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Cloning of *p*-Hydroxybenzoate Degradation Genes and the Overexpression of Protocatechuate 4,5-Dioxygenase from *Pseudomonas* sp. K82

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Abstract *Pseudomonas* sp. K82 cultured in *p*-hydroxybenzoate induces protocatechuate 4,5-dioxygenase (PCD 4,5) for *p*-hydroxybenzoate degradation. In this study, a 6.0-kbp EcoRI fragment containing *p*-hydroxybenzoate degradation genes was cloned from the genome of *Pseudomonas* sp. K82. Sequence analysis identified four genes, namely, *pcaD*, *pcaA*, *pcaB*, and *pcaC* genes known to be involved in *p*-hydroxybenzoate degradation. Two putative 4-hydroxyphenylpyruvate dioxygenases and one putative oxidoreductase were closely located by the *p*-hydroxybenzoate degradation genes. The gene arrangement and sequences of these *p*-hydroxybenzoate degradation genes were similar to those of *Comamonas testosteroni* and *Pseudomonas ochraceae*. PcaAB (PCD4,5) was overexpressed in the expression vector pGEX-4T-3, purified using a GST column, and confirmed to have protocatechuate 4,5-dioxygenase activity. The *N*-terminal amino acid sequences of overexpressed PCD4,5 were identical with those of purified PCD4,5 from *Pseudomonas* sp. K82.

Key words: *p*-Hydroxybenzoate, protocatechuate 4,5-dioxygenase, *pcaA* and *pcaB*, *Pseudomonas* sp. K82

Soil bacteria are known to have developed three ring-cleavage mechanisms for diol metabolites generated by the biodegradation of aromatic compounds; namely, intradiol cleavage (*ortho*), extradiol cleavage (*meta*), and gentisate 1,2-dioxygenase [2, 4]. Various monocyclic aromatic compounds (4-hydroxybenzoate, 3- and 4-nitrobenzoate, phthalate, vanillate, and *p*-toluate) and polycyclic aromatic compounds (anthracene, phenanthrene, naphthalene, and fluorene) have been reported to be converted into protocatechuate and then degraded through extradiol cleavage [12, 16]. Protocatechuate 4,5-dioxygenase (PCD4,5) is the first enzyme in the protocatechuate degradation pathway and belong to a type III extradiol dioxygenase [14]. PCD4,5 catalyzes the extradiol cleavage of protocatechuate to produce 2-hydroxy-4-carboxy-muconic semialdehyde, which is finally converted into oxaloacetate and pyruvate by *meta* pathways (Fig. 1). Although PCD4,5 is crucially required for the microbial biodegradation of monocyclic and polycyclic aromatic compounds, the instability of the enzyme *in vitro* makes enzyme purification and characterization difficult. Therefore, only a few purifications of PCD4,5 have been reported [1, 3, 11, 12]. Recently the crystal structure of PCD4,5 from *Sphingomonas paucimobilis* SYK-6 was described at a resolution of 2.2 Å [15]. In our previous paper, *Pseudomonas* sp. K82 was reported to be an aniline-, benzoate-, and *p*-hydroxybenzoate-assimilating soil bacterium, and the major metabolic pathways of these monocyclic aromatic compounds were elucidated using a proteomics/2-DE/MS approach [5]. Because two subunits (PcaA and

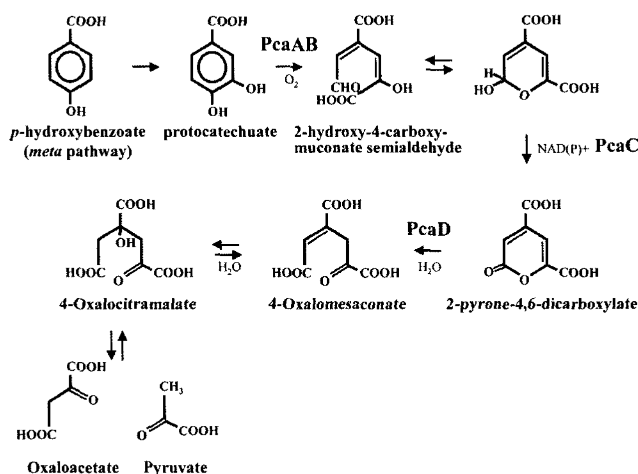

Fig. 1. *Meta* pathway for *p*-hydroxybenzoate degradation by *Pseudomonas* sp. K82.

