Characterization of *ligV* Essential for Catabolism of Vanillin by *Sphingomonas paucimobilis* SYK-6

Eiji Masai,¹,²† Yuko Yamamoto,¹ Tomohiko Inoue,¹ Kazuhiro Takamura,¹ Hirofumi Hara,¹ Daisuke Kasai,¹ Yoshihiro Katayama,² and Masao Fukuda¹

¹Department of Bioengineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2188, Japan
²Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan

Received May 2, 2007; Accepted June 15, 2007; Online Publication, October 7, 2007

The vanillin dehydrogenase gene (*ligV*), which conferred the ability to transform vanillin into vanillate on *Escherichia coli*, was isolated from *Sphingomonas paucimobilis* SYK-6. The *ligV* gene consists of a 1,440-bp open reading frame encoding a polypeptide with a molecular mass of 50,301 Da. The deduced amino acid sequence of *ligV* showed about 50% identity with the known vanillin dehydrogenases of *Pseudomonas* vanillin degraders. The gene product of *ligV* (LigV) produced in *E. coli* preferred NAD⁺ to NADP⁺ and exhibited a broad substrate preference, including vanillin, benzaldehyde, protocatechualdehyde, m-anisaldehyde, and p-hydroxybenzaldehyde, but the activity toward syringaldehyde was less than 5% of that toward vanillin. Insertional inactivation of *ligV* in SYK-6 indicated that *ligV* is essential for normal growth on vanillin. On the other hand, growth on syringaldehyde was only slightly affected by *ligV* disruption, indicating the presence of a syringaldehyde dehydrogenase gene or genes in SYK-6.

Key words: *Sphingomonas*: lignin degradation; aldehyde dehydrogenase; vanillin; syringaldehyde

*Sphingomonas paucimobilis* SYK-6 can utilize various types of lignin-derived biaryls, including β-aryl ether, biphenyl, and diarylpropane, as sole carbon and energy source.¹ Due to the useful and functional organic materials that can be produced from lignin, there is great potential for the utilization of lignin as a bioresource. Recently, our research group established production of 2-pyrene-4,6-dicarboxylate (PDC), an intermediate metabolite of the PCA 4,5-cleavage pathway, from PCA, and the synthesis of highly functional PDC-based polyesters has been developed.²–⁴) Because the chemical oxidation of lignin yields large amounts of vanillin and syringaldehyde, these compounds are among the ultimate starting compounds for the production of PDC, and the conversion of vanillin to PCA is an essential step in achieving successful PDC production.

In general, vanillin is oxidized to vanillate by vanillin dehydrogenase (Fig. 1A). The vanillin dehydrogenase gene, *vdh*, was initially isolated from *Pseudomonas* sp. HR199,⁵) *Pseudomonas putida* WCS358,⁶) and *Pseudomonas fluorescens* AN103.⁷) Recently, the hydroxybenzaldehyde dehydrogenase gene (*hcaB*) of *Acinetobacter* sp. ADP1⁸) and the vanillin dehydrogenase genes found in the genome sequences of *P. putida* KT2440⁹) and *Rhodococcus* strains¹⁰) were reported, but the substrate specificity and the roles of vanillin dehydrogenase in the catabolism of lignin-derived compounds have not been extensively investigated.

In our previous study,¹¹) an open reading frame (ORF, accession no. BAC56974), which showed about 30% identity with *Pseudomonas* vanillin dehydrogenases, was found just upstream of *ligW* encoding 5-carboxyvanillate decarboxylase involved in 5,5'-dehydrodi- vanillate catabolism, but disruption of this gene did not affect vanillin transformation activity in SYK-6. In this study, we isolated the vanillin dehydrogenase gene of SYK-6 and characterized the gene products and the role of the gene in the catabolism of vanillin, syringaldehyde, and ferulate in SYK-6.

Materials and Methods

Strains and culture conditions. *S. paucimobilis* SYK-
suspended in the same buffer containing 1 mM vanillin. 612) is the wild-type strain. It was grown at 30 °C with 50 mM Tris–HCl buffer (pH 7.5). Then cells were centrifuged at 5000 g for 10 min at 4 °C for 10 min. The 3.4-kb BstXI fragment carrying the ligV mutant (DLV). Abbreviations: A, Apal; B, BamHI; Bs, BstXI; EV, EcoRV; NcoI, Nt, NotI; Pv, PvuII; S, SalI; X, XhoI.

