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A Second 5-Carboxyvanillate Decarboxylase Gene, *ligW2*, Is Important for Lignin-Related Biphenyl Catabolism in *Sphingomonas paucimobilis* SYK-6

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A lignin-related biphenyl compound, 5,5′-dehydrodivanillate (DDVA), is degraded to 5-carboxyvanillate (5CVA) by the enzyme reactions catalyzed by DDVA *O*-demethylase (LigX), *meta*-cleavage oxygenase (LigZ), and *meta*-cleavage compound hydrolase (LigY) in *Sphingomonas paucimobilis* SYK-6. 5CVA is then transformed to vanillate by a nonoxidative 5CVA decarboxylase and is further degraded through the protocatechuate 4,5-cleavage pathway. A 5CVA decarboxylase gene, *ligW*, was isolated from SYK-6 (X. Peng, E. Masai, H. Kitayama, K. Harada, Y, Katayama, and M. Fukuda, Appl. Environ. Microbiol. 68:4407–4415, 2002). However, disruption of *ligW* slightly affected the 5CVA decarboxylase activity and the growth rate on DDVA of the mutant, suggesting the presence of an alternative 5CVA decarboxylase gene. Here we isolated a second 5CVA decarboxylase gene, *ligW2*, which consists of a 1,050-bp open reading frame encoding a polypeptide with a molecular mass of 39,379 Da. The deduced amino acid sequence encoded by *ligW2* exhibits 37% identity with the sequence encoded by *ligW*. Based on a gas chromatography-mass spectrometry analysis of the reaction product from 5CVA catalyzed by LigW2 in the presence of deuterium oxide, LigW2 was indicated to be a nonoxidative decarboxylase of 5CVA, like LigW. After disruption of *ligW2*, both the growth rate on DDVA and the 5CVA decarboxylase activity of the mutant were decreased to approximately 30% of the wild-type levels. The *ligW ligW2* double mutant lost both the ability to grow on DDVA and the 5CVA decarboxylase activity. These results indicate that both *ligW* and *ligW2* contribute to 5CVA degradation, although *ligW2* plays the more important role in the growth of SYK-6 cells on DDVA.

Lignin is the most abundant natural aromatic compound that consists of phenylpropane units with various types of linkages. Lignin helps to strengthen and stiffen plant cell walls and is resistant to breakdown by microorganisms, but many microorganisms have evolved the ability to degrade lignin. The specific reactions of lignin metabolism by microorganisms are expected to enable the use of lignin for industrial purposes in order to supplement or replace traditional methods of chemical synthesis.

Lignin-derived compounds and their metabolic intermediates (e.g., hydroxylated benzoate derivatives) include carboxyl groups, and therefore the removal of carboxyl groups from the benzene nucleus by decarboxylases is an important reaction in lignin biodegradation. The aromatic acid decarboxylases have been classified into two groups, the nonoxidative and oxidative decarboxylases. Nonoxidative decarboxylation of aromatic acids involves the removal of the carboxyl group from the benzene nucleus via an enzymatic reaction that requires neither oxygen nor cofactors (26). The nonoxidative process results in complete removal of the carboxyl group, in contrast to the oxidative reaction, which substitutes a hydroxyl group at the relevant carbon atom (7). Nonoxidative decarboxylases are used not only in the degradation of lignin-related compounds, including 4-hydroxybenzoate (22, 25, 31), protocatechuate (PCA) (23), gallate (19, 58), vanillate (10, 24, 46), ferulate (57), and *p*-coumarate (8), but also in the degradation of such artificial compounds as phthalates (9, 15, 30, 38, 45, 47).

Sphingomonas paucimobilis SYK-6 was isolated with a lignin-related biphenyl compound, 5,5′-dehydrodivanillate (DDVA), as the sole carbon and energy source (28). This strain is able to mineralize various lignin-derived biaryls through the PCA 4,5-cleavage pathway using unique and specific enzymatic systems (21), which are thought to be good tools for conversion of lignin to valuable intermediate metabolites. The DDVA catabolic pathway of this strain has been determined, as shown in Fig. 1. DDVA is initially converted to a diol compound, 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl (OH-DDVA), by multicomponent DDVA *O*-demethylase, the oxygenase component of which is encoded by *ligX* (52). The ring fission of OH-DDVA to produce a *meta*-cleavage compound is catalyzed by OH-DDVA oxygenase encoded by *ligZ* (39). The resulting *meta*-cleavage compound is hydrolyzed to form 5-carboxyvanillate (5CVA) by a *ligY*-encoded hydrolase (40). 5CVA is further degraded to vanillate, which is finally converted to pyruvate and oxaloacetate via the PCA 4,5-cleavage pathway.

