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Cloning and Characterization of Two *catA* Genes in *Acinetobacter lwoffii* K24

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Two novel type I catechol 1,2-dioxygenases inducible on aniline media were isolated from *Acinetobacter lwoffii* K24. Although the two purified enzymes, CD I_1 and CD I_2, had similar intradiol cleavage activities, they showed different substrate specificities for catechol analogs, physicochemical properties, and amino acid sequences. Two *catA* genes, *catA*$_1$ and *catA*$_2$, encoding by CD I_1 and CD I_2, respectively, were isolated from the *A. lwoffii* K24 genomic library by using colony hybridization and PCR. Two DNA fragments containing the *catA*$_1$ and *catA*$_2$ genes were located on separate regions of the chromosome. They contained open reading frames encoding 33.4- and 30.4-kDa proteins. The amino acid sequences of the two proteins matched well with previously determined sequences. Interestingly, further analysis of the two DNA fragments revealed the locations of the *catB* and *catC* genes as well. Moreover, the DNA fragment containing *catA*$_1$ had a cluster of genes in the order *catB*$_1$-*catC*$_1$-*catA*$_1$ while the *catB*$_2$-*catA*$_2$-*catC*$_2$ arrangement was found in the *catA*$_2$ DNA fragment. These results may provide an explanation of the different substrate specificities and physicochemical properties of CD I_1 and CD I_2.

Catechol 1,2-dioxygenase is the initial enzyme of one branch of the β-ketoadipate pathway. This enzyme cleaves catechol intermediates into *cis,cis*-muconate (19, 29). There are two types of catechol 1,2-dioxygenases, type I catechol 1,2-dioxygenase and type II catechol 1,2-dioxygenase (chlorocatechol 1,2-dioxygenase), which are distinguished by their substrate specificities. Type I catechol 1,2-dioxygenase has little or no cleavage activity against chlorocatechols, whereas type II catechol 1,2-dioxygenase can cleave these compounds and is found mainly in degradative pathways for chlorinated aromatic compounds (13). Catechol 1,2-dioxygenases (types I and II) have been purified, cloned, and characterized intensively in a number of different bacteria (7, 22–24, 27, 31). The organization and regulation mechanisms of *cat* genes (type I catechol 1,2-dioxygenase) have been reported in *Acinetobacter calcoaceticus* and *Pseudomonas putida* (1, 2, 15, 26, 30, 34). In addition, several isozymes of catechol 1,2-dioxygenases were found in *Pseudomonas arvilla* C-1, *Frateuria* sp. strain ANA-18, *Pseudomonas acidovorans* CA28, and *Pseudomonas* sp. strain B13 (4, 8, 14, 23). *P. acidovorans* CA28 (14) and *Pseudomonas* sp. strain B13 (8) were found to have both types of catechol 1,2-dioxygenases, which were separately induced when introduced into chloroaromatic compound and nonchloroaromatic compound media, indicating their involvement in different regulation and metabolism processes. But *P. arvilla* C-1 (23) and *Frateuria* sp. strain ANA-18 (4) induced three and two isozymes of type I catechol 1,2-dioxygenases in media containing nonchloroaromatic compounds such as benzoate and aniline, respectively. It has been proposed that two isozymes of *Frateuria* sp. strain ANA-18 should be complementary to each other in the cell because of their different chemical and catalytic characteristics (4). However, the regulation mechanisms and physiological significances of several isozymes of type I catechol 1,2-dioxygenases are not clearly understood. In this report, we describe the purification, characterization, sequential analysis and cloning of two novel type I catechol 1,2-dioxygenases, CD I_1 and CD I_2 from *Acinetobacter lwoffii* K24, which induced two enzymes on aniline media. We found that the 33.4- and 30.4-kDa polypeptides of catechol 1,2-dioxygenases are expressed from distinct genes. The two purified enzymes exhibit different substrate specificities for catechol analogs, physicochemical properties, and amino acid sequences in the partially determined N-terminal regions. In addition, we identified the two ORFs (open reading frames) of *catB* (encoding muconate-lactonizing enzyme) and *catC* (encoding muconolactone isomerase) in the vicinity of each of the *catA* genes. The *catB* and *catC* gene products are involved in the second and third steps of the β-ketoadipate pathway (29). These genes were arranged separately and in different patterns on the chromosome. To our knowledge, this is the first report on the finding of two *cat* gene clusters in one organism which are differently arranged in the order *catB*$_1$-*catC*$_1$-*catA*$_1$ and the order *catB*$_2$-*catA*$_2$-*catC*$_2$.

Purification and characterization of the two catechol 1,2-dioxygenases. *A. lwoffii* K24, a wild-type strain capable of growing on a medium containing aniline as a sole carbon and nitrogen source, was screened from a field in the Kyunggi district of Korea. *A. lwoffii* K24 was cultured on an aniline-containing culture medium at 27°C with continuous shaking (16). The culture medium included 6.5 mM potassium phosphate buffer (pH 6.25) containing 3.4 mM $MgSO_4$, 0.3 mM $FeSO_4$, 0.2 mM $CaCO_3$, and 1.022 g of aniline per liter. The cultures were grown to an optical density of 1.0 at 600 nm, harvested and stored at −20°C until use. When *A. lwoffii* K24 was grown on aniline-containing medium, two catechol 1,2-dioxygenases were detected by DEAE chromatography of the cell extract (Fig. 1A). *A. lwoffii* K24 cultured in 10 mM succinate medium (27) had no detectable catechol 1,2-dioxygenase activity by spectroscopic methods (3), but two catechol 1,2-dioxygenase activities appeared when *A. lwoffii* K24 was transferred from succinate medium to aniline-containing medium. Therefore, it is suggested that the two enzymes are induced by aniline. The purification of two enzymes is summarized in

