

**Bacterial catabolism of  $\beta$ -hydroxypropiovanillone and  $\beta$ -hydroxypropiosyringone produced in the reductive cleavage of arylglycerol- $\beta$ -aryl ether in lignin**

Running title: *Downstream pathway of the  $\beta$ -aryl ether catabolism*

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1 **ABSTRACT**

2 *Sphingobium* sp. strain SYK-6 converts four stereoisomers of arylglycerol- $\beta$ -guaiacyl  
3 ether into achiral  $\beta$ -hydroxypropiovanillone (HPV) via three stereospecific reaction  
4 steps. Here we determined the HPV catabolic pathway and characterized the HPV  
5 catabolic genes involved in the first two steps of the pathway. In SYK-6 cells, HPV was  
6 oxidized to vanilloyl acetic acid (VAA) via vanilloyl acetaldehyde (VAL). The resulting  
7 VAA was further converted into vanillate through the activation of VAA by coenzyme  
8 A. A syringyl-type HPV analog,  $\beta$ -hydroxypropiosyringone (HPS), was also catabolized  
9 via the same pathway. SLG\_12830 (*hvpZ*), which belongs to the glucose-methanol-  
10 choline oxidoreductase family, was isolated as the HPV-converting enzyme gene. An  
11 *hvpZ* mutant completely lost the ability to convert HPV and HPS, indicating that *hvpZ* is  
12 essential for the conversion of both the substrates. HpvZ produced in *Escherichia coli*  
13 oxidized both HPV and HPS, and other 3-phenyl-1-propanol derivatives. HpvZ  
14 localized to both the cytoplasm and membrane of SYK-6 and used ubiquinone  
15 derivatives as electron acceptors. Thirteen gene products of the 23 aldehyde  
16 dehydrogenase (ALDH) genes in SYK-6 were able to oxidize VAL into VAA. Mutant  
17 analyses suggested that multiple ALDH genes, including SLG\_20400, contribute to the  
18 conversion of VAL. We examined whether the genes encoding feruloyl-CoA synthetase  
19 (*ferA*) and feruloyl-CoA hydratase/lyase (*ferB* and *ferB2*) are involved in the conversion  
20 of VAA. Only FerA exhibited activity toward VAA; however, disruption of *ferA* did not  
21 affect VAA conversion. These results indicate that another enzyme system is involved in  
22 VAA conversion.

23 **IMPORTANCE**

24 Cleavage of the  $\beta$ -aryl ether linkage is the most essential process in lignin  
25 biodegradation. Although the bacterial  $\beta$ -aryl ether cleavage pathway and catabolic  
26 genes have been well documented, there have been no reports regarding the catabolism  
27 of HPV or HPS, the products of cleavage of  $\beta$ -aryl ether compounds. HPV and HPS  
28 have also been found to be obtained from lignin by chemoselective catalytic oxidation  
29 by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone/*tert*-butyl nitrite/O<sub>2</sub>, followed by  
30 cleavage of the  $\beta$ -aryl ether with zinc. Therefore, value-added chemicals are expected to  
31 be produced from these compounds. In this study, we determined the SYK-6 catabolic  
32 pathways for HPV and HPS, and identified the catabolic genes involved in the first two  
33 steps of the pathways. Since SYK-6 catabolizes HPV through 2-pyrone-4,6-  
34 dicarboxylate, which is a building block for functional polymers, characterization of  
35 HPV catabolism is important not only for understanding the bacterial lignin catabolic  
36 system but also for lignin utilization.

37

38 **KEYWORDS**

39 *Sphingobium*, lignin,  $\beta$ -aryl ether, glucose-methanol-choline oxidoreductase, aldehyde  
40 dehydrogenase

## 41 INTRODUCTION

42 Lignin, one of the major components of plant cell walls, is a complex phenolic  
43 heteropolymer produced from hydroxycinnamyl alcohols by radical coupling (1, 2).  
44 Lignin is the second most abundant bioresource on earth after cellulose and is expected  
45 to be used as an industrial raw material. However, the current industrial applications of  
46 lignin are limited to low value applications such as production of solid fuels and  
47 concrete additives (3, 4). On the other hand, it has been reported that the building blocks  
48 for functional polymers, such as 2-pyrone-4,6-dicarboxylic acid (5-7), *cis,cis*-muconic  
49 acid (8-10), and medium chain-length polyhydroxyalkanoic acid (11), can be obtained  
50 from lignin-derived aromatic compounds such as vanillic acid, vanillin, ferulic acid, and  
51 *p*-coumaric acid through microbial catabolism. Therefore, production of value-added  
52 chemicals from lignin through transformations systems that consist of chemical lignin  
53 decomposition and microbial catabolism of lignin-derived aromatics has attracted  
54 attention.

55  $\beta$ -Aryl ether is the most abundant linkage in lignin, comprising 45%–50% of all  
56 linkages in softwood lignin and 60%–62% in hardwood lignin (4). Accordingly,  
57 degradation of this structure is considered a crucial step in lignin biodegradation.  $\beta$ -Aryl  
58 ether-type biaryls have two distinct isomeric forms, *erythro* and *threo*, each of which  
59 has enantiomeric forms (12). To date, the whole picture of the enzyme system for the  
60 cleavage of  $\beta$ -aryl ether in *Sphingobium* sp. strain SYK-6 has been determined (13).  
61 SYK-6 is able to degrade all the stereoisomers of  $\beta$ -aryl ether-type biaryl,  
62 guaiacylglycerol- $\beta$ -guaiacyl ether (GGE). In SYK-6 cells, four stereoisomers of GGE  
63 are converted to two enantiomers of  $\alpha$ -(2-methoxyphenoxy)- $\beta$ -hydroxypropiovanillone  
64 (MPHPV) through the oxidation of the GGE  $\alpha$ -carbon atom catalyzed by C $\alpha$ -

65 dehydrogenases (LigD, LigL, and LigN) (Fig. 1) (14). LigD oxidizes ( $\alpha R, \beta S$ )-GGE and  
66 ( $\alpha R, \beta R$ )-GGE into ( $\beta S$ )-MPHPV and ( $\beta R$ )-MPHPV, respectively, while LigL/LigN  
67 converts ( $\alpha S, \beta R$ )-GGE and ( $\alpha S, \beta S$ )-GGE into ( $\beta R$ )-MPHPV and ( $\beta S$ )-MPHPV,  
68 respectively (14). The ether linkage of the resulting MPHPV is cleaved by  
69 enantioselective glutathione *S*-transferases (GSTs), LigF, LigE, and LigP, to produce  $\alpha$ -  
70 glutathionyl- $\beta$ -hydroxypropiovanillone (GS-HPV) and guaiacol via nucleophilic attack  
71 of glutathione on the MPHPV  $\beta$ -carbon atom (15, 16). LigF and LigE/LigP attack ( $\beta S$ )-  
72 MPHPV and ( $\beta R$ )-MPHPV to produce ( $\beta R$ )-GS-HPV and ( $\beta S$ )-GS-HPV, respectively.  
73 Another GST, LigG, catalyzes the cleavage of the thioether linkage in ( $\beta R$ )-GS-HPV by  
74 transferring glutathione of ( $\beta R$ )-GS-HPV to another glutathione molecule to produce  
75 HPV and glutathione disulfide (15, 17). On the other hand, LigG had little to no activity  
76 with ( $\beta S$ )-GS-HPV, suggesting involvement of an alternative GST in the conversion of  
77 ( $\beta S$ )-GS-HPV (15, 18). Recently, further detailed biochemical characterization of the  $\beta$ -  
78 aryl ether catabolic enzymes of SYK-6 and their orthologs in other bacterial strains, and  
79 structural analyses of LigD, LigL, LigE, LigF, and LigG have been performed (17-29).  
80 In addition, *Erythrobacter* sp. strain SG61-1L and *Novosphingobium* sp. strain  
81 MBES04, which are capable of cleaving  $\beta$ -aryl ether, have recently been isolated, and  
82 similar enzyme systems have been characterized (23, 24).

83 Although many investigations of the cleavage of  $\beta$ -aryl ether have been  
84 performed, there are no reports on the characterization of the catabolism of HPV, the  
85 product of the cleavage of  $\beta$ -aryl ether. Therefore, for understanding bacterial  $\beta$ -aryl  
86 ether catabolism, it is essential to elucidate the HPV catabolic system. Recently, the  
87 production of HPV and  $\beta$ -hydroxypropiosyringone (HPS; an intermediate metabolite of  
88 syringyl-type  $\beta$ -aryl ether) from lignin has been attempted through biological and

89 chemical processes for the purpose of obtaining phenolic monomers from lignin (27,  
90 30). Ohta et al. reported that HPV and HPS could be obtained from milled wood lignin  
91 from Japanese cedar (*Cryptomeria japonica*) and *Eucalyptus globulus* after reactions  
92 with MBES04 enzymes (27). Lancefield et al. reported an isolation method for HPV  
93 and HPS from Birch lignin via catalytic oxidation of the  $\beta$ -aryl ether linkage in lignin  
94 followed by zinc-mediated cleavage of the ether bonds (30). By combining these  
95 decomposition methods with microbial catabolism of HPV and HPS, development of a  
96 production system for value-added chemicals from lignin is expected.

97 In this study, we determined the catabolic pathway of HPV and HPS in SYK-6,  
98 and characterized the genes involved in the first two steps of the pathway.

## 99 RESULTS AND DISCUSSION

100 **Determination of the pathway for the catabolism of HPV and HPS in *Sphingobium***  
101 **sp. SYK-6.** In order to determine the catabolic pathway of HPV in SYK-6, intermediate  
102 metabolites generated during the incubation of HPV with resting cells of SYK-6 were  
103 identified. Resting cells of SYK-6 grown in Wx minimal medium (31) containing 10  
104 mM sucrose, 10 mM glutamate, 0.13 mM methionine, and 10 mM proline (Wx-SEMP)  
105 were incubated with 1 mM HPV for 6 h, and the reaction mixtures were analyzed by  
106 high-performance liquid chromatography-mass spectrometry (HPLC-MS). This analysis  
107 indicated that HPV was converted into compound I with a retention time of 3.1 min  
108 (Fig. 2B). Based on a comparison of the retention time and  $m/z$  value of the  
109 deprotonated ion of compound I with those of the authentic sample, this compound was  
110 identified as vanillic acid (molecular weight [Mw], 168) (Fig. 2C and Fig. S1A and B).  
111 Next, a cell extract (>10 kDa) of SYK-6 cells grown in Wx-SEMP was incubated with  
112 200  $\mu$ M HPV for 24 h. HPLC-MS analysis of the reaction mixture showed that HPV  
113 was converted into compound II with a retention time of 2.9 min (Fig. 2E). Positive  
114 electrospray ionization (ESI)-MS analysis of compound II showed a major fragment at  
115  $m/z$  298 (Fig. 2F). Based on the molecular weight deduced from the major fragment ion  
116 and additives in the reaction mixture, compound II was identified as an imine derivative  
117 of vanilloyl acetaldehyde (VAL), 2-((3-hydroxy-3-(4-hydroxy-3-  
118 methoxyphenyl)allylidene)amino)-2-(hydroxymethyl)propane-1,3-diol or its  
119 oxazolidine product (VAL-Tris; Mw, 297; Fig. S1C and D). It is known that some  
120 aldehyde substrates such as glyceraldehyde 3-phosphate, acetaldehyde, and  
121 benzaldehyde react with tris(hydroxymethyl)aminomethane (Tris) to form imine  
122 product, which is then trapped by one of the free hydroxyl groups forming an

123 oxazolidine product (32). Furthermore, Fukuzumi et al. attempted to synthesize VAL by  
124 Claisen-Wislicenus hydroxymethylene condensation between acetovanillone and ethyl  
125 formate in the presence of metallic sodium; however, *cis*-vanilloyl vinyl alcohol, a  
126 tautomer of VAL, was obtained as a major product (33). It was thought that VAL had  
127 five possible tautomers, and the form of hydroxyl vinyl ketone structure (vanilloyl vinyl  
128 alcohol) was stable (33). These observations suggested that VAL-Tris (imine product)  
129 was produced from HPV through oxidation of C $\gamma$ -alcohol catalyzed by the SYK-6 cell  
130 extract to generate VAL, and to result in the isomerization of VAL and condensation  
131 between a VAL isomer and Tris in the reaction buffer. VAL-Tris (imine product) was  
132 then possibly converted to the oxazolidine product.