In a previous study, we isolated and characterized the *ligW* gene, the product of which is a nonoxidative decarboxylase that

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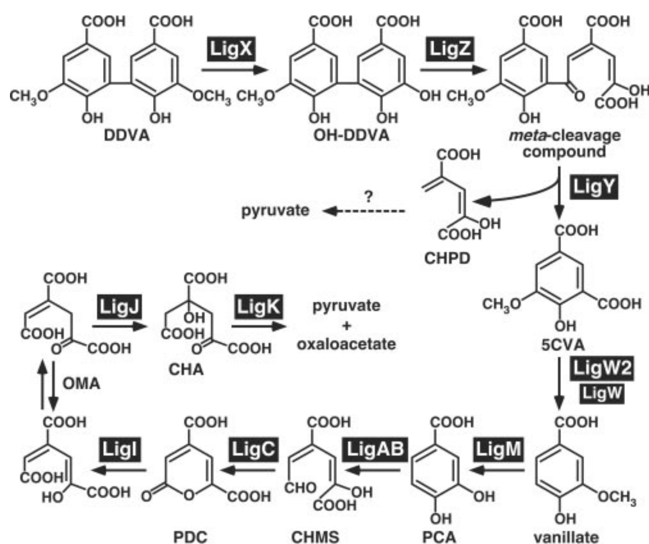

FIG. 1. Proposed DDVA catabolic pathway of *S. paucimobilis* SYK-6. The following gene products are involved in this pathway: LigX, a component of DDVA *O*-demethylase; LigZ, OH-DDVA *meta*-cleavage dioxygenase; LigY, OH-DDVA *meta*-cleavage compound hydrolase; LigW and LigW2, 5CVA decarboxylase; LigM, vanillate/3-*O*-methylgallate *O*-demethylase; LigA and LigB, small and large subunits, respectively, of PCA 4,5-dioxygenase; LigC, 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase; LigI, 2-pyrone-4,6-dicarboxylate hydrolase; LigJ, 4-oxalomesaconate hydratase; LigK, 4-carboxy-4-hydroxy-2-oxoadipate aldolase. Abbreviations: CHMS, 4-carboxy-2-hydroxymuconate-6-semialdehyde; PDC, 2-pyrone-4,6-dicarboxylate; OMA, 4-oxalomesaconate; CHA, 4-carboxy-4-hydroxy-2-oxoadipate.

catalyzes the conversion of 5CVA to vanillate (41). However, we found that *ligW* is not essential for the degradation of 5CVA by SYK-6. To identify the reaction step that converts 5CVA to vanillate, we isolated and characterized a second 5CVA decarboxylase gene, *ligW2*. The roles played by both *ligW* and *ligW2* in DDVA catabolism were examined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and substrates. The strains and plasmids used in this study are listed in Table 1. *S. paucimobilis* SYK-6 and its derivative strains were grown at 30°C in W minimal medium (39) containing 10 mM vanillate or 5 mM DDVA or in Luria-Bertani (LB) medium (Bacto Tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter). The methods used for preparation of DDVA (39) and 5CVA (41) have been described previously. Isophthalate, 4-hydroxyisophthalate, 3-methoxysalicylate, and deuterium oxide (D_2O) (99.75%) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cloning procedure. A gene library constructed with pVK100 and the partially SalI-digested DNA of SYK-6 (41) was introduced into *Pseudomonas putida* PpY101 by triparental mating. The transconjugants were grown in 3 ml of LB medium containing 25 mg of nalidixic acid/liter and 50 mg of kanamycin/liter at 30°C for 12 h. Cells were centrifuged at 4,500 × *g* for 10 min at 4°C, and they were washed twice with W medium. Then cells were suspended in 1 ml of the same medium containing 50 μM 5CVA. After incubation with shaking for 7 days at 30°C, each 200 μl of culture was acidified with 10 μl of 6 N hydrochloric acid and extracted with 60 μl of ethyl acetate. The disappearance of 5CVA from the resultant samples was analyzed by thin-layer chromatography (TLC) using Silica Gel 60 F254 (E. Merck, Darmstadt, Germany) with the solvent chloroform-ethyl acetate-formic acid (10:8:2). Compounds were visualized under UV light at 254 nm. 5CVA has an R_f of 0.52 in this system.

DNA manipulations and nucleotide sequencing. DNA manipulations were carried out essentially as described previously (2, 48). Nucleotide sequences were determined by the dideoxy termination method with a Thermosequenase fluorescence-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and an ALF express DNA sequencer (Pharmacia Biotech, Milwaukee, Wis.). Sequencing analysis and multiple alignment were carried out with the GeneWorks programs (IntelliGenetics, Inc., Mountain View, Calif.). Pairwise alignment was performed with the EMBOSS alignment tool at the homepage of the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/align).

Construction of insertion mutants of *S. paucimobilis* SYK-6. To disrupt *ligW*, *ligW2*, *orf1*, and *orf3* in SYK-6, the kanamycin resistance gene (*kan*) from pUC4K was inserted into each gene by the gene replacement technique. The *ligW*-, *ligW2*-, *orf1*-, and *orf3*-disrupted plasmids pDW, pDW2Km, pDorf1, and pDorf3, constructed with pK19*mobsacB*, were introduced into *Escherichia coli* S17-1 and then introduced into SYK-6 by conjugation. To disrupt both *ligW* and *ligW2* in SYK-6, pDW2Ap, in which *ligW2* was inactivated by the ampicillin resistance gene (*bla*) from pUC18, was introduced into a *ligW* mutant (DW). Selection of each gene-disrupted mutant was performed as described previously (35). To examine the disruption of each gene, a Southern hybridization analysis was carried out. Total DNA of the candidates for *ligW*, *ligW2*, *ligW* and *ligW2*, *orf1*, and *orf3* insertion mutants were digested with SacI and XhoI, SalI, SalI, BamHI, and SalI, respectively. The 1.3-kb SalI fragment carrying *kan*, the 1.9-kb SacI-XhoI fragment carrying *ligW*, the 3.6-kb SalI fragment carrying *ligW2* and *orf3*, the 1.0-kb BspHI fragment carrying *bla*, and the 2.8-kb BamHI fragment carrying *orf1* were labeled with the DIG system (Roche Molecular Biochemicals, Mannheim, Germany) and used as probes.