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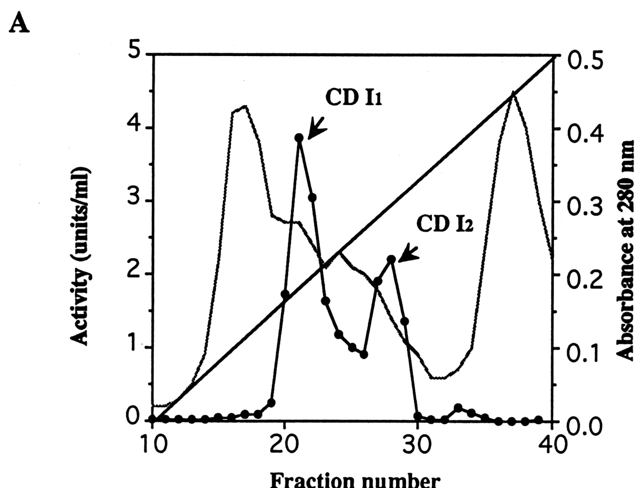

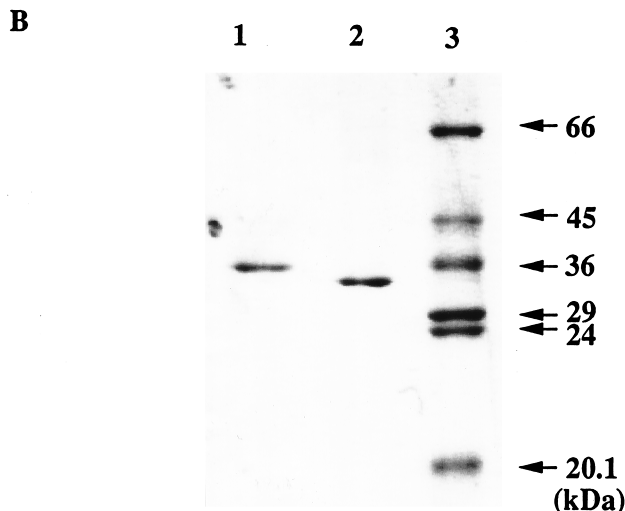

FIG. 1. Purification of two catechol 1,2-dioxygenases, CD I_1 and CD I_2, from *A. lwoffii* K24. (A) Wet cells (18 g) were suspended in 50 ml of buffer A (50 mM Tris-HCl, pH 8.0) and disrupted with a French pressure cell press (SLM AMINCO, Urbana, Ill.) at 20,000 lb/in^2. The supernatant was subjected to 30 to 55% ammonium sulfate precipitation and dialyzed against buffer A. The enzyme solution was applied to a DEAE-Sepharose column (1.5 by 15 cm; Pharmacia, Uppsala, Sweden) and eluted with a 0 to 0.5 M NaCl gradient at a flow rate of 2 ml/min for 200 min. Ten-milliliter fractions were collected, and the enzyme activity (closed circles) and A_{280} (broken line) of each fraction were determined. The enzyme activity was determined by the method of Aoki et al. (3). Protein concentration was determined by the method of Bradford (6). Two active peaks were eluted from a DEAE-Sepharose column at 0.26 and 0.3 M NaCl, and the two separated enzymes were named CD I_1 and CD I_2, respectively. (B) SDS–10% PAGE. Lanes: 1, CD I_1; 2, CD I_2; 3, molecular size marker. The molecular masses of the denatured enzymes were determined by the method of Laemmli (20), by using a Mini-Protean II cell (Bio-Rad, Hercules, Calif.).

Table 1. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, purified CD I_1 and CD I_2 gave single protein bands of approximately 36 and 34 kDa, respectively (Fig. 1B). The molecular masses of the native proteins were determined by use of a Superose 12 fast protein liquid chromatography column (1.0 by 30 cm; Pharmacia). The molecular masses of purified CD I_1 and CD I_2 were both determined to be approximately 62 kDa. These results suggest that CD I_1 and CD I_2 consist of two identical subunits. Both CD I_1 and CD I_2 were able to cleave 4-methyl-, 4-chloro-, and 3-methoxycatechol at lower rates than catechol, but neither enzyme had activity with 4-nitrocatechol or protocatechuic acid. With 3-methylcatechol as the substrate, the intradiol cleavage activity of CD I_2 was about eight times that of CD I_1 (Table 2). Some catechol 1,2-dioxygenases were reported to catalyze both intradiol and extradiol cleavages of some 3-substituted catechols (12). For the extradiol cleavage activity with 3-methylcatechol, CD I_1 had 49% and CD I_2 showed only 4.3% of the intradiol cleavage activity (Table 2). Isoelectric focusing was done with a Phast system (Pharmacia) by using Phast Gel IEF 3-9. An isoelectric focusing calibration kit (Pharmacia) was used for standard markers. An inhibitory test was performed by the methods of Aoki et al. (3). The isoelectric points of CD I_1 and CD I_2 were 5.2 and 4.7, and the K_m values were 4.2 and 5.2 μM, respectively. $AgNO_3$ showed differential effects on the enzyme activities of CD I_1 and CD I_2. At 0.1 mM, $AgNO_3$ almost completely inhibited CD I_1 but had no effect on CD I_2. The two enzymes showed maximal enzyme activities with catechol as the substrate at pH 8.0 and 40°C. CD I_1 and CD I_2 were very stable in buffer A at 4°C for more than 4 weeks, but at room temperature CD I_1 was much more stable than CD I_2 (data not shown). The NH_2-terminal sequences of the purified enzymes were determined by direct sequencing with an automatic sequencer (476A; Applied Biosystems, Foster City, Calif.). The NH_2-terminal sequence of CD I_1 was determined to be SIKVFGTKEVQDLLKAATNLEGKGGNARSKQIVHR. The NH_2-terminal sequence of CD I_2 was MNKQAIDA

TABLE 1. Purification of catechol 1,2-dioxygenases from *A. lwoffii* K24

Purification step	Total protein (mg) CD I_1/CD I_2	Total activity (U^a) CD I_1/CD I_2	Sp ac (U/mg) CD I_1/CD I_2	Recovery (%) CD I_1/CD I_2
Cell extract	540[b]	248	0.46	100
Ammonium sulfate	342	155	0.45	62.5
DEAE-Sepharose	58.1/23.9	102.7/54.8	1.77/2.29	41.4/22.1
Phenyl Sepharose	10.7/3.02	66.0/30.0	6.17/9.99	26.6/12.1
MonoQ	4.4/1.38	63/23.9	14.3/17.3	25.4/9.6

[a] One unit of enzyme activity was defined as the amount which produced 1 μmol of *cis,cis*-muconic acid per min at 24°C.
[b] Single values represent a mixture of CD I_1 and CD I_2.

