Characterization of the 4-Carboxy-4-Hydroxy-2-Oxoadipate Aldolase Gene and Operon Structure of the Protocatechuate 4,5-Cleavage Pathway Genes in Sphingomonas paucimobilis SYK-6

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The protocatechuate (PCA) 4,5-cleavage pathway is the essential metabolic route for degradation of low-molecular-weight products derived from lignin by Sphingomonas paucimobilis SYK-6. In the 10.5-kb EcoRI fragment carrying the genes for PCA 4,5-dioxygenase (ligAB), 2-pyrene-4,6-dicarboxylate hydrolase (ligC), 4-oxalomesaconate hydratase (ligI), and a part of 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase (ligJ), we found the ligK gene, which encodes 4-carboxy-4-hydroxy-2-oxoadipate (CHA) aldolase. The ligK gene was located 1,183 bp upstream of ligI and transcribed in the same direction as ligI. We also found the ligR gene encoding a LysR-type transcriptional activator, which was located 174 bp upstream of ligK. The ligK gene consists of a 684-bp open reading frame encoding a polypeptide with a molecular mass of 24,131 Da. The deduced amino acid sequence of ligK showed 57 to 88% identity with those of the corresponding genes recently reported in Sphingomonas sp. strain LB126, Comamonas testosteroni BR6020, Arthrobacter keyseri 12B, and Pseudomonas ochraceae NGJ. The ligK gene was expressed in Escherichia coli, and the gene product (LigK) was purified to near homogeneity. Electrospray-ionization mass spectrometry indicated that LigK catalyzes not only the conversion of CHA to pyruvate and oxaloacetate but also that of oxaloacetate to pyruvate and CO2. LigK is a hexamer, and its isoelectric point is 5.1. The Km for CHA and oxaloacetate are 11.2 and 136 μM, respectively. Inactivation of ligK in S. paucimobilis SYK-6 resulted in the growth deficiency of vanillate and syringate, indicating that ligK encodes the essential CHA aldolase for catabolism of these compounds. Reverse transcription-PCR analysis revealed that the PCA 4,5-cleavage pathway genes of S. paucimobilis SYK-6 consisted of four transcriptional units, including the ligK-orf4-ligI-ligA cluster, the ligAB cluster, and the monocistronic ligR and ligC genes.

Protocatechuate (PCA) is one of the most important intermediate metabolites in the bacterial degradation pathways for various aromatic compounds, including low-molecular-weight products derived from lignin. Sphingomonas paucimobilis SYK-6 is able to degrade a wide variety of dimeric lignin compounds, including β-aryl ether (24, 25), biphenyl (33, 34), pinoresinol, phenylcoumarane, and diarylpropene. In S. paucimobilis SYK-6, dimeric lignin compounds with guaiacyl (4-hydroxy-3-methoxyphenyl) and syringyl (4-hydroxy-3,5-dimethoxyphenyl) moieties are thought to be converted to vanillate and syringate, respectively (26). Vanillate and syringate are converted into PCA 4,5-dioxygenase (3,4-PCD) product, the three dioxygenase species: PCA 3,4-dioxygenase (3,4-PCD), 4-oxalomesaconate hydratase (ligI), and a part of 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase (ligJ), we found the ligK gene, which encodes 4-carboxy-4-hydroxy-2-oxoadipate (CHA) aldolase. The ligK gene was located 1,183 bp upstream of ligI and transcribed in the same direction as ligI. We also found the ligR gene encoding a LysR-type transcriptional activator, which was located 174 bp upstream of ligK. The ligK gene consists of a 684-bp open reading frame encoding a polypeptide with a molecular mass of 24,131 Da. The deduced amino acid sequence of ligK showed 57 to 88% identity with those of the corresponding genes recently reported in Sphingomonas sp. strain LB126, Comamonas testosteroni BR6020, Arthrobacter keyseri 12B, and Pseudomonas ochraceae NGJ. The ligK gene was expressed in Escherichia coli, and the gene product (LigK) was purified to near homogeneity. Electrospray-ionization mass spectrometry indicated that LigK catalyzes not only the conversion of CHA to pyruvate and oxaloacetate but also that of oxaloacetate to pyruvate and CO2. LigK is a hexamer, and its isoelectric point is 5.1. The Km for CHA and oxaloacetate are 11.2 and 136 μM, respectively. Inactivation of ligK in S. paucimobilis SYK-6 resulted in the growth deficiency of vanillate and syringate, indicating that ligK encodes the essential CHA aldolase for catabolism of these compounds. Reverse transcription-PCR analysis revealed that the PCA 4,5-cleavage pathway genes of S. paucimobilis SYK-6 consisted of four transcriptional units, including the ligK-orf4-ligI-ligA cluster, the ligAB cluster, and the monocistronic ligR and ligC genes.