133         When a SYK-6 cell extract (>10 kDa) was incubated with 200  $\mu$ M HPV in the  
134 presence of 500  $\mu$ M NAD<sup>+</sup> for 2 h, an accumulation of compound III with a retention  
135 time of 4.7 min was observed (Fig. 2G). Negative ESI-MS analysis of compound III  
136 showed fragments at *m/z* 209 and 165 (Fig. 2I). Since these fragments seemed to  
137 represent the deprotonated ion and its decarboxylated ion, respectively compound III  
138 was identified to be vanilloyl acetic acid (VAA; Mw, 210; Fig. S1E and F). VAL seems  
139 to be transformed to VAA by NAD<sup>+</sup>-dependent aldehyde dehydrogenase(s) (ALDHs)  
140 (Fig. 1). Additionally, when the same reaction mixture was incubated for 24 h,  
141 compound IV with a retention time of 5.8 min was generated (Fig. 2H). A comparison  
142 of the retention time and *m/z* value of the protonated ion with those of the authentic  
143 sample indicated that compound IV was acetovanillone (Mw, 166; Fig. 2L and Fig. S1G  
144 and H). Previously, Niwa and Saburi reported that VAA was spontaneously  
145 decarboxylated to acetovanillone (Fig. 1) (34).

146         Because lignin-derived aromatic acids such as ferulate, *p*-coumarate, and caffeate

147 are catabolized via CoA-dependent pathways, we predicted that VAA is catabolized  
148 through its C $\gamma$  activation by coenzyme A (CoA) (35-39). A SYK-6 cell extract (>10  
149 kDa) was therefore incubated with 200  $\mu$ M HPV in the presence of 500  $\mu$ M NAD<sup>+</sup>, 2  
150 mM CoA, 2.5 mM MgSO<sub>4</sub>, and 2.5 mM ATP. After incubation for 2 h, a decrease in  
151 HPV and an accumulation of VAA (compound III) were observed (Fig. 2J). After further  
152 incubation for 24 h, vanillic acid (compound I) and acetovanillone (compound IV) were  
153 observed (Fig. 2K). Vanillic acid was generated only when CoA, ATP, and MgSO<sub>4</sub> were  
154 present. This result strongly suggested that VAA was converted to vanillate through the  
155 CoA derivative of VAA (Fig. 1).

156 Similarly, we examined the SYK-6 catabolic pathway of HPS. Resting SYK-6  
157 cells grown in Wx-SEMP were incubated with 1 mM HPS for 4 h, and the reaction  
158 mixtures were analyzed by HPLC-MS. This analysis indicated that HPS was converted  
159 into compound V with a retention time of 2.6 min (Fig. 3B). Negative ESI-MS analysis  
160 of compound V showed a major fragment at  $m/z$  197 (Fig. 3C). Based on a comparison  
161 of the retention time and  $m/z$  value of the deprotonated ion with those of the authentic  
162 sample, compound V was identified as syringic acid (Mw, 198; Fig. S1I and J). In order  
163 to clarify the more detailed catabolic pathway of HPS, cell extracts (>10 kDa) prepared  
164 from SYK-6 cells grown in Wx-SEMP were incubated with 200  $\mu$ M HPS for 16 h. An  
165 accumulation of compound VI with a retention time of 1.8 min was observed (Fig. 3E).  
166 Negative ESI-MS analysis of compound VI showed a major fragment at  $m/z$  326 (Fig.  
167 3F), suggesting the formation of an imine derivative of 3-(4-hydroxy-3,5-  
168 dimethoxyphenyl)-3-oxopropanal (designated SAL), 2-((3-hydroxy-3-(4-hydroxy-3,5-  
169 dimethoxyphenyl)allylidene)amino)-2-(hydroxymethyl)propane-1,3-diol or its  
170 oxazolidine product (SAL-Tris; Mw, 327).

171 In the presence of 500  $\mu\text{M}$   $\text{NAD}^+$ , the same cell extract ( $>10$  kDa) converted HPS  
172 into SAL-Tris (compound VI) and compound VII with a retention time of 3.3 min (Fig.  
173 3H). Negative ESI-MS analysis of compound VII showed fragments at  $m/z$  239 and  
174 195, which seemed to represent the deprotonated ion and its decarboxylated ion,  
175 respectively (Fig. 3I). From these results, compound VII was identified as 3-(4-  
176 hydroxy-3,5-dimethoxyphenyl)-3-oxopropanoic acid (designated SAA; Mw, 240).  
177 These results indicate that HPS was oxidized to SAA via SAL, and may be degraded by  
178 the same enzyme system involved in HPV catabolism (Fig. 1).

179

180 **Basic properties of the enzyme involved in the conversion of HPV.** In order to  
181 characterize the enzymes involved in the catabolism of HPV in SYK-6, cofactor  
182 requirements and induction profiles of the HPV-transforming activities in SYK-6 were  
183 examined. The effects of the addition of 1-methoxy-5-methylphenazinium methylsulfate  
184 (PMS), flavin adenine dinucleotide (FAD) + PMS, and  $\text{NAD}^+$  on the enzyme activities  
185 in converting 200  $\mu\text{M}$  HPV were investigated. When an extract of SYK-6 cells grown in  
186 Wx-SEMP was incubated with HPV in the presence of PMS, the extract showed 1.8-  
187 fold higher activity ( $4.2 \pm 0.7$   $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ) than that in the absence of cofactors  
188 ( $2.4 \pm 0.8$   $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ). On the other hand, the addition of FAD or  $\text{NAD}^+$  had no  
189 effect on the activity. These results suggested that an oxidase(s) that requires an electron  
190 acceptor is involved in HPV oxidation. The HPV-oxidizing activity of an extract of  
191 SYK-6 cells grown with GGE was also measured in the presence of PMS. However, no  
192 activation was observed, suggesting the constitutive expression of the gene(s)  
193 responsible for the oxidation of HPV.

194

195           **Isolation of the gene involved in the conversion of HPV.** A cosmid library of  
196 SYK-6 constructed in *Sphingomonas sanguinis* IAM 12578 was screened for clones  
197 capable of degrading HPV. Of the 1,000 clones tested, three transconjugants degraded  
198 HPV, and cosmids pSA53, pSA88, and pSA684 were isolated. Southern hybridization  
199 analysis of the cosmid clones using SallI-digested pSA53 as a probe suggested that a 3.6  
200 kb, a 2.0 kb, and two 1.0 kb SallI fragments were commonly present in the above  
201 cosmids. Subcloning and nucleotide sequencing showed that these SallI fragments were  
202 present in a 17.9 kb DNA fragment that contained 13 genes corresponding to  
203 SLG\_12790 through to SLG\_12910. In this fragment, SLG\_12830 revealed 36%–39%  
204 amino acid sequence identity with the glucose-methanol-choline (GMC) oxidoreductase  
205 family enzymes, including 3-(2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-7-  
206 methoxy-2,3-dihydrobenzofuran-5-yl)acrylic acid (DCA-C) oxidases (PhcC and PhcD)  
207 involved in the catabolism of dehydrodiconiferyl alcohol (DCA) in SYK-6 (40), AlkJ,  
208 which is involved in the oxidation of primary alcohols to aldehydes in *Pseudomonas*  
209 *putida* GPo1 (41), and polyethylene glycol dehydrogenase (PegA) of *Sphingopyxis*  
210 *terrae* (42).

211

212           **The gene product of SLG\_12830 catalyzes oxidation of HPV.** SLG\_12830  
213 fused with a His tag at the 5' terminus was co-expressed in *E. coli* with the trigger factor  
214 chaperone. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)  
215 of a cell extract prepared from *E. coli* harboring pCold12830 and pTf16 (*hvpZ*-  
216 expressing *E. coli*) showed the expression of SLG\_12830 (Fig. S2). In order to  
217 determine the reaction product, resting cells of the *E. coli* transformant were incubated  
218 with 200  $\mu$ M HPV in Tris-HCl buffer. HPLC-MS analysis showed that HPV was almost

219 completely converted, and that VAL-Tris and compound VIII with a retention time of  
220 5.4 min were produced (Fig. 4A). When the same incubation was performed in water, a  
221 significant amount of compound VIII was observed without the generation of VAL-Tris  
222 (Fig. 4B). Positive ESI-MS analysis of compound VIII showed a major fragment at  $m/z$   
223 195 (Fig. 4C), suggesting that compound VIII was VAL (Mw, 194). These results  
224 indicated that the gene product of SLG\_12830 has the ability to oxidize HPV into VAL.  
225

226 **Role of SLG\_12830 in HPV and HPS catabolism.** In order to examine whether  
227 SLG\_12830 is indeed involved in the conversion of HPV in SYK-6, an SLG\_12830  
228 mutant (SME059) was created (Fig. S3A and B). The ability of SME059 to convert  
229 HPV was assessed using resting cells. SME059 was no longer able to convert HPV,  
230 whereas the wild type completely converted 200  $\mu$ M HPV within 3 h (Fig. 4D). When  
231 GGE was used as a substrate, the conversion rates of the wild type and SME059 were  
232 almost identical (Fig. 4E). However, only SME059 accumulated HPV at a concentration  
233 approximately equimolar to the added GGE (Fig. 4E). In addition, SME059 also  
234 completely lost the ability to convert HPS (Fig. 4F). The HPV conversion defect of  
235 SME059 was complemented by the introduction of pJB12830 carrying SLG\_12830  
236 (Fig. 4G). These results demonstrated that GGE is catabolized through HPV in SYK-6,  
237 and SLG\_12830 is essential for the catabolism of HPV and HPS; thus, we designated  
238 this gene *hvpZ*.

239

240 **Cellular localization of HvpZ.** In order to determine the cellular localization of  
241 HvpZ, HPV-transforming activities of soluble and membrane fractions of SYK-6 cells  
242 were compared. The HPV transforming activity in the cytoplasmic and membrane

243 fractions were estimated to be  $0.6 \pm 0.1$  nmol·min<sup>-1</sup> (7.5 mg of protein) and  $0.5 \pm 0.1$   
244 nmol·min<sup>-1</sup> (0.9 mg of protein), respectively, based on the results that the ratio of the  
245 amount of proteins in the soluble and membrane fractions was 75:9.4 (40). These results  
246 indicated that HpvZ is localized to cytoplasm and cytoplasmic membrane. Similarly,  
247 GMC oxidoreductase family proteins, PhcC and PhcD from SYK-6, and PegA from *S.*  
248 *terrae*, have been suggested to localize to both the soluble and membrane fractions (40,  
249 43). Another GMC oxidoreductase family protein, AlkJ from *P. putida* GPO1, and  
250 glucose dehydrogenase from *Pseudomonas fluorescens*, were localized to the membrane  
251 (41, 44). Since there are no predicted signal sequences or hydrophobic transmembrane  
252 segments in the deduced amino acid sequence of HpvZ, this enzyme appears to be a  
253 peripheral cytoplasmic membrane protein like other membrane-associated GMC  
254 oxidoreductase family enzymes.

