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The 4-Oxalomesaconate Hydratase Gene, Involved in the Protocatechuate 4,5-Cleavage Pathway, Is Essential to Vanillate and Syringate Degradation in *Sphingomonas paucimobilis* SYK-6

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***Sphingomonas paucimobilis* SYK-6 is able to grow on various dimeric lignin compounds, which are converted to vanillate and syringate by the actions of unique lignin degradation enzymes in this strain. Vanillate and syringate are degraded by the *O*-demethylase and converted into protocatechuate (PCA) and 3-*O*-methylgallate (3MGA), respectively. PCA is further degraded via the PCA 4,5-cleavage pathway, while the results suggested that 3MGA is degraded through another pathway in which PCA 4,5-dioxygenase is not involved. In a 10.5-kb *Eco*RI fragment carrying the genes for PCA 4,5-dioxygenase (*ligAB*), 2-pyrone-4,6-dicarboxylate hydrolase (*ligI*), and a portion of 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase (*ligC*), we found the *ligJ* gene encoding 4-oxalomesaconate (OMA) hydratase, which catalyzes the conversion of OMA into 4-carboxy-4-hydroxy-2-oxoadipate. The *ligJ* gene is transcribed in the same direction as *ligABC* genes and consists of an 1,023-bp open reading frame encoding a polypeptide with a molecular mass of 38,008 Da, which is located 73-bp upstream from *ligA*. The *ligJ* gene product (LigJ), expressed in *Escherichia coli*, was purified to near homogeneity and was estimated to be a homodimer (69.5 kDa) by gel filtration chromatography. The isoelectric point was determined to be 4.9, and the optimal temperature is 30°C. The K_m for OMA and the V_{max} were determined to be 138 μM and 440 U/mg, respectively. LigJ activity was inhibited by the addition of thiol reagents, suggesting that some cysteine residue is part of the catalytic site. The *ligJ* gene disruption in SYK-6 caused the growth defect on and the accumulation of common metabolites from both vanillate and syringate, indicating that the *ligJ* gene is essential to the degradation of these two compounds. These results indicated that syringate is converted into OMA via 3MGA, and it enters the PCA 4,5-cleavage pathway.**

Lignin is the most abundant aromatic compound on the earth, and its mineralization is a fundamental step in the terrestrial carbon cycle. It is expected that lignin can be used as biomass by converting it to valuable materials. Bacterial enzyme systems for lignin degradation and modification are of great use for this purpose. *Sphingomonas paucimobilis* SYK-6 is able to grow on various dimeric lignin compounds, including β-aryl ether, biphenyl, and diarylpropane, as sole carbon and energy sources (18). We have characterized the enzymes and genes involved in β-aryl ether cleavage (16, 17) and biphenyl degradation (23, 24), which include essential and late limiting steps of lignin degradation, respectively. These unique specific lignin degradation enzymes in SYK-6 would be suitable tools for conversion of lignin to useful intermediate metabolites.

Vanillate and syringate are important intermediate metabolites from lignin, having guaiacyl and syringyl moieties, respectively. In SYK-6, vanillate and syringate are converted to protocatechuate (PCA) and 3-*O*-methylgallate (3MGA), respectively, by the *O*-demethylase encoded by *ligH* (21). PCA is a key intermediate metabolite among various aromatic degradation pathways. Three kinds of dioxygenases are involved in the aromatic ring cleavage of PCA: PCA 3,4-dioxygenase (5, 6, 37), PCA 4,5-dioxygenase (22, 31), and PCA 2,3-dioxygenase (35). In the case of SYK-6, PCA is metabolized through the PCA 4,5-cleavage pathway (Fig. 1), which was enzymatically characterized in 1980s by Kersten *et al.* (8) and Maruyama and colleagues (11–15). PCA is initially transformed to 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS) by PCA 4,5-dioxygenase (LigAB). CHMS is nonenzymatically converted to an intramolecular hemiacetal form and then oxidized by CHMS dehydrogenase (11, 15). The resulting intermediate, 2-pyrone-4,6-dicarboxylate (PDC), is hydrolyzed by PDC hydrolase to yield the keto form and enol form (4-carboxy-2-hydroxymuconate) of 4-oxalomesaconate (OMA), which are in equilibrium (8, 12, 19). OMA is converted to 4-carboxy-4-hydroxy-2-oxoadipate (CHA) by OMA hydratase (13). Finally, CHA is cleaved by CHA aldolase to produce pyruvate and oxaloacetate (14, 32). We previously characterized the PCA 4,5-dioxygenase gene (*ligAB*) (22, 31) and PDC hydrolase gene (*ligI*) (19); recently, the CHMS dehydrogenase gene (*ligC*) was also characterized (E. Masai, K. Momose, H. Hara, S. Nishikawa, Y. Katayama, and M. Fukuda, submitted for publication). However, the PCA 4,5-cleavage pathway has not been genetically characterized in detail.

