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# Bacterial catabolism of β-hydroxypropiovanillone and β-hydroxypropiosyringone produced in the reductive cleavage of arylglycerol-β-aryl ether in lignin

Running title: *Downstream pathway of the β-aryl ether catabolism*

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1 **ABSTRACT**

2 *Sphingobium* sp. strain SYK-6 converts four stereoisomers of arylglycerol-β-guaiacyl
3 ether into achiral β-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, β-hydroxypropiosyringone (HPS), was also catabolized
9 via the same pathway. SLG_12830 (*hpvZ*), which belongs to the glucose-methanol-
10 choline oxidoreductase family, was isolated as the HPV-converting enzyme gene. An
11 *hpvZ* mutant completely lost the ability to convert HPV and HPS, indicating that *hpvZ* 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 β-aryl ether linkage is the most essential process in lignin
25 biodegradation. Although the bacterial β-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 β-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/$O_2$, followed by
30 cleavage of the β-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, β-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 β-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. β-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 β-aryl ether in *Sphingobium* sp. strain SYK-6 has been determined (13).
61 SYK-6 is able to degrade all the stereoisomers of β-aryl ether-type biaryl,
62 guaiacylglycerol-β-guaiacyl ether (GGE). In SYK-6 cells, four stereoisomers of GGE
63 are converted to two enantiomers of α-(2-methoxyphenoxy)-β-hydroxypropiovanillone
64 (MPHPV) through the oxidation of the GGE α-carbon atom catalyzed by Cα-

65 dehydrogenases (LigD, LigL, and LigN) (Fig. 1) (14). LigD oxidizes (α*R*,β*S*)-GGE and
66 (α*R*,β*R*)-GGE into (β*S*)-MPHPV and (β*R*)-MPHPV, respectively, while LigL/LigN
67 converts (α*S*,β*R*)-GGE and (α*S*,β*S*)-GGE into (β*R*)-MPHPV and (β*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 α-
70 glutathionyl-β-hydroxypropiovanillone (GS-HPV) and guaiacol via nucleophilic attack
71 of glutathione on the MPHPV β-carbon atom (15, 16). LigF and LigE/LigP attack (β*S*)-
72 MPHPV and (β*R*)-MPHPV to produce (β*R*)-GS-HPV and (β*S*)-GS-HPV, respectively.
73 Another GST, LigG, catalyzes the cleavage of the thioether linkage in (β*R*)-GS-HPV by
74 transferring glutathione of (β*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 (β*S*)-GS-HPV, suggesting involvement of an alternative GST in the conversion of
77 (β*S*)-GS-HPV (15, 18). Recently, further detailed biochemical characterization of the β-
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 β-aryl ether, have recently been isolated, and
82 similar enzyme systems have been characterized (23, 24).
83 Although many investigations of the cleavage of β-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 β-aryl ether. Therefore, for understanding bacterial β-aryl
86 ether catabolism, it is essential to elucidate the HPV catabolic system. Recently, the
87 production of HPV and β-hydroxypropiosyringone (HPS; an intermediate metabolite of
88 syringyl-type β-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 β-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 μM 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 Cγ–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 μM HPV in the
134 presence of 500 μM $NAD^+$ 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 $NAD^+$-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 Cγ activation by coenzyme A (CoA) (35-39). A SYK-6 cell extract (>10
149 kDa) was therefore incubated with 200 μM HPV in the presence of 500 μM $NAD^+$, 2
150 mM CoA, 2.5 mM $MgSO_4$, 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 $MgSO_4$ 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 μM 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 μM $NAD^+$, 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 $NAD^+$ on the enzyme activities
185 in converting 200 μM 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 ($4.2 \pm 0.7$ nmol·min$^{-1}$·mg$^{-1}$) than that in the absence of cofactors
188 ($2.4 \pm 0.8$ nmol·min$^{-1}$·mg$^{-1}$). On the other hand, the addition of FAD or $NAD^+$ 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 SalI-digested pSA53 as a probe suggested that a 3.6
200 kb, a 2.0 kb, and two 1.0 kb SalI fragments were commonly present in the above
201 cosmids. Subcloning and nucleotide sequencing showed that these SalI 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 (*hpvZ*-
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 μM 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 μM 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 *hpvZ*.
239

240 **Cellular localization of HpvZ.** In order to determine the cellular localization of
241 HpvZ, 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 ± 0.1 nmol·min$^{-1}$ (7.5 mg of protein) and 0.5 ± 0.1