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PcaB) of protocatechuate 4,5-dioxygenase were overly induced under *p*-hydroxybenzoate, *Pseudomonas* sp. K82 was presumed to use an extradiol cleavage pathway for *p*-hydroxybenzoate. However, the enzymatic characterization of PCD4,5 of *Pseudomonas* sp. K82 was previously studied in a partially purified state [17]. In this study, a 6.0-kbp EcoRI fragment that contained the *p*-hydroxybenzoate degradation genes were cloned from the genome of *Pseudomonas* sp. K82 and the sequences were analyzed. The two subunits (PcaA and PcaB) of PCD4,5, were overexpressed in pGEX-4T-3 vector, purified by GST column, and their dioxygenase activities were confirmed.

Pseudomonas sp. K82 was precultured at 28°C in potassium phosphate buffer (pH 6.25) containing 3.4 mM $MgSO_4$, 0.2 mM $CaCO_3$, 0.3 mM $FeSO_4$, 10 mM NH_4Cl, and 10 mM sodium succinate, and then cultured in the same medium containing *p*-hydroxybenzoate (5 mM) for activity assays and enzyme characterization. The PCRs of two genes of PCD4,5 (*pcaA* and *pcaB*) were performed using four primers, which were designed using the *N*-terminal and internal amino acid sequences: α subunit-F1, 5'-AAACCGTATCTGGACGTGCCCGGCA-3'; α subunit-R1, 5'-CAGGAAGTAGATATTGC CGCCGGT-3'; β subunit-F2, 5'-ATGGCTCGCATCACCGCATCCGTT-3'; β subunit-R2, 5'-GGC ACCACGCGCAATCA GCCACAT-3' [17]. In the present study, the 1,200- and 750-bp PCR products were confirmed to have originated from *pcaAB* and *pcaB* by DNA sequencing. Therefore, the 750-bp PCR products were used as a probe for Southern hybridization. The 760-bp PCR product of the protocatechuate 4,5-dioxygenase (PCD4,5) gene was labeled using an AlkPhos Direct Labeling and Detection system (Amersham Biosciences). After preparing the genomic DNA using QIAGEN Genomic DNA kits (Qiagen, Hilden, Germany), it was digested with EcoRI (New England Bio-Labs, Beverly, MA, U.S.A.), run on a 1% agarose gel, and transferred to Hybond-N+ nylon membranes (Amersham Biosciences). Hybridization with the labeled 760-bp PCR product was performed at 68°C and membranes were developed using a phosphoimager (Amersham Biosciences). Southern hybridization confirmed that *pcaAB* is located in a 6-kb EcoRI fragment of the *Pseudomonas* sp. K82 chromosome (data not shown). In order to increase cloning yield, 5.5 kb to 6.5 kb DNA fragments of EcoRI-treated chromosome were prepared and ligated with pBluescript II SK (+). *E. coli* transformants were used as DNA templates for the PCR of *pcaB* in positive clone screening. Transformants were screened by PCR using the a subunit-F1 and β subunit-R2 (1.2 kb). By screening 70 recombinant transformants, a single positive clone was detected. Plasmid pB*pcaAB* was purified prior to sequence analysis of the inserted DNA fragment from a positive clone (Fig. 2).

A DNA sequence of an approximately 6-kb EcoRI fragment containing the PCD4,5 gene cluster of *Pseudomonas* sp. K82 was sequenced on denatured double-stranded DNA templates. Homology searches (BLAST) were carried using the network of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Analyses of sequences and ORFs identified by BLAST were done using the ExPASy Proteomics Server (http://kr.expasy.org). ClustalW (http://www.ebi.ac.kr/clustalw/) was used to analyze phylogenic relationships of the genes. The nucleotide sequence of the PCD4,5 gene cluster reported in this paper has been submitted to the GenBank database under accession