On the other hand, the pathway for 3MGA degradation is ambiguous. PCA 4,5-dioxygenase was reported to catalyze the ring cleavage of 3MGA to form PDC, and metabolism of 3MGA through the PCA 4,5-cleavage pathway was suggested (8). However, two mutant strains of SYK-6, in which the *ligAB* and *ligI* genes were insertionally inactivated could grow on syringate but not on vanillate (19; H. Aoshima, E. Masai, S. Nishikawa, Y. Katayama, and M. Fukuda, Abstr. 8th Int. Symp. Microb. Ecol., abstr. 93, 1998). These results indicated that 3MGA generated from syringate is predominantly metabolized via a pathway other than the PCA 4,5-cleavage pathway.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
Sphingomonas paucimobilis		
SYK-6	Wild type; Nal^r Sm^r	7
DLJ	Mutant derivative of SYK-6; Km^r gene insertion mutant of *ligJ*; Nal^r Sm^r Km^r	This study
Escherichia coli		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	36
BL21(DE3)	*hsdS gal*(λcI*ts857 ind1 Sam7 nin5 lac*UV5-T7 *gene 1*)	30
Plasmids		
pUC18 and pUC19	Cloning vectors; Ap^r	36
pBluescript II KS(+)	Cloning vector; Ap^r	29
pT7-blue(R)	TA cloning vector; Ap^r	3
pET21(+)	Expression vector; Ap^r T7 promoter	Novagen
pUC4K	Source of Km^r cassette; Ap^r Km^r	33
pK19*mobsacB*	*oriT sacB* Km^r	27
pHN139F	pUC18 with a 10.5-kb *Eco*RI fragment of pVA01 carrying *ligJAB*, a part of *ligC*, and *ligI*	19
pHN139R	pUC18 carrying the same fragment as pHN139F in the opposite direction	19
pSS30F	pBluescript II KS(+) with a 3.0-kb *Sal*I fragment carrying *ligJ* of pHN139F	This study
pSS30R	pBluescript II KS(+) carrying the same fragment as pSS30F in the opposite orientation	This study
pSS73F, pSS73R, pSS50F, pSS50R, pSS17F, pSS17R, pSS20F, pSS20R	pBluescript II KS(+) with deletion fragments of pHN139F and pHN139R (Fig. 1)	19, this study
pSXB17	pBluescript II KS(+) with a 1.7-kb *Sma*I-*Xba*I fragment carrying a portion of *ligJ*	This study
pETJ	pET21(+) with a 1.1-kb PCR-amplified fragment carrying *ligJ*	This study
pSXB17K	pSXB17 with an insertion of the Km^r gene of pUC4K replacing a 500-bp *Eco*47III fragment	This study
pLJD	pK19*mobsacB* with a 2.4-kb *Sma*I-*Xba*I fragment of pSXB17K	This study

In this study, we characterized the OMA hydratase gene and the enzymatic properties of the gene product to obtain detailed genetic information on the PCA 4,5-cleavage pathway and insight into the metabolism of syringate in SYK-6. We also present evidence that the OMA hydratase gene is essential to the metabolism of both vanillate and syringate and that OMA is the common intermediate metabolite of vanillate and syringate.

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 (23) containing 0.2% (wt/vol) vanillate or syringate or in Luria-Bertani (LB) medium (Bacto Tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter).

Preparation of substrate. PDC was prepared from PCA by using cells of *Pseudomonas putida* PpY1100 harboring pVAD4, which conferred transformation activity from PCA to PDC as described earlier (19). To obtain OMA, 1 mmol of PDC was hydrolyzed by 0.057 N NaOH at room temperature for 3 h and then neutralized with 0.5 N HCl by the method of Maruyama (12). In a previous study, we identified the trimethylsilyl (TMS) derivatives of two isomeric enol forms of OMA (19). In this study, the OMA preparation was derivatized with methoxyamine hydrochloride to identify the keto form of OMA because α-keto acids are difficult to analyze by gas chromatography-mass spectrometry (GC-MS). Methoxyamine hydrochloride (final concentration, 10 mg/ml) was added to 200 μM OMA solution. The resultant solution was alkalinized (pH 11 to 12) and kept at 60°C for 1 h (34). This solution was acidified by 2 N HCl, and the derivatized OMA was extracted with ethyl acetate. The extract was dried in vacuo and trimethylsilylated. The gas chromatogram of the sample showed two peaks with retention times of 29.5 min (compound I) and 30.2 min (compound II). The mass spectrum of compound I corresponded to that of the enol form OMA (19). On the other hand, the mass spectrum of compound II showed the major fragments at *m/z* 447, 432, 416, and 330, which seemed to correspond to the molecular ions of methoxime-TMS derivatives of the keto form OMA (M), M-CH_3, M-OCH_3, and M-COOTMS, respectively. Thus, the OMA preparation contains the two isomeric enol forms and the keto form as shown in Fig. 1.

Vanillate, syringate, 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, 25). 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 (26) was carried out by using a 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 DDBJ database was used for searching homologous proteins. Southern hybridization analysis of SYK-6 and its OMA hydratase gene (*ligJ*) insertion mutants were performed with the DIG system (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the procedure recommended by the manufacturer.

Subcloning of *ligJ* gene. To overexpress the *ligJ* gene, the *ligJ* coding region in pHN139F was amplified by PCR using the Ex *Taq* polymerase (Takara Shuzo), pHN139F as a template, and primers ligJF and ligJR. Primers ligJF (forward; GAGACGATCACGAGAGGTAACC) and ligJR (reverse; GAAATCACGGG AAACAAAGC) were designed from the pHN139F *ligJ* coding sequence.

The 1.1-kb PCR product was inserted into pT7-blue(R) (3). Then the *Eco*RI-*Hin*dIII fragment of the resulting plasmid was ligated to pET21(+) to generate pETJ.

Enzyme assay. Using the method of Maruyama (13), OMA hydratase activity was spectrophotometrically determined by measuring the decrease in the absorbance at 265 nm ($\varepsilon_{265} = 2.6 \times 10^3\ M^{-1}\ cm^{-1}$; pH 8.0) with a DU-7500 spectrophotometer (Beckman, Fullerton, Calif.). The enzyme reaction was carried out at 30°C in a cuvette. The 1-ml reaction mixture contained 200 μM OMA and the enzyme in 0.1 M Tris-acetate buffer (pH 8.0). The OMA preparation contained the keto form and enol form (4-carboxy-2-hydroxymuconate), which are in equilibrium. One unit of enzyme activity was defined as the amount that degrades 1 μmol of substrate per min at 30°C. Specific activity was expressed as units per milligram of protein. K_m and V_{max} values were obtained from 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.).

(i) Preparation of cell extract. Cells were grown in 100 ml of 2×YT medium (Bacto Tryptone, 20 g/liter; yeast extract, 10 g/liter; NaCl, 5 g/liter) containing 100 mg of ampicillin/liter. Expression of *ligJ* was induced for 12 h at 30°C by the addition of isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM). Cells were harvested by centrifugation and resuspended in 20 mM Tris-HCl buffer (pH 8.0) (buffer A). The cells were broken by two passages through a French pressure cell. The cell lysate was centrifuged at 15,000 × *g* for 15 min. Streptomycin (final concentration, 1%) was added to the supernatant, which was recentrifuged at 15,000 × *g* for 15 min to remove nucleic acids. The supernatant was recovered and then centrifuged again at 170,000 × *g* for 60 min at 4°C. The