Preparation of cell extracts. *E. coli* JM109 cells harboring pW2 were grown in LB medium containing 100 mg of ampicillin/liter. Expression of *ligW2* was induced for 4 h by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM when the turbidity of the culture at 600 nm reached 0.5. Cells were harvested by centrifugation at 3,000 × *g* for 10 min and were ruptured by passage through a French pressure cell in 50 mM Tris-HCl buffer (pH 7.5). The cell lysate was centrifuged at 15,000 × *g* for 15 min, and the supernatant was used as a crude enzyme. The expression of the enzymes was examined by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by the method of Bradford (6).

For preparation of cell extracts of *S. paucimobilis* SYK-6 and its mutants, cells were grown in W medium containing 10 mM sucrose, 10 mM glutamate, and 50 mg of methionine/liter. The expression of 5CVA decarboxylase genes was induced by addition of 5 mM DDVA when the turbidity of the culture at 600 nm reached 0.5, and the cells were incubated for 4 h. Cells were harvested by centrifugation at 5,000 × *g* for 10 min and washed with 50 mM Tris-HCl buffer (pH 7.5). Cells suspended in the same buffer were sonicated, and the cell lysate was centrifuged at 15,000 × *g* for 15 min. The resulting supernatant was used as the cell extract.

Enzyme assays. The 5CVA decarboxylase activity was spectrophotometrically determined by measuring the decrease in the absorbance at 312 nm ($\varepsilon_{312} = 3.5 \times 10^3\ M^{-1}\ cm^{-1}$; pH 7.5) with a DU-7500 spectrophotometer (Beckman, Fullerton, Calif.) at 30°C. Each 1-ml assay mixture contained 50 mM Tris-HCl buffer (pH 7.5), 100 μM 5CVA, and cell extract of *E. coli* JM109 harboring pW2 (100 μg of protein) or *S. paucimobilis* SYK-6 and its mutants (200 μg of protein). One unit of enzyme activity was defined as the amount that degraded 1 μmol of substrate per min at 30°C

To determine the activity of LigW2 toward 3-methoxysalicylate, 4-hydroxyisophthalate, and isophthalate, a high-pressure liquid chromatography system (Alliance 2690 separation module; Waters, Milford, Mass.) equipped with an octadecyl silica reverse-phase column (4.6 by 100 mm; Waters) was employed. The reaction was performed as described above, and filtrates of the reaction mixture were analyzed by high-performance liquid chromatography. The mobile phase consisted of 0.1% phosphoric acid in water (solvent A) and 0.1% phosphoric acid in acetonitrile (solvent B). The flow rate was 1 ml/min. After injection of a sample, solvent A was run through the column for 2 min, the solvent gradient was programmed to increase from 0 to 70% solvent B over 6 min, and then this mixture was maintained for 5 min. The absorbance between 230 and 280 nm of eluent was monitored with a photodiode array detector (Waters 2996) for the reaction mixtures containing 4-hydroxyphthalate and isophthalate. Absorbance at 276 nm and 317 nm was monitored for the reaction mixture containing 3-methoxysalicylate. The retention times of 4-hydroxyisophthalate, 4-hydroxybenzoate, isophthalate, benzoate, 3-methoxysalicylate, and guaiacol were 6.36, 5.67, 6.17, 6.94, 6.93, and 6.80 min, respectively.