TABLE 2. Substrate specificities and different extradiol cleavage activities of catechol 1,2-dioxygenases[a]

Substrate	Relative activity (%)		Intradiol/Extradiol cleavage	
	CD I_1	CD I_2	CD I_1	CD I_2
Catechol	100	100	100/0	100/0
3-Methylcatechol	2.8	21.9	100/49	100/4.3
4-Methylcatechol	39.1	51.4		
4-Chlorocatechol	5.5	7.8		
3-Methoxycatechol	9.9	15.3		
4-Nitrocatechol	0	0		
Protocatechuic acid	0	0		

[a] Intradiol cleavage activities of catechol analogs and protocatechuic acid were measured spectrophotometrically with UV-visible light spectrophotometer (DU 7500; Beckman Palo Alto, Calif.) by the methods of Broderick and O'Halloran (7). Extradiol cleavage activities of catechol and 3-methyl catechol were determined by the methods of Aoki et al. and Nozaki (3, 28). All substrates were used at a concentration of 0.33 mM.

LLQKINDSAINEGNPRTKQIVN. Interestingly, there was no sequence homology in the 24 NH_2-terminal residues of CD I_1 and CD I_2, except for three amino acid residues. To obtain further sequence information, the proteins were digested with trypsin, endopeptidase Glu-C, and Lys-C (Boehringer GmbH, Mannheim, Germany) after reduction and carboxamidomethylation (21). Peptide fragments resulting from the protease digestion were purified by a proRPC column (5 by 200 mm) with a linear acetonitrile gradient of 10 to 60% and then used for sequence analysis (data not shown). Peptide peaks were numbered according to their order of elution from the proRPC column. Peptides derived by trypsin, endoproteinase Lys-C, and Glu-C digestion were designated T, L, and G, respectively. Together with the NH_2-terminal sequences of two enzymes, peptides L-11 (A IDDL<u>DITPDEV</u>WAGVNYLNKLGQDGEAT) and L-6 (G FYS<u>HFDPTGA</u>QSDFNLRG) of CD I_1 and peptides L-11 (QIVNRIVRDLFYTIEDLD<u>VQPDEFW</u>TALNYLGDAG RSGE) and T-7 (LTT<u>QINIDGDP</u>YLXDDFAF) of CD I_2 were used to design degenerate primers for PCR. Degenerate primers were synthesized with a DNA synthesizer (392; ABI, Foster City, Calif.) according to the underlined amino acid sequences above.

Two *catA* genes separated on the chromosome. Degenerate oligomers for CD I_1 and CD I_2 were synthesized as follows: EGKGGNA, primer CD1-1 (20-mer; 5′-GA[AG]GGNAA [AG]GGNGGNAA[CT]GC-3′), DITPDEV, primer CD1-2 (20-mer; 5′-GA[CT]AT[ATC]ACNCCNGA[CT]GA[AG]GT-3′), HFDPTGA, primer CD1-3 (20-mer; 5′-GCNCCNGT NGG[AG]TC[AG]AA[AG]TG-3′; MNKQAIDA, primer CD2-1 (21-mer; 5′GAA[CT]AA[AG]CA[AG]GCNAT[ATC]GA[C T]GC-3′); VQPDEFW, primer CD2-2 (21-mer; 5′-GTNCA [AG]CCNGA[CT]GA[AG]TT[CT]TG G-3′); QINIDGDP, primer CD2-3 (22-mer; 5′-GG[AG]TCNCC[AG]TC[AGT]AT [AG]TT[AGT]AT[CT]TG-3′). Degenerate oligonucleotideswere used as primers for PCR (95°C for 1 min, 55°C for 2 min, 72°C for 3 min, 30 cycles) containing 100 ng of genomic DNA of *A. lwoffii* K24. The sizes of PCR products for CD I_1 and CD I_2 were as expected when run on a 1.0% agarose gel (Fig. 2A). We subcloned 450 and 590 bp from CD I_1 and CD I_2, respectively, into plasmid pCR-Script SK(+) (Stratagene, La Jolla, Calif.) and sequenced them by the dideoxy-chain termination method (33) with a 373A automated DNA sequencer (ABI) by using a PRISM Ready Reaction Dye Terminator Cycle Sequencing Kit (ABI). The resulting nucleotide sequences and the deduced amino acid sequences matched known CD I_1 and CD I_2 sequences precisely. This strongly indicates that both of the *catA* genes are present. Chromosomal DNAs (about 10 μg) were digested with *Bam*HI, *Pst*I, and *Sal*I (New England BioLabs, Beverly, Mass.), run on a 1% agarose gel, and transferred to Zeta-probe blotting membranes. Hybridization with ^{32}P-labeled PCR products was performed at 65°C. We observed different sizes of DNA fragment bands for the CD I_1 and CD I_2 probes in same endonuclease-treated lanes, suggesting that the $catA_1$ and $catA_2$ genes are not compactly clustered but separately located on the chromosome (Fig. 2B). Two *catA* genes ($catA_\alpha$ for the α subunit and $catA_\beta$ for the β subunit) of *P. arvilla* C-1 were also assumed to be located apart (22), while the α and β subunit genes (*pcaH* and *pcaG*) of protocatechuate 3,4-dioxygenase of *P. putida* were compactly clustered and may be under the control of the same cotranscription system using the same promoter site (11). The fact that the two *catA* genes of *A. lwoffii* K24 are not clustered on the chromosome suggests the possibility of divergent regulation of the two *catA* genes.