In the case of S. paucimobilis SYK-6, PCA is degraded via the PCA 4,5-cleavage pathway (Fig. 1). This pathway was enzymatically characterized by Kersten et al. (16) and Maruyama and coworkers (18-22). In this pathway, PCA is initially transformed to 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS) by 4,5-PCD (LigAB). CHMS is nonenzymatically converted to an intramolecular hemiacetal form and then oxidized by CHMS dehydrogenase. The resulting intermediate, 2-pyrene-4,6-dicarboxylate (PDC), is hydrolyzed by PDC hydrolase to yield the keto form and enol form of 4-oxalomesaconate (OMA), which are in equilibrium. OMA is converted to 4-carboxy-2-hydroxy-2-oxoadipate (CHA) by OMA hydrolase. Finally, CHA is cleaved by CHA aldolase to produce pyruvate and oxaloacetate. Recently, we have identified and characterized all of the gene products and genes except the CHA aldolase gene in the SYK-6 PCA 4,5-cleavage pathway (11, 27, 28, 31, 44). These genes are essential to PCA degradation, while the 3MGA degradation is suggested to go through both the PCA 4,5-cleavage pathway and an alternative ring-cleavage pathway. In this alternative pathway, 3MGA was finally converted to OMA and then entered the PCA 4,5-cleavage pathway. Thus, the PCA 4,5-cleavage pathway genes play a key role in PCA and 3MGA degradation (11).

Recently, cloning of the PCA 4,5-cleavage pathway genes has been reported in Sphingomonas sp. strain LB126 (48), Comamonas testosteroni BR6020
FIG. 1. Catabolic pathway of vanillate and syringate by *S. paucimobilis* SYK-6 (A) and organization of the PCA 4,5-cleavage pathway genes (B). (A) LigA and LigB, the small and large subunits of 4,5-PCD (31, 44); LigH, an essential gene product for vanillate and syringate O demethylations (30); LigC, CHMS dehydrogenase (27); LigI, PDC hydrolase (28); LigJ, OMA hydratase (11); LigK, CHA aldolase/oxaloacetate (OA) decarboxylase (in this study). The degradation pathway for syringate indicated by a dashed line was suggested in our previous study (11, 27, 28). (B) orf1, orf2, ligI, ligJ, ligK, ligR, and lignostilbene α,β-dioxygenase homolog (lsdA) genes are demonstrated by the filled arrows. Vertical bars above the restriction map indicate the positions of the Km^r^ gene insertion of orf1 mutant (DF1), lKg mutant (DLK), lKgR mutant (DLR), and orf2 mutant (DF2). Double-headed arrows indicate locations of amplified RT-PCR products shown in Fig. 5. Abbreviations for restriction enzymes: C, ClaI; E, EcoRI; Ec, Eco47III; P, PstI; Pu, PpuMI; Sh, SphI; Sl, SalI; Sm, SmaI; X, XhoI; Xb, XbaI.
(36), and *Pseudomonas ochracea* NGJ1 (23). However, detailed information is not available in regard to the actual role and property of each of the corresponding gene products. In this study, we characterized the structure and functions of the CHA aldolase gene, which is involved in the final step of the PCA 4,5-cleavage pathway. We also examined the involvement of the two open reading frames (ORFs) found among the CHA aldolase gene, which is involved in the 4,5-cleavage pathway enzymes, and the operon structure of this pathway genes was estimated.

**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 (33) containing 10 mM vanillate or syringate or in Luria-Bertani (LB) medium (1). 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 (33) containing 10 mM vanillate or syringate or in Luria-Bertani (LB) medium (1).