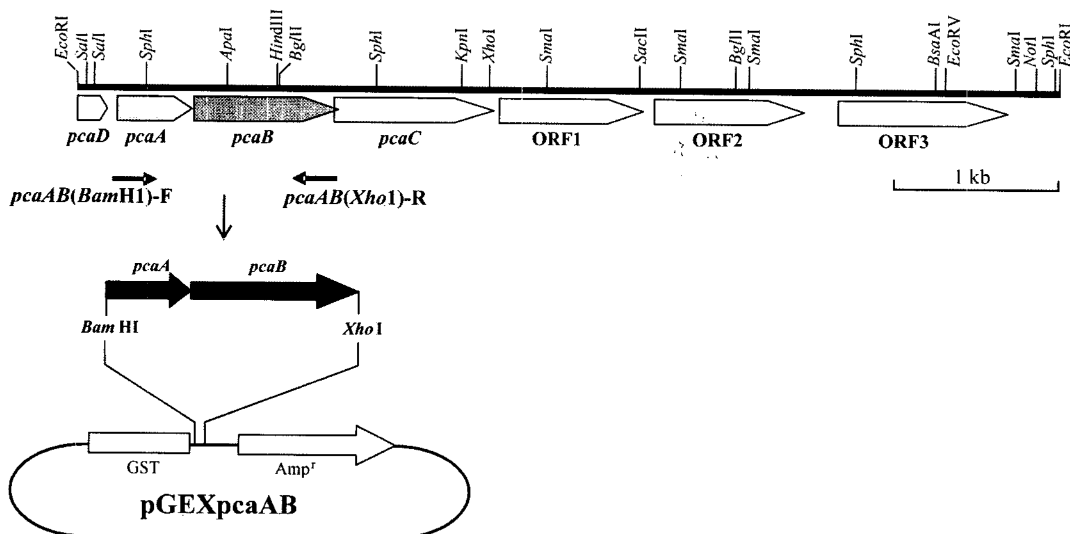

Fig. 2. Physical and restriction map of 6.0-kbp EcoRI fragments harboring protocatechuate *meta* pathway genes from the chromosome of *Pseudomonas* sp. K82.

Four *p*-hydroxybenzoate degradation genes and three putative ORFs were analyzed. For the overproduction of protocatechuate 4,5-dioxygenase (PcaAB), the 1.3-kb PCR product of *pcaAB* was ligated into the expression vector pGEX-4T-3. No BamH1 or Xho1 site was detected in this region.

metabolism. It is surprising that the vitality and high pigment content of *P. tricornutum* can be sustained for level of inorganic nutrients. Inhibition of growth, and chlorosis, of microalgae is usually caused by starvation of

number DQ397304. DNA sequence analysis showed that the insert fragment was consisted of 5,905 bp and contained six complete ORFs and one truncated ORF (Fig. 2). Four *pca* genes were elucidated in the order *pcaD-pcaA-pcaB-pcaC*. The *pca* gene structure of *Pseudomonas* sp. K82 is similar to that of *C. testosterone* BR6020 and *P. ochraceae* NGJ1 [8, 12] but differs from those of *S. paucimobilis* SYK-6 (accession no. AB073227) and *Sphingomonas* sp. LB126C (accession no. AJ277295). The *N*-terminal domain of PcaD was truncated. PcaA shared more than 90% identity with *C. testosterone* BR6020 (accession no. AAK73572) and *Comamonas* sp. DJ-12 (accession no. AAW66636) (Fig. 3), and PcaB shared more than 85% identity with *C. testosterone* BR6020 (accession no. AAK73573) and *Comamonas* sp. DJ-12 (accession no. AAW66637). However, PcaC was closer to *R. palustis* CGA009 (accession no. CAE30143) and *Bradyrhizobium* sp. BTAil (accession no. EAP31963) than *C. testosterone* BR6020 (accession no.