255

256 **Enzyme properties of HpvZ.** Cell extracts prepared from *hpvZ*-expressing *E.*  
257 *coli* were fractionated into the soluble and membrane fractions. SDS-PAGE of both  
258 fractions showed the expression of *hpvZ* (Fig. S2). The specific activity for HPV of the  
259 membrane fraction was estimated to be  $26 \pm 6$  nmol·min<sup>-1</sup>·mg<sup>-1</sup>. However, the soluble  
260 fraction showed no activity even in the presence of PMS. The membrane fraction was  
261 treated with each of ten different detergents, and the solubilized HpvZ was obtained  
262 from the membrane fractions treated with *n*-dodecylphosphocholine and 5-cyclohexyl-  
263 1-pentyl- $\beta$ -D-maltoside. However, purified HpvZ was not obtained due to the lack of  
264 adsorption of the enzyme to a nickel affinity column. Therefore, the enzyme properties  
265 of HpvZ were examined using the membrane fraction prepared from extracts of *hpvZ*-  
266 expressing *E. coli* cells.

267 The optimum pH and temperature for the activity of HpvZ were determined to be  
268 pH 8.5–9.0 and at 40°C–45°C, respectively (Fig. S4).

269 The substrate range of HpvZ was examined using 200 μM of HPV, HPS, coniferyl  
270 alcohol, sinapyl alcohol, cinnamyl alcohol, 3-(4-hydroxyphenyl)-1-propanol,  
271 homovanillyl alcohol, vanillyl alcohol, GGE, MPHPV, DCA, and DCA-C (Fig. S5).  
272 HPLC analyses of the reaction mixtures indicated that HpvZ showed no activity for a  
273 C<sub>6</sub>–C<sub>2</sub> monomeric alcohol (homovanillyl alcohol), a C<sub>6</sub>–C<sub>1</sub> monomeric alcohol (vanillyl  
274 alcohol), and lignin-derived biaryls, including GGE, MPHPV, and DCA-C (Table 1). On  
275 the other hand, HpvZ showed activities toward all of the C<sub>6</sub>–C<sub>3</sub> monomeric alcohols (3-  
276 phenyl-1-propanol derivatives; Table 1). Generally, GMC oxidoreductase family  
277 enzymes act on hydroxyl groups of alcohols, carbohydrates, or sterols (45). For  
278 example, an aryl-alcohol oxidase from *Pleurotus eryngii* is able to oxidize a variety of  
279 aromatic alcohols and aldehydes, including coniferyl alcohol and cinnamyl alcohol (46).  
280 On the contrary, PhcC and PhcD specifically oxidize the alcohol group at C<sub>γ</sub> of the A-  
281 ring side chain of DCA-C and DCA, although PhcC has a weak activity for coniferyl  
282 alcohol (40). HpvZ was able to oxidize the alcohol group at C<sub>γ</sub> of the B-ring side chain  
283 of DCA, which is different from the regioselectivity of PhcC and PhcD (data not  
284 shown).

285 Among the enzymes belonging to the GMC oxidoreductase family, an FAD-  
286 binding domain (GMC\_oxred\_N; PF00732) is conserved in the N-terminal region. This  
287 domain includes the typical GxGxxG/A sequence motif, which is indicative of the  
288 Rossmann fold involved in binding the ADP moiety of FAD (47). A substrate-binding  
289 domain is also conserved in the C-terminal region, although this domain is less  
290 conserved. In addition, an active-site histidine, which can assist in substrate oxidation

291 and FAD reoxidation by molecular oxygen, is generally conserved (45, 47). These  
292 domains and the residue are also conserved in HpvZ (Fig. S6). To identify the flavin  
293 cofactor in HpvZ, a supernatant obtained by heat treating the membrane fraction  
294 containing HpvZ was analyzed by HPLC. However, a significant peak was not  
295 observed. Furthermore, the specific activities in the presence of FAD ( $26 \pm 8 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ )  
296 or flavin mononucleotide ( $27 \pm 1 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ) were almost equivalent to  
297 that in the absence of any flavin cofactors ( $26 \pm 6 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ). GMC  
298 oxidoreductase family enzymes generally contain either covalently or non-covalently  
299 bound FAD (45). Choline oxidase from *Arthrobacter globiformis* (48) and pyranose  
300 dehydrogenase (*AmPDH*) from *Agaricus meleagris* (49) have covalently bound FAD.  
301 Our results suggested that HpvZ may also covalently bind to FAD. Moreover, although  
302 purified inactive HpvZ was obtained from the soluble fraction of the *hvpZ*-expressing *E.*  
303 *coli* cells, UV-visible spectra of the enzyme showed no absorption at 454 nm, indicating  
304 the absence of flavin cofactor in the enzyme (data not shown). This result suggested that  
305 HpvZ produced in the *E. coli* cytoplasm lacked FAD as a prosthetic group.

306

307 ***In vivo* electron acceptor of HpvZ.** AlkJ and PegA are able to use ubiquinone  
308 (CoQ<sub>10</sub>) and its derivatives (CoQ<sub>0</sub> and CoQ<sub>1</sub>) as electron acceptors for the oxidation of  
309 their substrates (41, 42). PhcC and PhcD have also been shown to be able to use CoQ<sub>0</sub>  
310 and CoQ<sub>1</sub> as electron acceptors as well as PMS (40). Furthermore, electron transport  
311 from AlkJ to cytochrome *c* in the presence of CoQ<sub>1</sub> has been observed (41). Therefore,  
312 the electrons that are removed from the substrate by AlkJ, PegA, and PhcC/PhcD are  
313 thought to be transferred to the respiratory chain. Based on these observations, we  
314 predicted that HpvZ could use ubiquinone as an electron acceptor in the oxidation of

315 HPV. When using a membrane fraction of SYK-6 cells harboring pJB12830 (*hpvZ*-  
316 expressing SYK-6), HpvZ showed 1.2- and 1.6-fold higher specific activities in the  
317 presence of CoQ<sub>0</sub> ( $27 \pm 2 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ) and CoQ<sub>1</sub> ( $36 \pm 6 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ),  
318 respectively, than that in the absence of cofactors ( $22 \pm 1 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ). The  
319 specific activity in the presence of CoQ<sub>1</sub> was almost equivalent to that obtained using  
320 PMS ( $40 \pm 3 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ). These results suggested that HpvZ is able to use  
321 ubiquinone as an electron acceptor *in vivo*. However, no increase in HpvZ activity was  
322 observed when the membrane fraction of *hpvZ*-expressing *E. coli* was used. The  
323 difference in the increase in HpvZ activities between the membrane fractions of *hpvZ*-  
324 expressing SYK-6 and *E. coli* cells in the presence of ubiquinone derivatives may be  
325 caused by a difference in the abundance of ubiquinone in their membrane fractions  
326 employed.

327

328 **Identification of the ALDH genes responsible for the conversion of VAL.** To  
329 obtain more information on the properties of the enzymes involved in the conversion of  
330 VAL in SYK-6, cofactor requirements of the enzyme activity were examined. Cell  
331 extracts (>10 kDa) of SYK-6 grown in lysogeny broth (LB) were incubated with 200  
332  $\mu\text{M}$  HPV in the presence of FAD, NAD<sup>+</sup>, and NADP<sup>+</sup> for 2 h. In the presence of NAD<sup>+</sup>  
333 and NADP<sup>+</sup>, VAA accumulated to levels of  $95 \pm 9 \mu\text{M}$  and  $86 \pm 6 \mu\text{M}$ , respectively,  
334 while the accumulation of VAA was not observed in the presence of FAD. These results  
335 suggested that NAD<sup>+</sup>/NADP<sup>+</sup>-dependent ALDHs play a major role in VAL oxidation.

336 Previously, 23 ALDH genes were predicted to be present in the SYK-6 genome  
337 (50, 51). To examine the ability of the putative 23 ALDH gene products to oxidize  
338 VAL, these genes were expressed in *E. coli* using expression plasmids constructed in a

339 previous study (51). SDS-PAGE showed sufficient gene expression except for  
340 SLG\_32240 and SLG\_34940 (Fig. S7). Since HPV was converted to VAL-Tris during  
341 incubation with HpvZ in Tris-HCl buffer, we employed HEPES buffer (pH 7.5) and  
342 sodium phosphate buffer (pH 7.5) to prepare VAL from HPV using HpvZ. However,  
343 HPV was converted to unknown products in HEPES buffer, and the reaction product  
344 was not detected in sodium phosphate buffer (data not shown). In addition, HPV  
345 conversion rate was significantly decreased in HEPES buffer (approximately 60%) and  
346 sodium phosphate buffer (approximately 50%). Therefore, we examined the ability of  
347 these 21 ALDHs to convert VAL by measuring the amount of VAA produced from  
348 HPV when HPV was reacted with both HpvZ and each ALDH. Resting cells of *E. coli*  
349 expressing each ALDH gene and *hpvZ*-expressing *E. coli* were mixed and incubated  
350 with 100  $\mu$ M HPV for 12 h. When resting cells of *E. coli* harboring a vector and the  
351 *hpvZ*-expressing *E. coli* were incubated with HPV, VAA was not generated, while VAL-  
352 Tris accumulated. On the other hand, when *E. coli* cells carrying each of the 13 ALDH  
353 genes [SLG\_07270, SLG\_07610, SLG\_07790, SLG\_09510, SLG\_11410, SLG\_12800,  
354 SLG\_18210, SLG\_20400, SLG\_27910 (*bzaA*), SLG\_27920 (*bzaB*), SLG\_28150,  
355 SLG\_31150, SLG\_38120] were used instead of the control vector, HPV was converted  
356 into VAA (Fig. 5A). Of the 13 ALDH genes, when incubating with *E. coli* carrying  
357 SLG\_07270, SLG\_12800, SLG\_18210, SLG\_20400, *bzaA*, *bzaB*, and SLG\_31150,  
358 larger amounts of VAA accumulated (Fig. 5A). We, therefore, measured the VAA  
359 production time course using *E. coli* carrying these 7 ALDH genes and the *hpvZ*-  
360 expressing *E. coli*. Of these, *E. coli* carrying SLG\_07270, SLG\_12800, SLG\_20400,  
361 and *bzaB* produced a greater amount of VAA than *E. coli* carrying one of the 3 other  
362 ALDH genes (Fig. 5B). Specifically, when using *E. coli* carrying SLG\_20400, the

363 amount of VAA produced was the greatest, and no VAL-Tris was detected at any of the  
364 sampling points (Fig. 5B).

365 In order to examine whether SLG\_07270, SLG\_12800, SLG\_20400, and *bzaB* are  
366 involved in the conversion of VAL in SYK-6, SLG\_07270 mutant (SME092) and  
367 SLG\_20400 mutant (SME061) were created (Fig. S3C–F). Resting cells of SME092,  
368 SME061, and the previously created SLG\_12800 mutant (SME031) and *bzaB* mutant  
369 (SME045) were incubated with 1 mM HPV in the presence of *hpvZ*-expressing *E. coli*  
370 cells. SME061 accumulated a 1.8-fold greater amount of VAL-Tris than the wild type or  
371 the other mutants after 20 h incubation (Fig. 5C). These results suggested that  
372 SLG\_20400 is involved in VAL oxidation. However, SME061 accumulated only 75  $\mu$ M  
373 VAL-Tris at 20 h of incubation. Therefore, multiple ALDHs including SLG\_20400  
374 appear to be involved in the conversion of VAL. Our previous phylogenetic analysis of  
375 the 23 ALDH genes in SYK-6 and other known ALDH genes indicated that 13 SYK-6  
376 ALDH genes, the products of which showed VAL oxidation activities, are  
377 phylogenetically diverse (51). SLG\_20400 clustered with *calB*, which encodes coniferyl  
378 aldehyde dehydrogenase from *Pseudomonas* sp. strain HR199, which shared 33%  
379 amino acid sequence identity with SLG\_20400 (51, 52). Involvement of multiple ALDH  
380 genes in SYK-6 was also shown in the oxidation of vanillin, syringaldehyde, and an  
381 intermediate metabolite of DCA (DCA-L) (50, 51). Another example of the involvement  
382 of multiple ALDH genes in the conversion of vanillin has also been reported for *P.*  
383 *putida* KT2440 (53). Since ALDHs exhibit broad substrate ranges in general, multiple  
384 ALDHs are likely to play roles in the oxidation of aromatic aldehydes to their acids.