244 nmol·min$^{-1}$ (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 ± 6 nmol·min$^{-1}$·mg$^{-1}$. 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-β-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 $C_6$–$C_2$ monomeric alcohol (homovanillyl alcohol), a $C_6$–$C_1$ 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 $C_6$–$C_3$ 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 Cγ 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 Cγ of the B-ring side chain
283 of DCA, which is different from the regiospecificity 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 ± 8 $nmol·min^{-1}$
296 $·mg^{-1}$) or flavin mononucleotide (27 ± 1 $nmol·min^{-1}·mg^{-1}$) were almost equivalent to
297 that in the absence of any flavin cofactors (26 ± 6 $nmol·min^{-1}·mg^{-1}$). 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 (*Am*PDH) 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 *hpvZ*-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 ($CoQ_{10}$) and its derivatives ($CoQ_0$ and $CoQ_1$) as electron acceptors for the oxidation of
309 their substrates (41, 42). PhcC and PhcD have also been shown to be able to use $CoQ_0$
310 and $CoQ_1$ as electron acceptors as well as PMS (40). Furthermore, electron transport
311 from AlkJ to cytochrome *c* in the presence of $CoQ_1$ 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 $CoQ_0$ (27 ± 2 nmol·min$^{-1}$·mg$^{-1}$) and $CoQ_1$ (36 ± 6 nmol·min$^{-1}$·mg$^{-1}$),
318 respectively, than that in the absence of cofactors (22 ± 1 nmol·min$^{-1}$·mg$^{-1}$). The
319 specific activity in the presence of $CoQ_1$ was almost equivalent to that obtained using
320 PMS (40 ± 3 nmol·min$^{-1}$·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 μM HPV in the presence of FAD, $NAD^+$, and $NADP^+$ for 2 h. In the presence of $NAD^+$
333 and $NADP^+$, VAA accumulated to levels of 95 ± 9 μM and 86 ± 6 μM, respectively,
334 while the accumulation of VAA was not observed in the presence of FAD. These results
335 suggested that $NAD^+$/$NADP^+$-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 μM 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 μM
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 μM 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 μM VAA in the presence of CoA,
397 $MgSO_4$, 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 μM
404 VAA in the presence of CoA, $MgSO_4$, 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 μM 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-β-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 *hpvZ* in other bacteria.** Since HpvZ 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 β-aryl ether compounds. BLAST searches of
434 *hpvZ* 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 β-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 $NH_4Cl$ (2 × 300 ml). The organic layer was further washed with water (500
471 ml), brine (300 ml), dried with $MgSO_4$, 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). $^{1}H$ NMR (500
475 MHz, $CDCl_3$) δ 9.79 (s, 1H), 7.07 (s, 2H), 3.84 (s, 6 H), 0.98 (s, 9H), 0.13 (s, 6H); $^{13}C$
476 NMR (125 MHz, $CDCl_3$) δ 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°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°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 $NH_4Cl$ (2 × 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 $MgSO_4$, 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%). $^1$H NMR (500 MHz, $CDCl_3$) δ 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); $^{13}$C
491 NMR (125 MHz, $CDCl_3$) δ 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-dimethyoxyphenyl)propane-*
494 *1,3-diol (C)* A suspension of $LiAlH_4$ (1.4 g, 37.8 mmol, 2.2 eq) in THF (100 ml) was
495 cooled to -20°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°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 $Na_2S_2O_3$ (2 × 300 ml). The organic layer was washed brine (300 ml), dried
500 with $MgSO_4$, 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%). $^1$H NMR (500 MHz, $CDCl_3$) δ 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 $^{13}$C NMR (125 MHz, $CDCl_3$) δ 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-butyldimethylsilyl)oxy)-3,5-dimethyoxyphenyl)-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 $Na_2S_2O_3$ (2 × 300 ml). The
513 organic layer was washed water (300 ml), brine (300 ml), dried with $MgSO_4$, 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%). $^1$H
516 NMR (500 MHz, $CDCl_3$) 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); $^{13}$C NMR
518 (125 MHz, $CDCl_3$) 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 $NH_4Cl$ (2 × 300 ml). The organic
525 layer was washed water (300 ml), brine (300 ml), dried with $MgSO_4$, 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 $[M - H]^+$ calcd. For $C_{11}H_{13}O_5$ 225.0800; found
530 225.0766; $^1$H NMR (500 MHz, DMSO-$d_6$) 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); $^{13}$C NMR (125 MHz,

