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Genetic and Biochemical Characterization of a 2-Pyrone-4,6-Dicarboxylic Acid Hydrolase Involved in the Protocatechuate 4,5-Cleavage Pathway of *Sphingomonas paucimobilis* SYK-6

EIJI MASAI,[1] SHOUJI SHINOHARA,[1] HIROFUMI HARA,[1] SEIJI NISHIKAWA,[2] YOSHIHIRO KATAYAMA,[3] AND MASAO FUKUDA[1]*

Department of Bioengineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2188,[1] New Products and Technology Laboratory, Cosmo Research Institute, Satte, Saitama 340-0193,[2] and Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509,[3] Japan

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***Sphingomonas paucimobilis* SYK-6 is able to grow on a wide variety of dimeric lignin compounds with guaiacyl moieties, which are converted into protocatechuate by the actions of lignin degradation enzymes in this strain. Protocatechuate is a key metabolite in the SYK-6 degradation of lignin compounds with guaiacyl moieties, and it is thought that it degrades to pyruvate and oxaloacetate via the protocatechuate 4,5-cleavage pathway. In a 10.5-kb *Eco*RI fragment carrying the protocatechuate 4,5-dioxygenase gene (*ligAB*) (Y. Noda, S. Nishikawa, K. Shiozuka, H. Kadokura, H. Nakajima, K. Yoda, Y. Katayama, N. Morohoshi, T. Haraguchi, and M. Yamasaki. J. Bacteriol. 172:2704–2709, 1990), we found the *ligI* gene encoding 2-pyrone-4,6-dicarboxylic acid (PDC) hydrolase. PDC hydrolase is a member of this pathway and catalyzes the interconversion between PDC and 4-carboxy-2-hydroxymuconic acid (CHM). The *ligI* gene is thought to be transcribed divergently from *ligAB* and consists of an 879-bp open reading frame encoding a polypeptide with a molecular mass of 32,737 Da. The *ligI* gene product (LigI), expressed in *Escherichia coli*, was purified to near-homogeneity and was estimated to be a monomer (31.6 kDa) by gel filtration chromatography. The isoelectric point was determined to be 4.9. The optimum pH for hydrolysis of PDC is 8.5, the optimum pH for synthesis of PDC is 6.0 to 7.5, and the K_m values for PDC and CHM are 74 and 49 μM, respectively. LigI activity was inhibited by the addition of thiol reagents, suggesting that the cysteine residue is a catalytic site. LigI is more resistant to metal ion inhibition than the PDC hydrolases of *Pseudomonas ochraceae* (K. Maruyama, J. Biochem. 93:557–565, 1983) and *Comamonas testosteroni* (P. J. Kersten, S. Dagley, J. W. Whittaker, D. M. Arciero, and J. D. Lipscomb, J. Bacteriol. 152:1154–1162, 1982). The insertional inactivation of the *ligI* gene in *S. paucimobilis* SYK-6 led to the complete loss of PDC hydrolase activity and to a growth defect on vanillic acid; it did not affect growth on syringic acid. These results indicate that the *ligI* gene is essential for the growth of SYK-6 on vanillic acid but is not responsible for the growth of SYK-6 on syringic acid.**

Protocatechuate (PCA) is one of the most important intermediate metabolites in the bacterial pathways for various phenolic compounds, including lignin, which is the most abundant aromatic material in nature. *Sphingomonas paucimobilis* SYK-6 is able to degrade a wide variety of dimeric lignin compounds, including β-aryl ether, biphenyl, and diarylpropane (20). The resulting lignin degradation enzymes are expected to be useful tools for the utilization of lignin as biomass. Dimeric lignin compounds with guaiacyl (4-hydroxy-3-methoxyphenyl) moieties are converted into PCA by the action of the various lignin degradation enzymes, including β-etherase (LigF and LigE) (20, 21), ring cleavage dioxygenase for biphenyl (LigZ) (27), and demethylases for 5,5′-dehydrodivanillic acid (LigX) (unpublished data) and vanillic acid (LigH) (23), as well as side chain-cleaving enzymes. Thus, PCA is the key intermediate metabolite in the lignin degradation pathway in *S. paucimobilis* SYK-6, and the PCA metabolic pathway plays a key role in lignin degradation by this strain. It is generally known that the aromatic ring of PCA is opened in reactions catalyzed by three kinds of dioxygenases: PCA 3,4-dioxygenase (3,4-PCD) (5, 6, 40), PCA 4,5-dioxygenase (4,5-PCD) (24), and PCA 2,3-dioxygenase (2,3-PCD) (38). Among these dioxygenases, 3,4-PCD is the most commonly characterized enzyme, and its three-dimensional structure has been elucidated (25). The β-ketoadipate pathway genes (*pca* genes), including that for 3,4-PCD, have been characterized in detail in *Acinetobacter calcoaceticus*, *Pseudomonas putida*, and *Agrobacterium tumefaciens* (8).

In the case of *S. paucimobilis* SYK-6, PCA is subjected to ring cleavage by 4,5-PCD and metabolized through the PCA 4,5-cleavage pathway proposed by Kersten et al. (13). In the PCA 4,5-cleavage pathway (Fig. 1), 4,5-PCD catalyzes 4,5-cleavage of PCA to form 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS), which is nonenzymatically converted into an intramolecular hemiacetal form and then dehydrogenated by CHMS dehydrogenase (19). The resulting intermediate, 2-pyrone-4,6-dicarboxylic acid (PDC) (15), is hydrolyzed by PDC hydrolase to yield 4-oxalomesaconic acid (OMA) or its tautomer, 4-carboxy-2-hydroxymuconic acid (CHM) (13, 16). OMA is converted into 4-carboxy-4-hydroxy-2-oxoadipic acid (CHA) by OMA hydratase (17). Finally, CHA is cleaved by CHA aldolase to produce pyruvate and oxaloacetate (18, 36). Each enzyme catalyzing the last four steps in *Pseudomonas ochraceae* (15–19) has been purified and characterized. Additionally, the PDC hydrolase and CHA aldolase of *Comamonas*

* Corresponding author. Mailing address: Department of Bioengineering, Nagaoka University of Technology, Kamitomioka, Nagaoka, Niigata 940-2188, Japan. Phone: 81-258-47-9405. Fax: 81-258-47-9450. E-mail: masao@vos.nagaokaut.ac.jp.