**Preparation of substrate.** PDC and OMA were prepared as described earlier (28). CHA was prepared by incubating 1 mmol of OMA with 500 U of purified OMA hydratase for 5 min (11). Electrospray-ionization mass spectrometry (ESI-MS) analysis revealed that the m/z 201 showing [M-H]⁻ of OMA (where M is a molecular ion of OMA) was completely converted into m/z 201, indicating [M-H]⁻ of CHA by LigJ. The reaction product of OMA (where M is a molecular ion of OMA) was completely converted into m/z 201 showing [M-H]⁻ of OMA (where M is a molecular ion of OMA) was completely converted into m/z 201, indicating [M-H]⁻ of CHA by LigJ. Then, the reaction product of OMA catalyzed by LigJ was used as a substrate.

**DNA manipulations and nucleotide sequencing.** DNA manipulations were performed according to the method described below by using a BioCAD700E apparatus (PerSeptive Biosystems, Framingham, Mass.).

**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**

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<th>Strains or plasmid</th>
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<td>Mutant derivative of SYK-6; Km⁺ gene insertion mutant of <em>ligK</em>; Na⁺ Sm⁺ Km⁺</td>
<td>This study</td>
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<tr>
<td>DF1</td>
<td>Mutant derivative of SYK-6; Km⁺ gene insertion mutant of <em>orf1</em>; Na⁺ Sm⁺ Km⁺</td>
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<tr>
<td>DF2</td>
<td>Mutant derivative of SYK-6; Km⁺ gene insertion mutant of <em>orf2</em>; Na⁺ Sm⁺ Km⁺</td>
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*Abbreviations: Na⁺, Sm⁺, Km⁺, and Ap⁺, resistance to nalidixic acid, streptomycin, kanamycin, and ampicillin, respectively.*
buffer (pH 8.0) and analyzed by ESI-MS as described above. On the other hand, the metabolites were extracted by ethylacetate, trimethylsilylated, and analyzed by GC-MS.

**Dispersion of orf1, ligK, ligR, and orf2.** The 4.0-kb XhoI-SmalI fragment carrying ligR and orf2 was cloned into pBluescript II SK(+) to generate pSX4, and it was digested with PvuII for ligK disruption or with SalI for orf2 disruption. The 1.2-kb PstI fragment containing the kanamycin resistance gene from pUC4K (47) was inserted into the PvuII or SalI site of the 4.0-kb XhoI-SmalI fragment to construct pSX4K and pSX5K, respectively. pSX4K and pSX5K were digested with BamHI and KpnI, and their inserts were cloned into pK19mobSacB (40) to generate pLDK and pFID, respectively. The 1.4-kb ClaI-SmalI fragment carrying ligR was cloned into pUC19 to generate pCS8, and it was digested with EcoRI7HIII. The kanamycin resistance gene was inserted into this Eco47III site. The resultant plasmid, pPS18K, was digested with KpnI and SalI, and the insert containing the inactivated ligR gene was cloned into pK19mobSacB to generate pLD.

Each of plasmids, pLDK, pFID, pLDR, and pF2D was introduced into SYK-6 cells by electroporation, and the candidates for mutants were isolated as described previously (28). To examine the disruption of each gene, Southern hybridization analysis was carried out. The total DNA of the candidates for ligK, ligR, and orf2 mutants was digested with PstI, and those for orf1 and SmalI. The 1.2-kb PstI fragment carrying ligK and orf2 was cloned into pUC19 to generate pPS17, and it was digested with SmalI. The kanamycin resistance gene was inserted into the SmalI site. The resultant plasmid, pPS17K, was digested with BamHI and KpnI, and the insert containing the inactivated orf2 gene was cloned into pK19mobSacB to generate pF2D.