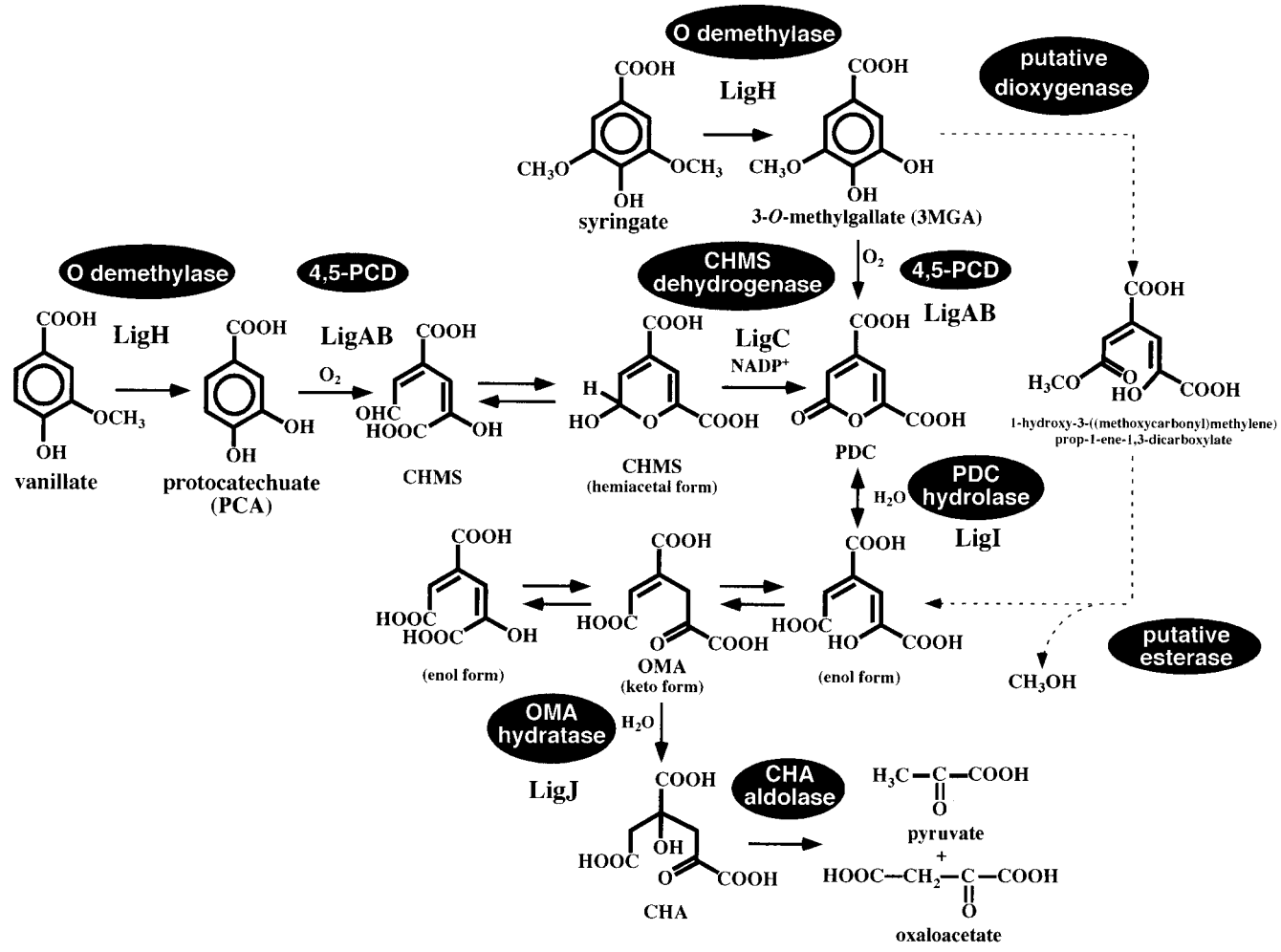

FIG. 1. The proposed degradation pathway of vanillate and syringate via the PCA 4,5-cleavage pathway in *S. paucimobilis* SYK-6. LigA and LigB, the small and large subunits of PCA 4,5-dioxygenase (4,5-PCD) (22); LigH, a gene product essential for vanillate and syringate O-demethylations (21); LigC, CHMS dehydrogenase (Masai et al., submitted); LigI, PDC hydrolase (19); LigJ, OMA hydratase (this study). The PCA 4,5-cleavage pathway is illustrated according to findings from previous studies (11–15, 19, 22). The degradation pathway for syringate indicated by a dashed line was suggested on the basis of the results obtained in this and a previous (4) study.

crude extract was obtained after concentration by ultrafiltration using a Minicon B15 (Amicon, Beverly, Mass.).

(ii) POROS PI anion-exchange chromatography. The crude extract was applied to a POROS PI (polyethyleneimine) column (7.5 by 100 mm; PerSeptive Biosystems) previously equilibrated with buffer A. The enzyme was eluted with 88 ml of linear gradient of 0 to 0.5 M NaCl. The OMA hydratase was eluted at approximately 0.34 M.

(iii) POROS HQ anion-exchange chromatography. The fractions containing OMA hydratase activity eluted from a PI column were pooled, desalted, and concentrated by ultrafiltration using a Minicon B15. The resulting solution was applied to a POROS HQ (quaternized PI) 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 OMA hydratase activity that eluted at approximately 0.28 M were pooled.

(iv) POROS PE hydrophobic interaction chromatography. The fractions containing OMA hydratase activity eluted from a HQ column were pooled, desalted, and concentrated. Ammonium sulfate was added to the enzyme solution to a final concentration of 2 M. After centrifugation at 15,000 × *g* for 10 min, the supernatant was recovered and applied to a POROS PE 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 OMA hydratase activity that eluted at approximately 1.1 M were pooled, desalted, and concentrated as described above. Glycerol was added to a final concentration of 10%, and the purified enzyme was stored at −80°C until use.

Analytical method. The protein concentration was determined by the method of Bradford (2). The purity of the enzyme preparation was examined by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) (10). The molecular mass of the native enzyme was determined by Superdex 200 HR10/30 (Pharmacia Biotech) gel filtration column chromatography using a BioCAD700E apparatus. Elution was performed with 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 0.8 ml/min. The molecular weight was estimated on the basis of calibration curve of reference proteins.