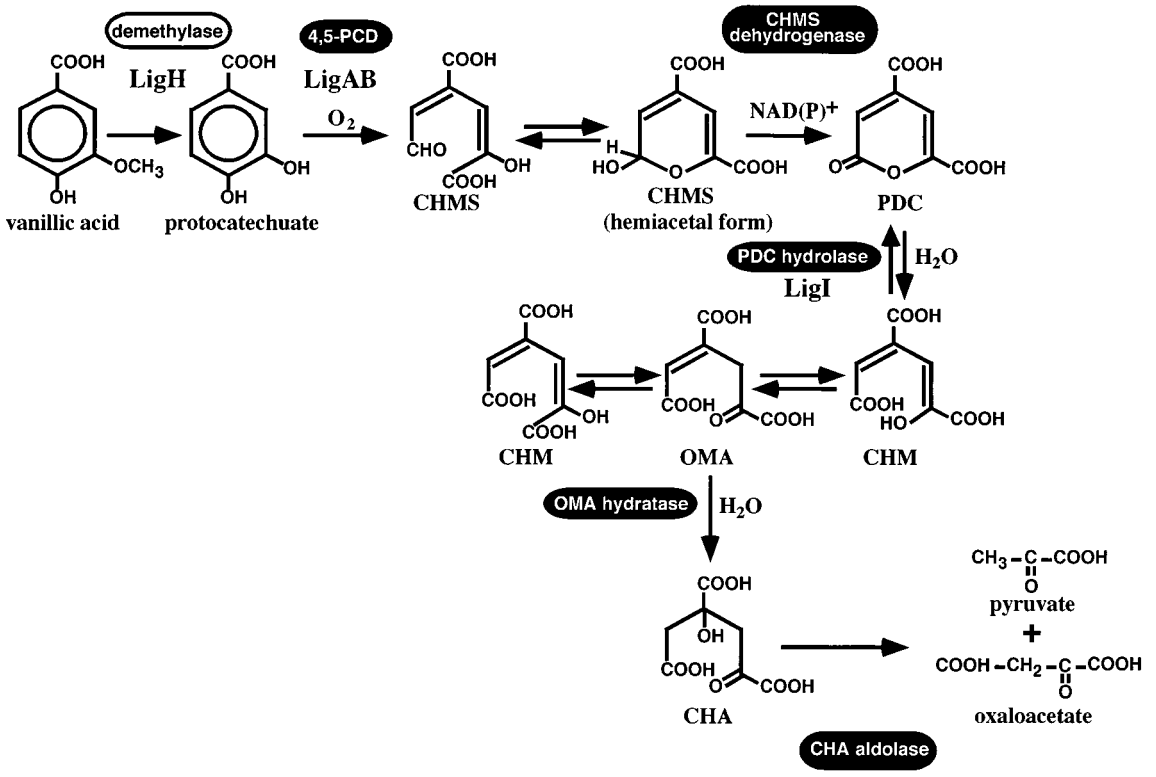

FIG. 1. The proposed degradation pathway of vanillic acid, including the protocatechuate 4,5-cleavage pathway in *S. paucimobilis* SYK-6. LigA and B, small and large subunits of 4,5-PCD (24); LigH, *O*-demethylase for vanillic acid and syringic acid (23); LigI, PDC hydrolase (this study). The PCA 4,5-cleavage pathway is illustrated according to findings from previous studies (13, 15–18).

testosteroni (13, 36) have been purified. However, little is known about the genes encoding these four enzymes.

In this paper, we present the structure of the PDC hydrolase gene and the biochemical properties of the gene product. The actual role of PDC hydrolase in the degradation of model lignin compounds by *S. paucimobilis* SYK-6 is also discussed.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *S. paucimobilis* SYK-6 was grown at 30°C in W minimal salt medium (27) containing 0.2% model lignin compounds, including vanillic acid and syringic acid, and in LB medium (Bacto Tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter).

Preparation of substrates. PDC was prepared from PCA by using cells of *P. putida* PpY1100 harboring pVAD4, which conferred transformation activity from PCA to PDC, but no PDC conversion activity. PCA appeared to be converted into PDC by 4,5-PCD and CHMS dehydrogenase encoded in pVAD4. PpY1100(pVAD4) was grown in 2 liters of W medium containing 0.2% succinate and 25 mg of kanamycin/liter for 20 h at 28°C. Cells were harvested by centrifugation, resuspended in 800 ml of W medium containing 10.4 mmol of PCA, and incubated for 20 h at 28°C. A culture was acidified to pH 1 and centrifuged to remove cells. Metabolites were extracted twice with 400 ml of ethyl acetate and dried in vacuo. The residue was dissolved in water, acidified to pH 1, and kept at 4°C for 3 days. The resultant white crystals were recovered, washed with 2 N HCl, and air dried. Gas chromatography and mass spectrometry (GC-MS) analysis of the trimethylsilylated (TMS) derivative of the product was carried out. The gas chromatogram showed a major peak at the retention time of 30.0 min. The mass spectrum of this peak had a molecular ion (M) at *m/z* 328, which corresponded to the expected molecular mass of the PDC TMS derivative. The product showed an absorption maximum at 312 nm in 50 mM Tris-HCl buffer (pH 8.5). In 1 N NaOH, the absorbance at 312 nm decreased and that at 353 nm increased. These characteristics correspond to the features of PDC reported by Maruyama (15, 16). Thus, it was concluded that the product obtained was PDC. Finally, 8.9 mmol of PDC was obtained from 10.4 mmol of PCA.