Vanillin dehydrogenase activity was measured by the method of Gasson et al. (18). Each 1-ml reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), cell extract of *E. coli* JM109 harboring pUCI1 (200 μg of protein), 50 μM vanillin, 500 μM NAD^+, 1.2 mM pyruvate, and 1.1 U lactate dehydrogenase.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
S. paucimobilis strains		
SYK-6	Wild type; Nal^r Sm^r	29
DW	SYK-6 derivative, *ligW*::*kan* Nal^r Sm^r Km^r	This study
DW2	SYK-6 derivative, *ligW2*::*kan* Nal^r Sm^r Km^r	This study
DDW	SYK-6 derivative, *ligW*::*kan ligW2*::*bla* Nal^r Sm^r Km^r Cb^r	This study
Dorf1	SYK-6 derivative, *orf1*::*kan* Nal^r Sm^r Km^r	This study
Dorf3	SYK-6 derivative, *orf3*::*kan* Nal^r Sm^r Km^r	This study
P. putida PpY101	Nal^r Sm^r	17
E. coli strains		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	56
S17-1	*recA*; harbors the *tra* genes of plasmid RP4 in the chromosome, *proA thi*	51
HB101	*supE44 hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	5
Plasmids		
pUC18	Cloning vector; Ap^r	56
pBluescript II KS(+)	Cloning vector; Ap^r	50
pVK100	Broad-host-range cosmid vector, Km^r Tet^r	14
pRK2013	Km^r Tra$^+$ Mob$^+$	16
pDEC6	pVK100 with an approximately 20-kb fragment carrying *ligW2*	This study
pU351	pUC18 with a 3.6-kb SalI fragment carrying *ligW2*	This study
pU352	pUC18 carrying the same fragment as pU351 in the opposite direction	This study
pA1, pA5, pA9, pA13, pA14	Deletion derivatives of pU351	This study
pB15, pB16, pB19, pB26, pB28, pB32	Deletion derivatives of pU352	This study
pW2	KS(+) with a 1.1-kb SmaI-SacII fragment carrying *ligW2*	This study
pVKW2	pVK100 carrying the same fragment as pW2	This study
pVK7E2	pVK100 with a 7.0-kb EcoRI fragment carrying *ligW*	41
pUC4K	Ap^r/Km^r	53
pK19*mobsacB*	*oriT sacB* Km^r	49
pU351Km	pU351 with insertion of *kan* from pUC4K into an EcoRV site	This study
pDW2Km	pK19*mobsacB* with a 4.8-kb SalI fragment from pU351Km	This study
pK19U351	pK19*mobsacB* with a 3.6-kb SalI fragment from pU351	This study
pDW2Ap	pK19U351 with insertion of *bla* from pUC18 into an EcoRV site	This study
pKS7E2	KS(+) with a 7.0-kb EcoRI fragment of pVK7E2	41
pKV44	Deletion derivative of pKS7E2 carrying *ligW*	41
pKV44Km	pKV44 with insertion of *kan* from pUC4K into a SalI site	This study
pDW	pK19*mobsacB* with a 3.6-kb SacI-XbaI fragment from pKV44Km	This study
pUC11	pUC19 with a 2.8-kb BamHI fragment carrying *orf1*	This study
pUC11Km	pUC11 with insertion of *kan* from pUC4K into a SalI site	This study
pDorf1	pK19*mobsacB* with a 4.0-kb BamHI fragment from pUC11Km	This study
pSV322	KS(+) with a 2.8-kb EcoRV-SalI fragment carrying *orf3*	This study
pSV322Km	pSV322 with insertion of *kan* from pUC4K into a HindIII site	This study
pDorf3	pK19*mobsacB* with a 4.0-kb EcoRI-SalI fragment from pSV322Km	This study
pHN139F	pUC18 with a 10.5-kb EcoRI fragment carrying *ligJAB*, part of *ligC*, *ligK*, and *ligI*	35
pDE20	pVK100 with an approximately 20-kb EcoRI fragment carrying part of *ligM*, *metF*, and *ligH*	37

[a] Abbreviations: Nal^r, Sm^r, Km^r, Cb^r, Tet^r and Ap^r, resistance to nalidixic acid, streptomycin, kanamycin, carbenicillin, tetracycline, and ampicillin, respectively.

The reaction was carried out at 30°C, and the decrease in absorbance at 340 nm was monitored.

Conversion of 5CVA to vanillate by LigW2 in D_2O. 5CVA (400 μM) and cell extract of *E. coli* JM109 harboring pW2 (100 μg of protein) were incubated in 1-ml reaction mixtures containing 0 or 96% D_2O. After incubation for 30 min at 30°C, the reaction product was acidified with 6 N hydrochloric acid and extracted with ethyl acetate. The organic phase was dried in vacuo and was treated with the trimethylsilyl reagent. The trimethylsilylated (TMS) derivatives of the reaction product were analyzed by gas chromatography-mass spectrometry (GC-MS) using a model 5971A instrument (Agilent Technologies, Palo Alto, Calif.) with an Ultra-2 capillary column (50 m by 0.2 mm; Agilent Technologies). The analytical conditions used have been described previously (41).

Mapping of *ligW2*. Total DNA of SYK-6 prepared in an agarose block was digested with AseI and separated with a CHEF DRIII apparatus (Bio-Rad, Hercules, Calif.) as described in a previous study (41). For Southern hybridization analysis, the digoxigenin-labeled 3.6-kb SalI fragment carrying *ligW2* was used as a probe. To determine the location of *ligW2* and *ligM-metF-ligH* or the PCA 4,5-cleavage pathway genes, Southern hybridization of the SalI digests of pDEC6 was performed with digoxigenin-labeled pDE20 and pHN139F digested with SalI as probes.

Nucleotide sequence accession number. The nucleotide sequences of *ligW2*, *orf1*, and *orf3* have been deposited in the DDBJ, EMBL, and GenBank sequence databases under accession no. AB089690.