To determine the N-terminal amino acid sequence, a cell extract of *Escherichia coli* BL21(DE3) harboring pETJ was subjected to SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif.). The area at 35 kDa was cut out and analyzed on a PPSQ-21 protein sequencer (Shimadzu, Kyoto, Japan). The isoelectric point of LigJ was determined by isoelectric point focusing on 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 products were detected and identified by GC-MS using model 5971A with an Ultra-2 capillary column (50 m by 0.2 mm; Hewlett-Packard Co., Palo Alto, Calif.) and electrospray ionization (ESI)-MS using HP1100 series LC-MSD (Hewlett-Packard Co.). The analytical conditions for GC-MS were the same as described previously (19). In ESI-MS analysis, mass spectra were obtained by negative-mode ESI, with a needle voltage of −3.5 kV and a source temperature at 350°C. The sample was injected into the flow system; the water/methanol ratio was 90:10 (vol/vol), and the flow rate was 0.2 ml/min.

Identification of the reaction product. OMA was incubated with purified LigJ (0.5 μg) in 0.1 M Tris-acetate buffer (pH 8.0) for 10 min. The reaction mixture was acidified and extracted with ethyl acetate, and then the extract was trimethylsilylated. The resultant TMS derivatives are analyzed by GC-MS as described above.

In the case of ESI-MS analysis, the reaction mixture was diluted to 1/10 with

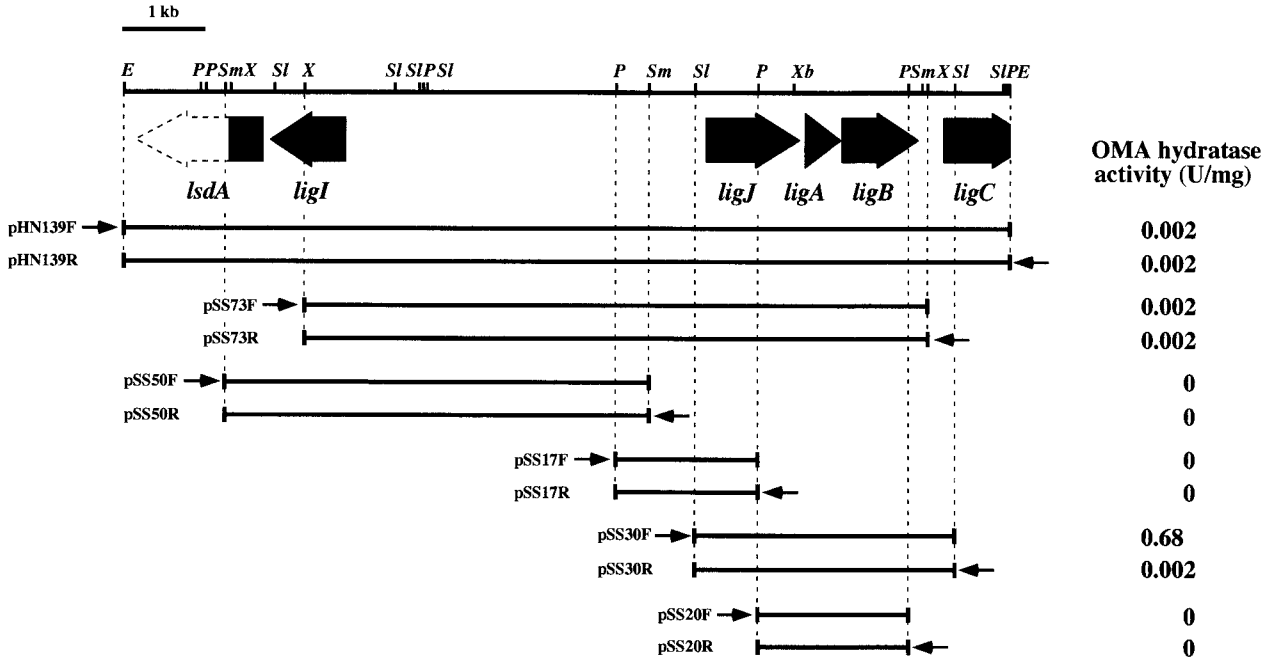

FIG. 2. Deletion analysis of the 10.5-kb *Eco*RI fragment and *lig* gene organization. The OMA hydratase activity of cells containing each subclone is presented on the right. Arrows indicate the direction of transcription from the *lac* promoters. The *ligI, ligJ, ligA, ligB*, and *ligC* genes are indicated by filled arrows; a partly filled arrow represents the part of the ORF of the lignostilbene α, β-dioxygenase homolog (*lsdA*). Each plasmid was introduced in *E. coli* JM109. *E*, *Eco*RI; *P*, *Pst*I; *Sl*, *Sal*I; *Sm*, *Sma*I; *X*, *Xho*I; *Xb*, *Xba*I.

10 mM Tris-acetate buffer (pH 8.0), and 5 μl of the mixture was injected into the flow system.

Insertional inactivation of the *ligJ* gene. The 1.7-kb *Sma*I-*Xba*I fragment carrying a portion of *ligJ* was cloned into pBluescript II KS(+) to generate pSXB17. pSXB17 was digested with *Eco*47III, and the 500-bp fragment in the middle of *ligJ* was deleted. The 1.2-kb *Pst*I fragment containing the kanamycin resistance (Km^r) gene from pUC4K (33) was inserted into this *Eco*47III site. The resultant plasmid, pSXB17K, was digested with *Eco*RI and *Kpn*I, and the insert containing the inactivated *ligJ* gene was cloned into pK19*mobsacB* (27) to generate pLJD.

pLJD was introduced into SYK-6 cells by electroporation as described previously (19). Km^r 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 50 mg of kanamycin/liter and 10% sucrose. The candidates for mutants were isolated on an LB agar plate containing 10% sucrose and kanamycin. Southern hybridization analyses of the *Sal*I digests of total DNA prepared from the candidates for mutants were carried out with the 1.2-kb *Sal*I-*Xba*I and 1.2-kb *Pst*I fragment probes containing a portion of *ligJ* and the Km^r gene, respectively.

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. AB035121.