The PDC hydrolysate which is the substrate for the PDC synthesis was prepared according to the method of Maruyama (16). One millimole of PDC was incubated in 0.057 N NaOH at room temperature for 3 h and then neutralized with 0.5 N HCl. Vanillic acid and other chemicals were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan) or Wako Pure Chemical Industries (Osaka, Japan).

DNA manipulations and nucleotide sequencing. DNA manipulations were carried out essentially as described elsewhere (1, 29). A Kilosequence kit (Takara Shuzo Co., Ltd., Kyoto, Japan) was used to construct a series of deletion derivatives, whose nucleotide sequences were determined by the dideoxy termination method with an ALFexpress DNA sequencer (Pharmacia Biotech, Milwaukee, Wis.).

A Sanger reaction (30) was carried out by using the Thermosequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). Sequence analysis and homology alignment were carried out with the GeneWorks programs (IntelliGenetics, Inc., Mountain View, Calif.). The GenBank and SwissProt databases were used for searching homologous proteins. Southern hybridization analyses of SYK-6 and its PDC hydrolase gene (*ligI*) insertion mutants were performed with the DIG System (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the procedure recommended by the manufacturer.

Enzyme assays. According to the method of Maruyama (16), PDC hydrolysis and synthesis were spectrophotometrically determined by measuring the decrease and increase in the absorbance at 312 nm (ε_{312} = 6,600 M^{-1} cm^{-1}; pH 8.5), respectively, with a DU-7500 spectrophotometer (Beckman, Fullerton, Calif.). The reaction was carried out at 30°C in a cuvette. The 1-ml reaction mixture for PDC hydrolysis contained 100 μM PDC and the enzyme in 50 mM Tris-HCl buffer (pH 8.5). That for PDC synthesis contained 100 μM CHM and the enzyme in 50 mM sodium phosphate buffer (pH 7.0). One unit of the enzyme was defined as the amount that degraded 1 μmol of substrate per min at 30°C. Specific activity was expressed as units per milligram of protein. The optimum pHs for PDC hydrolysis and synthesis were examined in the pH range of 4.0 to 10.0 by using buffers consisting of 50 mM sodium acetate (pH 4.0 to 5.0), GTA (16.7 mM [each] 3,3-dimethylglutaric acid, Tris, and 2-amino-2-methyl-1,3-propanediol) (pH 4.0 to 8.5), sodium phosphate (pH 6.0 to 8.0), Tris-HCl (pH 8.0 to 9.0), or sodium borate (pH 9.0 to 10.0). K_m and V_{max} values were obtained from the Hanes-Woolf plots and expressed as means from at least three independent experiments.

Enzyme purification. Enzyme purification was performed according to the method described below by using a BioCAD700E apparatus (PerSeptive Biosystems, Framingham, Mass.).

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
S. paucimobilis		
SYK-6	Wild type; Nal^r Sm^r	12
DLI	Mutant derivative of SYK-6; Km^r gene insertion mutant of *ligI*; Nal^r Sm^r Km^r	This study
P. putida PpY1100	Nal^r Sm^r	12
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	39
Plasmids		
pUC18 and pUC19	Cloning vectors; Ap^r	39
pBluescript II KS(+)	Cloning vector; Ap^r	35
pK19*mobsacB*	*oriT sacB* Km^r	31
pKT230	Broad-host-range vector; Km^r	2
pHN139F	pUC18 with a 10.5-kb *Eco*RI fragment of SYK-6 carrying *ligAB* and *ligI*	This study
pHN139R	pUC18 carrying the same fragment as pHN139F in the opposite direction	This study
pSS50F	KS(+) with a 5.0-kb *Sma*I fragment carrying *ligI* of pHN139	This study
pSS50R	KS(+) carrying the same fragment as pSS50F in the opposite direction	This study
pDS15	Deletion derivative of pSS50R carrying *ligI*[a]	This study
pSS32F, pSS14F, pSS55F, pSS73F, pSS32R, pSS14R, pSS55R, pSS73R	Deletion derivatives of pHN139F and pHN139R[a]	This study
pUC1923	pUC19 with a 2.3-kb *Pst*I-*Sma*I fragment carrying *ligI*	This study
pUC1923K	pUC1923 with insertion of the Km^r gene of pUC4K into a *Stu*I site	This study
pLID1	pK19*mobsacB* with a 3.6-kb *Eco*RI-*Sma*I fragment of pUC1923K	This study
pVA01	pKT230 with a 10.5-kb *Eco*RI fragment carrying *ligAB* and *ligI*	12
pVAD4	Deletion plasmid of pVA01; *Sal*I fragments in the middle of the insert were deleted[a]	22

[a] See Fig. 2.

(i) Preparation of cell extract. Cells were grown in 2 liters of LB medium containing 100 mg of ampicillin/liter. Expression of the *ligI* gene was induced for 3.5 h by adding isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM) when the optical density at 660 nm (OD_{660}) of the culture reached 0.5. Cells were harvested by centrifugation and sonicated in 50 mM Tris-HCl buffer (pH 8.0) (buffer A). The cell lysate was centrifuged at 15,000 × *g* for 15 min. Streptomycin (final concentration, 1%) was added to the supernatant, and it was recentrifuged at 15,000 × *g* for 15 min to remove nucleic acids. The supernatant was then