385

386 **Candidate genes for the catabolism of VAA.** In a previous report, Palamuru et

387 al. detected vanillin as a metabolite when SYK-6 cells were incubated with GGE (24).  
388 However, vanillin was not observed during the conversion of HPV (Fig. 2). In order to  
389 examine whether vanillin is an actual intermediate in HPV catabolism, resting cells of a  
390 *desV ligV* double mutant (SME077), which has a weak ability to convert vanillin (51),  
391 were incubated with 100  $\mu$ M VAA or ferulate. Only the accumulation of vanillate and  
392 acetovanillone was observed at any of the sampling points (1, 2, 4, 6, and 24 h) in the  
393 mixture for the VAA conversion, whereas the mixture for the ferulate conversion  
394 accumulated a significant amount of vanillin in addition to vanillate (Fig. 6;  
395 chromatograms at 2 and 4 h of incubation are shown). In addition, when a cell extract  
396 (>10 kDa) of SME077 was incubated with 100  $\mu$ M VAA in the presence of CoA,  
397 MgSO<sub>4</sub>, and ATP, only the accumulation of vanillate and acetovanillone was observed  
398 (Fig. S8). These results suggested that VAA was catabolized to vanillate without passing  
399 through vanillin as an intermediate.

400 We hypothesized that the feruloyl-CoA synthetase gene (*ferA*) and feruloyl-CoA  
401 hydratase/lyase genes (*ferB* and *ferB2*) may be involved in VAA catabolism based on  
402 the structural similarity between VAA and ferulic acid (36). These genes were  
403 adequately expressed in *E. coli* (Fig. S9). When crude FerA was incubated with 100  $\mu$ M  
404 VAA in the presence of CoA, MgSO<sub>4</sub>, and ATP for 60 min, compound IX with a  
405 retention time of 1.7 min was generated (Fig. S10B). Negative ESI-MS analysis of  
406 compound IX showed fragments at  $m/z$  959 ( $[M - H]^-$ ) and 479 ( $[M - 2H]^{2-}$ ), suggesting  
407 that compound IX was the CoA derivative of VAA (VAA-CoA; Mw, 960) (Fig. S10E).  
408 However, no other peak except VAA-CoA was observed when crude enzymes of FerA +  
409 FerB and FerA + FerB2 were incubated with VAA, respectively (Fig. S10C and D).  
410 Therefore, FerB and FerB2 appear to be not involved in the conversion of VAA-CoA.

411 In order to examine whether *ferA* is indeed involved in the conversion of VAA in  
412 SYK-6, resting cells of a previously created *ferA* mutant (SME009) grown in LB were  
413 incubated with 100  $\mu$ M VAA. SME009 showed a higher conversion rate for VAA than  
414 that of the wild type (Fig. S11), suggesting that *ferA* is not essential for the catabolism  
415 of VAA. The reason for the high conversion rate of VAA of SME009 is not clear but  
416 disruption of *ferA* may cancel the substrate competition between FerA and (an)  
417 unidentified true VAA-converting enzyme(s).

418 Recently, the catabolic pathway of *p*-hydroxycinnamate derivatives, such as  
419 dihydroferulate, ferulate, and *p*-coumarate in *Rhodococcus jostii* RHA1, were  
420 characterized (37). In this pathway, dihydroferulate was catabolized to vanillate via  
421 VAA-CoA. VAA-CoA was converted into vanillate and acetyl-CoA by the gene product  
422 of *couO* (ro0512), which encodes 4-hydroxyphenoxy- $\beta$ -ketoacyl-CoA hydrolase. CouO  
423 was predicted to be a zinc-dependent metalloenzyme belonging to amidohydrolase  
424 superfamily. Orthologs of *couO*, showing 53%–58% amino acid sequence identity, have  
425 also been found and characterized in *Agrobacterium fabrum* C58 (Atu1421) (38) and  
426 *Corynebacterium glutamicum* (*phdC*) (39). In the SYK-6 genome, we found  
427 SLG\_12680, which exhibited 50% amino acid sequence identity with CouO. We are  
428 currently investigating the function of the gene product of SLG\_12680 and exploring  
429 the actual gene encoding CoA ligase for VAA.

430

431 **Genome search for orthologs of *hvpZ* in other bacteria.** Since HvpZ is essential  
432 for the catabolism of HPV and HPS, the presence of this gene determines whether  
433 bacteria can utilize the A-ring portion of  $\beta$ -aryl ether compounds. BLAST searches of  
434 *hvpZ* were carried out to determine the distribution of its orthologs among bacteria. The

435 *hpvZ* orthologs that showed high amino acid sequence identity (62%–92%) were found  
436 in *Altererythrobacter* sp. strain Root672 (ASD76\_15935), *Altererythrobacter atlanticus*  
437 (WYH\_02786), *Erythrobacter* sp. strain SG61-1L (SZ64\_15220), *Sphingomonas*  
438 *hengshuiensis* WHSC-8 (TS85\_07880), and *Sphingobium* sp. strain 66-54  
439 (BGP16\_16810). All these bacteria possess orthologs of the genes responsible for the  
440 conversion of GGE into HPV. Among these strains, SG61-1L was reported to be able to  
441 utilize GGE as the sole source of carbon and energy (24). By contrast,  
442 *Novosphingobium* sp. strain MBES04 accumulated HPV from GGE (23). Consistent  
443 with this observation, MBES04 possesses GMC oxidoreductase family enzyme genes,  
444 whose products showed less than 37% amino acid sequence identity with HpvZ.  
445 Similarly, no *hpvZ* orthologs were found in *Novosphingobium* sp. strain PP1Y and  
446 *Novosphingobium aromaticivorans* DSM 12444, which possess orthologs of the genes  
447 responsible for converting GGE into HPV. Due to the lack of *hpvZ*, PP1Y and DSM  
448 12444 also appear to be able to utilize only the portion of the B-ring of  $\beta$ -aryl ether  
449 compounds as carbon and energy sources.

450

451

## 452 **MATERIALS AND METHODS**

453 **Bacterial strains, plasmids, and culture conditions.** The strains and plasmids  
454 used in this study are listed in Table 2. *Sphingobium* sp. strain SYK-6 and its mutants  
455 were grown in LB, Wx-SEMP, and Wx-SEMP containing 5 mM GGE at 30°C. *S.*  
456 *sanguinis* IAM 12578 was grown in LB at 30°C. When necessary, 50 mg  
457 kanamycin/liter, 100 mg streptomycin/liter, or 300 mg carbenicillin/liter were added to  
458 the cultures. *E. coli* strains were grown in LB at 37°C. For cultures of cells carrying

459 antibiotic resistance markers, the media for *E. coli* transformants were supplemented  
460 with 100 mg ampicillin/liter, 25 mg kanamycin/liter, or 12.5 mg chloramphenicol/liter.

461

#### 462 **Synthesis of HPS (S-Hibbert-Westwood-Lancefield ketone; Fig. 7)**

463 *Synthesis of 4-((tert-butyldimethylsilyl)oxy)-3,5-dimethoxybenzaldehyde (A)* To a  
464 stirring solution of syringaldehyde (20.0 g, 109.8 mmol, 1.0 eq) in dichloromethane  
465 (DCM) (600 ml,  $c = 0.18$  M) was added 4-dimethylaminopyridine (4-DMAP) (13.4 g,  
466 109.8 mmol, 1.0 eq), and imidazole (Imid) (14.9 g, 219.6, 2.0 eq). The resulting mixture  
467 was allowed to stir for 5 min, and then butyldimethylchlorosilane (TBSCl) (17.3 g,  
468 115.3 mmol, 1.1 eq) was added. The mixture was stirred at room temperature for 1 h.  
469 After the reaction had reached completion, it was neutralized with a saturated aqueous  
470 solution of  $\text{NH}_4\text{Cl}$  ( $2 \times 300$  ml). The organic layer was further washed with water (500  
471 ml), brine (300 ml), dried with  $\text{MgSO}_4$ , filtered, and concentrated in vacuo. Purification  
472 by silica gel chromatography using 5–10% ethyl acetate (EtOAc) in petroleum ether  
473 afforded compound A as a white solid (26.6 g, 89.8 mmol, 82%). Analytical data for  
474 compound A agreed with that previously reported in the reference (54).  $^1\text{H}$  NMR (500  
475 MHz,  $\text{CDCl}_3$ )  $\delta$  9.79 (s, 1H), 7.07 (s, 2H), 3.84 (s, 6 H), 0.98 (s, 9H), 0.13 (s, 6H);  $^{13}\text{C}$   
476 NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  191.1, 152.0, 140.7, 129.4, 106.7, 55.8, 25.8, 18.9, -4.46.

477 *Synthesis of Ethyl 3-(4-((tert-butyldimethylsilyl)oxy)-3,5-dimethoxyphenyl)-3-*  
478 *hydroxypropanoate (B)* ethyl acetate (4.2 g, 47.5 mmol, 1.3 eq) was added to a cooled  
479 solution of lithium bis(trimethylsilyl)amide (LiHMDS) [1.0 M in tetrahydrofuran  
480 (THF); 47.5 ml, 47.5 mmol, 1.3 eq] in THF (100 ml) at  $-78^\circ\text{C}$ . After 15 min, a solution  
481 of compound A (10.8 g, 36.6 mmol, 1.0 eq) in THF (20 ml, overall  $c = 0.30$  M) was  
482 added at  $-78^\circ\text{C}$  and the resulting mixture was left to stir at this temperature for 1 h. After

483 the reaction had reached completion, it was quenched with saturated aqueous solution of  
484  $\text{NH}_4\text{Cl}$  ( $2 \times 300$  ml) and extracted with ethyl acetate (500 ml). The organic layer was  
485 further washed with water (500 ml), brine (300 ml), dried with  $\text{MgSO}_4$ , filtered and  
486 concentrated in vacuo. Purification by silica gel chromatography using 10–20% ethyl  
487 acetate in petroleum ether afforded compound B as a light-yellow solid (12.5 g, 32.7  
488 mmol, 89%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  6.51 (s, 2H), 5.00 (dt,  $J = 9.0, 3.5$  Hz, 1H),  
489 4.13 (q,  $J = 7.0$  Hz, 2H), 3.74 (s, 6H), 3.36 (m, 1H), 2.70 (dd,  $J = 16.0, 9.0$  Hz, 1H),  
490 2.63 (dd,  $J = 16.0, 3.5$  Hz, 1H), 1.22 (t,  $J = 7.0$  Hz, 3H), 0.97 (s, 9H), 0.08 (s, 6H);  $^{13}\text{C}$   
491 NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  172.4, 151.5, 135.2, 133.6, 102.5, 70.6, 60.8, 55.7, 43.7,  
492 25.8, 18.7, 14.2, -4.7.

