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Characterization of *Sphingomonas paucimobilis* SYK-6 genes involved in degradation of lignin-related compounds

E Masai[1], Y Katayama[2], S Nishikawa[3] and M Fukuda[1]

[1]*Department of Bioengineering, Nagaoka University of Technology, Nagaoka, Niigata 940–2188;* [2]*Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183–8509;* [3]*New Products and Technology Laboratory, Cosmo Research Institute, Satte, Saitama 340–0193, Japan*

Sphingomonas paucimobilis **SYK-6 is able to grow on a wide variety of dimeric lignin compounds. These compounds are degraded via vanillate and syringate by a unique enzymatic system, composed of etherases, O demethylases, ring cleavage oxygenases and side chain cleaving enzymes. These unique and specific lignin modification enzymes are thought to be powerful tools for utilization of the most abundant aromatic biomass, lignin. Here, we focus on the genes and enzymes involved in *β*-aryl ether cleavage and biphenyl degradation. Two unique etherases are involved in the reductive cleavage of *β*-aryl ether. These two etherases have amino acid sequence similarity with the glutathione *S*-transferases, and use glutathione as a hydrogen donor. It was found that 5,5′-dehydrodivanillate, which is a typical lignin-related biphenyl structure, was transformed into 5-carboxyvanillate by the reaction sequence of O-demethylation, *meta*-ring cleavage, and hydrolysis, and the genes involved in the latter two reactions have been characterized. Vanillate and syringate are the most common intermediate metabolites in lignin catabolism. These compounds are initially O-demethylated and the resulting diol compounds, protocatechuate (PCA) and 3-*O*-methylgallate, respectively, are subjected to ring cleavage catalyzed by PCA 4,5-dioxygenase. The ring cleavage products generated are further degraded through the PCA 4,5-cleavage pathway. We have isolated and characterized genes for enzymes involved in this pathway. Disruption of a gene for 2-pyrone-4,6-dicarboxylate hydrolase (*ligI*) in this pathway suggested that an alternative route for 3-*O*-methylgallate degradation, in which *ligI* is not involved, would play a role in syringate catabolism. In this article, we describe the genetic and biochemical features of the *S. paucimobilis* SYK-6 genes involved in degradation of lignin-related compounds. A possible application of the SYK-6 lignin degradation system to produce a valuable chemical material is also described.**

Keywords: *Sphingomonas paucimobilis*; lignin degradation; *β*-aryl ether cleavage; biphenyl degradation; O-demethylation; protocatechuate 4,5-cleavage pathway

Introduction

Lignin is the most abundant aromatic material in nature and its effective utilization would be desirable. One of the practical procedures to utilize lignin is its conversion into useful chemical materials using microbial lignin degradation systems. It is well known that degradation of native ligin is initiated by the attack by lignin peroxidase, manganese peroxidase and laccase secreted by white rot fungi [7]. The resulting low molecular weight lignin is further degraded and mineralized by bacteria [56,64]. Lignin degradation is therefore accomplished by the cooperative actions of the fungal and bacterial enzyme systems. Bacterial lignin degradation systems consist of many reaction steps, which are catalyzed by unique and specific enzymes. Thus, bacterial enzyme systems are expected to serve as useful tools for conversion of lignin into intermediate metabolites, which are valuable for commercial use.

Sphingomonas paucimobilis SYK-6 (formerly *Pseudomonas paucimobilis* SYK-6) which grows on 5,5′-dehydrodivanillate (DDVA) had been isolated from pulp effluent [21]. *S. paucimobilis* SYK-6 has the ability to degrade a wide variety of dimeric lignin compounds including *β*-aryl ether, phenylcoumarane, pinoresinol, and diarylpropane in addition to biphenyl [20,21,32] (Figure 1). These dimeric lignin compounds having guaiacyl or syringyl moieties are degraded to vanillate or syringate, respectively, by the action of the SYK-6 lignin degradation enzyme system, which is composed of ether cleavage [32–34], sidechain cleavage, O-demethylation [39], and aromatic ring cleavage enzymes [40,42] (Figure 1). Vanillate and syringate are initially O-demethylated, and then converted into protocatechuate (PCA) and 3-*O*-methygallate, respectively. The resulting PCA is further degraded to pyruvate and