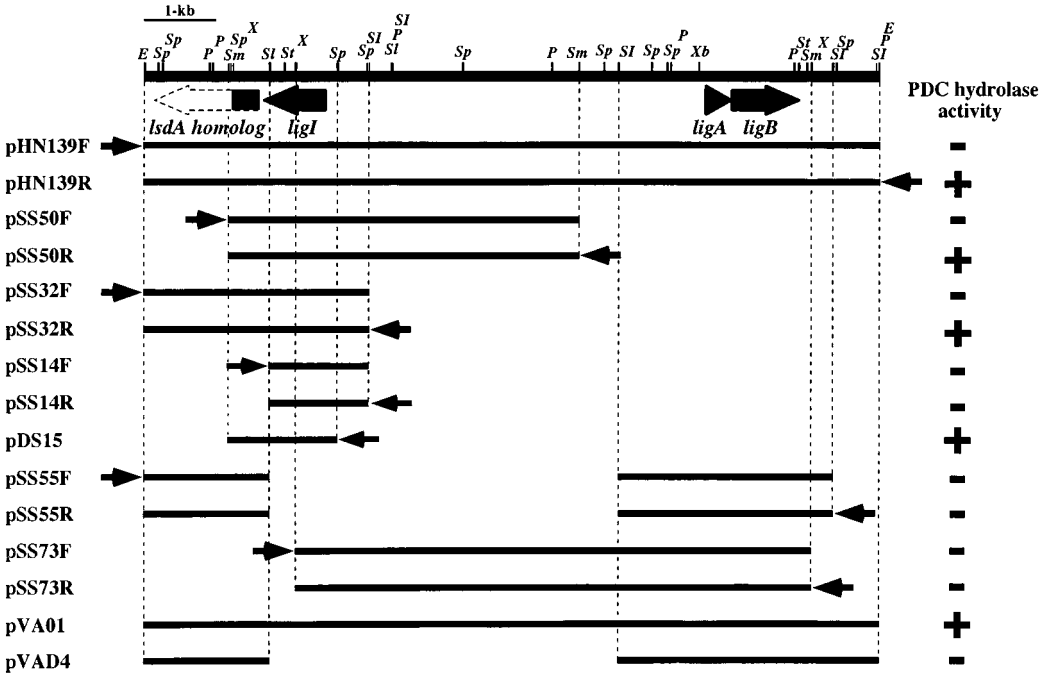

FIG. 2. Deletion analysis of the 10.5-kb *Eco*RI fragment and *lig* gene organization. The PDC hydrolase activities of the cells containing each subclone are presented on the right. The small arrows indicate the direction of transcription from the *lac* promoter. Large filled arrows, *ligI*, *ligA*, and *ligB* genes. A large partly filled arrow, the part of the ORF which showed a similarity with the LSD gene (*lsdA*) (9, 10). *E. coli* JM109 was used as a host strain except for pVA01 and pVAD4, for which *P. putida* PpY1100 was used. *E*, *Eco*RI; *P*, *Pst*I; *Sl*, *Sal*I; *Sm*, *Sma*I; *Sp*, *Sph*I; *St*, *Stu*I; *X*, *Xho*I; *Xb*, *Xba*I.

TABLE 2. Purification of PDC hydrolase from *E. coli* harboring pDS15

Fraction	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Fold
Crude extract	200	673	3.40	100	1.0
PI	14.0	158	11.3	23	3.3
HQ	2.00	104	52.7	15	16
HP2	0.50	66.0	128	10	38

centrifuged at 150,000 × *g* for 60 min at 4°C, and the crude extract was obtained after concentration by ultrafiltration using a YM-10 membrane (Amicon, Beverly, Mass.).

(ii) POROS PI anion-exchange chromatography. The crude extract was applied to a POROS PI (polyethyleneimine) column (4.6 by 100 mm) (PerSeptive Biosystems) previously equilibrated with buffer A. The enzyme was eluted with 25 ml of a linear gradient of 0 to 0.5 M NaCl. The PDC hydrolase was eluted at approximately 0.26 M.

(iii) POROS HQ anion-exchange chromatography. The fractions containing PDC hydrolase activity eluted from a PI column were pooled, desalted, and concentrated by ultrafiltration using a YM-10 filter. The resulting solution was applied to a POROS HQ (quaternized polyethyleneimine) column (4.6 by 100 mm) (PerSeptive Biosystems) previously equilibrated with buffer A. The enzyme was eluted with 33 ml of a linear gradient of 0 to 0.5 M NaCl. The fractions containing PDC hydrolase activity that eluted at approximately 0.2 M were pooled.

(iv) POROS HP2 hydrophobic interaction chromatography. The fractions containing PDC hydrolase activity were pooled, desalted, and concentrated by ultrafiltration using a YM-10 filter. Ammonium sulfate was added to the enzyme solution to a final concentration of 2 M. After centrifugation (at 3,000 × *g* for 10 min), the supernatant was collected and applied to a POROS HP2 (phenyl) column (4.6 by 100 mm) (PerSeptive Biosystems) equilibrated with buffer B (buffer A containing 2 M ammonium sulfate). The enzyme was eluted with 25 ml of a linear gradient of 2.0 to 0 M ammonium sulfate. The fractions containing PDC hydrolase activity that eluted at approximately 1.3 M were pooled. Glycerol was added to a final concentration of 10%, and the purified enzyme was stored at −80°C until use.

Analytical methods. The protein concentration was determined by the method of Bradford (3). The purity of the enzyme preparation was examined by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) (14). The molecular mass of the native enzyme was determined by Superdex200 HR10/30 (Pharmacia Biotech) gel filtration column chromatography using LC module 1 (Waters Corp., Milford, Mass.). Elution was performed with 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl with a flow rate of 0.25 ml/min. The molecular weight was estimated on the basis of the calibration curve of reference proteins.

To determine the N-terminal amino acid sequence, the purified enzyme was subjected to SDS–12% PAGE and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif.). The enzyme band was cut out and analyzed on a Procise 492 protein sequencer (Perkin-Elmer, Norwalk, Conn.). The isoelectric point of LigI was determined by isoelectric focusing with an Ampholine PAG plate (pH 3.5 to 9.5) (Pharmacia Biotech) using a model Multiphor II Electrophoresis system (Pharmacia Biotech).