493 *Synthesis of 1-(4-((tert-butyldimethylsilyl)oxy)-3,5-dimethoxyphenyl)propane-*  
494 *1,3-diol (C)* A suspension of  $\text{LiAlH}_4$  (1.4 g, 37.8 mmol, 2.2 eq) in THF (100 ml) was  
495 cooled to  $-20^\circ\text{C}$ . After 15 min, a solution of compound B (6.6 g, 17.2 mmol, 1.0 eq) in  
496 THF (20 ml, overall  $c = 0.14$  M) was added at  $-20^\circ\text{C}$  and the resulting mixture was left  
497 to stir at this temperature for 1 h. After the reaction had reached completion, it was  
498 poured slowly into a stirring mixture of ethyl acetate (300 ml) and saturated aqueous  
499 solution of  $\text{Na}_2\text{S}_2\text{O}_3$  ( $2 \times 300$  ml). The organic layer was washed brine (300 ml), dried  
500 with  $\text{MgSO}_4$ , filtered and concentrated in vacuo. Purification by silica gel  
501 chromatography using 5–10% methanol in DCM afforded compound C as colorless oil  
502 (5.62 g, 16.4 mmol, 96%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  6.49 (s, 2H), 4.79 (dd,  $J = 8.5,$   
503 3.5, 1H), 3.77 (m, 2H), 3.75 (s, 6H), 3.32 (s, 1H), 2.93 (s, 1H), 1.94 (dddd,  $J = 14.0,$   
504 8.5, 7.0, 5.0 Hz, 1H), 1.84 (ddt,  $J = 14.0, 5.5, 4.0$  Hz, 1H), 0.98 (s, 9H), 0.09 (s, 6H);  
505  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  151.6, 137.2, 133.5, 102.6, 74.6, 61.5, 55.8, 40.6, 25.9,  
506 18.8, -4.6.

507            *Synthesis of 1-(4-((tert-butyl)dimethylsilyloxy)-3,5-dimethoxyphenyl)-3-*  
508 *hydroxypropan-1-one (D)* 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (4.1 g, 18.1  
509 mmol, 1.05 eq) was added to a stirring solution of compound C (5.6 g, 16.4 mmol, 1.0  
510 eq) in THF (160 ml,  $c = 0.10$  M) at 22°C and the resulting mixture was left to stir at this  
511 temperature for 12 h. Afterwards, the reaction mixture was diluted with ethyl acetate  
512 (300 ml) and washed with saturated aqueous solution of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (2 × 300 ml). The  
513 organic layer was washed water (300 ml), brine (300 ml), dried with MgSO<sub>4</sub>, filtered  
514 and concentrated in vacuo. Purification by silica gel chromatography using 2–5%  
515 methanol in DCM afforded compound D as a white solid (5.1 g, 14.9 mmol, 91%). <sup>1</sup>H  
516 NMR (500 MHz, CDCl<sub>3</sub>) δ 7.16 (s, 2H), 3.97 (app q,  $J = 5.5$  Hz, 2H), 3.80 (s, 6H), 3.15  
517 (t,  $J = 5.5$  Hz, 2H), 3.01 (app t,  $J = 6.0$  Hz, 1H), 0.97 (s, 9H), 0.11 (s, 6H); <sup>13</sup>C NMR  
518 (125 MHz, CDCl<sub>3</sub>) δ 199.1, 151.4, 139.9, 129.4, 105.6, 58.3, 55.8, 40.0, 25.7, 18.8, -  
519 4.6.

520            *Synthesis of HPS* Tetrabutylammonium fluoride (TBAF) (1.0 M in THF, 8.8 ml,  
521 8.8 mmol, 3.0 eq) was added to a stirring solution of compound D (1.0 g, 2.9 mmol, 1.0  
522 eq) in THF (15 ml,  $c = 0.20$  M) at 22°C and the resulting mixture was left to stir at this  
523 temperature for 1 h. Afterwards, the reaction mixture was diluted with ethyl acetate (300  
524 ml) and washed with saturated aqueous solution of NH<sub>4</sub>Cl (2 × 300 ml). The organic  
525 layer was washed water (300 ml), brine (300 ml), dried with MgSO<sub>4</sub>, filtered and  
526 concentrated in vacuo. The crude material was recrystallized in petroleum ether and  
527 washed with a minimum amount of ethyl acetate to afford compound HPS as a white  
528 solid (0.5 g, 2.3 mmol, 77%). Spectroscopic data agreed with that previously reported in  
529 the reference (30). High-resolution MS [M - H]<sup>+</sup> calcd. For C<sub>11</sub>H<sub>13</sub>O<sub>5</sub> 225.0800; found  
530 225.0766; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 9.34 (s, 1H), 7.24 (s, 2H), 4.59 (s, 1H),

531 3.83 (s, 6H), 3.77 (t,  $J = 6.5$  Hz, 2H), 3.10 (t,  $J = 6.5$  Hz, 2H);  $^{13}\text{C}$  NMR (125 MHz,  
532 DMSO- $d_6$ )  $\delta$  197.4, 147.6, 140.8, 127.5, 106.0, 57.3, 56.1, 41.0.

533 **Synthesis of HPV (G-Hibbert-Westwood-Lancefield ketone; Fig. 7)**

534 *Synthesis of 4-((tert-butyldimethylsilyl)oxy)-3-methoxybenzaldehyde (E)*

535 Compound E was synthesized following the same experimental procedure as described  
536 for the synthesis of compound A. To vanillin (19.2 g, 126.0 mmol, 1.0 eq) in DCM (500  
537 ml,  $c = 0.25$  M) was added 4-DMAP (15.4 g, 126.0 mmol, 1.0 eq), imidazole (17.1 g,  
538 252.0 mmol, 2.0 eq), and TBSCl (1.2 eq). Purification by silica gel chromatography  
539 using 5–10% ethyl acetate in petroleum ether afforded compound E as colorless oil  
540 (30.0 g, 113.1 mmol, 90%). Spectroscopic data was in agreement with the reference  
541 (55).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  9.73 (s, 1H), 7.27 (m, 2H), 6.85 (d,  $J = 8.0$  Hz, 1H),  
542 3.74 (s, 3H), 0.90 (s, 9H), 0.09 (s, 6H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  190.5, 151.4,  
543 151.0, 130.9, 125.8, 120.5, 110.0, 55.1, 25.4, 18.3, -4.78.

544 *Synthesis of Ethyl 3-(4-((tert-butyldimethylsilyl)oxy)-3-methoxyphenyl)-3-*  
545 *hydroxypropanoate (F)* Compound F was synthesized following the same experimental  
546 procedure as described for the synthesis of compound B. Compound E (10.9 g, 41.0  
547 mmol, 1.0 eq) in THF (140 ml,  $c = 0.29$  M). LiHMDS (1.0 M in THF; 53.2 ml, 53.2  
548 mmol, 1.3 eq). ethyl acetate (4.7 g, 53.2, 1.3 eq). Purification by silica gel  
549 chromatography using 10–30% ethyl acetate in petroleum ether afforded compound F as  
550 light-yellow oil (11.9 g, 33.6 mmol, 81%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  6.84 (d,  $J =$   
551 1.5 Hz, 1H), 6.72 (m, 2H), 4.98 (dt,  $J = 5.0, 3.5$  Hz, 1H), 4.09 (q,  $J = 7.0$  Hz, 2H), 3.73  
552 (s, 3H), 3.50 (m, 1H), 2.68 (dd,  $J = 16.0, 9.5$  Hz, 1H), 2.59 (dd,  $J = 16.0, 4.0$  Hz, 1H),  
553 1.18 (t,  $J = 7.0$  Hz, 3H), 0.95 (s, 9H), 0.09 (s, 6H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$   
554 172.3, 150.9, 144.4, 136.3, 120.6, 118.0, 109.5, 70.2, 60.7, 55.3, 43.6, 25.7, 18.4, 14.1, -

555 4.7.

556 *Synthesis of 1-(4-((tert-butyldimethylsilyl)oxy)-3-methoxyphenyl)propane-1,3-*  
557 *diol (G)* Compound G was synthesized following the same experimental procedure as  
558 described for the synthesis of compound C. LiAlH<sub>4</sub> (1.6 g, 40.1 mmol, 2.2 eq) in THF  
559 (80 ml). Compound F (6.6 g, 18.6 mmol, 1.0 eq) in THF (20 ml, overall *c* = 0.18 M).  
560 Purification by silica gel chromatography using 5–10% methanol in DCM afforded  
561 compound G as light-yellow oil (5.4 g, 17.4 mmol, 94%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  
562 δ 6.89 (d, *J* = 1.5 Hz, 1H), 6.80 (m, 2H), 4.86 (m, 1H), 3.81 (s, 3H), 3.23 (s, 1H), 2.88  
563 (s, 1H), 2.00 (m, 1H), 1.88 (m, 1H), 1.01 (s, 9H), 0.16 (s, 6H); <sup>13</sup>C NMR (125 MHz,  
564 CDCl<sub>3</sub>) δ 151.0, 144.4, 138.1, 120.8, 118.1, 109.6, 74.3, 61.5, 55.6, 40.6, 25.8, 18.6, -  
565 4.5.

566 *Synthesis of 1-(4-((tert-butyldimethylsilyl)oxy)-3-methoxyphenyl)-3-*  
567 *hydroxypropan-1-one (H)* Compound H was synthesized following the same  
568 experimental procedure as described for the synthesis of compound D. DDQ (4.3 g,  
569 19.2 mmol, 1.05 eq). Compound G (5.4 g, 17.4 mmol, 1.0 eq) in THF (170 ml, *c* = 0.10  
570 M). Purification by silica gel chromatography using 2–5% methanol in DCM afforded  
571 compound H as light-yellow oil (4.1 g, 13.3 mmol, 76%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  
572 δ 7.43-7.35 (m, 2H), 6.82 (d, *J* = 8.0 Hz, 1H), 3.95 (t, *J* = 5.5 Hz, 2H), 3.79 (s, 3H), 3.12  
573 (t, *J* = 5.5 Hz, 2H), 0.94 (s, 9H), 0.13 (s, 6H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 199.1,  
574 151.1, 150.4, 130.8, 122.7, 120.3, 110.9, 58.2, 55.4, 40.0, 25.6, 18.5, -4.6.

575 *Synthesis of HPV* HPV was synthesized following the same experimental  
576 procedure as described for the synthesis of HPS. TBAF (1.0 M in THF; 15.6 ml, 15.6  
577 mmol, 1.2 eq). Compound H (4.13 g, 13.3 mmol, 1.0 eq) in THF (100 ml, *c* = 0.13 M).  
578 Purification by silica gel chromatography afforded HPV as a white solid (1.0 g, 5.1

579 mmol, 38%). Spectroscopic analysis was in agreement with the reference (30). High-  
580 resolution MS  $[M + Na]^+$  calcd. For  $C_{10}H_{12}O_4Na$  196.0700; found 219.0625;  $^1H$  NMR  
581 (500 MHz,  $CDCl_3$ )  $\delta$  7.58-7.50 (m, 2H), 6.95 (d,  $J = 8.0$  Hz, 1H), 6.12 (s, 1H), 4.01 (q,  
582  $J = 5.0$  Hz, 2H), 3.95 (s, 3H), 3.18 (t,  $J = 5.0$  Hz, 2H), 2.73 (app t,  $J = 5.9 =$  Hz, 1H);  $^{13}C$   
583 NMR (125 MHz,  $CDCl_3$ )  $\delta$  199.2, 151.0, 146.9, 129.7, 123.8, 114.1, 109.7, 58.5, 56.2,  
584 39.9.

585

586 **Preparation of other substrates.** MPHPV, DCA, and DCA-C were prepared as  
587 described previously (50, 56). For preparation of VAA, HPV was added into 15 ml of  
588 the cell suspensions of *hvpZ*-expressing *E. coli* cells ( $OD_{600}$  of 10.0) and SLG\_20400-  
589 expressing *E. coli* cells ( $OD_{600}$  of 2.0) to a final concentration of 200  $\mu$ M. After  
590 incubation with shaking for 12 h at 30°C, the culture was centrifuged, and the  
591 supernatant was filtered using an Amicon ultra spin filter unit (3-kDa cutoff, Millipore).  
592 The resulting filtrate was used as a preparation of 200  $\mu$ M VAA. For preparation of  
593 VAL-Tris, HPV was added into 15 ml of the cell suspension of *S. sanguinis* IAM 12578  
594 harboring pJB12830 cells ( $OD_{600}$  of 1.0) to a final concentration of 1 mM. After  
595 incubation with shaking for 45 h at 30°C, the culture was centrifuged, and the  
596 supernatant was collected. The supernatant was extracted with ethyl acetate, and then  
597 the extract was finally dissolved in the dimethyl sulfoxide. The compounds obtained  
598 were analyzed by HPLC-MS.