RESULTS

Nucleotide sequence of the OMA hydratase gene. We have already isolated the SYK-6 10.5-kb *Eco*RI fragment carrying *ligAB* (22), *ligI* (19), and a part of *ligC* (18; Masai et al., submitted). We examined the OMA hydratase activity on this fragment to see whether the OMA hydratase gene is located in it. The OMA hydratase activity was observed in *E. coli* JM109 harboring pHN139F, which contained this 10.5-kb *Eco*RI fragment (Fig. 2). In the deletion analysis, the DNA region that conferred OMA hydratase activity was limited to the 2.7-kb *Sal*I-*Xho*I fragment, which spanned the most of the insert of pSS30F and pSS30R. The *ligAB* gene was included in this 2.7-kb fragment as indicated in Fig. 2. Therefore, the OMA hydratase gene seemed to be located upstream from the *ligA* gene. Based on the difference of the activity between pSS30F and pSS30R, the direction of transcription of OMA hydratase gene was suggested to be the same as that of *ligAB*. The deletion derivatives of pSS30F were constructed, and the nucleotide sequence of the region except for the *ligAB* gene, whose sequence had been established previously (22), was determined. The only open reading frame (ORF) found was 73 bp upstream from *ligA* and designated *ligJ*. The start codon of *ligJ* could not be deduced because the 5′ end of the *ligJ* sequence has three consecutive ATG codons. The *ligJ* gene spans bp 1017 to 1023 and has a putative ribosome binding sequence upstream.

Expression of *ligJ* in *E. coli*. The *ligJ* gene was amplified by PCR and subcloning in pET21(+) to generate plasmid pETJ as described in Materials and Methods. The *ligJ* gene was expressed in *E. coli* BL21(DE3) harboring plasmid pETJ with the aid of its T7 promoter. OMA hydratase activity of the cell extract of *E. coli* BL21(DE3) harboring pETJ was 24.3 U/mg, indicating the high level of expression of *ligJ*. In SDS-PAGE analysis, the molecular mass of a subunit of the *ligJ* product (LigJ) was estimated to be 35 kDa (Fig. 3, lane 3), this 35-kDa protein was subjected to the N-terminal amino acid sequencing. The sequence of the first 15 residues was determined to be Met-Met-Met-Ile-Ile-Asp-Xaa-His-Gly-Xaa-Tyr-Thr-Val-Leu-Pro, which corresponded to the deduced amino acid sequence of *ligJ* translated from the first ATG of the three consecutive ATG codons. Therefore, the *ligJ* gene was estimated to be an ORF of 1,023 bp, encoding 341 amino acid residues. The molecular mass deduced from the amino acid sequence of LigJ (M_r, 38,008) is close to the value estimated by SDS-PAGE (35 kDa). A homology search with the deduced amino acid sequence of *ligJ* in the SwissProt and DAD databases showed identity only with LigY (37%), which is a hydrolase for the *meta*-cleavage compound 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl, (OH-DDVA), involved in the catabolism of the lignin-related biphenyl by *S. paucimobilis* SYK-6 (24).

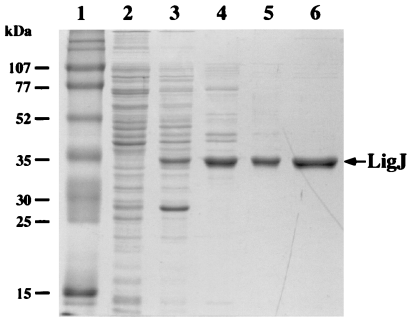

FIG. 3. SDS-PAGE analysis of protein fractions. Proteins were separated on an SDS–12% polyacrylamide gel and stained with Coomassie brilliant blue. Lanes: 1, molecular weight markers; 2, crude extract of *E. coli* BL21(DE3) harboring pET21(+) (10 μg of protein); 3, crude extract of *E. coli* BL21 (DE3) harboring pETJ (10 μg of protein); 4, PI fraction (5 μg of protein); 5, HQ fraction (3 μg of protein); 6, PE fraction (2 μg of protein). Molecular masses are given on the left.

Purification of OMA hydratase. LigJ protein expressed in *E. coli* BL21(DE3) harboring pETJ was purified to near homogeneity (Fig. 3) by a series of column chromatographyies with PI, HQ, and PE (Table 2 and Fig. 3). LigJ was purified approximately 13-fold, with a recovery of 20%.

Identification of the reaction product. To identify the reaction product of OMA catalyzed by the purified LigJ, the reaction mixture was analyzed by ESI-MS. Figure 4A shows the mass spectrum of the substrate. The major fragment at *m/z* 201 in Fig. 4A was estimated to be the deprotonated molecular ion ($[M\text{-}H]^-$) of OMA (where M is a molecular ion of OMA). In the mass spectrum of the reaction mixture shown in Fig. 4B, the fragment at *m/z* at 201 of OMA decreased to 65% of its initial intensity, and the new major fragment at *m/z* 219 corresponding to $[M\text{-}H]^-$ of CHA (where M is the molecular ion of CHA) was observed. Thus, the results strongly suggested that OMA was converted to CHA by incorporation of a water molecule.

Enzyme properties. Gel filtration column chromatography using Superdex 200 indicated that the molecular mass of the native LigJ was 69.5 kDa. This result suggested that LigJ is a homodimer. The isoelectric point of LigJ was determined by isoelectric focusing gel electrophoresis to be 4.9. The optimal temperature for LigJ hydratase activity on OMA was determined to be 30°C; the optimal pH was not determined since OMA was unstable in at high pH. The K_m for OMA and the V_{max} were determined to be 138 μM and 440 U/mg, respectively.

The influence of thiols and thiol reagents on LigJ was also examined. Purified LigJ (0.5 μg) was preincubated with 1 mM cysteine, reduced glutathione, and dithiothreitol individually at 30°C for 10 min, and the remaining activities were determined. OMA hydratase activity was activated to 147, 135, and 120% in the presence of cysteine, reduced glutathione, and dithiothreitol, respectively. On the other hand, the addition of 1 mM $HgCl_2$ completely inhibited the activity. These results suggested that some cysteine residue in LigJ is involved in the enzyme reaction.