Cloning, expression, and sequencing of the two *catA* genes. *A. lwoffii* K24 genomic DNA was prepared from saturated cultures in which aniline was the sole source of carbon and

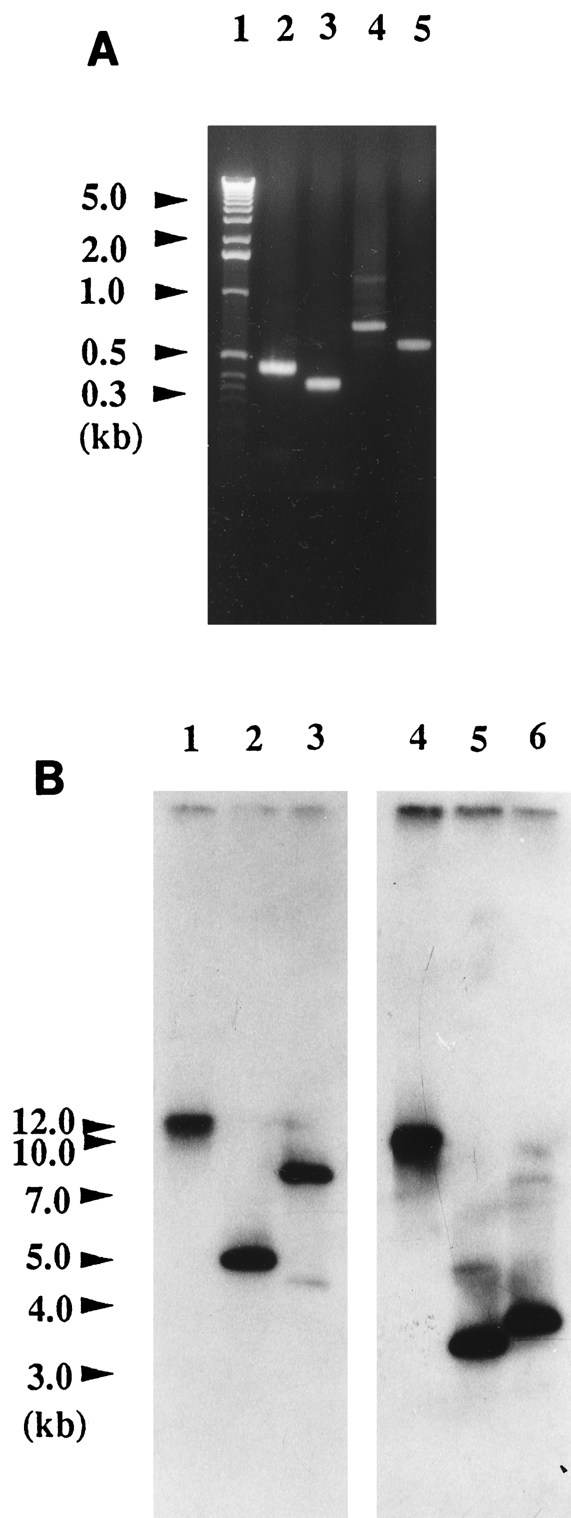

FIG. 2. (A) PCR products of the genes specifying catechol 1,2-dioxygenases CD I_1 and CD I_2. Lanes: 1, molecular size marker; 2, PCR product (450 bp) amplified with degenerated primers CD1-1 and CD1-3; 3, 360-bp PCR product obtained with primers CD1-2 and CD1-3; 4, 590-bp PCR product obtained with primers CD2-2 and CD2-3; 5, 720-bp PCR product obtained with primers CD2-1 and CD2-3. (B) Southern blotting of digested genomic DNA of *A. lwoffii* K24. Lanes 1 to 3 were probed with 450 bp of CD I_1, and lanes 4 to 6 were probed with 590 bp of CD I_2. Restriction enzymes used: lanes 1 and 4, *Bam*HI; lanes 2 and 5, *Sal*I; lanes 3 and 6, *Pst*I.

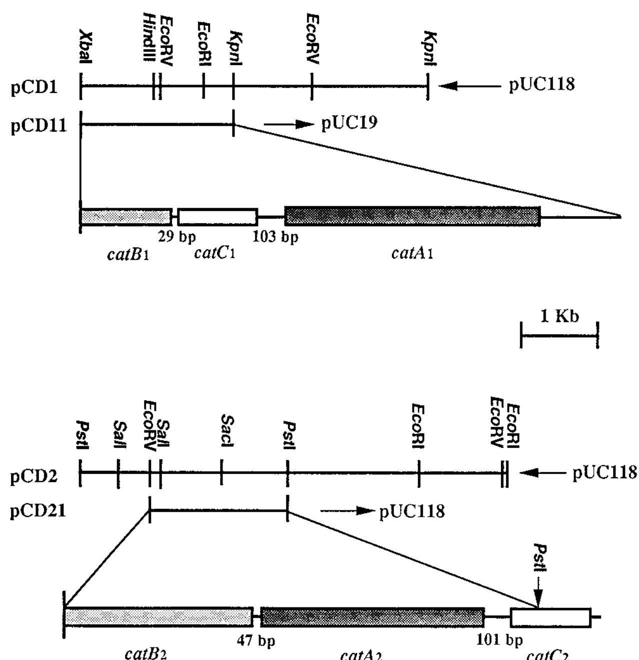

FIG. 3. Restriction maps of the DNA fragments carrying the *cat* genes on plasmids pCD1 and pCD2. Arrows indicate the direction of transcription. Plasmids pCD11 and pCD21 contain the *Xba*I-*Kpn*I fragment of pCD1 and the *Eco*RV-*Pst*I fragment of pCD2 inserted into pUC19 and pUC118, respectively.