599 Other aromatic compounds were purchased from Tokyo Chemical Ind., Co., Ltd.,  
600 Sigma-Aldrich Co., Llc., and Wako Pure Chemical Ind., Ltd.

601

602           **Preparation of resting cells and cell extracts, and fractionation of cell**  
603 **extracts.** Cells of SYK-6 and its mutants grown in Wx-SEMP for 16 h or LB for 24 h  
604 were collected by centrifugation ( $5,000 \times g$  for 5 min), and then washed twice with 50  
605 mM Tris-HCl buffer (pH 7.5; buffer A). The cells were resuspended in the same buffer  
606 and used as resting cells. Cells were broken by an ultrasonic disintegrator (57), and the  
607 supernatants of cell lysates were obtained as cell extracts after centrifugation ( $19,000 \times$   
608  $g$  for 15 min). To examine the cofactor requirements, cell extracts were filtered using an  
609 Amicon ultra spin filter unit (10 kDa cutoff, Millipore) and then washed 5 times with  
610 buffer A. The filtrates were used as cell extracts ( $>10$  kDa). For fractionation of cell  
611 extracts, they were further centrifuged at  $124,000 \times g$  for 30 min at  $4^\circ\text{C}$ , and the  
612 resulting supernatants were used as the soluble fraction. The pellets were washed twice  
613 with buffer A, resuspended in the same buffer, and used as the membrane fraction.

614

615           **Identification of the metabolites.** SYK-6 resting cells ( $\text{OD}_{600}$  of 5.0) were  
616 incubated with 1 mM HPV or HPS in buffer A at  $30^\circ\text{C}$  with shaking. SME077 resting  
617 cells ( $\text{OD}_{600}$  of 0.5 or 5.0) were incubated with 100  $\mu\text{M}$  VAA or 1 mM ferulic acid in  
618 buffer A at  $30^\circ\text{C}$  with shaking. Following incubation, portions of the reaction mixtures  
619 were collected, and reactions were stopped by centrifugation. Methanol was added to  
620 the resulting supernatants (final concentration, 40%), and the filtrated samples were  
621 analyzed by HPLC-MS.

622           SYK-6 cell extracts ( $>10$  kDa; 500  $\mu\text{g}$  protein/ml) were incubated with 200  $\mu\text{M}$   
623 HPV or HPS in the presence and absence of 500  $\mu\text{M}$   $\text{NAD}^+$  or 500  $\mu\text{M}$   $\text{NAD}^+$  + 2 mM  
624 CoA + 2.5 mM  $\text{MgSO}_4$  + 2.5 mM ATP in buffer A at  $30^\circ\text{C}$ . SME077 cell extracts ( $>10$   
625 kDa; 500  $\mu\text{g}$  protein/ml) were incubated with 100  $\mu\text{M}$  VAA in the presence of 1 mM

626 CoA + 1.25 mM MgSO<sub>4</sub> + 1.25 mM ATP in buffer A at 30°C. The reactions were  
627 stopped by the addition of methanol or acetonitrile (final concentration, 50%) at various  
628 sampling time points. Precipitated proteins were removed by centrifugation at 19,000 ×  
629 g for 15 min. The resulting supernatants of the reaction mixtures were analyzed by  
630 HPLC-MS.

631

632 **Enzyme assays using cell extracts of SYK-6.** HPV-oxidizing activities of SYK-  
633 6 cell extracts were determined by measuring the decrease in the amount of HPV by  
634 HPLC analysis. In order to examine the effect of GGE on enzyme induction, SYK-6  
635 cells grown in LB were inoculated into Wx-SEMP to an OD<sub>600</sub> of 0.2, and grown at  
636 30°C. GGE (5 mM) was added when the OD<sub>600</sub> of the culture reached 0.5, and the  
637 culture was then further incubated for 12 h. SYK-6 cell extracts (500 µg protein/ml)  
638 were incubated with 200 µM HPV in the presence and absence of cofactors (500 µM  
639 PMS, 500 µM FAD + PMS, or 500 µM NAD<sup>+</sup>) in buffer A for 30 min at 30°C. The  
640 reaction mixtures were analyzed by HPLC, and HPV was detected at 276 nm. The  
641 specific activity was expressed in moles of HPV converted per min per milligram of  
642 protein.

643 In order to examine the activity of VAL-oxidizing activity, production of VAA  
644 from HPV was measured when HPV was reacted with SYK-6 cell extracts (>10 kDa).  
645 The cell extracts (>10 kDa; 500 µg protein/ml) were incubated with HPV in the  
646 presence of cofactors (500 µM FAD, 500 µM NAD<sup>+</sup>, or 500 µM NADP<sup>+</sup>) in buffer A  
647 for 2 h at 30°C. The supernatant of the reaction mixtures was analyzed by HPLC, and  
648 compounds were detected at 280 nm.

649

650           **Analytical methods.** HPLC-MS analysis was performed with the ACQUITY  
651 UPLC system (Waters) coupled with an ACQUITY TQ detector as described previously  
652 (58). Reaction products of HPV, coniferyl alcohol, sinapyl alcohol, cinnamyl alcohol, 3-  
653 (4-hydroxyphenyl)-1-propanol, homovanillyl alcohol, vanillyl alcohol, GGE, MPHPV,  
654 DCA, and DCA-C were analyzed using TSKgel ODS-140HTP column (2.1 × 100 mm;  
655 Tosoh). Reaction products of HPS were analyzed using ACQUITY UPLC BEH C18  
656 column (2.1 × 100 mm; Waters). Reaction products of VAA was analyzed using both  
657 columns. All analyses were carried out at a flow rate of 0.5 ml/min. The mobile phase  
658 was a mixture of solution A (acetonitrile containing 0.1% formate) and B (water  
659 containing 0.1% formate) with the following conditions: *Detection of the reaction*  
660 *products of HPV:* 0–4.2 min, 5% A; 4.2–6.0 min, linear gradient from 5% to 30% A; 6.0–  
661 6.5 min, decreasing gradient from 30% to 5% A; 6.5–7.0 min, 5% A. *Detection of VAL*  
662 *generated from HPV:* 0–4.7 min, 5% A; 4.7–4.9 min, linear gradient from 5% to 80%;  
663 4.9–7.0 min, 80% A. *Detection of VAA-CoA generated from VAA:* 0–0.8 min, 10% A; 0.8–  
664 1.0 min, linear gradient from 10% to 25% A; 1.0–1.5 min, 25% A; 1.5–1.8 min,  
665 decreasing gradient from 25% to 10% A; 1.8–3.0 min, 10% A. *Detection of vanillate*  
666 *generated from VAA:* 0–3.0 min, linear gradient from 5% to 15% A; 3.0–4.0 min,  
667 decreasing gradient from 15% to 5%. *Detection of the reaction product of vanillyl*  
668 *alcohol:* 0–5.0 min, 5% A. *Detection of the reaction products of coniferyl alcohol, sinapyl*  
669 *alcohol, cinnamyl alcohol, homovanillyl alcohol, and HPS:* 0–7.0 min, 10% A. *Detection*  
670 *of the reaction products of 3-(4-hydroxyphenyl)-1-propanol, GGE, and ferulate:* 0–5.0  
671 min, 15% A. *Detection of the reaction products of MPHPV, DCA, and DCA-C:* 0–5.0 min,  
672 25% A. HPV, HPS, VAL, VAL-Tris, VAA, VAA-CoA, vanillate, syringate, acetovanillone,  
673 coniferyl alcohol, sinapyl alcohol, cinnamyl alcohol, 3-(4-hydroxyphenyl)-1-propanol,

674 homovanillyl alcohol, vanillyl alcohol, GGE, MPPHV, DCA, and DCA-C were detected  
675 at 276, 300, 310, 352, 280, 300, 260, 276, 275, 263, 273, 250, 276, 279, 279, 277, 280,  
676 277, and 326 nm, respectively. In ESI-MS analysis, MS spectra were obtained using the  
677 positive- and negative-ion modes with the settings described in our previous study (58).  
678 Protein concentrations were determined by the Bradford method using the Bio-Rad  
679 protein assay kit or by Lowry's assay using the DC protein assay kit (Bio-Rad  
680 Laboratories). The expression of the genes was analyzed by SDS-PAGE. Protein bands  
681 in gels were stained with Coomassie Brilliant Blue.

682

683 **DNA manipulations and sequence analysis.** PCR primers used in this study are  
684 listed in Table 3. Nucleotide sequences were determined using a CEQ 2000XL genetic  
685 analysis system (Beckman Coulter). Sequence analysis was performed with the  
686 MacVector program (MacVector, Inc.). Sequence similarity searches, pairwise  
687 alignments, and multiple alignments were carried out using the BLASTP program (59),  
688 the EMBOSS Needle program through the EMBL-EBI server (60), and the Clustal  
689 Omega program (61), respectively.

690

691 **Cloning of *hpvZ*.** A partially SalI-digested gene library of SYK-6 constructed  
692 with pVK100 was introduced into a host strain, *S. sanguinis* IAM 12578, by triparental  
693 mating (62). The ability of 1000 transconjugants grown in diluted LB to transform 15  
694  $\mu$ M HPV was analyzed by HPLC. Southern hybridization analysis of the SalI digests of  
695 positive clones with pSA53 as a probe was carried out using the digoxigenin (DIG)  
696 system (Roche Diagnostics). The hybridized SalI fragments were cloned in pBluescript  
697 II SK(+), and the nucleotide sequences of both ends of the inserts were determined.

698

699           **Expression of SYK-6 genes in *E. coli* and SYK-6.** A DNA fragment carrying  
700 *hvpZ* was amplified by PCR using SYK-6 total DNA as a template. The amplified  
701 fragment was ligated into pT7Blue, and the NdeI-BamHI fragment of the resulting  
702 plasmid was then inserted in pCold I to generate pCold12830. DNA fragments carrying  
703 *ferB* and *ferB2* were amplified by PCR. The amplified fragments were ligated into pET-  
704 16b to obtain pE16FB and pE16FB2. Nucleotide sequences of their inserts were  
705 confirmed by nucleotide sequencing. Expression plasmids for SYK-6 ALDH genes and  
706 *ferA* were prepared in previous studies (31, 51). The expression plasmids were  
707 introduced into *E. coli* BL21(DE3), and the transformed cells were grown in LB. In the  
708 case of *hvpZ* expression, pTf16 encoding the trigger factor chaperone was introduced  
709 into *E. coli* BL21(DE3) in addition to pCold12830, and the resulting transformant was  
710 grown in the presence of 0.5 mg/ml L-arabinose. Expression of *hvpZ* and other genes  
711 were induced for 24 h at 16°C and for 4 h at 30°C, respectively, by adding 0.1–1 mM  
712 isopropyl-β-D-thiogalactopyranoside when the OD<sub>600</sub> of the cultures reached 0.5. Cells  
713 were then harvested by centrifugation and washed with buffer A. pJB12830 was created  
714 by inserting the 2.3-kb BamHI-SacII (blunted) fragment carrying *hvpZ* from pBH37F  
715 into pJB864. pJB12830 was introduced into SYK-6, and the transformed cells were  
716 grown in LB containing 1 mM *m*-toluate for 24 h. Resting cells and cell extracts were  
717 prepared as described above.