The substrate and the reaction product compounds were identified by using a GC-MS (model 5971A) with an Ultra-2 capillary column (50 m by 0.2 mm) (Hewlett-Packard Co., Palo Alto, Calif.). The column temperature was increased initially from 100 to 150°C, and then from 150 to 300°C, at rates of 20 and 3°C per min, respectively. Temperatures of injection and detection were 220 and 300°C, respectively.

Identification of the reaction product. PDC was incubated with purified LigI (0.5 μg) in 50 mM Tris-HCl buffer (pH 8.5) for 1 h. After the decrease of the absorbance at 312 nm derived from PDC, the reaction mixture was acidified, extracted with ethyl acetate, and then trimethylsilylated. GC-MS analysis was carried out as described above.

Insertional inactivation of the *ligI* gene. The 2.3-kb *Pst*I-*Sma*I fragment carrying *ligI* was cloned into pUC19 to generate pUC1923. The 1.3-kb *Pst*I fragment containing the kanamycin resistance gene from pUC4K was inserted into *Stu*I in the middle of the *ligI* gene in pUC1923. The resultant plasmid, pUC1923K, was digested with *Eco*RI and *Pst*I, and the insert containing the inactivated *ligI* gene was cloned into pK19*mobsacB* (31) to generate pLID1.

When the cell density of SYK-6 cultured in 10 ml of LB reached 0.5 OD_{660} unit, the cells were harvested, washed twice with 1 ml of ice-cold 0.3 M sucrose, and resuspended with 300 μl of ice-cold 0.5 M sucrose. One microgram of pLID1 was mixed with 100 μl of cells. Electroporation was performed with a Gene Pulser (Bio-Rad) under the following conditions: 12-kV/cm field strength, 800-Ω resistor, and a 25-μF capacitor. After incubation in LB medium for 12 h, kanamycin-resistant transformants were selected on an LB agar plate containing 50 mg of kanamycin/liter. They were cultured for 12 h in LB liquid medium containing 10% sucrose. The candidates for mutants were isolated on an LB agar plate containing 10% sucrose and kanamycin in order to select the cells in which the *sacB*-containing vector portion was deleted by a double crossover. Southern hybridization analyses of the *Pst*I digests of total DNA prepared from the candidates for mutants were carried out with the 2.3-kb *Pst*I-*Sma*I and 1.3-kb *Pst*I fragment probes containing the *ligI* and the kanamycin resistance gene, respectively.

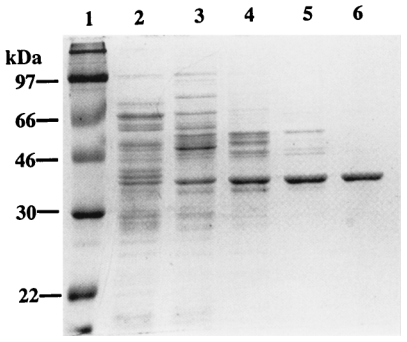

FIG. 3. SDS-PAGE analysis of protein fractions. Proteins were separated on an SDS–12% polyacrylamide gel and stained with Coomassie brilliant blue. Lanes: 1, molecular weight markers; 2, crude extract of *E. coli* JM109 harboring pBluescript II KS(+) (5 μg of protein); 3, crude extract of *E. coli* JM109 harboring pDS15 (5 μg of protein); 4, PI fraction (5 μg of protein); 5, HQ fraction (2 μg of protein); 6, HP2 fraction (2 μg of protein). Molecular masses are given on the left.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper was deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB015964.

RESULTS

Nucleotide sequence of the PDC hydrolase gene. The *ligAB* genes are located near the 3′ end of the SYK-6 10.5-kb *Eco*RI fragment (24). We examined PDC hydrolase activity on this 10.5-kb fragment, expecting to see evidence of the existence of the PDC hydrolase gene in it. PDC hydrolase activity was initially observed in *P. putida* PpY1100 containing pVA01, which has a 10.5-kb fragment inserted in a broad-host-range vector, pKT230. When *Sal*I fragments in the middle of the 10.5-kb insert were deleted from pVA01, no PDC hydrolase activity was detected or in the resultant plasmid, pVAD4. PDC hydrolase activity was found in *Escherichia coli* JM109 harboring pHN139R, which contained the 10.5-kb *Eco*RI fragment (Fig. 2). Among the deletion derivatives, pSS50R, pSS32R, and pDS15 conferred PDC hydrolase activity. These depended on the transcription from the *lac* promoter. The results obtained with the deletion derivatives showed the following features of the PDC hydrolase gene: (i) its direction of transcription is the same as that indicated for pSS32R in Fig. 2 and opposite that of the *ligAB* genes; (ii) it resides in the *Sph*I fragment of pDS15; and (iii) the 3′-terminal deletion beyond the *Sal*I site, which was accomplished in pSS14R, eliminated its function. The first feature suggests that the PDC hydrolase gene belongs to a transcriptional unit different from that of *ligAB*.

Deletion derivatives of pSS50F and pSS50R were generated, and the nucleotide sequence of the 1.5-kb *Sph*I fragment was determined. Only one open reading frame (ORF) of 879 bp, encoding 293 amino acid residues, had the three features mentioned above and was therefore designated *ligI*. A homology search for the deduced amino acid sequence with the SwissProt database revealed no similarity with any other proteins. This suggests that PDC hydrolase may constitute a unique class of hydrolases.