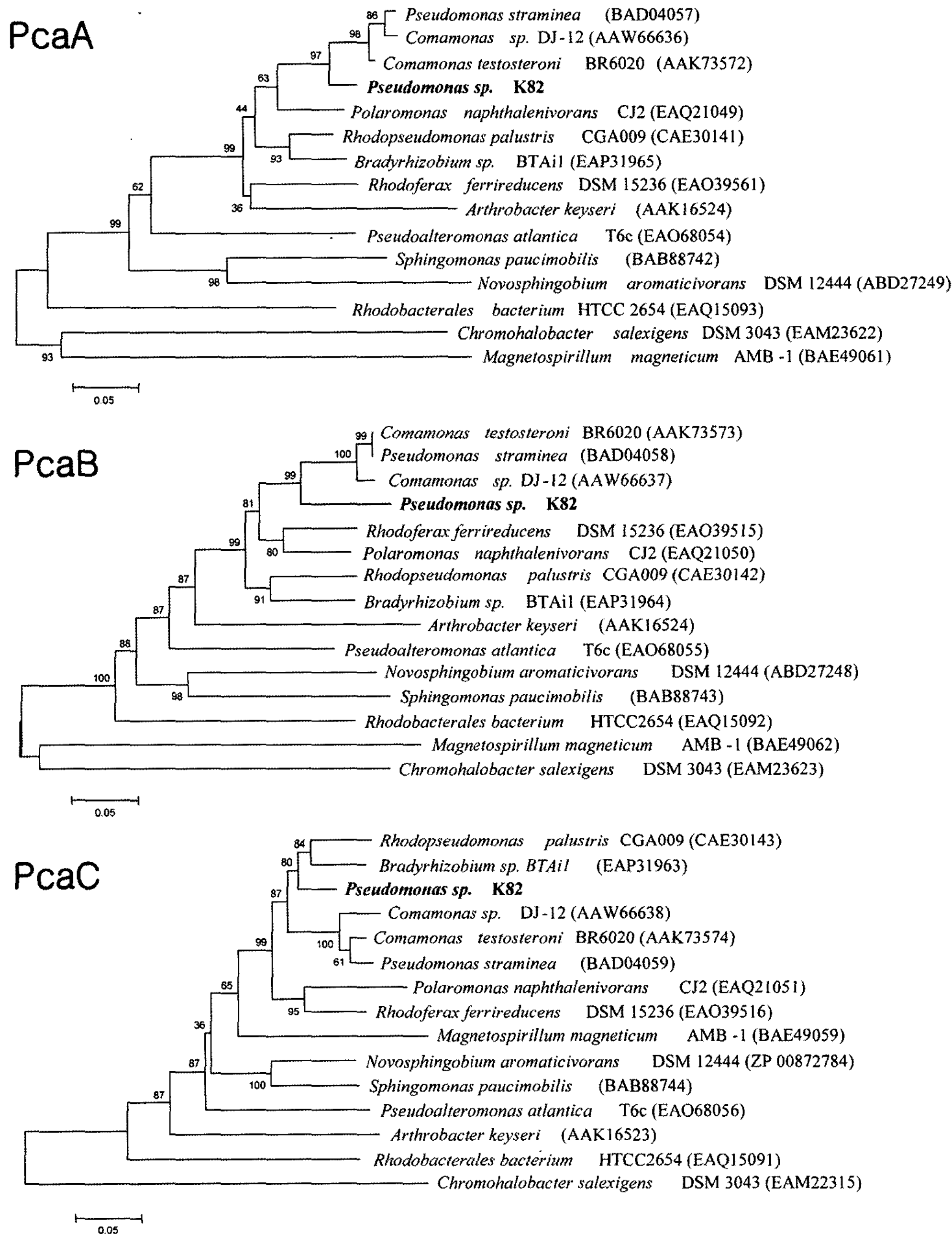

Fig. 3. Dendrograms showing the phylogenic relationships of PcaA (α subunit of PCD4,5), PcaB (β subunit of PCD4,5), and PcaC (4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase) from *Pseudomonas* sp. K82.

This dendrogram was constructed using ClustalW (http://www.ebi.ac.kr/clustalw/) based on the deduced amino acid sequences.