Correspondence: E Masai, Department of Bioengineering, Nagaoka University of Technology, Kamitomioka, Nagaoka, Niigata 940–2188, Japan
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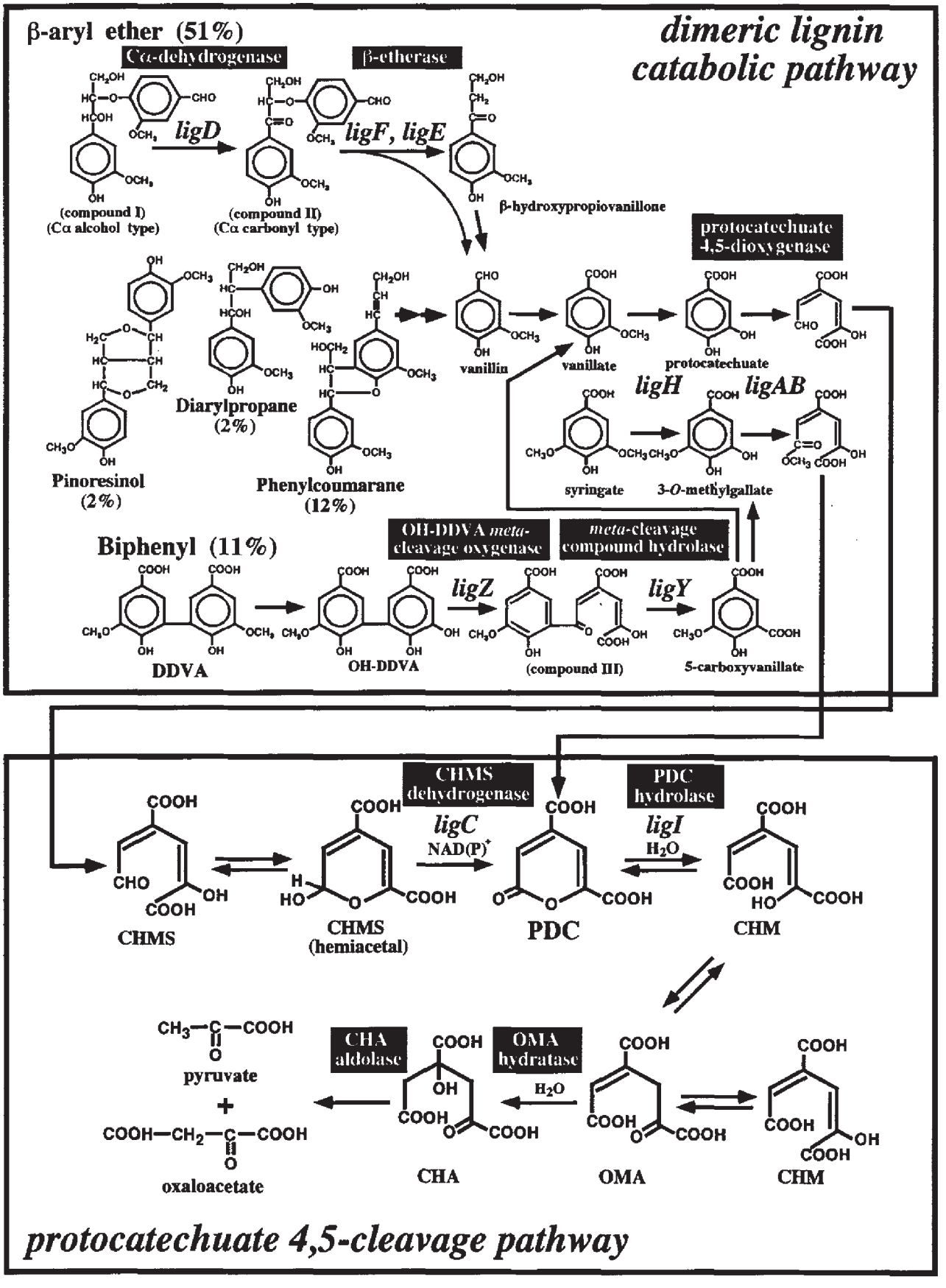
Figure 1 Proposed degradation pathway of various lignin-related compounds and the protocatechuate 4,5-cleavage pathway in *S. paucimobilis* SYK-6. Dimeric lignin compounds having guaiacyl or syringyl moieties are converted into vanillate or syringate, respectively, by actions of the specific lignin degradation enzymes shown in the upper section. Vanillate and syringate were O-demethylated and transformed into protocatechuate (PCA) and 3-*O*-methylgallate, respectively. Both compounds were subjected to PCA 4,5-dioxygenase (LigAB) and the resulting products were further metabolized via the PCA 4,5-cleavage pathway shown in the lower section. An alternative suggested route for 3-*O*-methylgallate degradation is illustrated in Figure 6b. The *lig* genes indicated above the arrows have been characterized and their functions are summarized in Table 1. DDVA, 5,5′-dehydrodivanillate; OH-DDVA, 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl; CHMS, 4-carboxy-2-hydroxymuconate-6-semialdehyde; PDC, 2-pyrone-4,6-dicarboxylate; CHM, 4-carboxy-2-hydroxymuconate; OMA, 4-oxalomesaconate; CHA, 4-carboxy-4-hydroxy-2-oxoadipate.