Reverse transcription (RT)-PCR. Cells of *S. paucimobilis* SYK-6 were grown in W minimal salt medium containing 10 mM vanillate until they reached the turbidity at 660 nm of 0.5. Total RNA was prepared from 10 ml of culture by using RNasy Mini columns (Qiagen Inc, Chatsworth, Calif.). To remove any contaminating genomic DNA, the RNA samples were incubated with 1 unit of RNase-free DNase (Takara Shuzo Co., Ltd.) in 40 mM Tris-HCl (pH 7.9) containing 1 unit of RNase inhibitor (Takara Shuzo Co., Ltd.), 10 mM NaCl, 10 mM CaCl₂, and 6 mM MgSO₄ for 30 min at 37°C. RT-PCR was carried out with a BioBEST RNA PCR kit (Takara Shuzo Co., Ltd.). A cDNA library was obtained by an RT reaction using a hexanucleotide random priming mix. The cDNA was used as a template for subsequent PCRs with specific primers, which amplify the boundaries of *lig*orf-1-lig-ligA and ligorf-2-lig-ligB-ligC. The forward and reverse primers used were as follows: lis4-forward (nucleotide positions from 1,363 to 1,383 in the 10.5-kb EcoRI fragment) and ligg-reverse (positions 1,924 to 1,944); ligg-forward (positions 2,528 to 2,548) and orf1-reverse (positions 2,999 to 3,019); orf1-forward (positions 3,499 to 3,509) and ligg-reverse (positions 3,740 to 3,760); internal ligg-forward (positions 4,633 to 4,533) and internal ligg-reverse (positions 5,215 to 5,235); ligg-forward (positions 5,770 to 5,790) and orf2-reverse (positions 6,476 to 6,496); internal orf2-forward (positions 5,843 to 5,863) and orf2-reverse; orf2-forward (positions 6,536 to 6,556) and ligg-reverse (positions 6,943 to 6,963); ligg-forward (positions 7,662 to 7,682) and ligg-reverse (positions 8,162 to 8,182); ligg-forward (positions 8,182 to 8,192) and ligg-reverse (positions 8,870 to 8,726); ligg-forward (positions 9,119 to 9,139) and ligg-reverse (positions 9,598 to 9,608); internal ligg-forward (positions 9,609 to 9,629) and internal ligg-reverse (positions 10,098 to 10,118). Control samples in which reverse transcriptase was omitted in RT-PCR and in which genomic DNA was used as a template in PCRs were run in parallel with RT-PCRs.

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. AB073227.

**RESULTS AND DISCUSSION**

**Nucleotide sequence analysis.** We detected the *cha* aldolase activity in *E. coli* JM109 harboring pHNI139F, which contained the 10.5-kb EcoRI fragment carrying ligAB (31), ligg (28); liggC (11), and a part of *ligC* (27) (Fig. 1B). In the deletion analysis, the DNA region that conferred *cha* aldolase activity to *E. coli* was limited to the 1.0-kb SauI-SphI fragment. The nucleotide sequences of the 5.0-kb *SmaI* fragment and the overlapping 1.7-kb *PstI* fragment were determined, and an
ORF of 684 bp was revealed in the 1.0-kb SalI-SphI fragment. This ORF encodes 228 amino acid residues with a molecular mass of 24,131 Da and was designated ligK. The deduced amino acid sequence of ligK shared the highest degree of identity (88%) with that of fldZ, which is located in the putative PCA 4,5-cleavage pathway genes of the fluorene degrader Sphingomonas sp. strain LB126 (48). The deduced amino acid sequence of ligK also showed 66, 66, and 57% identity with those of the CHA aldolase genes recently identified in C. testosteroni BR6020 (36), P. ochraceae NGJ1 (23), and A. keyseri 12B (6), respectively. Three ORFs were also found adjacent to ligK (Fig. 1B). orf1 was located downstream of ligK with a direction of transcription identical to that of ligK. On the other hand, ligR and orf2 were located upstream of ligK with a transcription orientation opposite that of ligK. The orf1 product showed 78 and 44% identity to FldA of LB126 and a putative transmembrane protein of YbhH in E. coli (GenBank accession no. D90715), whose functions are unknown. This similarity may suggest that orf1 encodes a transporter for substrates such as PCA, vanillate, and/or syringate. LigR has 28% identity with various LysR-type transcriptional regulators, including PcaQ of α proteobacterium Y3F (3), and 22% identity with SdsB, which positively regulates alkyl sulfatase (SdsA) of Pseudomonas sp. strain ATCC 19151 (5). LigR might be involved in the transcriptional control of ligK-orf1-ligI-lsdA, because LysR-type transcriptional regulators generally control the divergently transcribed operon (41). We are unable to conjecture about the function of orf2, although its deduced amino acid sequence showed 50% identity with FldX from Sphingomonas sp. strain LB126 (48) and 39% identity with a conserved hypothetical protein from Sinorhizobium melliloti (8), whose functions are unknown.