nitrogen (5). Construction of the genomic library was constructed in accordance with the procedures of Kaufman et al. (17). Genomic DNA (80 μg) was partially digested with *Sau*3AI. DNA fragments (5 to 10 kb) were fractionated by use of a 10 to 40% sucrose density gradient and ligated into the *Bam*HI site of pUC118. For colony hybridization, about 10^4 transformed cells were transferred to colony/plaque screen hybridization transfer membranes (DuPont) and screened by use of the supplier's protocols. Ten colonies were finally selected after secondary screening of the first screened colonies. By using PCR with primers CD1-2 and -3 and primers CD2-2 and -3, we confirmed that eight colonies had the $catA_1$ gene and two colonies had the $catA_2$ gene, while no colony containing both genes was detected. We selected two plasmids for the $catA_1$ and $catA_2$ genes and designated them pCD1 and pCD2, respectively. pCD1 and pCD2 had 4.5- and 5.8-kb inserts, respectively. Enzyme mapping and PCR were used to localize the *catA* genes (Fig. 3). The 2.0-kb *Xba*I-*Kpn*I fragment in pCD1 was found to contain the $catA_1$ gene. The fragment was subcloned into pUC19 and designated pCD11. *Escherichia coli* DH5α containing pCD11 showed catechol 1,2-dioxygenase activity in LB medium (17) with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The 1.8-kb *Eco*RV-*Pst*I fragment of pCD2 had the $catA_2$ gene. This fragment was also subcloned into pUC118 and designated pCD21. pCD21 could induce catechol 1,2-dioxygenase activity under the same conditions. The specific activities of CD I_1 in pCD11 and CD I_2 in pCD21 were 0.34 and 0.059 U/mg, respectively, which are considerably lower than those of the other *catA* genes expressed. Frazee et al. (11) suggested that the reasons for the lower expression of *P. putida* protocatechuate 3,4-dioxygenase are higher GC content (62%) than *E. coli* (50%) and differently biased codon usage between *P. putida* and *E. coli*. We also found that the GC contents of the *Xba*I-*Kpn*I fragment of pCD1 and the *Eco*RV-*Pst*I fragment of pCD2 were 59 and 62%. The 2.0-kb *Xba*I-*Kpn*I fragment in pCD12 was sequenced (Fig. 4A). The nucleotide sequences revealed an ORF of 311 amino acids, the deduced amino acid sequences of which matched perfectly with previously determined amino acid sequences, except for cysteine. Cysteine could not be identified under our high-pressure liquid chromatography condition because carboxamidomethylated phenylthiohydantoin (PTH)-Cys coeluted with PTH-Glu and was misread as PTH-Glu. Direct comparison of the deduced NH_2-terminal amino acid sequences of $catA_1$ and the NH_2-terminal amino acid sequences of CD I_1 revealed that the NH_2-terminal amino acid, Met, of CD I_1 was posttranslationally removed in the active enzyme in *A. lwoffii* K24. This is consistent with the previous finding on the $catA_\beta$ gene of *P. arvilla* C-1 (22). However, no posttranslational modification was observed with CD I_2. The deduced molecular mass of the $catA_1$ gene product was 33,376 Da. The 1.8-kb *Eco*RV-*Pst*I fragment of pCD21 was also sequenced (Fig. 4B). $catA_2$ had a ORF of 275 amino acids and a deduced molecular mass of 30,397 Da. Comparison of the deduced amino acid sequences of $catA_1$ and $catA_2$ revealed that the two enzymes had approximately 47% identity but that the NH_2-terminal and COOH-terminal regions had no sequences in common (data not shown), which explains their different characteristics. The differential effects of $AgNO_3$ on CD I_1 and CD I_2 could be explained on the basis of the amino acid sequences of the two enzymes. CD I_1 has two cysteines (Cys^{155} and Cys^{202}), whereas CD I_2 has none. $AgNO_3$ is believed to react with a sulfhydryl group (4). A homology search of other type I catechol 1,2-dioxygenases (9, 18, 22, 25) and type II catechol 1,2-dioxygenases (10, 32, 35) suggested that $catA_1$ and $catA_2$ are more similar to type I catechol 1,2-dioxygenases (data not shown) and have four amino acids (Y^{164}, Y^{200}, H^{224}, and H^{226}) with an iron ligand function that are conserved in both types of catechol 1,2-dioxygenases. The two enzymes have more than 40% homology with type I catechol 1,2-dioxygenases, but less than 30% of their sequences are identical to those of type II catechol 1,2-dioxygenases.

Differential arrangement of the two *cat* gene clusters. Further analysis of the nucleotide sequence upstream of the $catA_1$ gene produced some interesting results. The deduced amino acid sequence analyses revealed two ORFs (Fig. 4A). One 96-amino-acid ORF was located 103 bp upstream of $catA_1$, and its deduced amino acid sequences were highly identical to those of the *catC* gene products (muconolactone isomerase) of *A. calcoaceticus* (68% identity) and *P. putida* (55% identity). We named it $catC_1$. The other ORF contained only the C-terminal region, and it was highly homologous to the *catB* gene products (muconate-lactonizing enzyme) of *A. calcoaceticus* (57% identity) and *P. putida* (55% identity). We named it $catB_1$. $catB_1$ and $catC_1$ were separated by only 29 bp. These results revealed that the cat_1 genes were clustered with $catB_1$ and $catC_1$ in the order $catB_1$-$catC_1$-$catA_1$. The DNA sequences of the cat_1 genes revealed higher homology with those genes in *A. calcoaceticus*, although their gene arrangements are different in that the *catA* gene lies several kilobases upstream of *catB-catC* (27). The same arrangement was reported in *P. putida* PRS 2000, i.e., *catR-catB-catC-catA* (15), and *catA* and *catBC* of *P. putida* PRS2000 were suggested to be divergently activated by *catR*, functioning as a transcriptional activator. Therefore, the immediate objectives of our research are to investigate the upstream region of $catB_1$ and the presence of *catR*. Additional analysis of the sequences on both sides of the $catA_2$ gene also showed that the ORFs exist (Fig. 4B). The ORF located upstream of $catA_2$ has high homology with the *catB* gene products of *A. calcoaceticus* (59% identity) and *P.*