AAK73574) and *Comamonas* sp. DJ-12 (accession no. AAW66638) (Fig. 3). In our previous report, the *de novo* internal amino acid sequences of PcaA, PcaB, PcaE, and *p*-hydroxybenzoate hydroxylase were deduced by MS/MS analysis and used for protein identification [5]. The DNA sequence determined in the present study confirmed that the deduced amino acid sequences of PcaA and PcaB were matched with the amino acid sequences of PcaA and PcaB, obtained from proteome analysis, except for a small number of amino acids (*i.e.*, $T^{57}\rightarrow S^{57}$ in PcaA; $Q^{254}\rightarrow A^{254}$ and $A^{261}P^{262}\rightarrow P^{261}A^{262}$ in PcaB). The internal sequence of PcaE and *p*-hydroxybenzoate hydroxylase will be helpful information for the further gene cloning [5]. The 3D structure of protocatechuate 4,5-dioxygenase was elucidated from the PCD4,5 from *S. paucimobilis* SYK-6 [15]. A homology search showed that two subunits of PCD4,5 from *Pseudomonas* sp. K82 share 59% and 64% identity with the PCD4,5 from *S. paucimobilis* SYK-6 and that active sites are conserved. This result suggests a 3D structural similarity between the two enzymes. From sequence analysis, we obtained three ORFs with significant homology with the genes of *Polaromonas naphthalenivorans* CJ2. ORF1 (294 amino acids, 32,497 Da) had 58% identity with putative 4-hydroxyphenylpyruvate dioxygenase oxidoreductase, ORF2 (290 amino acids, 32,032 Da) 69% identity with 4-hydroxyphenylpyruvate dioxygenase, and ORF 3 (341 amino acids, 37,093 Da) 60% identity with aldo/keto reductase. 4-Hydroxyphenylpyruvate dioxygenase (HPPD) is involved in the catabolism of tyrosine and catalyzes the conversion of 4-hydroxyphenylpyruvate to homogentisate [9].

The *pcaAB* gene was expressed using the GST Gene Fusion System (Amersham Biosciences) [6]. The *pcaAB* coding region was cloned into a pGEX 4T-3 plasmid and expressed in *Escherichia coli* BL21 as a glutathione-S-transferase-tagged fusion protein. A summary of steps used for the construction of pGEX*pcaAB* and a schematic diagram are provided in Fig. 2, respectively. For PCR amplification of the PCD4,5 gene, two oligonucleotides were designed, oligo *pcaAB* (BamHI)-F 5'-CGCGGATCC-ATGGCATTGGACAAACCCTATC-3' and oligo *pcaAB* (XhoI)-R 5'-TTGCCTCGAGTCATTGGGGGT TCTCCA-GGA-3'. PCR reactions were performed for 30 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C. PCR products were purified on 1% agarose gels using gel extraction kits (Qiagen, Hilden, Germany). Then, the *pcaAB* genes were amplified, treated with BamH1 and XhoI, and ligated into expression vector pGEX-4T-3 (Fig. 2). The recombinant plasmid was named as pGEXpcaAB. *E. coli* harboring recombinant plasmid pGEX*pcaAB* was cultured at 37°C in 500 ml of Luria-Bertani medium containing ampicillin (50 µg/ml). When the A_{600} reached 0.2–0.3, isopropyl β-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM and the culture was continued for 6 h at 25°C. Harvested cells were suspended in 20 mM Tris-HCl buffer (pH 8.0) and disrupted using a French pressure cell (SLM AMINCO, Urbana, IL, U.S.A.) at 20,000 lb/in^2. Supernatants (crude cell extracts) were collected by centrifugation at 15,000 ×g for 45 min and used to determine PCD4,5 activity. Two subunits (*pca A* and *B*) of PCD4,5 were reported to be co-translated in *C. testosterone* and *P. paucimobilis* [10, 12]. In the presence of IPTG, GST-fused PcaA (43 kDa) and PcaB (34 kDa) were induced in the crude extract of *E. coli* BL21 transformants containing pGEXpcaAB (Fig. 4, lane 3). Two subunits of PCD4,5 (GST fused PcaA and PcaB) were successfully purified from a GST column after thrombin cleavage (Fig. 4, lane 5). Dimerization of GST-fused PcaA and PcaB seemed to be stable in our experimental and buffer conditions and the GST fusion part of PcaA did not have any negative effect on dimerization with PcaB. Therefore, affinity chromatography using GST column was useful for the purification of PcaB. PCD4,5 was purified from the resulting supernatant using Glutathione-Sepharose 4B, which was packed into a disposable column with a 200 µl/mg bed volume. This column was washed and equilibrated with ice-chilled 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3). Cell lysate was applied (flow rate 20 ml/h) to the column, which was then washed with 1× PBS. PCD4.5 was separated from GST by thrombin cleavage. Fusion proteins bound to the column were digested overnight with thrombin solution (10 cleavage units/mg) at 4°C, and the digested proteins were then eluted. The samples were separated by 12% SDS-PAGE, and the enzyme activities