532 DMSO-$d_6$) δ 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). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 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); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 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%). $^{1}$H NMR (500 MHz, $CDCl_3$) δ 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); $^{13}$C NMR (125 MHz, $CDCl_3$) δ

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-methyoxyphenyl)propane-1,3-*

557 *diol (G)* Compound G was synthesized following the same experimental procedure as

558 described for the synthesis of compound C. $LiAlH_4$ (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%). $^1$H NMR (500 MHz, $CDCl_3$)

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); $^{13}$C NMR (125 MHz,

564 $CDCl_3$) 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-methyoxyphenyl)-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%). $^1$H NMR (400 MHz, $CDCl_3$)

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); $^{13}$C NMR (125 MHz, $CDCl_3$) 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, $CDCl_3$) δ 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, $CDCl_3$) δ 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 *hpvZ*-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 μM. 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 μM 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 × *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 ×
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 × *g* for 30 min at 4°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 ($OD_{600}$ of 5.0) were
616 incubated with 1 mM HPV or HPS in buffer A at 30°C with shaking. SME077 resting
617 cells ($OD_{600}$ of 0.5 or 5.0) were incubated with 100 μM VAA or 1 mM ferulic acid in
618 buffer A at 30°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 μg protein/ml) were incubated with 200 μM
623 HPV or HPS in the presence and absence of 500 μM $NAD^+$ or 500 μM $NAD^+$ + 2 mM
624 CoA + 2.5 mM $MgSO_4$ + 2.5 mM ATP in buffer A at 30°C. SME077 cell extracts (>10
625 kDa; 500 μg protein/ml) were incubated with 100 μM VAA in the presence of 1 mM

626 CoA + 1.25 mM $MgSO_4$ + 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 $OD_{600}$ of 0.2, and grown at
636 30°C. GGE (5 mM) was added when the $OD_{600}$ 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 $NAD^+$) 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 $NAD^+$, or 500 μM $NADP^+$) 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, MPHPV, 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 μM 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 *hpvZ* 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 *hpvZ* 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 *hpvZ* 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 $OD_{600}$ 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 *hpvZ* 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 SalI-BamHI

720 fragment carrying the kanamycin resistance gene (*kan*) of pIK03 was inserted into the

721 XhoI-BglII sites in *hpvZ* of pUC12830. The 3.1-kb SalI 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 *hpvZ*
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 ($OD_{600}$ of 10.0), SYK-6 ($OD_{600}$ of 0.5, 1.0, or 5.0), SYK-6 harboring pJB864 ($OD_{600}$ of
738 1.0), SME059 ($OD_{600}$ of 0.5, 1.0, or 5.0), SME059 harboring pJB864 ($OD_{600}$ of 1.0),
739 SME059 harboring pJB12830 ($OD_{600}$ of 1.0), and SME009 ($OD_{600}$ 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 HpvZ.** To determine the cellular localization of HpvZ, 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 μg of protein/ml), soluble
747 fraction (500 μg mg of protein/ml), and membrane fraction (500 μg of protein/ml) were
748 incubated with 200 μM HPV and 500 μM 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 μg of protein/ml) of *E. coli* cells harboring pCold12830 and pTf16 with
752 200 μM HPV and 500 μM 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 μM 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 μM 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 $CoQ_0$
765 and $CoQ_1$ were determined. The enzyme reactions were carried out by incubating
766 membrane fractions of *E. coli* cells harboring pCold12830 and pTf16 (300 μg of
767 protein/ml) or SYK-6 cells harboring pJB12830 (300 μg of protein/ml) with 200 μM
768 HPV and 500 μM $CoQ_0$ or $CoQ_1$ 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 ($OD_{600}$ of 1.0) and *E. coli*
775 harboring pCold12830 and pTf16 ($OD_{600}$ 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 ($OD_{600}$ of 0.5) and resting cells of *E. coli* harboring pCold12830 and pTf16 ($OD_{600}$ 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 $MgSO_4$, 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-β-aryl ether in *Sphingobium* sp.
1072 strain SYK-6. The pathway for both guaiacyl (R = H) and syringyl (R = $OCH_3$)-type β-
1073 aryl ether compounds is shown. Enzymes: LigD, LigL, and LigN, Cα-dehydrogenases;
1074 LigF, LigE, and LigP, β-etherases; LigG, glutathione *S*-transferase; HpvZ, HPV oxidase;
1075 ALDHs, aldehyde dehydrogenases; Abbreviations: GGE, guaiacylglycerol-β-guaiacyl
1076 ether; MPHPV, α-(2-methoxyphenoxy)-β-hydroxypropiovanillone; GS-HPV, α-
1077 glutathionyl-β-hydroxypropiovanillone; HPV, β-hydroxypropiovanillone; HPS, β-
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 ($OD_{600}$ of 5.0)
1084 and SYK-6 cell extracts (>10 kDa; 500 μg of protein/ml) were incubated with 1 mM
1085 HPV (A and B) and 200 μM HPV (D and E), respectively. The same cell extracts (>10
1086 kDa) were incubated with HPV in the presence of 500 μM $NAD^+$ (G and H) and in the
1087 presence of 500 μM $NAD^+$ + 2 mM CoA + 2.5 mM $MgSO_4$ + 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 ($OD_{600}$ 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 $NAD^+$ (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 *hpvZ* 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 ($OD_{600}$ of 1.0) were incubated with