A

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      TC GAA CAG AGT GGT GGG CTA TTC GCC GCG CAG CGC GTG GCG GCG ATC GCC GAC GCG GCC GGC ATC GAG CTG TAC GGT GGC    80
catB1     E   Q   S   G   G   L   F   A   A   Q   R   V   A   A   I   A   D   A   A   G   I   E   L   Y   G   G
      ACG ATG CTC GAA GGC GCG TTC AGC ACG GTC GCG TCA GCG CAT CTA TTC GCG AGC TTC GCG AAC CTG CAA TGG GGC ACC GAA   161
       T   M   L   E   G   A   F   S   T   V   A   S   A   H   L   F   A   S   F   A   N   L   Q   W   G   T   E
      CTG TTT GGC CCG CTG TTG ATC ACC GAA GAA ATT CTG ACC AAG CCG CTA GAT TAC AGT GAT TAC CAG TTG ACC GTG CCA GAC   242
       L   F   G   P   L   L   I   T   E   E   I   L   T   K   P   L   D   Y   S   D   Y   Q   L   T   V   P   D
      GGT CCT GGC CTT GGC ATC GAA CTC GAC GAA GAG AAG GTC CGG CGT TTC ACG CGC GAC GGG CTG ATC AAG GTC ACG AAG GCG   323
       G   P   G   L   G   I   E   L   D   E   E   K   V   R   R   F   T   R   D   G   L   I   K   V   T   K   A
      TAA  CCCCGGACGAAAAAAAAGAGAGGATGACG ATG CTT TTC CAT GTA CGC ATG GAT GTG AAT ATT CCG GAC GAT ATG CCG GTC GAG   409
       *                       catC1       M   L   F   H   V   R   M   D   V   N   I   P   D   D   M   P   V   E
      GTC GCA GAC GAA ATC AAG GCA CGC GAG AAG GCG TAT TCG CAG GCG TTG CAA AAG AGC GGC AAA TGG CCT CAC ATC TGG CGC   490
       V   A   D   E   I   K   A   R   E   K   A   Y   S   Q   A   L   Q   K   S   G   K   W   P   H   I   W   R
      CTC GTT GGT GAG TAT GCG AAC TAC AGC ATC TTC GAT GTT GAG AGC AAT GCG GAA CTG CAC GGC ATC CTG ACC GGA CTG CCG   571
       L   V   G   E   Y   A   N   Y   S   I   F   D   V   E   S   N   A   E   L   H   G   I   L   T   G   L   P
      CTG TTT TCA TAT ATG AAG ATC GAG GTG ACG CCG CTG TGC CGT CAC CCG TCG TCG ATT CGC GAC GAC GAG TCG TGA  TCGGAT   652
       L   F   S   Y   M   K   I   E   V   T   P   L   C   R   H   P   S   S   I   R   D   D   E   S   *
       GATTCAAACGAGGGCGCGCTTCCGAGGGCGAGCGCGCTTCCAACGGGTTCGTTACCCGATATGTGCGCCCCAAAACTACTCGATGGAGACTGGCATC  ATG  AGC   755
                                                                                           catA1         M    S
      ATC AAA GTG TTC GGT ACG AAG GAA GTG CAA GAC CTG CTG AAG GCG GCG ACC AAT CTC GAA GGC AAG GGC GGC AAC GCC CGC   836
       I   K   V   F   G   T   K   E   V   Q   D   L   L   K   A   A   T   N   L   E   G   K   G   G   N   A   R
      TCC AAG CAG ATC GTA CAT CGA CTG CTT TCC GAT CTG TTC AAG GCG ATT GAC GAT CTC GAC ATC ACG CCC GAC GAA GTG TGG   917
       S   K   Q   I   V   H   R   L   L   S   D   L   F   K   A   I   D   D   L   D   I   T   P   D   E   V   W
      GCC GGC GTG AAC TAC CTG AAC AAG CTT GGC CAG GAC GGC GAG GCG ACT CTG CTT GCA GCC GGT TCC GGC CTC GAG AAG TAT   998
       A   G   V   N   Y   L   N   K   L   G   Q   D   G   E   A   T   L   L   A   A   G   S   G   L   E   K   Y
      CTT GAT ATC CGG TTG GAC GCG GCG GAC AAA GCT GAG GGT ATC GAG GGC GGT ACA CCG CGC ACG ATC GAG GGT CCG CTG TAC  1079
       L   D   I   R   L   D   A   A   D   K   A   E   G   I   E   G   G   T   P   R   T   I   E   G   P   L   Y
      GTC GCC GGT GCA ACG GTG CAT GAC GGT GTG TCG AAG ATC GAC ATC AAC CCG GAC GAG GAT GCC GGC CCC TTG GTG ATC CAC  1160
       V   A   G   A   T   V   H   D   G   V   S   K   I   D   I   N   P   D   E   D   A   G   P   L   V   I   H
      GGC ACG GTC ACT GGT CCG GAC GGC AAG CCG GTA GCT GGC GCG GTA GTG GAA TGC TGG CAC GCG AAC TCA AAG GGC TTT TAC  1241
       G   T   V   T   G   P   D   G   K   P   V   A   G   A   V   V   E   C   W   H   A   N   S   K   G   F   Y
      TCG CAT TTC GAC CCG ACC GGA GCG CAG AGT GAT TTC AAC CTG CGT GGC GCG GTT AAG ACC GGT GCG GAT GGC AAG TAC GAG  1322
       S   H   F   D   P   T   G   A   Q   S   D   F   N   L   R   G   A   V   K   T   G   A   D   G   K   Y   E
      TTC CGC ACG CTG ATG CCG GTC GGC TAT GGC TGC CCG CCG CAA GGT GCG ACG CAG CAG CTC CTG AAT GTA CTC GGG CGT CAC  1403
       F   R   T   L   M   P   V   G   Y   G   C   P   P   Q   G   A   T   Q   Q   L   L   N   V   L   G   R   H
      GGA AAC CGC CCG GCA CAC GTG CAC TTC TTC GTT TCG TCC GAT AGT GCG CGC AAG TTG ACC ACG CAG TTC AAC ATC GAG GGC  1484
       G   N   R   P   A   H   V   H   F   F   V   S   S   D   S   A   R   K   L   T   T   Q   F   N   I   E   G
      GAT CCG CTC ATC TGG GAC GAC TTT GCA TAT GCG ACG CGT GAA GAG CTG ATC CCG CCG GTG ACC GAG AAG AAG GGT GGT ACG  1565
       D   P   L   I   W   D   D   F   A   Y   A   T   R   E   E   L   I   P   P   V   T   E   K   K   G   G   T
      GCG CTT GGC CTG AAG GCG GAC ACG TAC AAG GAC ATC GAA TTC AAC CTC ACG CTG ACG TCC CTG GTG AAG GGC AAG GAC AAT  1646
       A   L   G   L   K   A   D   T   Y   K   D   I   E   F   N   L   T   L   T   S   L   V   K   G   K   D   N
      CAG GTC GTC CAC CGG TTG CGT GCC GAA GTC GCA GCC TAA CCGATCTAGGCGGCGGGGGGCATACCGCCGCCAATAGGCGTGAGTGGTGCGAGA  1739
       Q   V   V   H   R   L   R   A   E   V   A   A   *
      TGTGACGAGAGGATGTGGGGGCGTCATTGTCAGCCACTGACAGTCGCTGGCTAAATCTCCTCCCTTTACGACATTTGATTGCGAAAACGGGCACGGCAAGCGCGCCG  1846
      GAACGACCATGGAGTTCTTGCATGTATGAAGGCGAACGGCTGCAATTGCCGAACCACCACCTGCATTACCGTCTCGACGGCGATGCAACCGATAGCGCGAAGCATGT  1953
      TGCTATTCTGCAACTCCCTTGGTACC                                                                                   1979
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B