718

719           **Construction of mutants.** To construct pKmb12830K, the 1.3-kb Sall-BamHI  
720 fragment carrying the kanamycin resistance gene (*kan*) of pIK03 was inserted into the  
721 XhoI-BglII sites in *hvpZ* of pUC12830. The 3.1-kb Sall fragment of the resulting

722 plasmid was ligated into the same site of pK19*mobsacB* to obtain pKmb12830K. To  
723 construct pKmb07270K, the 1.5-kb HindIII-XbaI fragment of pKS0727 was ligated into  
724 the same sites of pK18*mobsacB*, yielding pKmb07270. pKmb07270K was constructed  
725 by inserting *kan* into the NruI site in SLG\_07270 of pKmb07270. pKmb12830K and  
726 pKmb07270K were independently introduced into SYK-6 cells by electroporation, and  
727 candidate mutants were isolated as described previously (63). Disruption of each gene  
728 was examined by Southern hybridization analysis (Fig. S3). To construct pAK20400,  
729 upstream and downstream regions (ca. 1.0 kb each) of SLG\_20400 were amplified by  
730 PCR. The resulting fragments were cloned into pAK405 by In-fusion cloning (In-Fusion  
731 HD Cloning Kit; Takara Bio). pAK20400 was introduced into SYK-6 cells by  
732 triparental mating (62), and the mutant strain was selected as described previously (64).  
733 Disruption of the gene was confirmed by colony PCR. For the complementation of *hvpZ*  
734 in SME059, pJB12830 was introduced into cells by electroporation.

735

736 **Resting cell assays.** Resting cells of *E. coli* harboring pCold12830 and pTf16  
737 (OD<sub>600</sub> of 10.0), SYK-6 (OD<sub>600</sub> of 0.5, 1.0, or 5.0), SYK-6 harboring pJB864 (OD<sub>600</sub> of  
738 1.0), SME059 (OD<sub>600</sub> of 0.5, 1.0, or 5.0), SME059 harboring pJB864 (OD<sub>600</sub> of 1.0),  
739 SME059 harboring pJB12830 (OD<sub>600</sub> of 1.0), and SME009 (OD<sub>600</sub> of 1.0) prepared  
740 from LB-grown cultures were incubated with substrates (200 μM HPV, 200 μM HPS,  
741 200 μM GGE, or 100 μM VAA) at 30°C with shaking. Portions of the cultures were  
742 collected and the amounts of substrates were measured by HPLC.

743

744 **Enzyme properties of HvpZ.** To determine the cellular localization of HvpZ, the  
745 HPV-oxidizing activities of the soluble and membrane fractions prepared from cell

746 extracts of SYK-6 were measured. The cell extracts (500  $\mu\text{g}$  of protein/ml), soluble  
747 fraction (500  $\mu\text{g}$  mg of protein/ml), and membrane fraction (500  $\mu\text{g}$  of protein/ml) were  
748 incubated with 200  $\mu\text{M}$  HPV and 500  $\mu\text{M}$  PMS in buffer A for 10 or 30 min at 30°C.  
749 The amounts of HPV were measured by HPLC.

750 The enzyme reaction was typically carried out by incubating the membrane  
751 fraction (300  $\mu\text{g}$  of protein/ml) of *E. coli* cells harboring pCold12830 and pTf16 with  
752 200  $\mu\text{M}$  HPV and 500  $\mu\text{M}$  PMS in buffer A for 10 min at 30°C. Following incubation,  
753 the amount of substrate was measured by HPLC. The optimum pH was determined at  
754 pH ranges from 7.0 to 10.0 using 50 mM GTA buffer (50 mM 3,3-dimethylglutaric acid,  
755 50 mM Tris, and 50 mM 2-amino-2-methyl-1,3-propanediol; pH 7.0 to 9.0) and 50 mM  
756 CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid) buffer (pH 8.6 to 10) at 30°C. The  
757 optimum temperature was determined at temperature ranges from 25°C to 50°C using  
758 buffer A. To determine the substrate range, 200  $\mu\text{M}$  of HPV, HPS, coniferyl alcohol,  
759 sinapyl alcohol, cinnamyl alcohol, 3-(4-hydroxyphenyl)-1-propanol, homovanillyl  
760 alcohol, vanillyl alcohol, GGE, MPHPV, DCA, and DCA-C were used for the reaction,  
761 and the decrease in the amount of substrate was measured by HPLC. To examine the  
762 effect of flavin cofactors on HpvZ activity, the activities of HpvZ in the presence of 500  
763  $\mu\text{M}$  FAD or flavin mononucleotide were determined. To examine the effect of  
764 ubiquinone derivatives on HpvZ activity, the activities of HpvZ in the presence of CoQ<sub>0</sub>  
765 and CoQ<sub>1</sub> were determined. The enzyme reactions were carried out by incubating  
766 membrane fractions of *E. coli* cells harboring pCold12830 and pTf16 (300  $\mu\text{g}$  of  
767 protein/ml) or SYK-6 cells harboring pJB12830 (300  $\mu\text{g}$  of protein/ml) with 200  $\mu\text{M}$   
768 HPV and 500  $\mu\text{M}$  CoQ<sub>0</sub> or CoQ<sub>1</sub> in buffer A for 10 and 5 min, respectively, at 30°C.  
769 The decrease in the substrate was determined by HPLC analysis.

770

771           **Identification of the ALDH genes involved in VAL conversion.** The abilities of  
772 the 23 ALDHs of SYK-6 to convert VAL were examined by measuring the amount of  
773 VAA produced from HPV when HPV was reacted with both HpvZ and each ALDH.  
774 Resting cells of *E. coli* expressing each ALDH gene (OD<sub>600</sub> of 1.0) and *E. coli*  
775 harboring pCold12830 and pTf16 (OD<sub>600</sub> of 5.0 or 10.0) were incubated with 100 μM  
776 HPV in buffer A at 30°C with shaking. Portions of the cultures were collected at various  
777 sampling time points, and the supernatants of the reaction mixtures were analyzed by  
778 HPLC.

779           The mixtures of resting cells of SYK-6, SME031, SME045, SME061, or SME092  
780 (OD<sub>600</sub> of 0.5) and resting cells of *E. coli* harboring pCold12830 and pTf16 (OD<sub>600</sub> of  
781 10.0) were incubated with 1 mM HPV in buffer A at 30°C with shaking. Portions of the  
782 reaction mixtures were collected at various sampling time points. The supernatants  
783 prepared were analyzed by HPLC.

784

785           **Conversion of VAA by enzymes for ferulate catabolism.** Crude enzymes of  
786 FerA, FerB, and FerB2 were prepared from the *E. coli* transformants described above.  
787 FerA, FerA + FerB, and FerA + FerB2 (100 μg protein/ml of each) were incubated with  
788 100 μM VAA, 1 mM CoA, 1.25 mM MgSO<sub>4</sub>, and 1.25 mM ATP in buffer A for 60 min  
789 at 30°C. The supernatants prepared were analyzed by HPLC.

790

791

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1070 **Figure Legends**

1071 **FIG 1** Proposed catabolic pathway of arylglycerol- $\beta$ -aryl ether in *Sphingobium* sp.  
1072 strain SYK-6. The pathway for both guaiacyl (R = H) and syringyl (R = OCH<sub>3</sub>)-type  $\beta$ -  
1073 aryl ether compounds is shown. Enzymes: LigD, LigL, and LigN, C $\alpha$ -dehydrogenases;  
1074 LigF, LigE, and LigP,  $\beta$ -etherases; LigG, glutathione *S*-transferase; HpvZ, HPV oxidase;  
1075 ALDHs, aldehyde dehydrogenases; Abbreviations: GGE, guaiacylglycerol- $\beta$ -guaiacyl  
1076 ether; MPHPV,  $\alpha$ -(2-methoxyphenoxy)- $\beta$ -hydroxypropiovanillone; GS-HPV,  $\alpha$ -  
1077 glutathionyl- $\beta$ -hydroxypropiovanillone; HPV,  $\beta$ -hydroxypropiovanillone; HPS,  $\beta$ -  
1078 hydroxypropiosyringone; VAL, vanilloyl acetaldehyde; SAL, 3-(4-hydroxy-3,5-  
1079 dimethoxyphenyl)-3-oxopropanal; VAA, vanilloyl acetic acid; SAA, 3-(4-hydroxy-3,5-  
1080 dimethoxyphenyl)-3-oxopropanoic acid; CoA, coenzyme A; VAA-CoA, CoA derivative  
1081 of VAA.

1082

1083 **FIG 2** HPLC-MS analysis of HPV metabolites. Resting cells of SYK-6 (OD<sub>600</sub> of 5.0)  
1084 and SYK-6 cell extracts (>10 kDa; 500  $\mu$ g of protein/ml) were incubated with 1 mM  
1085 HPV (A and B) and 200  $\mu$ M HPV (D and E), respectively. The same cell extracts (>10  
1086 kDa) were incubated with HPV in the presence of 500  $\mu$ M NAD<sup>+</sup> (G and H) and in the  
1087 presence of 500  $\mu$ M NAD<sup>+</sup> + 2 mM CoA + 2.5 mM MgSO<sub>4</sub> + 2.5 mM ATP (J and K).  
1088 Portions of the reaction mixtures were collected at the start (A and D) and after 2 h (G  
1089 and J), 6 h (B), and 24 h (E, H, and K) of incubation, and analyzed by HPLC. ESI-MS  
1090 spectra of compounds I (negative mode), II (positive mode), III (negative mode), and IV  
1091 (positive mode) are shown in panels C, F, I, and L, respectively. An asterisk shown in  
1092 panel (I) indicates an unidentified MS fragment that appeared between retention times  
1093 of 2.0 and 7.0 min in the HPLC chromatogram (H).

1094

1095 **FIG 3** HPLC-MS analysis of HPS metabolites. SYK-6 resting cells (OD<sub>600</sub> of 5.0) and  
1096 SYK-6 cell extracts (>10 kDa; 500 μg of protein/ml) were incubated with 1 mM HPS  
1097 (A and B) and 200 μM HPS (D and E), respectively. The same cell extract (>10 kDa)  
1098 was incubated with HPS in the presence of 500 μM NAD<sup>+</sup> (G and H). Portions of the  
1099 reaction mixtures were collected at the start (A, D, and G) and after 4 h (B) and 16 h (E  
1100 and H) of incubation, and analyzed by HPLC. Negative-ion ESI-MS spectra of  
1101 compounds V, VI, and VII are shown in panels C, F, and I, respectively.

1102

1103 **FIG 4** Function and role of *hvpZ* in SYK-6. (A and B) Conversion of 200 μM HPV by  
1104 resting cells of *E. coli* harboring pCold12830 and pTf16 in Tris-HCl buffer (pH 7.5) (A)  
1105 and water (B). Portions of the reaction mixtures were collected after 6 h of incubation,  
1106 and analyzed by HPLC. ESI-MS spectra of compound VIII (positive mode) is shown in  
1107 panel C. (D–F) Conversions of 200 μM HPV (D), GGE (E), and HPS (F) by resting  
1108 cells of SYK-6 (open symbols) and SME059 (closed symbols). Circles, triangles, and  
1109 diamonds indicate the concentrations of HPV, GGE, and HPS, respectively. (G)  
1110 Complementation of SME059 with pJB12830. Cells of SYK-6 harboring pJB864  
1111 (circles), SME059 harboring pJB864 (triangles), and SME059 harboring pJB12830  
1112 (diamonds) were incubated with 200 μM HPV. Experiments shown in panels D–G were  
1113 performed in triplicate, and the data represent the averages ± standard deviations.