1117 100 μM HPV in the presence of resting cells of *E. coli* harboring pCold12830 and

1118 pTf16 ($OD_{600}$ 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 ($OD_{600}$ of 10.0) and *E. coli*

1123 carrying following ALDH genes ($OD_{600}$ 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 ($OD_{600}$ of 10.0) and the following mutants of the

1129 ALDH genes ($OD_{600}$ 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[a] (nmol·min$^{-1}$·mg$^{-1}$) |
|---|---|
| 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[b] |
| vanillyl alcohol | ND |
| GGE | ND |
| MPHPV | ND |
| DCA | 13 ± 2 |
| DCA-C | ND |

[a]The membrane fraction of *hpvZ* 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.
[b]ND, not detected

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

| Strains or plasmids | Relevant characteristic(s)[a] | Reference or source |
|---|---|---|
| *Sphingobium* sp. | | |
| SYK-6 | Wild type; Nal$^{r}$ Sm$^{r}$ | (65) |
| SME009 | SYK-6 derivative; *ferA*::*kan*; Nal$^{r}$ Sm$^{r}$ Km$^{r}$ | (36) |
| SME031 | SYK-6 derivative; SLG_12800::*kan*; Nal$^{r}$ Sm$^{r}$ Km$^{r}$ | (66) |
| SME045 | SYK-6 derivative; *bzaB*::*tet*; Nal$^{r}$ Sm$^{r}$ Tc$^{r}$ | (67) |
| SME059 | SYK-6 derivative; *hpvZ*::*kan*; Nal$^{r}$ Sm$^{r}$ Km$^{r}$ | This study |
| SME061 | SYK-6 derivative; ΔSLG_20400; Nal$^{r}$ Sm$^{r}$ | This study |
| SME077 | SYK-6 derivative; *desV*::*cat ligV*::*kan*; Nal$^{r}$ Sm$^{r}$ Cm$^{r}$ Km$^{r}$ | (51) |
| SME092 | SYK-6 derivative; SLG_07270::*kan*; Nal$^{r}$ Sm$^{r}$ Km$^{r}$ | This study |
| *Sphingomonas sanguinis* IAM 12578 | Nal$^{r}$ | (68) |
| *Escherichia coli* | | |
| BL21(DE3) | F$^{-}$ *ompT hsdS$_{B}$*(r$_{B}^{-}$ m$_{B}^{-}$) *gal dcm* (DE3); T7 RNA polymerase gene under the control of the *lacUV5* promoter | (69) |
| HB101 | *recA13 supE44 hsd20 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* | (70) |
| NEB 10-beta | *araD*139 Δ(*ara-leu*)7697 *fhuA lacX74 galK* (ϕ80 Δ*lacZ* Δ*M15*) *recA1 endA1 nupG rpsL* (Sm$^{r}$) Δ(*mrr-hsdRMS-mcrBC*) | New England Biolabs |
| Plasmids | | |
| pVK100 | Broad-host-range cosmid vector; Km$^{r}$ Tc$^{r}$ | (62) |
| pRK2013 | Tra$^{+}$ Mob$^{+}$ ColE1 replicon; Km$^{r}$ | (71) |
| pT7Blue | Cloning vector; Ap$^{r}$ | Novagen |
| pBluescript II KS(+) and SK(+) | Cloning vector; Ap$^{r}$ | (72) |
| pUC19 | Cloning vector; Ap$^{r}$ | (73) |
| pET-16b | Expression vector; T7 promoter, Ap$^{r}$ | Novagen |
| pET-21a(+) | Expression vector; T7 promoter, Ap$^{r}$ | Novagen |
| pCold I | Expression vector; *cspA* promoter, Ap$^{r}$ | Takara Bio |
| pTf16 | Expression vector for *tig*; *araB* promoter; Cm$^{r}$ | Takara Bio |