RESULTS AND DISCUSSION

Cloning of a second 5CVA decarboxylase gene. In order to examine the role played by *ligW* in the DDVA catabolism of *S. paucimobilis* SYK-6, *ligW* was disrupted by a gene replacement technique using pDW, in which *ligW* was inactivated by inser-

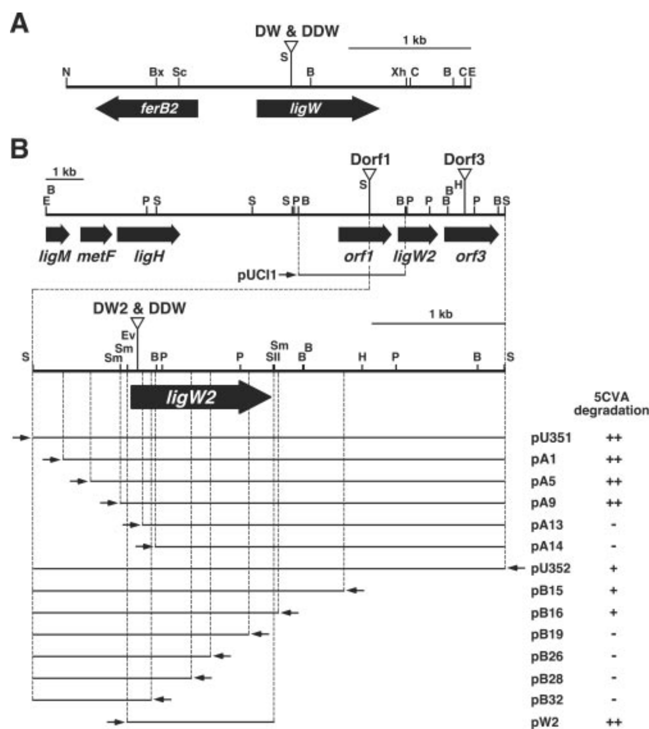

FIG. 2. Restriction map of the 3.3-kb NotI-EcoRI fragment carrying *ligW* (A) and deletion analysis of the 3.6-kb SalI fragment carrying *ligW2* (B). Triangles indicate the positions of the *kan* or *bla* gene insertion of the *ligW* mutant (DW), *ligW2* mutant (DW2), *ligW ligW2* double mutant (DDW), *orf1* mutant (Dorf1), and *orf3* mutant (Dorf3). Small arrows indicate the direction of transcription from the *lac* promoter. The 5CVA decarboxylase activities of *E. coli* containing each of the deletion plasmids are indicated on the right. ++, 5CVA was completely degraded; +, approximately one-half of 5CVA was degraded; −, 5CVA was not degraded. Abbreviations: B, BamHI; Bx, BstXI; C, ClaI; E, EcoRI; Ev, EcoRV; H, HindIII; N, NotI; P, PstI; S, SalI; Sc, SacI; Sm, SmaI; SII, SacII; Xh, XhoI.

tion of *kan* (Fig. 2). Disruption of *ligW* in the candidate mutants was confirmed by Southern hybridization analysis using a 1.9-kb SacI-XhoI fragment carrying *ligW* and a 1.3-kb SalI fragment carrying *kan*. The resulting *ligW* mutant (DW) and

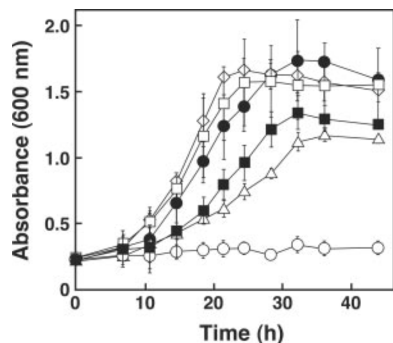

FIG. 3. Growth of the insertion mutants on DDVA. Cells of SYK-6 (open diamonds), DW (open squares), DW2 (open triangles), DDW (open circles), DDW harboring pVKW2 (solid circles), and DDW harboring pVK7E2 (solid squares) were grown in W medium containing 5 mM DDVA at 30°C. The values are averages ± standard deviations (error bars) of three independent experiments.

SYK-6 cells were grown in W medium containing 5 mM DDVA as the sole carbon and energy source. The growth rate of DW was slightly decreased ($k = 0.079$/h; 83% of the growth rate of the wild-type strain) (Fig. 3). Because the 5CVA decarboxylase activity of the SYK-6 cells incubated with DDVA (95 ± 15 mU/mg) was approximately three times higher than that of SYK-6 cells incubated without DDVA, the activity of DW was examined using cells incubated with 5 mM DDVA in order to induce the enzyme activity. It was found that the decarboxylase activity of the cell extract of DW was approximately 80% of the wild-type activity. These results indicated that *ligW* is not essential for the degradation of 5CVA, although it contributes to the total 5CVA decarboxylase activity in SYK-6 cells.

To isolate an alternative 5CVA decarboxylase gene, a cosmid gene library of SYK-6 constructed in *E. coli* was introduced into *P. putida* PpY101 by triparental mating. Approximately 2,000 transconjugants were screened for 5CVA transformation activity by TLC analysis. As a result, 13 positive clones that transformed 5CVA to vanillate were obtained. In a previous study, we obtained only one positive clone carrying *ligW*. This finding might have been due to the difference in the growth conditions used for the transconjugants in the assay. The transconjugants were grown in LB medium in this study, whereas vanillate-grown cells were used in the previous study. Because the growth of *P. putida* PpY101 on vanillate was somewhat poor, the activities of the positive clones might have been too weak for detection by TLC.