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      ATC GAC GAA GCC GAG CGC GTT TTC GAA GCG AAG CGG CAT CGC GTG TTC AAG CTG AAG ATC GGT TCG CGA GCG TTG GCC GAC    81
catB2  I   D   E   A   E   R   V   F   E   A   K   R   H   R   V   F   K   L   K   I   G   S   R   A   L   A   D
      GAC GTG GCG CAC GTT GTG GCG ATT CAG AAG GCG CTG CAA GGG CGC GGT GAA GTG CGG GTC GAC GTG AAC CAG GCG TGG ACC   162
       D   V   A   H   V   V   A   I   Q   K   A   L   Q   G   R   G   E   V   R   V   D   V   N   Q   A   W   T
      GAG TCC GAG GCG ATC TGG GCC GGT AAA CGG TTC GCC GAT GCG AGC GTT GCG CTG ATC GAG CAG CCG ATT GCC GCG GAA AAT   243
       E   S   E   A   I   W   A   G   K   R   F   A   D   A   S   V   A   L   I   E   Q   P   I   A   A   E   N
      CGC GCG GGT CTC AAA CGT CTG ACG GAT CTC GCT CAG GTG CCG ATC ATG GCG GAC GAA GCC TTG CAT GGG CCG GCC GAT GCC   324
       R   A   G   L   K   R   L   T   D   L   A   Q   V   P   I   M   A   D   E   A   L   H   G   P   A   D   A
      TTC GCA CTG GCG AGC GCG CGT GCC GCC GAC GTG TTC GCC GTG AAG ATC GCG CAA TCG GGC GGC CTG AGC GGC GCC GCG AAC   405
       F   A   L   A   S   A   R   A   A   D   V   F   A   V   K   I   A   Q   S   G   G   L   S   G   A   A   N
      GTG GCG GCG ATT GCG CTC GCC GCG AAT ATC GAC CTG TAC GGC GGC ACG ATG CTC GAA GGC GCG GTG GGC ACG ATC GCC TCG   486
       V   A   A   I   A   L   A   A   N   I   D   L   Y   G   G   T   M   L   E   G   A   V   G   T   I   A   S
      GCG CAA CTC TTC AGC ACC TTC GGC GAA TTG AAG TGG GGC ACC GAA CTG TTC GGG CCG TTG CTG CTG ACC GAA GAA ATT CTC   567
       A   Q   L   F   S   T   F   G   E   L   K   W   G   T   E   L   F   G   P   L   L   L   T   E   E   I   L
      ACT GAG CCG CTG CGT TAC GAG AAT TTT GTT TTG CAT CTT CCA CAA GGA CCG GGT CTG GGC ATC ACG CTC GAC TGG GAC AAG   648
       T   E   P   L   R   Y   E   N   F   V   L   H   L   P   Q   G   P   G   L   G   I   T   L   D   W   D   K
      ATC GAC CGC TTA CGG CGC GAT ACG CGC AAG GGC GCA AGC ATC ACC ATG AAC TGA CGGTTCATTCGCTAACCGCCAAGAACATCAAACG   729
       I   D   R   L   R   R   D   T   R   K   G   A   S   I   T   M   N   *
      AAGGAGATACCCC  ATG AAC AAG CAA GCC ATC GAC GCG CTG CTG CAA AAA ATC AAC GAT AGC GCC ATC AAT GAA GGC AAT CCG   818
           catA2      M   N   K   Q   A   I   D   A   L   L   Q   K   I   N   D   S   A   I   N   E   G   N   P
      CGC ACG AAG CAG ATC GTC AAC CGC ATC GTC AGG GAT CTG TTC TAT ACG ATC GAA GAC CTC GAC GTG CAG CCG GAC GAG TTC   899
       R   T   K   Q   I   V   N   R   I   V   R   D   L   F   Y   T   I   E   D   L   D   V   Q   P   D   E   F
      TGG ACC GCG CTG AAT TAT CTC GGC GAC GCG GGC AGG AGC GGC GAG CTC GGT CTG TTG GCC GCC GGT CTC GGC TTC GAG CAT   980
       W   T   A   L   N   Y   L   G   D   A   G   R   S   G   E   L   G   L   L   A   A   G   L   G   F   E   H
      TTT CTC GAT CTG CGC ATG GAC GAA GCC GAA GCG AAG GCC GGC GTC GAA GGC GGC ACG CCG CGC ACC ATC GAA GGA CCG TTG  1061
       F   L   D   L   R   M   D   E   A   E   A   K   A   G   V   E   G   G   T   P   R   T   I   E   G   P   L
      TAT GTG GCG GGC GCG CCG GTT TCC GAC GGC CAC GCG CGG CTC GAT GAC GGC ACC GAT CCG GGT CAG ACG CTG GTG ATG CGC  1142
       Y   V   A   G   A   P   V   S   D   G   H   A   R   L   D   D   G   T   D   P   G   Q   T   L   V   M   R
      GGC CGC GTG TTC GGC GAA GAC GGC AAG CCG CTC GCG AAT GCG CTG GTT GAG GTG TGG CAC GCG AAT CAC CTC GGC AAC TAC  1223
       G   R   V   F   G   E   D   G   K   P   L   A   N   A   L   V   E   V   W   H   A   N   H   L   G   N   Y
      TCG TAC TTC GAC AAG TCG CAG CCG GCT TTC AAT CTG CGC CGC TCG ATT CGC ACC GAT GCC GAA GGC AAG TAC AGC TTC CGC  1304
       S   Y   F   D   K   S   Q   P   A   F   N   L   R   R   S   I   R   T   D   A   E   G   K   Y   S   F   R
      AGC GTG GTG CCG GTC GGT TAC TCG GTG CCG CCG CAA GGG CAG ACG CAG TTG CTG CTC GAT CAG TTG GGC CGC CAT GGG CAT  1385
       S   V   V   P   V   G   Y   S   V   P   P   Q   G   Q   T   Q   L   L   L   D   Q   L   G   R   H   G   H
      CGT CCG GCG CAT ATT CAC TTC TTC GTT TCG GCG CCG GGT TTC CGC AAG CTG ACC ACC CAG ATC AAC ATC GAC GGC GAT CCG  1466
       R   P   A   H   I   H   F   F   V   S   A   P   G   F   R   K   L   T   T   Q   I   N   I   D   G   D   P
      TAT CTG TGG GAC GAC TTT GCG TTT GCC ACG CGC GAC GGG CTC GTG CCC GCC GTC AGG CAG GCC GAG GTG CGG AAG GCA AAC  1547
       Y   L   W   D   D   F   A   F   A   T   R   D   G   L   V   P   A   V   R   Q   A   E   V   R   K   A   N
      CGT ACG GCG TGG ACG GTC AGT TCG CGT TGA TCGATTTCGACTTCACGCTGTTCAAGGAACGCGACAATCTGCCGGGCGCCGAAGTGGAGCGCGTGCG  1644
       R   T   A   W   T   V   S   S   R   *
      CGCCGAAGCCTGAGATAAGAAAAGGAGAGCAAGC ATG CTG TTT CAC GTA GAG ATG ACT GTC AAT CTG CCG TCC GAT ATG GAC GCG GAG  1732
                             catC2        M   L   F   H   V   E   M   T   V   N   L   P   S   D   M   D   A   E
      CGC GCC GCC CGT TTG AAG TCC GAT GAG AAA GCG ATG TCG CAA AAG CTG CAG CAG GAG GGC GTG TGG CGG CAC TTG TGG CGT  1813
       R   A   A   R   L   K   S   D   E   K   A   M   S   Q   K   L   Q   Q   E   G   V   W   R   H   L   W   R
      ATC GCC GGG CGC TAC GCG AAC ATC AGC GTG TTC GAC GTG GAA AGC CCA GCG CAT CTG CAT GAC GTG CTG AGC CAG TTG CCG  1894
       I   A   G   R   Y   A   N   I   S   V   F   D   V   E   S   P   A   H   L   H   D   V   L   S   Q   L   P
      CTG TTT CCG TAT ATG GAC GTC GAA GTG CGC GCG CTG TGC CGG CAT GCT TCA TCG ATC CAC GAC GAC GAT CGC TAA GGGCGC  1975
       L   F   P   Y   M   D   V   E   V   R   A   L   C   R   H   A   S   S   I   H   D   D   D   R   *
      AAGCAAGCCGG                                                                                                  1986
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FIG. 4. Nucleotide sequences of the DNA fragments inserted into pCD11 and pCD21 and predicted amino acid sequence. (A) DNA sequence of the *Xba*I-*Kpn*I fragment of pCD11 presented together with the amino acid sequences of truncated $catB_1$, $catC_1$, and $catA_1$. (B) DNA sequence of the *Eco*RI-*Pst*I fragment of pCD21 containing the truncated $catB_2$, $catA_2$, and $catC_2$ genes. The putative ribosome-binding sites are underlined. The sequences of the 450- and 580-bp PCR products are underlined.