TABLE 2. Purification of OMA hydratase from *E. coli* BL21(DE3) harboring pETJ

Fraction	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Fold purification
Crude extract	72.0	3,100	43.1	100	1.0
PI	8.21	1,380	168	45	3.9
HQ	3.00	921	307	30	7.1
PE	1.10	613	557	20	13

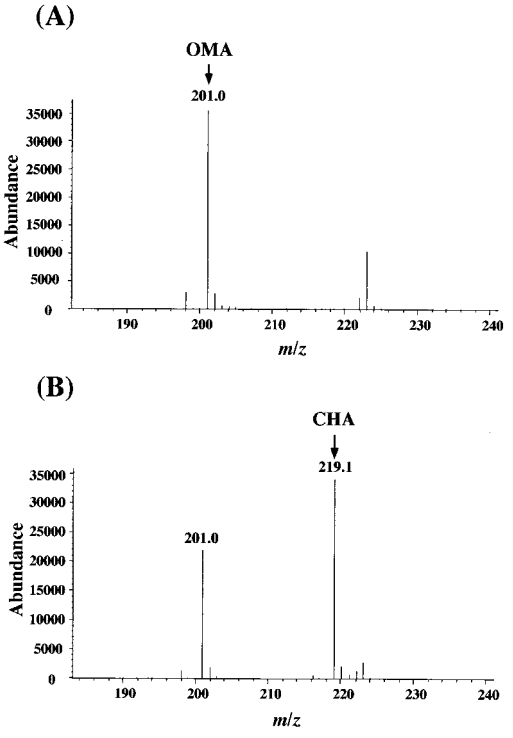

FIG. 4. Identification of the reaction product from OMA catalyzed by LigJ. (A) Negative-ion ESI-MS spectrum of OMA. The peak at *m/z* 201 was assigned to the deprotonated molecular ion $[M\text{-}H]^-$ of OMA. (B) Negative-ion ESI-MS spectrum of the reaction product generated from OMA catalyzed by purified LigJ. The peak at *m/z* 201 derived from OMA reduced, and the generation of the product peak at *m/z* 219 was observed.

Disruption of the *ligJ* gene in *S. paucimobilis* SYK-6. The *ligJ* gene was disrupted to investigate its role in the catabolism of vanillate and syringate by SYK-6. Gene inactivation was carried out using the *ligJ* disruption plasmid pLJD, which was constructed by replacing the internal segment of *ligJ* inserted in pK19*mobsacB* by a Km^r gene. The *ligJ* insertional mutation was confirmed by Southern hybridization analysis using the 1.2-kb *Sal*I-*Xba*I fragment carrying a portion of *ligJ* and the 1.2-kb *Pst*I fragment carrying the Km^r gene as probes (Fig. 5). The mutant strain DLJ obtained completely lost the ability to grow on both vanillate and syringate.

Vanillate and syringate (0.2 %) were independently incubated with whole cells of strain DLJ in W minimal medium. The metabolites produced from vanillate and syringate were examined and determined by GC-MS and ESI-MS. As shown in the gas chromatogram (Fig. 6), vanillate and syringate, detected with retention times of 21.2 and 25.2 min, respectively, disappeared completely, and accumulation of the enol form of OMA, PDC, and product I with a retention time of 30.5 min was observed in both cultures. The major fragments at *m/z* 475 and *m/z* 373 were identified in the mass spectrum of the TMS derivative of the enol form OMA as reported previously (Fig. 6D) (19). These fragments agree with M-CH_3 and M-COOTMS, respectively, where M is a molecular ion of TMS-OMA. Similarly, the fragments at *m/z* 477 and *m/z* 375 of product I are thought to be M-CH_3, and M-COOTMS, respec-

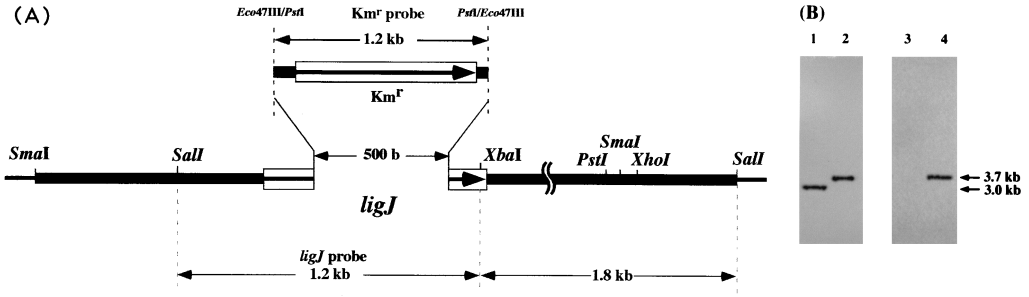

FIG. 5. The *ligJ* disruption in *S. paucimobilis* SYK-6. (A) Schematic representation of the insertional inactivation of *ligJ* by the Km^r gene. Thick arrows indicate orientations of transcription of the *ligJ* and Km^r genes. (B) Southern hybridization analysis of the *ligJ* insertion mutant (DLJ). Lanes 1 and 3, total DNA of SYK-6 digested with *Sal*I; lanes 2 and 4, total DNA of DLJ digested with *Sal*I. The 1.2-kb *Sal*I-*Xba*I fragment carrying a portion of *ligJ* (lanes 1 and 2) and the 1.2-kb *Pst*I fragment of the Km^r gene (lanes 3 and 4) were used as probes.

tively (Fig. 6C). On the other hand, ESI-MS analysis indicated the accumulation of the ions at *m/z* 183 and 201 in the metabolites from vanillate and syringate, which corresponded to the deprotonated molecular ions of PDC and OMA, respectively. In addition to these ions, another ion at *m/z* 203 accumulated from both vanillate and syringate. Thus, this ion seemed to be the deprotonated molecular ion of product I. A *ligJ* inactivated mutant, DLJ should have accumulated a significant amount of OMA. However, the amount of OMA accumulated was smaller than expected. In our previous study, we found that the keto form of OMA, which is an α-keto acid, could not be detected by GC-MS in the condition that we used (19). Thus, product I did not correspond to the keto form of OMA. Possibly, product I was generated from OMA by an unknown reaction in SYK-6. To examine this hypothesis, OMA was incubated with the DLJ cell extract prepared from cells grown in LB. The reaction was carried out in 0.1 M Tris-acetate buffer (pH 8.0) containing 100 μg DLJ cell extract, 1 mM NADPH, 200 μM OMA, and 1 mM $ZnSO_4$ at 30°C. Zn^{2+} was added to inhibit the reverse reaction of the PDC hydrolase, which catalyzes the conversion of OMA to PDC (19). The reaction product after 10 min of incubation was analyzed by ESI-MS and GC-MS. In the ESI-MS analysis, the peak at *m/z* 201 derived from OMA was converted to the peak at *m/z* 203 only