β-aryl ether (51%)
dimeric lignin catabolic pathway
Cα-dehydrogenase
β-etherase
ligD
ligF, ligE
(compound I)
(Cα alcohol type)
(compound II)
(Cα carbonyl type)
β-hydroxypropiovanillone
protocatechuate 4,5-dioxygenase
Diarylpropane (2%)
Pinoresinol (2%)
Phenylcoumarane (12%)
vanillin
vanillate
protocatechuate
ligH
ligAB
syringate
3-O-methylgallate
Biphenyl (11%)
OH-DDVA meta-cleavage oxygenase
meta-cleavage compound hydrolase
ligZ
ligY
DDVA
OH-DDVA
(compound III)
5-carboxyvanillate
CHMS dehydrogenase
ligC
NAD(P)+
PDC hydrolase
ligI
H2O
CHMS
CHMS (hemiacetal)
PDC
CHM
pyruvate
oxaloacetate
CHA aldolase
OMA hydratase
CHA
OMA
CHM
protocatechuate 4,5-cleavage pathway

oxaloacetate via the PCA 4,5-cleavage pathway. On the other hand, the degradation pathway of 3-*O*-methylgallate is not clear at present. To clarify all the details of the complex lignin degradation enzyme system in *S. paucimobilis* SYK-6, we have isolated the SYK-6 genes and analyzed their functions. In this article, we review the genetic and biochemical features of strain SYK-6 lignin degradation genes.

β-aryl ether cleavage

β-aryl ether cleavage is an essential step in lignin degradation because this linkage is most abundant (approximately 50%). Gas chromatography and mass spectrometry (GC-MS) analysis indicated that *β*-aryl ether (compound II in Figure 1) was converted into *β*-hydroxypropiovanillone and vanillin by strain SYK-6, indicating that the *β*-aryl ether cleaving activity in strain SYK-6 is a unique reductive reaction [34]. We chemically synthesized a substrate using 4-methylumbelliferone to construct a convenient and sensitive fluorescent assay system for the *β*-aryl ether cleavage catalyzed by *β*-etherase [34]. When the cell extract of strain SYK-6 was treated with a detergent, MEGA-8, *β*-etherase activity was detected using this assay substrate. This result suggested that the enzyme is associated with the cell membrane.

Cloning of the *β*-etherase gene was carried out by screening of the strain SYK-6 cosmid gene library for *S. paucimobilis* IAM12578 for the ether bond cleaving activity of the assay substrate [32,33]. The organization of the *β*-etherase gene cluster is shown in Figure 2. Interestingly, we found two kinds of *β*-etherase genes, designated *ligE* and *ligF*, in the cloned 24-kb *Sal*I fragment. Putative amino acid sequences deduced from *ligF* [33] and *ligE* [32] nucleotide sequences have 27% and 18% identities with the glutathione *S*-transferases (GST) of potato [accession No. AF002692–1 in DAD (DDBJ amino acid sequence database)] and carnation [14] (L05915–1 in DAD), respectively. The ORF3 of a gentisate degrader, *Sphingomonas* sp RW5 [59] (AJ224977–3 in DAD) was most similar to LigF. Cell extracts of *E. coli* carrying *ligE* or *ligF* showed *β*-etherase activity. In the presence of reduced glutathione (GSH), 15-fold and two-fold stimulations of *β*-etherase activity were observed in the reaction mixture containing LigF and LigE, respectively. Over 600-fold higher activity was detected in the reaction mixture containing LigF than that of LigE. This result strongly suggested that LigF plays a major role in the *β*-aryl ether cleavage in strain SYK-6. The substrate specificity of LigF and LigE are restricted to the *β*-aryl ether having C*α*-carbonyl (compound II in Figure 1). A carbonyl group in the *β*-aryl ether compounds is thought to be an important determinant for *β*-etherase activity. The carbon atom at position *α* (C*α*) may be attacked by GSH since the electron density of this carbon atom is thought to be reduced due to the presence of a carbonyl group. A *β*-aryl ether compound containing C*α*-hydroxyl (compound I) was oxidized by C*α*-dehydrogenase (LigD) [35] to generate a *β*-aryl ether compound having C*α*-carbonyl which is the substrate for *β*-etherase. The *ligD* gene locates just upstream from *ligF* and seems to constitute an operon with *ligF* and *ligE* genes based on their tandem arrangement. The *β*-etherases could cleave the *β*-aryl ether compound having side chains of C_2 and C_3. LigF and LigE showed no activity toward 1-chloro-2,4-dinitrobenzene (CDNB), a common substrate for GSTs but not a good substrate for the theta class of GSTs that was proposed to be the progenitor of the other classes of GST [57]. Recently, we purified LigF from a cell extract of *E. coli* carrying the *ligF* gene. LigF was estimated to be a tetramer (128 kDa) by gel filtration chromatography (unpublished data). This is a striking difference between LigF and other GSTs, which have been reported to be dimers.