Cloning procedure. A gene library constructed with pVK10015) and partially SalI-digested DNA of SYK-6 was introduced by conjugation into S. paucimobilis IAM 12578. Transconjugants were grown in 1 ml of LB containing kanamycin at 30 °C for 12 h. Cells were centrifuged at 4500 × g for 10 min at 4 °C and washed with 50 mM Tris–HCl buffer (pH 7.5). Then cells were suspended in the same buffer containing 1 mM vanillin. After shaking for 12 h at 30 °C, the cells were removed by centrifugation, and the amounts of vanillin in the supernatant were determined with a high-pressure liquid chromatography system (HP1100 series; Agilent Technologies, Palo Alto, CA), using a TSK gel ODS-80 column (6 by 150 mm; Tosoh, Tokyo) The mobile phase was a mixture of water (74%), acetonitrile (25%), and acetic acid (1%), and the flow rate was 1.0 ml/min. Compounds were detected at 280 nm. Six positive clones (pKT1V1-6) were isolated from transconjugants. Southern hybridization analysis of these cosmid clones digested with SalI was performed with digoxigenin (Roche Molecular Biochemicals, Mannheim, Germany)-labeled pKT1V3 and pKT6V as probes. The nucleotide sequence of ligV was determined with a CEQ 2000XL genetic analysis system (Beckman Coulter, Fullerton, CA). Analysis of the nucleotide sequence was performed as described in a previous study.16

Expression of ligV in E. coli. A 530-bp fragment carrying the 5′ portion of ligV was PCR-amplified using primers 5′-TCCCCGATATGGACTCAGC-3′ and 5′-CAGGTCTCGTCGGCTCAGG-3′, and the amplified products were TA cloned into pT7Blue (Novagen, Darmstadt, Germany) to obtain pTLV530. A 528-bp NdeI fragment of pTLV530 was cloned into the NdeI site of pET21a(+) (Novagen) to generate pTLV530a. The 1.5-kb NcoI-XhoI fragment carrying the rest of ligV was subcloned into pTLV530a to generate pLHV. E. coli BL21(DE3) cells harboring pLHV were grown in LB medium containing 100 mg/liter of ampicillin at 30 °C. Expression of ligV was induced for 7 h by adding 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) when the culture at 600 nm reached 0.5. Cells were harvested by centrifugation at 5000 × g for 10 min, suspended in 100 mM potassium phosphate buffer (pH 7.0), and washed with the same buffer. Cells suspended in the 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. Expression of the enzyme was examined by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS–PAGE). The protein concentration was measured with a protein assay kit with bovine serum albumin as the standard (Bio-Rad, Hercules, CA).

Enzyme assay. The assay to determine the vanillin dehydrogenase activity in the cell extracts was carried out principally according to a method described previously.17) The decrease in absorbance at 346 nm derived from vanillin (ε246 = 8697 M–1 cm–1, pH 7.0) was monitored in 100 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM NAD+, 1.2 mM pyruvate, 1.0 U lactate dehydrogenase, 100 μM substrate, and 100–500 μg/ml of cell extract at 30 °C. To test the substrate preference of LigV, the cell extract was incubated with 100 μM of the following substrates in the same reaction mixture as that described above: benzaldehyde (ε293 = 1.270 M–1 cm–1), p-hydroxybenzaldehyde (ε331 = 7.088 M–1 cm–1), protocatechualdehyde (ε341 = 9.070 M–1 cm–1), m-anisaldehyde (ε330 = 2.181 M–1 cm–1), veratraldehyde (ε330 = 3.536 M–1 cm–1), coniferyl aldehyde (ε410 = 3.330 M–1 cm–1), or syringaldehyde (ε364 = 8.936 M–1 cm–1).

Construction of the ligV mutant. The 3.4-kb BstXI fragment carrying ligV was cloned into pK19mobsacB18) to generate pKTC34B (Fig. 1B). In order to construct pK34D, the 0.7-kb Apal-NotI fragment carrying the internal region of ligV in pKTC34B was replaced with
the 1.2-kb ApaI-NotI fragment carrying the kanamycin resistance gene (kan) of pK03,9) pK34D was introduced into SYK-6 cells by electroporation.20) Southern hybridization analysis of the PvuII digests of mutant candidates grown on LB containing 50 mg/liter of kanamycin used as probes the 1.1-kb kan fragment and the 1.2-kb EcoRV fragment carrying ligV and kan respectively.