Southern hybridization analysis of the cosmid clones digested with SalI using a 3.3-kb NotI-EcoRI fragment carrying *ligW* as a probe suggested that 5 of the 13 clones contained *ligW*. The remaining eight clones were used for further experiments. These cosmid clones had a common 3.6-kb SalI fragment, and this fragment from pDEC6 was cloned into pUC18 in order to generate pU351 (Fig. 2). TLC analysis showed that the cell extract of *E. coli* JM109 harboring pU351 grown with IPTG was able to convert 5CVA to vanillate (Fig. 2), suggesting that the 3.6-kb SalI fragment contained a second 5CVA decarboxylase gene.

A series of subclones of the 3.6-kb SalI fragment was constructed by using restriction enzymes and *E. coli* exonuclease III, and these subclones were subjected to nucleotide sequencing. To determine the localization of the 5CVA decarboxylase gene in the 3.6-kb SalI fragment, the 5CVA decarboxylase activity of cell extracts of *E. coli* JM109 carrying each of the subclones was assayed by using TLC plates (Fig. 2). The 5CVA decarboxylase activity was detected in *E. coli* carrying pA9 and pB16 and yet was lost in *E. coli* carrying pA13 and pB19. This result suggested that the decarboxylase gene was limited to the DNA segment shared by pA9 and pB16. The presence of a promoter sequence upstream of *ligW2* was considered, since the *lac* promoter of the vector was located downstream of *ligW2* in pB16. Plasmid pW2 with the 1.1-kb SmaI-SacII fragment containing the DNA shared by pA9 and pB16 indeed conferred 5CVA decarboxylase activity on *E. coli*, and the specific activity for 5CVA decarboxylation was estimated to be 120 ± 32 mU/mg. The nucleotide sequence of the 3.6-kb SalI fragment revealed a 1,050-bp open reading frame (ORF) in the 1.1-kb SmaI-SacII segment, which was considered to be a second 5CVA decarboxylase gene and was designated *ligW2*. The

molecular mass of the *ligW2* gene product (LigW2) calculated from the deduced amino acid sequence was 39,379 Da.

Ishii et al. (27) reported that nonoxidative aromatic acid decarboxylases should be classified as members of the following enzyme families: the 3-octaprenyl-4-hydroxybenzoate carboxylyase (UbiD) family (59), which includes a 4-hydroxybenzoate decarboxylase, Ohb1 of *Clostridium hydroxybenzoicum* (25); the aldolase family, which includes 4,5-dihydroxyphthalate decarboxylases, including Pht5 of *P. putida* (38), PhtC of *Arthrobacter keyseri* (15), and PhtD of *Comamonas testosteroni* (30); the phenolic acid decarboxylase family, which includes the ferulic acid decarboxylase, Fdc, of *Bacillus pumilus* (57), and *p*-coumaric acid decarboxylase, PdcC, of *Lactobacillus plantarum* (8), as well as the phenolic acid decarboxylases of both *Bacillus* (3, 43) and *Pediococcus* (4); and a new family, which includes LigW and the 2,6-dihydroxybenzoic acid decarboxylase, Rdc, of *Rhizobium radiobacter* (27). LigW2 exhibited no homology with the enzymes in the UbiD, aldolase, and phenolic acid decarboxylase families, but it exhibited 37 and 28% identity with LigW and Rdc, respectively. In addition, LigW2 exhibited approximately 20% identity with the OH-DDVA *meta*-cleavage compound hydrolase (LigY) (40) and the 4-oxalomesaconate hydratase (LigJ) (15, 20, 32, 44, 55). Based on the finding that the decarboxylation activity of Rdc was inhibited by diethyl pyrocarbonate, a His residue was suggested to be one of the active site residues. The site-directed mutagenesis study suggested that His-164 and His-218 are essential for the decarboxylation activity catalyzed by Rdc. Interestingly, alignment of the amino acid sequences of Rdc with those of LigW2, LigW, LigY, and LigJ revealed that two corresponding His residues are completely conserved in these enzymes. Thus, these enzymes may have similar active sites.

Downstream of *ligW2*, 1,458-bp *orf3* was found. The deduced amino acid sequence encoded by *orf3* exhibited 51% and 30% identity with the sequences of the putative major facilitator superfamily transporters ZP_003051 and ZP_003018, respectively, of *Novosphingobium aromaticivorans* DSM 12444. However, *orf3* exhibited only 15 to 19% identity with the benzoate transporter (BenK) (11, 12) and PCA/4-hydroxybenzoate transporter (PcaK) (13, 36) of *P. putida* and *Acinetobacter*, which are members of the major facilitator superfamily.

To obtain the sequence upstream of *ligW2*, the nucleotide sequence of the 2.8-kb BamHI fragment of pDEC6, which overlapped the 3.6-kb SalI fragment, was determined (Fig. 2); 1,428-bp *orf1* was found in this region, and the deduced amino acid sequence encoded by *orf1* exhibited ca. 30% identity with vanillin dehydrogenase (18, 42, 54). However, no vanillin dehydrogenase activity was detected in the cell extract of *E. coli* JM109 cells harboring pUCI1, which contains *orf1* (data not shown).