Table 1 *S. paucimobilis* SYK-6 genes involved in the degradation of lignin-related compounds

Gene	No. of amino acids	Deduced mol mass (kDa)	Product and/or function	Accession No. (DAD[d] or DDBJ[e])	Reference(s)
ligA	139	15.5 (15)[b]	*α*-subunit of protocatechuate 4,5-dioxygenase	M34835–1[d]	[40]
ligB	302	33.2 (34)	*β*-subunit of protocatechuate 4,5-dioxygenase	M34835–2[d]	[40]
ligC	NA[a]		4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase		[38]
ligD	305	32.3 (ND[c])	C*α*-dehydrogenase involved in *β*-aryl ether cleavage	D11473–1[d]	[35]
ligE	280	32.0 (32)	*β*-etherase for *β*-aryl ether cleavage; similar to glutathione *S*-transferase	M11473–3[d]	[32]
ligF	257	29.7 (30)	*β*-etherase for *β*-aryl ether cleavage; similar to glutathione *S*-transferase	D11473–2[d]	[33]
ligG	265	30.2 (ND)	similar to glutathione *S*-transferase	AB026292[e]	This study
ligH	557	59.4 (60)	essential gene for O-demethylation of vanillate and syringate	AB006079–1[d]	[39]
ligI	293	32.7 (38)	2-pyrone-4,6-dicarboxylate hydrolase	AB015964[e]	[36]
ligY	332	37.2 (37)	OH-DDVA *meta*-cleavage compound hydrolase	AB018415[e]	[43]
ligZ	334	37.0 (38)	OH-DDVA *meta*-cleavage dioxygenase	AB007823–1[d]	[42]
lsdA	NA		similar to lignostilbene-*α*,*β*-dioxygenase	AB015964[e]	[36]

[a]NA, not available. The complete nucleotide sequences of *ligC* and *lsdA* are not available. Partial *lsdA* sequence had been deposited in the database.
[b]Mol mass estimated on SDS-PAGE is presented in the brackets.
[c]ND, not determined.
[d]DAD, DDBJ amino acid sequence database.
[e]DDBJ, DDBJ nucleotide sequence database. The deduced amino acid sequences of the *ligG*, *ligI*, *ligY* and *lsdA* have not been deposited in the amino acid sequence database.

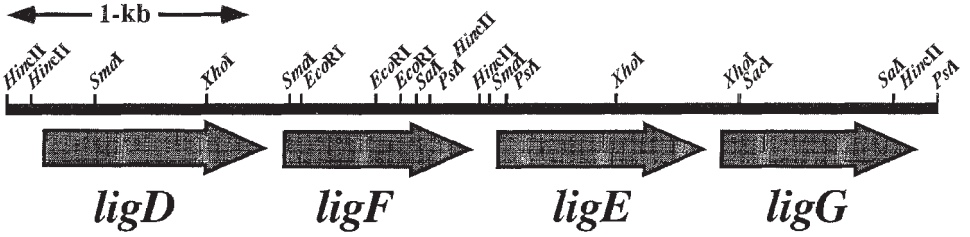


Figure 2 Organization of the β-aryl ether cleavage enzyme gene cluster of *S. paucimobilis* SYK-6. The nucleotide sequence of the *ligG* gene whose gene product shows similarity with glutathione *S*-transferases (GST) was determined in this study. The *ligG* gene is located 78 bp downstream from *ligE* and encodes 265 amino acid residues. The nucleotide sequence of *ligG* was deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession No. AB026292.