Results and Discussion

Isolation of the vanillin dehydrogenase gene

A cosmid library of SYK-6 was transferred to S. paucimobilis IAM 12578, and the vanillin transformation activities of the transformants were examined. Six positive clones harboring pKTV1-pKTV6, which accumulated vanillate, were isolated. Southern hybridization analysis using pKTV3 and pKTV6 as probes indicated that these clones can be divided into two groups. We focused on pKTV6, since the vanillin dehydrogenase activity of the cell extract of IAM 12578 harboring pKTV6 was 4 times higher than that of IAM 12578 harboring pKTV3.

Among the subclones of pKTV6, IAM 12578, carrying a 7.2-kb BamHI fragment in pVK100, showed vanillin transformation activity. A further subcloning experiment indicated that the 3.1-kb BamHI-EcoRV fragment conferred vanillin dehydrogenase activity on E. coli. The nucleotide sequence of the 3.1-kb BamHI-EcoRV fragment (accession no. AB287332) revealed an ORF that consisted of 1,440 bp and encoded a polyepitope with a molecular mass of 50,301 Da (Fig. 1B). The deduced amino acid sequence of this ORF (ligV) showed 84% and 73% identity with those of the putative aldehyde dehydrogenase genes of Novosphingobium aromaticivorans DSM 12444 (accession no. AB26108) and Sphingomonas wittichii RW1 (ZP_01605-804) respectively, but exhibited only 50–55% identity with those of the known vanillin dehydrogenase genes of P. putida WCS358,6) Pseudomonas sp. HR199,5) and P. fluorescens AN103.7)

Downstream of ligV, there exists a convergent ORF (orf1) of 1,011 bp. The deduced amino acid sequence of orf1 showed about 30% identity with the putative signal transduction histidine kinase of S. wittichii RW1 (EAW05188). The 3.5-kb SalI fragment located upstream of ligV was isolated and sequenced (accession no. AB287332). An orf2 of 1,590 bp and an incomplete ORF (orf3) showed 62% and 48% identity with the hypothetical proteins of S. wittichii RW1 (ZP_01609414 and ZP_01609416 respectively) (Fig. 1B), but the respective functions of these putative genes are unknown. In the case of Pseudomonas vanillin degraders, the vanillin dehydrogenase gene (vdh) is located between the feruloyl-coenzyme A (CoA) hydratase/lyase gene and the feruloyl-CoA synthetase gene,10) but the ferulate catabolic genes in SYK-6 (ferA, ferB, and ferB2), which have been characterized,21) were not located proximal to ligV. This arrangement is similar to the corresponding cases of Delftia acidovorans9) and Amycolatopsis sp. HR167.22)

Characterization of LigV

The ligV gene expression induced by IPTG in E. coli was examined. A 50-kDa protein was observed by SDS–PAGE of the cell extract from E. coli BL21(DE3) harboring pLVH, which carries ligV (Fig. 2). The vanillin dehydrogenase activities of LigV in the cell extract (100 μg/ml) toward 100 mM of vanillin in the presence of 0.5 mM NAD⁺ was approximately 90 times higher than those with 0.5 mM NADP⁺, indicating that LigV is highly specific for NAD⁺. The substrate preference of LigV was determined using various benzaldehyde derivatives. LigV exhibited the highest dehydrogenase activity toward vanillin, and showed relatively high levels of activity toward benzaldehyde, p-hydroxybenzaldehyde, protocatechualdehyde, and m-anisaldehyde (Table 1 and Fig. 3), but the activity of LigV in a reaction mixture with syringaldehyde, veratraldehyde, and coniferyl alcohol was less than 5% of the activity of the enzyme in the presence of vanillin (Table 1). These results suggest that the level of participation of ligV in syringaldehyde degradation is quite low.