Inactivation of *ligW2*, *orf1*, and *orf3* in *S. paucimobilis* SYK-6. In order to determine the involvement of *ligW2* in 5CVA and DDVA degradation by SYK-6, the *ligW2* gene in both SYK-6 and DW was disrupted by a gene replacement technique using *ligW2*-inactivated plasmids. The *kan* and *bla* genes were inserted into the *ligW2* coding region in order to generate plasmids pDW2Km and pDW2Ap, respectively. These two plasmids were introduced into SYK-6 and DW, respectively. Disruption of *ligW2* in the resulting *ligW2* mutant (DW2) and the *ligW ligW2* double mutant (DDW) was confirmed by Southern hybridization analysis using the 3.6-kb SalI fragment carrying *ligW2* and the 1.3-kb SalI fragment carrying *kan* or the 1.0-kb BspHI fragment carrying *bla* as probes.

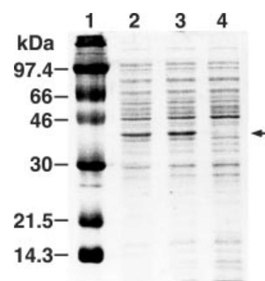

FIG. 4. Expression of *ligW2* in *E. coli* demonstrated by SDS-PAGE. Lane 1, molecular size markers; lane 2, cell extract of *E. coli* JM109 harboring pU351; lane 3, cell extract of *E. coli* JM109 harboring pW2; lane 4, cell extract of *E. coli* JM109 harboring pBluescript II KS(+).

To test the ability of DW2 and DDW to grow on DDVA, these mutants were cultured in W medium containing 5 mM DDVA. DW2 retained the ability to grow on DDVA; however, the growth rate of DW2 on DDVA ($k = 0.026$/h) was markedly decreased (27% of the growth rate of the wild-type strain), and DDW completely lost the ability to grow on DDVA (Fig. 3). These results indicated that both *ligW2* and *ligW* are involved in 5CVA degradation, but *ligW2* plays a more important role in 5CVA degradation. The 5CVA decarboxylase activities of the mutants incubated with DDVA were also examined. Cell extract of DW2 exhibited 28% (27 $\pm$ 5.7 mU/mg) of the activity of the wild-type cells, whereas DDW almost completely lacked this activity (2.7 $\pm$ 1.5 mU/mg). The 5CVA decarboxylase activities of DW and DDW were in accord with the growth rates of these strains. The introduction of pVKW2 and pVK7E2, which carried *ligW2* and *ligW*, respectively, into DDW restored the ability of DDW to grow on DDVA (Fig. 3), thus indicating that the deficiency of the 5CVA decarboxylase activity in DDW was truly caused by the disruption of *ligW2* and *ligW*. Further investigations are needed to clarify the reason for the difference in the levels of participation of *ligW2* and *ligW* in 5CVA degradation.

To gain a better understanding of the roles played by *orf1* and *orf3*, these ORFs were disrupted by the strategy described above, using pDorf1 and pDorf3, respectively. No differences in either the growth rate on DDVA or 5CVA decarboxylase activity were observed between the insertion mutants of *orf1* (Dorf1) and *orf3* (Dorf3) and the wild-type cells, indicating that these ORFs are not involved in DDVA degradation. Due to the poor growth of SYK-6 on 5CVA, the growth of the mutants on 5CVA could not be tested. Therefore, we cannot exclude the possibility that *orf3* encodes the transporter for 5CVA. On the other hand, it was suggested that *orf1* does not encode vanillin dehydrogenase because Dorf1 grew normally on vanillin (data not shown).

Characterization of LigW2. The *ligW2* gene expression induced by IPTG in *E. coli* JM109 was examined with plasmids pU351 and pW2. Production of a 37-kDa protein in these transformants was observed by SDS-PAGE, and this size was in good agreement with the value calculated from the deduced amino acid sequence of *ligW*2 (Fig. 4).

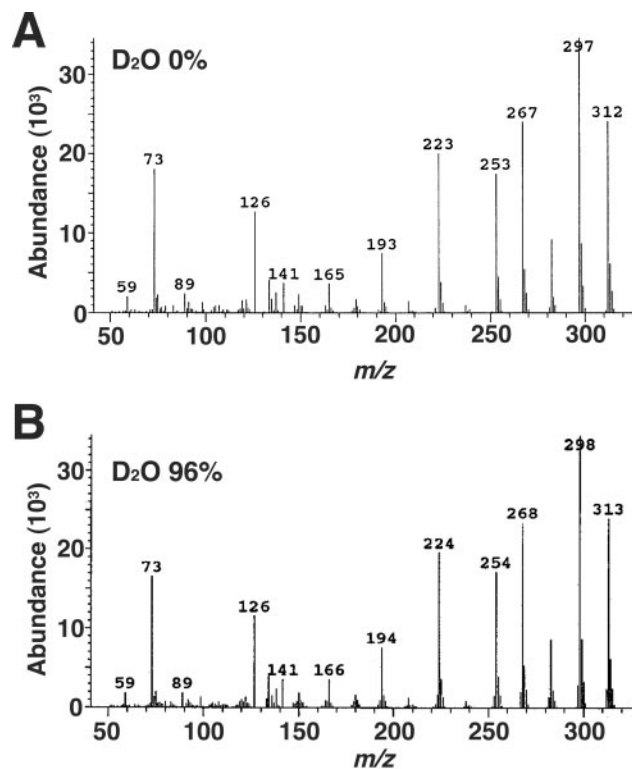

FIG. 5. GC-MS analysis of the reaction product from 5CVA catalyzed by LigW2 in the presence of D_2O. Mass spectra of TMS-vanillate generated in the presence of 0 and 96% D_2O are shown in panels A and B, respectively.