1114

1115 **FIG 5** Identification of ALDH genes involved in VAL conversion. (A) Resting cells of  
1116 *E. coli* carrying each of the 23 SYK-6 ALDH genes (OD<sub>600</sub> of 1.0) were incubated with  
1117 100 μM HPV in the presence of resting cells of *E. coli* harboring pCold12830 and

1118 pTf16 (OD<sub>600</sub> of 5.0). The amounts of VAA produced in the reaction mixtures  
1119 containing each of the ALDH gene-expressing cells are shown as the relative ratio to  
1120 that in the reaction mixture containing SLG\_20400-expressing cells. ND, VAA was not  
1121 detected. (B) Time course of the production of VAA during incubation of 100 μM HPV  
1122 with cells of *E. coli* harboring pCold12830 and pTf16 (OD<sub>600</sub> of 10.0) and *E. coli*  
1123 carrying following ALDH genes (OD<sub>600</sub> of 1.0): SLG\_20400 (closed circles),  
1124 SLG\_07270 (closed triangles), *bzaB* (closed squares), SLG\_12800 (closed diamonds),  
1125 SLG\_18210 (open circles), *bzaA* (open triangles), and SLG\_31150 (open squares). *E.*  
1126 *coli* cells harboring pET-21a(+) was used as a negative control (open diamonds). (C)  
1127 Accumulation of VAL-Tris during incubation of 1 mM HPV with cells of *E. coli*  
1128 harboring pCold12830 and pTf16 (OD<sub>600</sub> of 10.0) and the following mutants of the  
1129 ALDH genes (OD<sub>600</sub> of 0.5): SME061 (ΔSLG\_20400; closed circles), SME045 (Δ*bzaB*;  
1130 closed squares), SME092 (ΔSLG\_07270; closed triangles), and SME031  
1131 (ΔSLG\_12800; closed diamonds). SYK-6 cells were used as a control (open diamonds).  
1132 These experiments were performed in triplicate, and the data represent the averages ±  
1133 standard deviations.

1134

1135 **FIG 6** Conversion of VAA by *desV ligV* double mutant. Resting cells of *desV ligV*  
1136 double mutant (SME077) were incubated with 100 μM VAA (A–C) and 1 mM ferulate  
1137 (D–F). Portions of the reaction mixtures were collected at the start (A and D) and after 2  
1138 h (B and E) and 4 h (C and F) of incubation, and analyzed by HPLC using two different  
1139 analytical conditions described in the materials and methods. The retention times of  
1140 vanillin separated under the analytical conditions for the reaction mixtures of VAA (A–  
1141 C) and ferulate (D–F) were 2.9 and 2.4 min, respectively.

1142 **FIG 7** Synthetic routes to HPS and HPV.

**TABLE 1** Substrate range of HpvZ

Compounds	Specific activity <sup>a</sup> (nmol·min <sup>-1</sup> ·mg <sup>-1</sup> )
HPV	26 ± 6
HPS	40 ± 5
coniferyl alcohol	8.0 ± 0.4
sinapyl alcohol	52 ± 7
cinnamyl alcohol	40 ± 9
3-(4-hydroxyphenyl)-1-propanol	8 ± 2
homovanillyl alcohol	ND <sup>b</sup>
vanillyl alcohol	ND
GGE	ND
MPHPV	ND
DCA	13 ± 2
DCA-C	ND

<sup>a</sup>The membrane fraction of *hvpZ* expressing *E. coli* (300 µg of protein/ml) was incubated with 200 µM substrate in the presence of 500 µM PMS. The data represent the averages ± standard deviations of three independent experiments.

<sup>b</sup>ND, not detected

**TABLE 2** Strains and plasmids used in this study

Strains or plasmids	Relevant characteristic(s) <sup>a</sup>	Reference or source
<i>Sphingobium</i> sp.		
SYK-6	Wild type; Nal <sup>r</sup> Sm <sup>r</sup>	(65)
SME009	SYK-6 derivative; <i>ferA::kan</i> ; Nal <sup>r</sup> Sm <sup>r</sup> Km <sup>r</sup>	(36)
SME031	SYK-6 derivative; SLG_12800:: <i>kan</i> ; Nal <sup>r</sup> Sm <sup>r</sup> Km <sup>r</sup>	(66)
SME045	SYK-6 derivative; <i>bzaB::tet</i> ; Nal <sup>r</sup> Sm <sup>r</sup> Tc <sup>r</sup>	(67)
SME059	SYK-6 derivative; <i>hvpZ::kan</i> ; Nal <sup>r</sup> Sm <sup>r</sup> Km <sup>r</sup>	This study
SME061	SYK-6 derivative; ΔSLG_20400; Nal <sup>r</sup> Sm <sup>r</sup>	This study
SME077	SYK-6 derivative; <i>desV::cat ligV::kan</i> ; Nal <sup>r</sup> Sm <sup>r</sup> Cm <sup>r</sup> Km <sup>r</sup>	(51)
SME092	SYK-6 derivative; SLG_07270:: <i>kan</i> ; Nal <sup>r</sup> Sm <sup>r</sup> Km <sup>r</sup>	This study
<i>Sphingomonas sanguinis</i>		
IAM 12578	Nal <sup>r</sup>	(68)
<i>Escherichia coli</i>		
BL21(DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm</i> (DE3); T7 RNA polymerase gene under the control of the <i>lacUV5</i> promoter	(69)
HB101	<i>recA13 supE44 hsd20 ara-14 proA2 lacY1 galk2 rpsL20 xyl-5 mtl-1</i>	(70)
NEB 10-beta	<i>araD139 Δ(ara-leu)7697 fhuA lacX74 galK</i> (φ80 Δ <i>lacZ</i> Δ <i>M15</i> ) <i>recA1 endA1 nupG rpsL</i> (Sm <sup>r</sup> ) Δ( <i>mrr-hsdRMS-mcrBC</i> )	New England Biolabs
Plasmids		
pVK100	Broad-host-range cosmid vector; Km <sup>r</sup> Tc <sup>r</sup>	(62)
pRK2013	Tra <sup>+</sup> Mob <sup>+</sup> ColE1 replicon; Km <sup>r</sup>	(71)
pT7Blue	Cloning vector; Ap <sup>r</sup>	Novagen
pBluescript II KS(+) and SK(+)	Cloning vector; Ap <sup>r</sup>	(72)
pUC19	Cloning vector; Ap <sup>r</sup>	(73)
pET-16b	Expression vector; T7 promoter, Ap <sup>r</sup>	Novagen
pET-21a(+)	Expression vector; T7 promoter, Ap <sup>r</sup>	Novagen
pCold I	Expression vector; <i>cspA</i> promoter, Ap <sup>r</sup>	Takara Bio
pTf16	Expression vector for <i>tig</i> ; <i>araB</i> promoter; Cm <sup>r</sup>	Takara Bio
pK18 <i>mobsacB</i>	<i>oriT sacB</i> ; Km <sup>r</sup>	(74)
pK19 <i>mobsacB</i>	<i>oriT sacB</i> ; Km <sup>r</sup>	(74)
pIK03	KS(+) with a 1.3-kb EcoRV fragment carrying <i>kan</i> of pUC4K; Ap <sup>r</sup> Km <sup>r</sup>	(75)
pJB864	RK2 broad-host-range expression vector; Ap <sup>r</sup> Cb <sup>r</sup> P <sub>m</sub> <i>xyIS</i>	(76)
pAK405	Plasmid for allelic exchange and markerless gene deletions in <i>Sphingomonads</i> ; Km <sup>r</sup>	(64)
pSA53	pVK100 with partially Sall-digested fragments of SYK-6 carrying <i>hvpZ</i>	This study
pSA88	pVK100 with partially Sall-digested fragments of SYK-6 carrying <i>hvpZ</i>	This study
pSA684	pVK100 with partially Sall-digested fragments of SYK-6 carrying <i>hvpZ</i>	This study
pT7B12830	pT7Blue with a 1.7-kb PCR amplified fragment carrying <i>hvpZ</i>	This study
pCold12830	pCold I with a 1.7-kb NdeI-BamHI fragment carrying <i>hvpZ</i> from pT7B12830	This study
pBH37F	SK(+) with a 3.7-kb HindIII fragment carrying <i>hvpZ</i> from pSA53	This study
pUC12830	pUC19 with a 2.0-kb Sall fragment of pBH37F	This study
pUC12830K	pUC12830 with a 1.3-kb Sall-BamHI fragment of pIK03 carrying <i>kan</i>	This study
pKmb12830K	pK19 <i>mobsacB</i> with a 3.1-kb Sall fragment of pUC12830K	This study
pJB12830	pJB864 with a 2.3-kb BamHI-SacII (blunted) fragment carrying <i>hvpZ</i> from pBH37F	This study
pLVH	pET-21a(+) with a 1.9-kb NdeI-XhoI fragment carrying <i>ligV</i>	(77)
pT21-0727	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_07270	(51)
pT21-0761	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_07610	(51)
pT21-0779	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_07790	(51)
pT21-0940	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_09400	(51)
pT21-0951	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_09510	(51)
pT21-0992	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_09920	(51)
pT21-1141	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_11410	(51)
pT21-1202	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_12020	(51)
pT21-1219	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_12190	(51)
pT21-1280	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_12800	(51)
pT21-1821	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_18210	(51)
pT21-2040	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_20400	(51)

pT21-2721	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_27210	(51)
pT21-2791	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying <i>bzaA</i>	(51)
pT21-2792	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying <i>bzaB</i>	(51)
pT21-2815	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_28150	(51)
pT21-2832	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_28320	(51)
pT21-3115	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_31150	(51)
pT21-3224	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_32240	(51)
pT21-3494	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_34940	(51)
pT21-3812	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_38120	(51)
pT21-p0068	pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_p_00680	(51)
pKS0727	KS(+) with a 1.5-kb NdeI-BamHI fragment carrying SLG_07270	(51)
pKmb07270	pK18 <i>mobsacB</i> with a 1.5-kb HindIII-XbaI carrying SLG_07270 from pKS0727	This study
pKmb07270K	pKmb07270 with a 1.3-kb EcoRV fragment carrying <i>kan</i> from pK03 into NruI site of SLG_07270	This study
pAK20400	pAK405 with a 2.3-kb deletion cassette carrying up- and downstream regions of SLG_20400	This study
pE16FA	pET-16b with a 2.2-kb NdeI-BamHI fragment carrying <i>ferA</i>	(31)
pE16FB	pET-16b with a 0.9-kb NdeI-BamHI PCR amplified fragment carrying <i>ferB</i>	This study
pE16FB2	pET-16b with a 1.1-kb NdeI-BamHI PCR amplified fragment carrying <i>ferB2</i>	This study

<sup>a</sup> Km<sup>r</sup>, Nal<sup>r</sup>, Sm<sup>r</sup>, Ap<sup>r</sup>, Tc<sup>r</sup>, Cm<sup>r</sup>, and Cb<sup>r</sup>, resistance to kanamycin, nalidixic acid, streptomycin, ampicillin, tetracycline, chloramphenicol, and carbenicillin, respectively.

**TABLE 3** Primer sequences used for construction of plasmids and colony PCR

Plasmid or strain	Primer	Sequence (5' to 3')
Construction of plasmids		
pT7B12830	12830_pT7B_F	AGACAGGCATATGGTTGATG
	12830_pT7B_R	GGGCGGCATGGATCCGC
pAK20400	dis20400_Top_F	CGGTACCCGGGGATCCGGCTTCGGTGACAATCAT
	dis20400_Top_R	ATGTCCGTGGTGTCTGCGT
	dis20400_Bot_F	ACGCAGAACACCACGGACATTCTCCCCGTGATGACCTAT
	dis20400_Bot_R	CGACTCTAGAGGATCGTGGCGGCATCAACATATCG
pE16FB	ferB_exp_F	GGAAAATCATATGTCCGAGG
	ferB_exp_R	ATACTGGCGGATCCAGCC
pE16FB2	ferB2_exp_F	TGAGGATGCATATGTCCGATG
	ferB2_exp_R	GCCGGATCCCGGAATGC
Colony PCR		
SME061	dis20400_conf_F	CCTTCATCGCCATCATAAAT
	dis20400_Bot_R	CGACTCTAGAGGATCGTGGCGGCATCAACATATCG