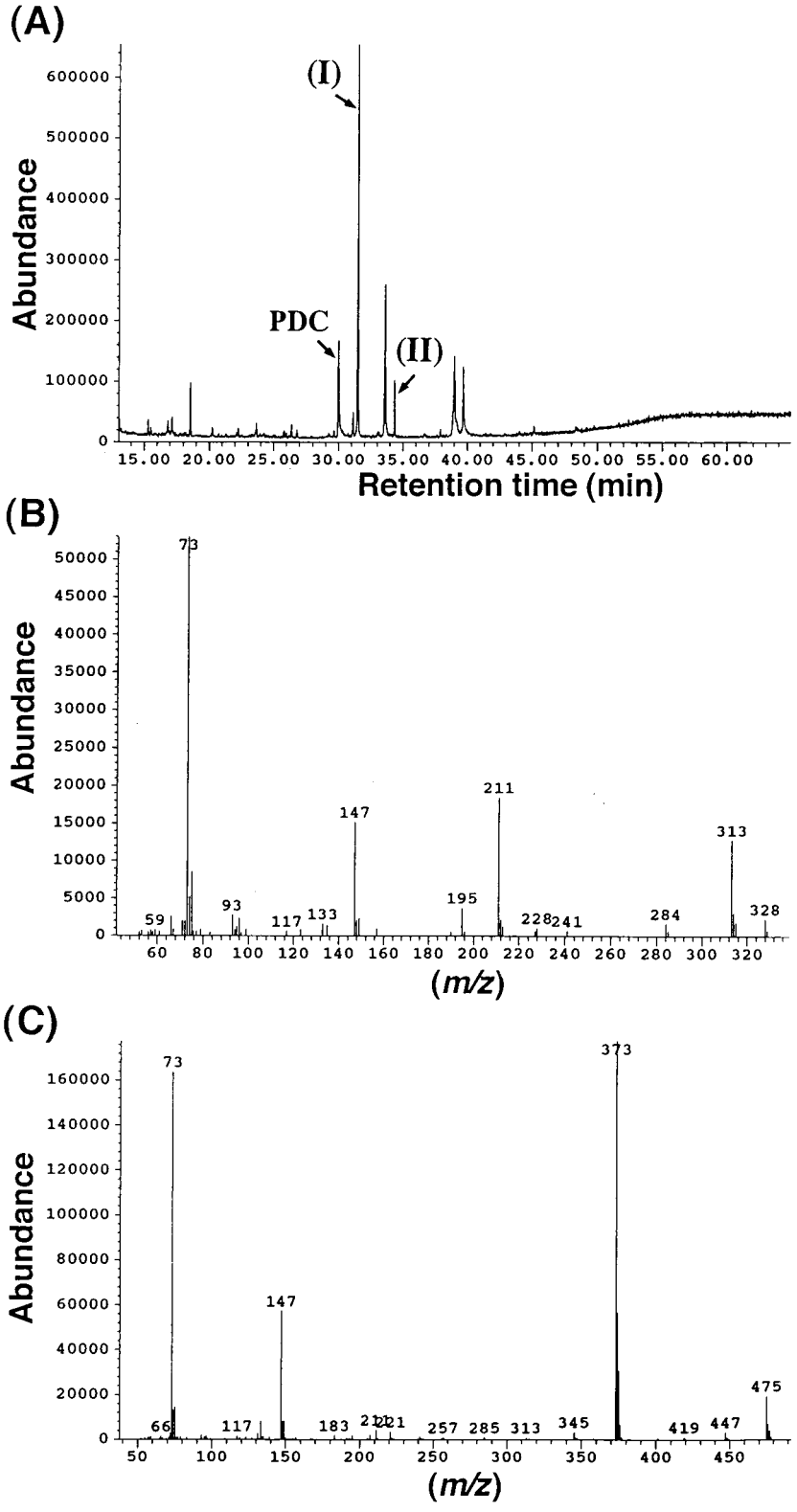

FIG. 4. Identification of the reaction product from PDC catalyzed by LigI. (A) Gas chromatogram of the TMS derivative of the reaction product from PDC catalyzed by purified LigI. PDC indicates the TMS derivative of PDC. Compounds I and II were the TMS derivatives of the reaction products originating from a substrate, PDC. (B) Mass spectrum of the TMS derivative of PDC. (C) Mass spectrum of compound I. The mass spectra of compounds I and II are identical. Compounds I and II were identified as isomeric forms of CHM.

TABLE 3. Effects of thiol reagents and metal ions on PDC hydrolase activity[a]

Reagent	Concn (mM)	Relative activity (%)	
		PDC hydrolysis	PDC synthesis
None		100	100
Ellman's reagent	0.1	10	20
N-Ethylmaleimide	1	5	6
Iodoacetic acid	1	90	90
$MnCl_2 \cdot 4H_2O$	0.2	100	100
$CoCl_2 \cdot 6H_2O$	0.2	100	100
$CuSO_4 \cdot 5H_2O$	0.2	100	40
$ZnSO_4 \cdot 7H_2O$	0.2	5	10

[a] Purified LigI (0.5 μg) was preincubated with each reagent at 30°C for 10 min, and the remaining activities were determined.

Purification of PDC hydrolase. *E. coli* JM109 harboring pDS15 was grown, and the *ligI* gene in pDS15 was expressed. The *ligI* gene product (LigI) was purified by a series of column chromatography with PI, HQ, and HP2. Table 2 and Fig. 3 summarize the results of a typical purification. LigI was purified approximately 38-fold, with a recovery of 10%. The purified enzyme was shown to be near homogeneity by SDS-PAGE (Fig. 3). The molecular mass of a subunit of LigI was estimated to be 38 kDa, which is close to the value deduced from the amino acid sequence of the *ligI* gene product (M_r, 32,737). The LigI protein in the HP2 fraction was subjected to N-terminal sequencing. The first six residues, M-T-N-D-E-R, corresponded to the deduced amino acid sequence of the *ligI* gene product.

Identification of the reaction product. Figure 4 shows the gas chromatogram and mass spectra of TMS derivatives of the reaction product of LigI from PDC. Two product peaks with retention times of 31.5 (I) and 34.4 (II) min were observed. The mass spectra of products I and II were identical. We extracted and analyzed the PDC alkaline hydrolysate according to the procedure reported by Maruyama (16). Two kinds of products yielding the same retention times and mass spectra as products I and II in Fig. 4 were observed (data not shown). The two major fragments at *m/z* 475 and 373 seemed to correspond to (M-CH_3) and (M-COOTMS), respectively (where M is a molecular ion of the TMS derivative of CHM and COO represents a carboxyl group). The M-CH_3 ion is generally found in mass spectra of TMS derivatives of organic acids. Based on these results, we concluded that products I and II are the stereoisomers of CHM.