To confirm that LigW2 also acts as a nonoxidative decarboxylase, the cell extract of *E. coli* harboring pW2 was incubated with 400 μM 5CVA for 30 min in the presence of 0 or 96% D_2O. The reaction product, vanillate, was extracted and subjected to GC-MS analysis. The mass spectrum of vanillate generated in the presence of 96% D_2O was compared with that of vanillate produced in the absence of D_2O (Fig. 5). Unlabeled TMS-vanillate showed an intense molecular ion at *m/z* 312, and the major ion fragments were observed at *m/z* 297 (M-CH_3), 282 (M-2CH_3), and 267 (M-3CH_3). On the other hand, the abundance of these ion fragments of TMS-vanillate produced in the presence of D_2O decreased, and that of the ion fragments at *m/z* 313, 298, 283, and 268 increased. This result indicated that the 5-carboxyl group in 5CVA was replaced by a deuterium atom that originated from D_2O. LigW2 was concluded to be a nonoxidative decarboxylase, like LigW.

No decarboxylation activity of LigW2 toward 5CVA analogs like 3-methoxysalicylate, 4-hydroxyisophthalate, and isophthalate was detected. The substrate specificity of LigW2 appeared to be restricted to 5CVA.

Localization of *ligW2* on the 95-kb AseI fragment. In our previous study (41), we demonstrated that the gene clusters for DDVA and β-aryl ether degradation lie in the 340-kb AseI fragment, and the PCA 4,5-cleavage pathway gene cluster and *ligH*, which is thought to encode the 10-formyltetrahydrofolate synthetase involved in one-carbon metabolism, are located in the 95-kb AseI fragment. Recently, we found that the vanillate/3-*O*-methylgallate *O*-demethylase gene (*ligM*) and the 5,10-methylenetetrahydrofolate reductase gene (*metF*) are located upstream of *ligH* (1). Southern hybridization analysis using the 3.6-kb SalI fragment carrying *ligW2* as a probe indicated that *ligW2* was located on the 95-kb AseI fragment (data not shown). To determine the localization of *ligW2* and other genes in the 95-kb AseI fragment, Southern hybridization analysis of pDEC6 with the pHN139F and pDE20 probes carrying the PCA 4,5-cleavage pathway genes and part of *ligM*, *metF*, and *ligH*, respectively, was carried out. The pDE20 probe hybridized to the 1.0-, 2.2-, 2.3-, and 3.6-kb SalI fragments of pDEC6, thus demonstrating the overlap between pDEC6 and pDE20. Comparison of the restriction map of pDEC6 and that of pDE20 revealed that *ligW2* was located approximately 6-kb downstream of *ligH* (Fig. 2). Further analysis is necessary in order to determine the distance between *ligW2* and the PCA 4,5-cleavage pathway genes.

Implications for the DDVA catabolic pathway. In this study, we found that two 5CVA decarboxylase genes are involved in 5CVA degradation, with *ligW2* playing the more important role in the growth of SYK-6 cells on DDVA. It should be noted that in the course of this study, the question was raised regarding whether SYK-6 is able to utilize 4-carboxy-2-hydroxypent-2,4-dienoate (CHPD), which appeared to be generated from the *meta*-cleavage compound of OH-DDVA resulting from the reaction catalyzed by LigY (Fig. 1). Our preliminary experiment indicated that the *ligI* (35), *ligJ* (20), and *ligK* (21) mutants grew well on DDVA (data not shown). Because vanillate generated from 5CVA is degraded through the PCA 4,5-cleavage pathway, a pathway already known to be essential for vanillate degradation, the present findings suggested that CHPD supports the growth of SYK-6. On the other hand, the *ligB* and *ligC* mutants (33) exhibited poor growth on DDVA (data not shown). Recently, we found that generation of 5-methyltetrahydrofolate in the vanillate O demethylation catalyzed by the tetrahydrofolate-dependent *O*-demethylase, LigM, is essential for methionine biosynthesis in this strain. Therefore, SYK-6 is able to grow on PCA only when methionine is added to the medium (1, 34). Disruption of *ligB* and *ligC* seemed to cause the deficiency in *ligM* induction; however, DDW could not grow on DDVA in the presence of 50 mg/liter of methionine (data not shown). These findings may suggest that a metabolite of 5CVA, probably 2-pyrone-4,6-dicarboxylate, is required for induction of CHPD catabolic gene expression. The lack of induction of CHPD catabolic gene expression might explain why DDW lacked the ability to grow on DDVA. However, further study, including isolation of the CHPD catabolic genes and regulation of the all of the genes involved in DDVA catabolism, is necessary to critically assess this possibility.

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