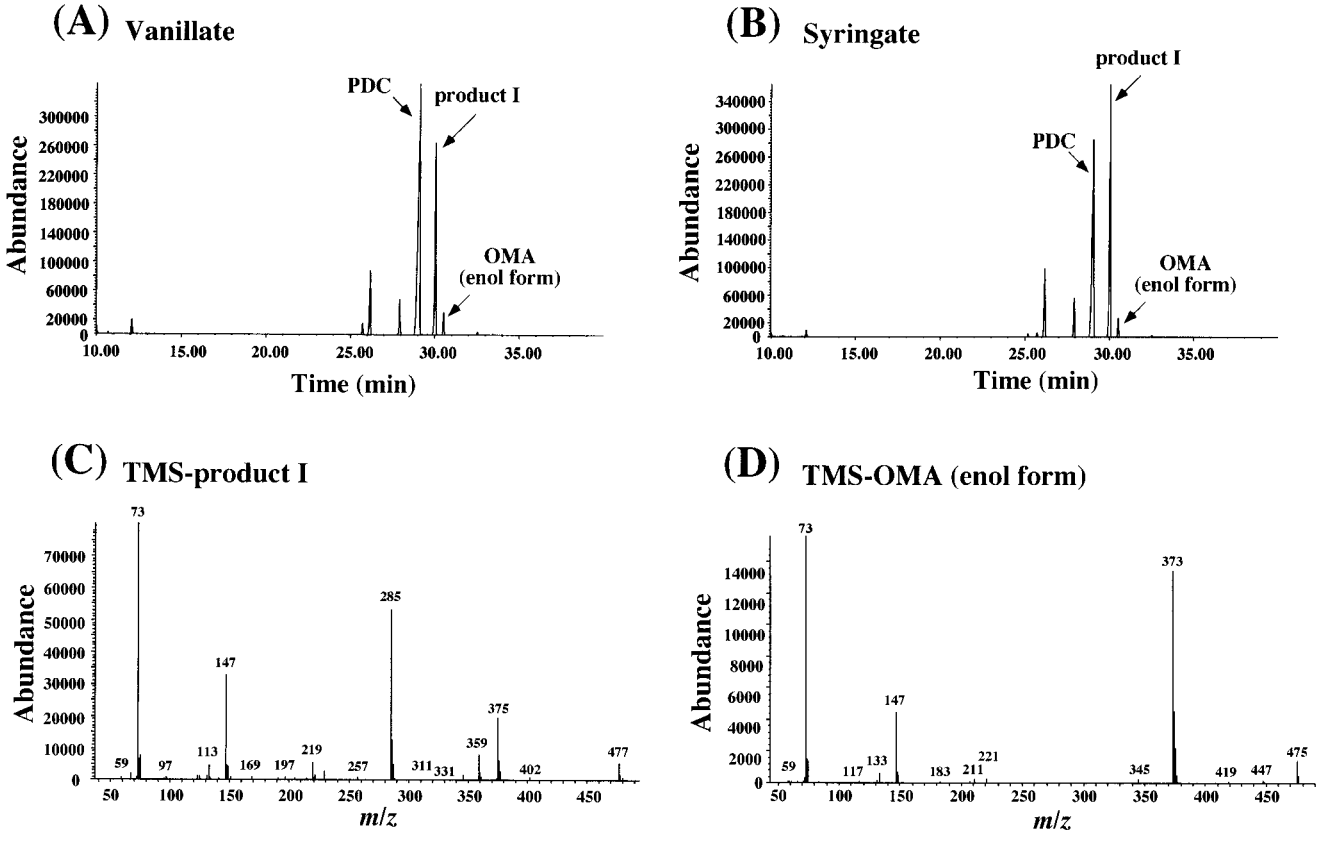

FIG. 6. Identification of accumulated products from vanillate and syringate by DLJ. (A and B) Gas chromatograms of TMS derivatives of the accumulated products from vanillate and syringate, respectively. In both cultures, PDC, the enol form of OMA, and the unidentified product I were observed. (C and D) Mass spectra of the TMS derivatives of product I and the enol form of OMA, respectively.