putida (55% identity). The $catB_2$ and $catA_2$ genes are separated by 47 bp. The deduced amino acid sequence downstream of the $catA_2$ gene has high homology with the *catC* gene products of *A. calcoaceticus* (58% identity) and *P. putida* (70% identity). A 101-bp sequence is located between $catA_2$ and $catC_2$. Therefore, the cat_2 genes are arranged in the order $catB_2$-$catA_2$-$catC_2$. The $catB_2$-$catA_2$-$catC_2$ gene arrangement has not been reported. It is notable that the deduced amino acids of the cat_1 ($catA_1$, $catB_1$, and $catC_1$) genes are closer to the *cat* gene products of *A. calcoaceticus* (percentages of homology: 56% with $catA_1$, 57% with $catB_1$, and 68% with $catC_1$) but the deduced amino acids of the cat_2 genes have higher homology with those of *P. putida* (51% with $catA_2$ and 70% with $catC_2$), except for $catB_2$. The reason why two different *catABC* gene clusters exist and the question of how they are regulated in *A. lwoffii* K24 need further investigation. However, we assume that the different arrangement and sequences of the two *cat* genes in *A. lwoffii* K24 occurred through genetic adaptation. In conclusion, the $catA_1$ and $catA_2$ genes are separate on the chromosome of *A. lwoffii* K24 and the two catechol 1,2-dioxygenases (CD I_1 and CD I_2) have different enzymatic properties and sequences. $catA_1$ is arranged in the order $catB_1$-$catC_1$-$catA_1$, while $catA_2$ is arranged in the order $catB_2$-$catA_2$-$catC_2$.

Nucleotide sequence accession number. The nucleotide sequences presented here have been deposited in the GenBank database under accession numbers U77658 and U77659.

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