggesting that CatR might competitively bind the promoter region of catI and initiate transcription more effectively in the wild-type ND6. On the other hand, we found that the β-galactosidase activity of ND2P1 (ND6-ΔcatRII containing pDN19lacII + catII promoter) was obviously higher than that of NP1, both on glucose and on naphthalene. This result suggested that CatR could bind the catII promoter and initiate transcription more efficiently with or without the inducer when CatR 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 and CatR have interactive regulation on the catI and catII gene clusters, and CatR has a stronger regulatory and activation effect on the gene clusters than CatR.

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 catI and catII of the ND6 strain. In the Pseudomonas spp. group, most second copies of the catI gene clusters, including catRII, CIΩ, AΩ, P. putida ND6, formed a monophyletic clade. By contrast, most catII gene clusters, including catRII, CIΩ, AΩ, P. putida ND6, formed a separate clade, indicating that catI and catII 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-catN/catC. Therefore, the expression of C2O, 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 CatRI to both the catIB and catII 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 + catI promoter); NP2 (ND6 containing pDN19lacΩ + catII promoter); ND1P1 (ND6-ΔcatRII containing pDN19lacII + catII promoter); ND1P2 (ND6-ΔcatRII containing pDN19lacII + catII promoter); ND2P1 (ND6-ΔcatRII containing pDN19lacII + catII promoter); ND2P2 (ND6-ΔcatRII containing pDN19lacII + catII promoter).
Interaction of Two Catechol-Degrading Gene Clusters in Pseudomonas putida ND6

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-ΔcatAnahH 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 catA2 is not indispensable for the

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growth on 2CB, the presence of the cat gene cluster is beneficial [44]. Similar observations were made in Acinetobacter lwofii K24, and Frutaria 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 observed in Fig. 10. Most of them are arranged in the order cat-B-catC-catA-catA-catC, while the catA 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-limit 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, CatRI and CatRII are the native regulatory proteins of the cat, cluster and cat cluster, respectively. Third, in the absence of CCM, CatRI and CatRII 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, CatRI and CatRII occupy an adjacent downstream site, designated as the ABS. Based on these conserved characters, CatRI and CatRII share functional similarities which allow them to complement each other’s mutants at the transcriptional level. Different from CatR and ClcR of P. putida, CatRI and CatRII 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 CatRI binds to both the cat and cat promoters with high affinity, while CatRII binds weakly. These observations were confirmed by lacZ transcriptional-fusion expression experiments, which indicated that CatRII might competitively bind the promoter region of catI and initiate transcription more effectively (Table 3). A mutation in the binding motif from T12-N11-A (catI) to G11-N11-A (cat) may explain the difference in 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/catCI and catA/catAI 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.

References

1. Linger JG, Vardon DR, Guarnieri MT, Karp EM, Hunsinger GB, Franden MA et al. 2014. Lignin valorization through integrated biological funneling and chemical catalysis. Proc. Natl. Acad. Sci. USA 111: 12013-12018.

2. Mallick S. 2019. Biodegradation of acenaphthene by Sphingobacterium sp. strain RTSB involving trans-3-carboxy-2-hydroxybenzylidenepyruvic acid as a metabolite. Chemosphere 219: 748-755.

3. Sethare B, Kumar A, Mokoena MP, Olaniran AO. 2018. Catechol 1,2-Dioxygenase is an analogue of Homogentisate 1,2-Dioxygenase and is involved in benzoate degradation in Burkholderia sp. strain TH2 and Paraburkholderia phytofirmans strain PsJN. This work was supported by the National Natural Science Foundation of China (Grant Nos. 21477163 and 31670512), and the Natural Science Basic Research Plan in Shaanxi Province of China (Grant No. 2018J0309).

4. Wackett LP. 2003. Catechol 1,2-Dioxygenase genes from Pseudomonas chlororaphis P. putida mt-2 with an enzymatic safety valve for excess of catechol. J. Bacteriol. 185: 6161-6170.

5. Harwood CS, Parales RE. 1996. The cat genes of Pseudomonas putida: probing the interaction of CatR and RNA polymerase through in vitro transcription. J. Bacteriol. 178: 2221-2227.