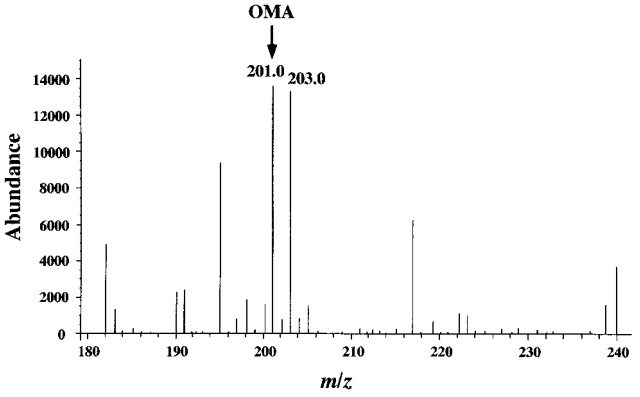

FIG. 7. Conversion of OMA by DLJ cell extract. OMA was converted to the product of which $[M-H]^-$ is *m/z* 203 only in the presence of NADPH.

```
LigJ   MMMIIDCHGHYTVLPKAHDEWREQQKAAFKAGQPAPPYPEISDDEI------RETIEAN   53
LigY    MIIDCHGHVSAPVEL---WAYKASLLAHRGSHGRGGVKVTDEQIIAAAHHKETWPDG   54

LigJ   QLRLIKERGADMTIFSPRASAMAPHVGDQSVAVPWAQACNNLIARVVDLFPETFAGVCM   112
LigY   HIELLHNHGTDMQLISPRTFQMMNSAKPARVVHWFCEEVNTLIHRQCTLIPEMFIPVAG   113

LigJ   LPQSPEADMTSSIAELERCVNELGFIGCNLNPDP-GGGHFKHPPLTDRFWYPFYEKMVE   170
LigY   LPQVAGEPIENVFAEMDRCVS-MGFKGFLLNPDPYENGAEEAPPLGDRYWYPLYEKLCE   171

LigJ   LDVPAMIHVSGSCNPAMHATGAYYLAADTIAFMQLLQGNLFADFPTLRFIIPHGGGAVP   229
LigY   LDLPAHIHATGS-QSERSPYSLHFINEETIATYNLCTSSVFDDFPQLKVVVSHGGGAIP   229

LigJ   YHWGRFRGLADMLKQPSLDTLLMNNVFFDTCVYHQPGINLLADVIDNKNILFGSEMVGA   288
LigY   YQLGRFESQSRRSKH--LFSERMAKLYFDTVLYTEGALRLLIETVGPERCLFGSECPGV   286

LigJ   VRGIDPTTGHYFDDTKRYIDALD-ISDQERHAIFEGNTRRVFPRLDAKLKARGL         341
LigY   GSTIDPATGKQMDHIAPFIQKFDFLSDADKKLIFEDNARKVFNLEV               332
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FIG. 8. Alignment of amino acid sequences between LigJ and LigY of *S. paucimobilis* SYK-6. A BLAST search indicated that the most similar protein whose function has been clarified is the OH-DDVA *meta*-cleavage compound hydrolase (LigY) of *S. paucimobilis* SYK-6 (24). Identical and similar amino acids are indicated by asterisks and colons, respectively.

in the presence of NADPH (Fig. 7). GC-MS analysis showed the generation of the product with the same retention time (30.5 min) as product I. Although the ion at *m/z* 477 observed with product I was not detected, the relative intensities of the mass fragments at *m/z* 375, 285, 147, and 73 were identical to those of product I (data not shown). These results strongly suggested that product I was produced from accumulated OMA possibly by the addition of two hydrogen atoms catalyzed by an unknown NADPH-dependent reductase in SYK-6 and that the accumulation of product I represents the accumulation of OMA. Thus, the results indicate that *ligJ* encodes OMA hydratase, which is essential for catabolism of both vanillate and syringate.

DISCUSSION

The OMA hydratase gene, designated as *ligJ*, encoding a protein of 38,008 Da (341 amino acids), was characterized in this study. LigJ showed no similarity to the functionally related 2-hydroxypent-2,4-dienoate hydratases of catechol (9, 28) and biphenyl (20) degradation pathways. However, LigJ showed 37% identity only with the OH-DDVA *meta*-cleavage compound hydrolase (LigY) involved in the degradation of 5,5′-dehydrodivanillate (DDVA) by *S. paucimobilis* SYK-6. LigY showed no similarity to other aromatic compound hydrolases involved in benzene, toluene, xylene, and biphenyl degradation and did not contain a lipase box (Gly-Xaa-Ser-Gly motif), which constitutes an active site in serine hydrolases. Alignment of the deduced amino acid sequences of *ligJ* and *ligY* is shown in Fig. 8. Similarity is distributed throughout the whole sequence; striking identity is found in their amino-terminal sequences, Met-Ile-Ile-Asp-Cys-His-Gly-His. It is interesting that these two proteins in the successive degradation pathway are highly similar. LigJ and LigY seem to evolve from the same ancestral origin. The structural similarity between the LigJ substrate (OMA) and the organic acid moiety of the LigY substrate might have contributed to the evolution of these proteins.

Only the OMA hydratase of *P. ochraceae* had been characterized. The OMA hydratases of both *S. paucimobilis* SYK-6 and *P. ochraceae* are dimeric proteins, and their subunit molecular masses and pIs are very similar. Various thiols and thiol reagents affected the activities of both enzymes. However, their kinetic parameters are considerably different. The K_m and V_{max} values of SYK-6 OMA hydratase are approximately 10 and 3.7 times higher than those of the *P. ochraceae* enzyme, respectively, indicating that the *P. ochraceae* enzyme has significantly higher affinity toward OMA than SYK-6 enzyme.

The production of CHA from OMA catalyzed by OMA hydratase was suggested by Maruyama (13). Kersten *et al.* suggested that OMA is in equilibrium among the two isomeric enol forms and keto form. Both enol forms of OMA (4-carboxy-2-hydroxymuconate) were easily detected as TMS derivatives by GC-MS; however, the keto form of OMA could be detected only when it was modified to the methoxime form as described in Materials and Methods. In the case of CHA, it could not be detected by GC-MS even in its methoxime form. Therefore, we used ESI-MS to detect these compounds and demonstrate the production of CHA from OMA by LigJ. It is difficult to specify which form of OMA is the real substrate; however, considering the chemical structure of the LigJ reaction product, CHA, the keto form is the most likely candidate.

The *ligJ* gene of SYK-6 was inactivated by gene replacement to clarify the catabolic role of the *ligJ* gene. In a previous study, each disruption of *ligB*, *ligC*, and *ligI* did not affect syringate metabolism, while these mutants could not grow on vanillate (19; Aoshima et al., Abstr. 8th Int. Symp. Microb. Ecol., 1998; Masai et al., submitted). On the other hand, the *ligJ* insertion mutant DLJ could not grow on vanillate and syringate. Thus, it appears that the syringate catabolic pathway adjoined the PCA 4,5-cleavage pathway at the LigJ reaction step. DLJ accumulated the enol form of OMA, PDC, and the unknown product I from vanillate and syringate (Fig. 6). Accumulation of PDC seemed to be a result of the reverse reaction of PDC hydrolase, which converts OMA to PDC (19). Product I was suggested to be generated from OMA probably by addition of two atoms of hydrogen by NADPH-dependent reductase in DLJ cells. Product I is estimated to be 4-hydroxybut-1-ene-1,2,4-tricarboxylate. Taken together, the results suggest that the *ligJ* gene is essential for both vanillate and syringate degradation and that the syringate degradation pathway joins to the PCA 4,5-cleavage pathway at OMA.

Donnelly and Dagley reported that *P. putida* TMC degrades 3MGA to oxaloacetate and pyruvate via OMA, with the release of methanol (4). They proposed the involvement of a 3MGA dioxygenase and an esterase in the transformation of 3MGA to OMA. Characterization of a putative 3MGA dioxygenase and an esterase is necessary for a better understanding of syringate catabolism in *S. paucimobilis* SYK-6.

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