Enzyme properties. The molecular mass of the native enzyme was estimated to be 31.6 kDa on a Superdex 200 gel filtration column. This suggests that LigI is a monomer. The isoelectric point of LigI was determined by isoelectric focusing to be 4.9.

It is known that PDC hydrolase catalyzes both PDC hydrolysis (production of CHM from PDC) and PDC synthesis (production of PDC from CHM) (13, 16). The pH optima for PDC hydrolysis and PDC synthesis by LigI were examined. The enzyme exhibited the highest activity at pH 8.5 for PDC hydrolysis and at pH 6.0 to 7.5 for PDC synthesis. These pH optima were similar to those of the PDC hydrolase of *P. ochraceae* (16). The ratio of PDC hydrolysis activity to PDC synthesis activity was 2.6 at pH 8.5. LigI showed maximum hydrolase activity at 50°C. The activity at 50°C, however, was only 1.2 times higher than that at 30°C. Therefore, the following experiments were carried out at 30°C, which is the physiological temperature for *S. paucimobilis* SYK-6.

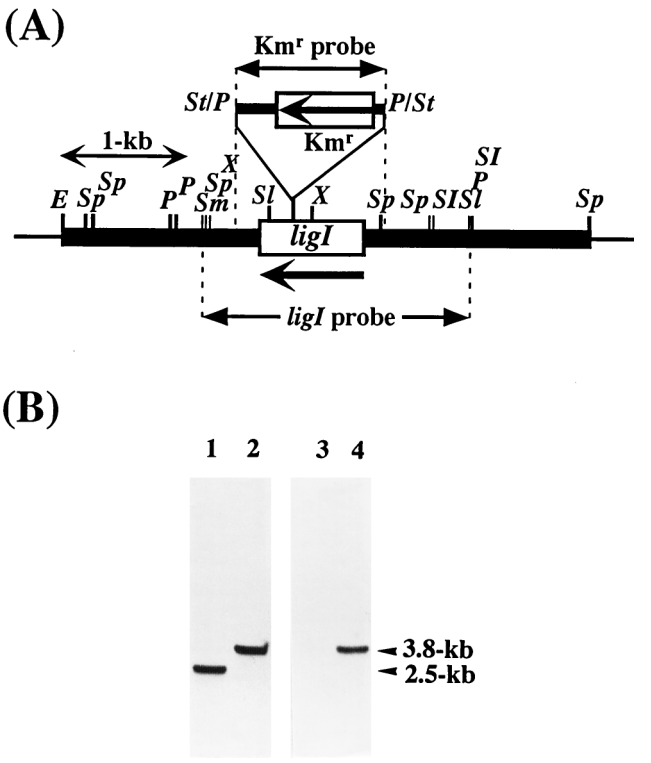

FIG. 5. Insertional inactivation of the *ligI* gene in *S. paucimobilis* SYK-6. (A) Schematic representation of the insertional inactivation of *ligI* by the kanamycin resistance gene from pUC4K. Thick arrows, orientation of transcription of the *ligI* and kanamycin resistance genes. *E*, *Eco*RI; *P*, *Pst*I; *Sl*, *Sal*I; *Sm*, *Sma*I; *Sp*, *Sph*I; *St*, *Stu*I; *X*, *Xho*I. (B) Southern hybridization analysis of the *ligI* insertion mutant (DLI). Lanes 1 and 3, total DNA of SYK-6 digested with *Pst*I; lanes 2 and 4, total DNA of DLI digested with *Pst*I. The 2.3-kb *Pst*I-*Sma*I fragment carrying *ligI* (lanes 1 and 2) and the 1.3-kb *Pst*I fragment of the kanamycin resistance gene (lanes 3 and 4) were used as probes.

The K_m and V_{max} values were estimated for PDC hydrolysis and PDC synthesis. The K_m for PDC was 73.8 μM. For CHM, it was 48.5 μM. The V_{max} for PDC hydrolysis was 506 U/mg. For PDC synthesis, it was 283 U/mg.

Effects of thiol reagents and metal ions. The influence of thiol reagents 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) and *N*-ethylmaleimide on the activity for PDC hydrolysis and PDC synthesis was examined (Table 3). These thiol reagents strongly inhibited both PDC hydrolysis and PDC synthesis activity, suggesting that the cysteine residue is involved in the enzyme reaction. The effects of metal ions on LigI activity are summarized in Table 3. Zn^{2+} strongly inhibited both PDC hydrolysis and PDC synthesis. Cu^{2+} inhibited only PDC synthesis. In the case of the *P. ochraceae* enzyme, Mn^{2+} and Co^{2+} inhibited PDC hydrolysis, and inhibition by Zn^{2+} and Cu^{2+} was similar to that for the SYK-6 enzyme (16). The SYK-6 PDC hydrolase was therefore shown to be more resistant to metal ions than the *P. ochraceae* enzyme.