| pK18*mobsacB* | *oriT sacB*; Km$^{r}$ | (74) |
| pK19*mobsacB* | *oriT sacB*; Km$^{r}$ | (74) |
| pIK03 | KS(+) with a 1.3-kb EcoRV fragment carrying *kan* of pUC4K; Ap$^{r}$ Km$^{r}$ | (75) |
| pJB864 | RK2 broad-host-range expression vector; Ap$^{r}$ Cb$^{r}$ P$_{m}$ *xylS* | (76) |
| pAK405 | Plasmid for allelic exchange and markerless gene deletions in Sphingomonads; Km$^{r}$ | (64) |
| pSA53 | pVK100 with partially SalI-digested fragments of SYK-6 carrying *hpvZ* | This study |
| pSA88 | pVK100 with partially SalI-digested fragments of SYK-6 carrying *hpvZ* | This study |
| pSA684 | pVK100 with partially SalI-digested fragments of SYK-6 carrying *hpvZ* | This study |
| pT7B12830 | pT7Blue with a 1.7-kb PCR amplified fragment carrying *hpvZ* | This study |
| pCold12830 | pCold I with a 1.7-kb NdeI-BamHI fragment carrying *hpvZ* from pT7B12830 | This study |
| pBH37F | SK(+) with a 3.7-kb HindIII fragment carrying *hpvZ* from pSA53 | This study |
| pUC12830 | pUC19 with a 2.0-kb SalI fragment of pBH37F | This study |
| pUC12830K | pUC12830 with a 1.3-kb SalI-BamHI fragment of pIK03 carrying *kan* | This study |
| pKmb12830K | pK19*mobsacB* with a 3.1-kb SalI fragment of pUC12830K | This study |
| pJB12830 | pJB864 with a 2.3-kb BamHI-SacII (blunted) fragment carrying *hpvZ* from pBH37F | This study |
| pLVH | pET-21a(+) with a 1.9-kb NdeI-XhoI fragment carrying *ligV* | (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 *bzaA* | (51) |
| pT21-2792 | pET-21a(+) with a 1.5-kb NdeI-BamHI fragment carrying *bzaB* | (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*mobsacB* with a 1.5-kb HindIII-XbaI carrying SLG_07270 from pKS0727 | This study |
| pKmb07270K | pKmb07270 with a 1.3-kb EcoRV fragment carrying *kan* from pIK03 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 *ferA* | (31) |
| pE16FB | pET-16b with a 0.9-kb NdeI-BamHI PCR amplified fragment carrying *ferB* | This study |
| pE16FB2 | pET-16b with a 1.1-kb NdeI-BamHI PCR amplified fragment carrying *ferB2* | This study |

[a] $Km^r$, $Nal^r$, $Sm^r$, $Ap^r$, $Tc^r$, $Cm^r$, and $Cb^r$, 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 | ATGTCCGTGGTGTTCTGCGT |
| | dis20400_Bot_F | ACGCAGAACACCACGGACATTCCTCCCCGTGATGACCTAT |
| | dis20400_Bot_R | CGACTCTAGAGGATCGTGGCGGCATCAACATATCG |
| pE16FB | ferB_exp_F | GGAAAATCATATGTCCGAGG |
| | ferB_exp_R | ATACTGGCGGATCCAGCC |
| pE16FB2 | ferB2_exp_F | TGAGGATGCATATGTCGGATG |
| | ferB2_exp_R | GCCGGATCCCGGAATGC |
| Colony PCR | | |
| SME061 | dis20400_conf_F | CCTTCATCGCCATCATAAAT |
| | dis20400_Bot_R | CGACTCTAGAGGATCGTGGCGGCATCAACATATCG |