The PCA 4,5-cleavage pathway genes of S. paucimobilis SYK-6 consisted of the two divergently transcribed gene clusters of ligK-orf1-ligI-lsdA and ligR-orf2-ligI-ligA-ligB-ligC (Fig. 1B). Recently, all or partial sequences of the PCA 4,5-cleavage pathway genes have been reported in Sphingomonas sp. strain LB126 (48), A. keyseri 12B (6), C. testosteroni BR6020 (36), and P. ochraceae NGJ1 (23). Two types of gene clusters can be clearly seen. Interestingly, the gene organization of the fluorene degrader, Sphingomonas sp. strain LB126, is essentially the same as that of SYK-6, although the identities of the corresponding genes between SYK-6 and LB126 vary from 50 to 88%. On the other hand, C. testosteroni BR6020 and A. keyseri 12B have a packed single gene cluster that seems to be an operon.

Purification of CHA aldolase. The 1.0-kb SalI-SphI fragment carrying ligK was cloned in pET21(+) to construct pETK, and ligK was expressed in E. coli BL21(DE3) under the control of the T7 promoter. Production of the 27-kDa protein was observed by SDS-PAGE (data not shown). The size of the product is close to the molecular mass calculated from the deduced amino acid sequence of ligK. LigK was purified from a cell extract of E. coli BL21(DE3) harboring pETK by a series of
column chromatography procedures with PI, HQ, and PE. LigK was purified approximately 52-fold to near homogeneity (>99%) with a recovery of 20%. N-terminal amino acid sequencing revealed that the first 15 residues, with the exception of the first methionine, Arg-Gly-Ala-Ala-Met-Gly-Val-Val-Val-Gln-Asn-Ile-Glu-Arg-Ala, corresponded to the deduced amino acid sequence of ligK.

**Identification of the reaction product.** To identify the reaction product of CHA catalyzed by the purified LigK, the reaction mixture was analyzed by ESI-MS. The fragment at m/z 219 in Fig. 2B was estimated to be the deprotonated molecular ion ([M-H]⁻) of CHA (where M is a molecular ion). The other peaks in the spectrum of CHA were originated from components of the reaction buffer containing LigK enzyme (Fig. 2A). After 1 min of reaction, the intensity of the fragment at m/z 219 of CHA decreased to 42% of its initial intensity, and the generation of two fragments at m/z 87 and at m/z 131 corresponding to [M-H]⁻ of pyruvate and [M-H]⁻ of oxaloacetate, respectively, was observed (Fig. 2C). This result indicated that LigK catalyzes the conversion of CHA to pyruvate and oxaloacetate. After 5 min of reaction, the intensity of the fragment at m/z 87 increased to 144% of that of the corresponding fragment in the 1-min reaction mixture, whereas the intensity of the fragment at m/z 131 decreased to 38% of that of the corresponding fragment in the 1-min reaction mixture (Fig. 2D). These results strongly suggested that oxaloacetate was converted into pyruvate by LigK. The activity for β-decarboxylation of oxaloacetate has been reported in CHA aldolase of *P. putida* (45), and 2-keto-4-hydroxyglutarate aldolase of *E. coli* (29). To examine whether LigK has this activity, oxaloacetate was incubated with LigK in the presence of lactate dehydrogenase and NADH. A decrease in absorbance at 340 nm derived from NADH was observed. It is concluded that LigK is able to decarboxylate oxaloacetate to generate pyruvate.