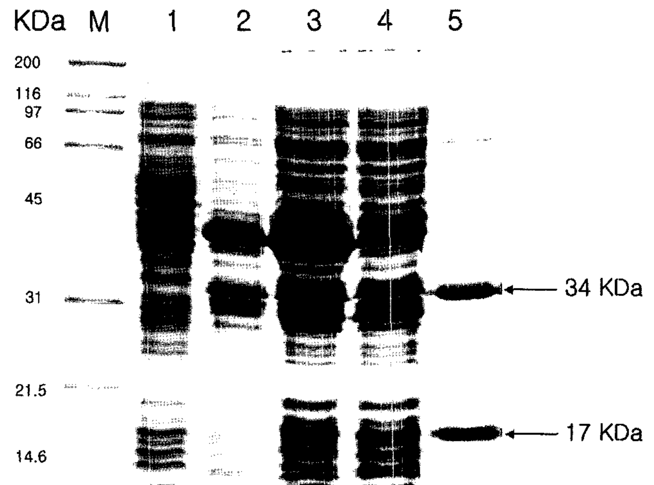

Fig. 4. Purification of overexpressed PCD4,5 using a GST column. *E. coli* BL21 transformants containing pGEXpcaAB were boiled in lysis buffer after IPTG induction and extracted proteins were subjected to 12% SDS-PAGE (lane 2). The extracted proteins from *E. coli* BL21 transformants, cultured in LB without IPTG induction, were used as control (lane 1). Supernatant (lane 3) from IPTG-treated *E. coli* BL21 transformant was prepared after cell disruption using a French pressure cell. Purified PCD4,5 (lane 5) was eluted from the GST column after thrombin treatment. Unbounded proteins flowed through the GST column (lane 4).

of PCD4,5 were measured spectrophotometrically using a Beckman DU7500 UV spectrometer at 25°C, as previously reported [17]. Edman sequencing confirmed the identities of these two subunits (PcaA, GSMALDKP; PcaB, MARITASV). In case of PcaA, sequences of the GST domain (GS) remained after thrombin cleavage. For *N*-terminal amino sequence analysis, purified enzymes were electrotransferred onto PVDF membranes, as previously described [17]. One interesting point is that two subunits were not equally expressed in the *E. coli* expression system; the PcaA (α subunit) was expressed twice as much (Fig. 4). This result suggests that the transcription or translation yield of the *pcaB* gene was attenuated. Moreover, this process also causes incomplete assembly of the two subunits for enzyme activity. In our previous study, we tried to purify the PCD4,5 of *Pseudomonas* sp. K82. However, because of instability in its activity, the purification was incomplete. Because of the overexpression of PCD4,5, the specific activity of the crude extract was increased 3.0-fold (0.09 U/mg) in the *E. coli* expression system as compared with the results of our previous purification experiment [17]. However, despite the complete purification of PCD4,5 using a GST column in the present study, its enzyme specific activity was not considerably increased (0.15 U/mg). All PCD4,5s were reported to be prone to lose the activities because of the oxidation of the active site ferrous iron group [9] and activities were particularly decreased in the state of purified enzymes [3]. Thus, some means to prevent the rapid oxidation of the active site should be found for maintaining the enzyme activity.

In summary, we cloned *pca* genes, overexpressed PCD4,5, and confirmed the protocatechuate 4,5-dioxygenase activity. From these and our previous results, we suggest that *Pseudomonas* sp. K82 uses extradiol cleavage pathways (CD2,3 and PCD4,5) as a primary means for biodegrading monocyclic aromatic compounds [5].

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