***ligI* disruption in *S. paucimobilis* SYK-6.** Inactivation of the *ligI* gene by the insertion of the kanamycin resistance gene was performed by using the *ligI* disruption plasmid pLID1 as described in Materials and Methods. The *ligI* insertion mutant was confirmed by Southern hybridization analysis (Fig. 5). The mutant strain DLI, grown on LB medium or syringic acid, showed no PDC transformation activity and no PDC hydrolysis. This strain completely lost the ability to grow on vanillic acid, even though it grew as well on syringic acid as the wild-

TABLE 4. Characteristics of PDC hydrolases of *S. paucimobilis* SYK-6, *P. ochraceae*, and *C. testosteroni*[a]

Strain or species	Molecular mass (kDa)	pI	Optimum pH		Kinetic parameter			
			PDC hydrolysis	PDC synthesis	PDC hydrolysis		PDC synthesis	
					K_m (μM)	V_{max} (U/mg)	K_m (μM)	V_{max} (U/mg)
SYK-6	32 (monomer)	4.90	8.5	6.0–7.5	74	506	49	283
P. ochraceae	31 (monomer)	5.49	8.5	6.0–7.5	87	476	26	127
C. testosteroni	37 (monomer)	ND[b]	8.4–8.8	ND	90	ND	ND	ND

[a] The results for the *P. ochraceae* and *C. testosteroni* enzymes have been reported in previous studies (13, 16).
[b] ND, not determined.

type strain. The metabolite of vanillic acid generated by the whole cells of DLI grown on LB medium was examined by GC-MS analysis. After 22 h of incubation, vanillic acid was transformed completely, and only the accumulation of PDC was observed.

DISCUSSION

This is the first report on the genetic analysis of PDC hydrolase, which is one of the protocatechuate 4,5-cleavage pathway enzymes. The PDC hydrolase gene of *S. paucimobilis* SYK-6, designated *ligI*, encodes a protein of 32,737 Da (293 amino acids). There was no similarity between the LigI amino acid sequence and those of the proteins in the databases, including the dienelactone hydrolase (4, 28, 37) and the β-ketoadipate enol-lactone hydrolase (7, 33), which seemed to be functionally related to LigI.

The *ligI* gene is located approximately 5.4 kb upstream of *ligA*. Interestingly, *ligI* is transcribed divergently from *ligAB*. This fact indicated that the PCA 4,5-cleavage pathway was composed of at least two distinct operons. We could not find other genes responsible for the PCA 4,5-cleavage pathway enzymes in the region sequenced. Downstream from *ligI*, an incomplete ORF which had the same direction of transcription as *ligI* was found. The predicted amino acid sequence of the product of this ORF showed significant similarity to those of the lignostilbene-α,β-dioxygenase (LSD) genes of *Pseudomonas paucimobilis* TMY1009 (9, 10). LSD has been reported to be a dioxygenase catalyzing the cleavage of the interphenyl double bond of lignostilbenes. The occurrence of the LSD gene homolog beside the PCA 4,5-cleavage pathway enzyme genes is interesting. Further nucleotide sequencing and functional analysis of the 10.5-kb *Eco*RI fragment may address the functions of a putative LSD and other enzymes of the PCA 4,5-cleavage pathway.

The reaction product from PDC, catalyzed by LigI, was estimated to be two stereoisomers of CHM. OMA, which is a tautomer of CHM was not detected. The production of OMA from PDC was suggested by Maruyama (16). OMA is an α-keto acid, and its TMS derivative is easily distinguishable in MS from that of CHM, because a keto group of OMA is not trimethylsilylated. OMA might have been degraded during the process of extraction, since α-keto acid is generally unstable.

The characteristics of the PDC hydrolases of *S. paucimobilis* SYK-6, *P. ochraceae*, and *C. testosteroni* are summarized in Table 4. All enzymes are monomeric proteins. The molecular mass and pH optima of the SYK-6 enzyme are very similar to those of the *P. ochraceae* enzyme. A higher affinity with CHM (or OMA) than with PDC and a higher V_{max} for PDC hydrolysis than for PDC synthesis were common features of the PDC hydrolases of SYK-6 and *P. ochraceae*. Thiol reagents, such as Ellman's reagent and *N*-ethylmaleimide, strongly inhibited activity, suggesting that the cysteine residue is the catalytic site of these three enzymes. A similar inhibition was also observed in the *P. ochraceae* and *C. testosteroni* enzymes. In the α/β hydrolase fold enzymes, the catalytic nucleophile is located in a highly conserved peptide, Gly-X-(Ser/Cys)-X-Gly. The three-dimensional structure of the dienelactone hydrolase from *Pseudomonas* sp. strain B13 was solved, and its catalytic nucleophile, cysteine residue 123, was shown to constitute part of a catalytic triad of residues (26). Recently, Schrag and Cygler reported that a consensus sequence of these enzymes might better be described as Sm (small amino acid)-X-Nuc (nucleophile)-X-Sm, since the glycine residues at Nuc −2 and Nuc +2 are sometimes substituted for other small amino acids, including alanine and serine (32). Among the three cysteine residues of LigI, Cys76 may be the catalytic site, since the sequence Ala74-Ser75-Cys76-His77-Gly78 corresponds to the consensus of Sm-X-Nuc-X-Sm.

The *ligI* gene insertion mutant, DLI, showed no PDC hydrolase activity when the DLI cells were grown on LB medium and on syringic acid. DLI was not able to grow on vanillic acid, and the cells of DLI grown on LB medium accumulated PDC from vanillic acid. These results indicated that the *ligI* gene is unique in conferring PDC hydrolysis and is essential for growth of SYK-6 on vanillic acid. According to the proposed metabolic pathway of syringic acid in SYK-6, syringic acid is converted to PDC via 3-*O*-methylgallic acid (11, 23). The production of 3-*O*-methylgallic acid from syringic acid was evident from the results obtained with the *ligH* gene. The mutation in the *ligH* gene, the product of which is involved in the conversion of syringic acid to 3-*O*-methylgallic acid, resulted in a growth defect on syringic acid (23). Kersten et al. reported the production of PDC from 3-*O*-methylgallic acid by 4,5-PCD (13). However, the *ligI* insertion mutant DLI grew on syringic acid and showed no PDC transformation activity and no PDC hydrolysis. These results obviously suggest that syringic acid is not metabolized via PDC and that neither 4,5-PCD nor LigI is involved in the metabolism of syringic acid.

The results obtained in this study strongly support the proposed PCA 4,5-cleavage pathway presented in Fig. 1. Further research is needed to elucidate the correct degradation pathway of syringic acid in SYK-6.

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