**Enzyme properties.** In accord with the previous study by Maruyama (21), the CHA aldolase activity was observed only when a divalent cation such as Mg²⁺ was present in the reaction mixture. We examined the effect of the various divalent cations for the enzyme activities of LigK. Addition of 1 mM Co²⁺, Zn²⁺, Ca²⁺, or Mn²⁺ resulted in 85, 65, 20, or 0% of the activity resulting from addition of 1 mM Mg²⁺, respectively. A similar metal dependency was observed in the decarboxylation of oxaloacetate by LigK. When 1 mM EDTA was added to the reaction mixture, both enzyme activities were completely lost in the presence of 1 mM metal ion. Aldolases are categorized as class I or class II based on their metal dependency. LigK was suggested to be one of the class II aldolases, which require the metal ion. Most class II aldolases show a significant rate enhancement in the presence of phosphate ion (37). Addition of 0.5 mM phosphate ion in the LigK reaction mixture caused 3.0- and 1.7-fold activation of CHA aldolase and oxaloacetate decarboxylase activity, respectively.

Gel filtration column chromatography using the Superdex200 indicated that the molecular mass of the native LigK was 160 kDa. This result suggested that LigK is a homohexamer. The isoelectric point of LigK was determined to be 5.1 by isoelectric focusing gel electrophoresis. The optimal tem-
perature of LigK for aldolase activity on CHA, and the decarboxylase activity on oxaloacetate were both determined to be 25°C. The optimal pH for aldolase activity and decarboxylase activity were estimated to be 8.0 and 7.0, respectively. The $K_m$ for oxaloacetate (136 μM) is 12 times higher than that for CHA (11.2 μM). The $V_{max}$ for CHA aldol cleavage (265 U/mg) is 20 times higher than that for oxaloacetate decarboxylation (13.2 U/mg).

We also examined the influence of sulphydryl reagents on LigK. One microgram of purified LigK was preincubated with 1 mM sulphydryl reagents for 10 min. HgCl₂ and N-ethylmaleimide inhibited 60 and 62% of the CHA aldolase and 92 and 88% of the oxaloacetate decarboxylase activities, respectively. These results suggested that some cysteine residues might be involved in the enzyme reaction. CHA aldolase activity was inhibited by oxaloacetate with a $K_i$ value of 23 μM. As suggested by Maruyama (21), the amount of oxaloacetate in the cells might control the production of oxaloacetate from CHA.

CHA aldolase has been biochemically characterized only in P. ochraceae (21) and P. putida (45). The molecular mass, subunit structure, and pI of LigK are very similar to those of aldolases of P. ochraceae and P. putida. In the P. ochraceae enzyme, the kinetics parameters are measured using the substrates $d$-CHA and $l$-CHA. The $K_m$ and $V_{max}$ values of the P. ochraceae enzyme for $l$-CHA were similar to those for LigK. In our experiment, CHA was prepared from OMA by using the purified OMA hydratase from E. coli carrying the SYK-6 ligJ gene. The physiological substrate for LigK might be an $l$-isomer. The $K_m$ for oxaloacetate of the P. ochraceae enzyme was twofold higher than that of LigK, although the $V_{max}$ values of these strains are similar. LigK has a significantly higher affinity for oxaloacetate than did the P. ochraceae enzyme.

Disruption of ligK in S. paucimobilis SYK-6. The ligK gene was disrupted to clarify the actual role of ligK in the catabolism of vanillate and syringate by SYK-6. Gene inactivation was carried out using the ligK disruption plasmid, pLKd. The ligK insertional mutation was confirmed by Southern hybridization analysis using the 2.3-kb PstI fragment carrying ligK and the 1.2-kb PstI fragment carrying the kanamycin resistance gene as probes (data not shown). The ligK and kanamycin resistance gene probes revealed that the ligK gene was inactivated by homologous recombination through the double crossover. This mutant strain was designated DLK and used for the following experiments. The obtained mutant strain DLK completely lost the ability to grow on both vanillate and syringate. This result is compatible with the deduced catabolic pathways of vanillate and syringate by SYK-6 shown in Fig. 1A.