Characterization of the ligV mutant of S. paucimobilis SYK-6

To investigate the roles of ligV in vanillin and syringaldehyde catabolism by SYK-6, ligV in SYK-6 was inactivated by the gene replacement technique with the ligV disruption plasmid pK34D, which was constructed by inserting the kan gene within ligV in pK19mobacB. The insertion mutant was confirmed by Southern hybridization analysis with the ligV and kan genes as probes (Figs. 1B and 4A). The growth of the ligV-inactivated mutant (DLV) on W medium containing 5 mM of vanillin was compared to that of SYK-6 (Fig. 4B). No growth of DLV on vanillin was observed after 30 h of incubation, while the optical density at
600 nm (OD$_{600}$) of the wild-type culture reached 1.0. However, DLV started to grow gradually after 40 h, and the OD$_{600}$ value of the culture finally reached 0.5 after 100 h of incubation (Fig. 4C). On the other hand, the growth of DLV on W medium containing 10 mM vanillate was almost the same as that of SYK-6 (data not shown). These results indicate that $ligV$ is essential for the normal growth of SYK-6 on vanillin, and that an alternative form of vanillin oxidation activity is also present in SYK-6 cells. The disruption of $ligV$ to some extent affected the ability of SYK-6 to grow on syringaldehyde (Fig. 4B). This fact might suggest that $ligV$ contributes to the total activity of syringaldehyde oxidation in SYK-6 cells. The vanillin and syringaldehyde oxidation activities (0.04–0.05 U/mg and 0.005–0.008 U/mg respectively) of the extract of SYK-6 cells grown on vanillin and syringaldehyde did not significantly differ from those of cells grown in LB. These results suggest that the vanillin and syringaldehyde dehydrogenase genes are constitutively expressed. Hence the activity of these enzymes in DLV and SYK-6 was examined using cells grown in LB.

### Table 1. Activities of the Cell Extracts of *E. coli* BL21(DE3) Harboring pLVH, SYK-6, and DLV against Various Benzaldehydes

<table>
<thead>
<tr>
<th>Strain</th>
<th>VAN</th>
<th>BAL</th>
<th>HBA</th>
<th>PAL</th>
<th>AAL</th>
<th>VAL</th>
<th>CAL</th>
<th>SAL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (pLVH)</td>
<td>1,100</td>
<td>970</td>
<td>370</td>
<td>790</td>
<td>590</td>
<td>23</td>
<td>27</td>
<td>44</td>
</tr>
<tr>
<td>SYK-6</td>
<td>43</td>
<td>41</td>
<td>18</td>
<td>27</td>
<td>28</td>
<td>2.8</td>
<td>5.7</td>
<td>7.9</td>
</tr>
<tr>
<td>DLV</td>
<td>4.8</td>
<td>11</td>
<td>6.3</td>
<td>3.7</td>
<td>15</td>
<td>2.3</td>
<td>5.7</td>
<td>6.4</td>
</tr>
</tbody>
</table>

*The data are averages of three independent experiments.

*Abbreviations:* VAN, vanillin; BAL, benzaldehyde; HBA, $p$-hydroxybenzaldehyde; PAL, protocatechualdehyde; AAL, $m$-anisaldehyde; VAL, veratraldehyde; CAL, coniferyl aldehyde; SAL, syringaldehyde; DLV, a $ligV$ insertional inactivated mutant of SYK-6

**Fig. 3.** Chemical Structures of Various Benzaldehydes Used in This Study.

I, vanillin; II, benzaldehyde; III, $p$-hydroxybenzaldehyde; IV, protocatechualdehyde; V, $m$-anisaldehyde; VI, veratraldehyde; VII, coniferyl aldehyde; VIII, syringaldehyde

**Fig. 4.** Disruption of $ligV$ in *S. paucimobilis* SYK-6.

A, Southern blot analysis of the insertion mutant. Lanes: 1 and 3, total DNA of SYK-6 digested with *Pvu*I; 2 and 4, total DNA of the $ligV$ mutant (DLV) digested with *Pvu*I. The 1.1-kb *Sal*I fragment carrying $ligV$ (lanes 1 and 2) and the 1.2-kb *EcoRV* fragment carrying *kan* (lanes 3 and 4) were used as probes. B, Growth of DLV and SYK-6 on vanillin and syringaldehyde. SYK-6 (open symbols) and DLV (closed symbols) were incubated in W medium containing 5 mM vanillin (triangles) or syringaldehyde (squares). C, An extended incubation period made possible the growth of DLV on vanillin. The data are averages ± standard deviations of three independent experiments.
Specifically, a cosmid clone, pKTV3, should be necessary to identify the aldehyde dehydrogenase gene syringaldehyde degradation in SYK-6. Further study is needed to identify the aldehyde dehydrogenase gene or genes involved in the degradation of syringaldehyde. Specifically, a cosmid clone, pKTV3, should be analyzed in the future.

Acknowledgments

This study was supported in part by an Industrial Technology Research Grant received in 2004 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

References

20) Masai, E., Shinohara, S., Hara, H., Nishikawa, S., Katayama, Y., and Fukuda, M., Genetic and biochemical characterization of a 2-pyrene-4,6-dicarboxylic acid