To determine the accumulated products from vanillate and syringate incubated with DLK, 10 mM concentrations of vanillate and syringate were independently incubated with the LB-grown whole cells of DLK in the W minimal medium, and the metabolites were identified by GC-MS and ESI-MS. As shown in the gas chromatogram (Fig. 3A and B), vanillate and syringate detected with retention times of 21.2 and 25.2 min, respectively, disappeared completely, and the accumulation of PDC, the enol form of OMA, product I with a retention time of 30.5 min, and product II with a retention time of 26.1 min was observed in both cultures. In a previous study, we identified product I as the compound generated from OMA by addition of two atoms of hydrogen by NADPH-dependent reductase of the ligJ insertion mutant of SYK-6 (11). The mass spectrum of product II accumulated in both cultures are identical but could not be assigned (data not shown). On the other hand, ESI-MS analysis indicated accumulation of the products whose deprotonated molecular ions appeared at $m/z$ 203 and at $m/z$ 221 in the metabolite from vanillate and syringate (Fig. 3C and D). In this analytical condition, neither PDC nor the enol form of OMA could be detected. We previously suggested that the ion at $m/z$ 203 was a deprotonated molecular ion of product I. We therefore estimated that the ion at $m/z$ 221 was generated from CHA accumulated by addition of two hydrogen atoms catalyzed by unidentified reductase(s) in DLK. To examine this hypothesis, CHA was incubated with the DLK crude extract prepared from cells grown in LB. ESI-MS of the reaction product after 10 min incubation showed that the peak at $m/z$ 219 derived from CHA was converted to that at $m/z$ 221 only in the presence of NADPH (data not shown). These

![FIG. 4. Growth of SYK-6 and ligR insertion mutant (DLR) on vanillate and syringate. Growth of SYK-6 (open circles) and DLR (filled circles) on 10 mM vanillate (A) and 10 mM syringate (B). The results are the means of the representative of three independent experiments.](http://jb.asm.org/Downloaded from)
results strongly suggested that product II was produced from accumulated CHA. Our preliminary experiment indicated that LigJ activity was not inhibited by the presence of CHA, and thus the reason why a large amount of OMA (product I) and PDC were also accumulated from vanillate and syringate in DLK is unknown.

Disruption of ligR, orf1, and orf2 in S. paucimobilis SYK-6.

To investigate whether ligR, orf1, and orf2 are involved in the catabolism of vanillate and syringate, each of these genes in SYK-6 was disrupted. Gene inactivation was carried out using the ligR, orf1, and orf2 disruption plasmids, pLRD, pF1D, and pF2D, respectively. The growth rates of the ligR disruption mutant, DLR, on both vanillate and syringate were decreased compared with those of SYK-6 (Fig. 4). Based on this result and the fact that LigR has similarity with LysR-type transcriptional regulator, LigR may positively regulate the expression of the PCA 4,5-cleavage pathway genes, although it is not essential to growth of SYK-6 on vanillate and syringate. However, the actual role of orf1 is remained to clarify together with that of ligR. On the other hand, the disruption of orf2 did not affect the growth of SYK-6 on both vanillate and syringate.

RT-PCR analysis of PCA 4,5-cleavage pathway genes.

To determine the operon structure of the genes included in the 10.5-kb EcoRI fragment, RT-PCR experiments were performed with total RNA isolated from SYK-6 grown on vanillate and primers complementary to neighboring ORFs. The amplification products of lsdA-ligI (581 bp), ligI-orf1 (491 bp), orf1-ligK (271 bp), ligJ-ligA (520 bp), and ligA-ligB (564 bp) were obtained. However, RT-PCR products using primer which span the ligR-orf2, orf2-ligJ, and ligB-ligC regions were not obtained (Fig. 5), while PCR using their primers with SYK-6 total DNA as a template gave the expected PCR products (data not shown). In order to confirm the presence of the ligR, orf2, and ligC transcripts in the RNA samples, RT-PCR was carried out using primers to amplify inside of each ORF. RT-PCR products of ligR (622 bp) and ligC (509 bp) with the
expected sizes were obtained (Fig. 5). On the other hand, the RT-PCR product of orf2 did not appear, indicating the DNA region of orf2 was not transcribed in SYK-6 cells grown on vanillate.

In conclusion, the PCA 4,5-cleavage pathway genes of S. paucimobilis SYK-6 consist of four transcriptional units, including the ligC-ligIJFBDKCHG cis-clusters are independently regulated by the IclR-type transcriptional activator PcaU in concert with the monocistronic orf2.