In this study, we found another putative GST gene downstream from *ligE*. It was designated *ligG* (accession No. AB026292 in DDBJ), and encodes 265 amino acid residues (Figure 2). The deduced amino acid sequence of *ligG* shows 23% identity with auxin-induced GST homolog of tobacco [55] (X56263–1 in DAD). We detected neither β-etherase activity nor GST activity on CDNB in the cell extract of *E. coli* carrying *ligG*. However the tandem arrangement of these three GST homologous genes is interesting. The N-terminal amino acid sequences of LigE, LigF, and LigG are aligned with those of alpha- [51], mu- [15], pi- [8], sigma- [16], theta-class [47,61], and *E. coli* [37] GSTs, for which three-dimensional structures have been reported (Figure 3). The tyrosine residue in the N-terminus had been thought to contribute to the activation of glutathione [62]. The tyrosine residue is conserved in the N-terminal region among LigE, LigF, and LigG (Figure 3). The N-terminal serine residue, which is well-conserved in the theta-class [4], is critical for enzyme activity in this class of GST, as has been found in the Australian sheep blowfly (*Lucilia cuprina*) [5] and dichloromethane dehalogenase from *Methylophilus* sp strain DM11 [58]. In *E. coli* GST, Cys10 has been implicated to be a catalytic residue [37]. These Ser and Cys residues are, however, ambiguous in the N-terminals of LigE, LigF, and LigG, and should be investigated.

Biphenyl degradation

Biphenyl structure is included at approximately 10% in spruce lignin, and so stable that its decomposition should be rate limiting in lignin degradation. 5,5′-Dehydrodivanillate (DDVA) has been used as a typical biphenyl compound.

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                β1          α1              β2              α2              β3
alpha     AEKPKLHYFNARGRMESTRWLLAA....AGVEFEEKFIKS.......AEDLDKLRNDGY.LMFQQVPMVEIDG  61
mu          PMILGYWNVRGLTHPIRLLLEY....TDSSYEEKRYAMGDAPDYDRSQWLNEKFKLG.LDFPNLPYLIDGS  66
pi        PPYTITYFPVRGRCEAMRMLLAD....QDQSWKEEVVTME........TWPPLKPSCL.FR..QLPKFQDGD  57
sigma     PKYTLHYFPLMGRAELCRFVLAA....HGEEFTDRVVEMA........DWPNLKATM..YS.NAMPVLDID.  56
insect      MDFYYLPGSAPCRSVLMTAKA....LGIELNKKLLNLQAG......EHLKPEFLKI.NPQHTIPTLVDGD  59
plant     AGIKVFGHPASIATRRVLIALHE....KNLDFELVHVE......LKDGEHKKEPFLSR.NPFGQVPAFEDGD  61
E. coli     MKLFYKPGA.CSLASHITLRE....SGKDFTLVSVDLMKKR.....LENGDDYFAV.NPKGQVPALLLDD  59
DM11      STKLRYLHHPASQPCRAVHQFMLE....NNIEFQEEIVDITTD......INEQPEFRERYNPTGQVPILVDGD  63
PcpC        PEVSLYNYTMSICSMKTRLAMEE....FGVDYDDKQVDIGFAL.....ENFEPDYVRL.NEKAVVPTLVVGD  62
LigF        MTLKLYSFGPGANSLKPLATLYE....KGLEFEQVFVDPSKF......EQHSDWFKKI.NPRGQVPAL.WHD  60
LigE    MARNNTITLYDLQLESGCTISPYVWRTKYALKHKGFDIDIV.PGGFTGIL..ERTG.......GRSERVPVIVD.D  65
LigG    MAEPQELTIYHIPGCPFSERVEIML.E....LKGLR.MKDVEIDISKPRP..DWLLAKT....GGTTALPLLDVEN  64

              β4        α3
alpha     .MKLVQTRAILNYIASKYN.....LYG  82
mu        .RKITQSNAIMRYLARKHH.....LCG  87
pi        .LTLYQSNAILRHLGRSFG.....LYG  78
sigma     GTKMSQSMCIARHLAREFG.....LDG  78
insect    .FALWESRAIMVYLVEKYGKNDS.LFP  84
plant     .LKLFESRAITQYIAHRYENQGTNLLQ  87
E. coli   GTLLTEGVAIMQYLADSVPDR..QLLA  84
DM11      .FTIWESAAIVYYLSEKYDCSSS.WWG  88
PcpC      .RVVTNSYNIVLEAANVGK....VGIP  84
LigF      GKVVTESTVICEYLEDVFPESGNSLRP  87
LigE      GEWVLDSWVIAEYLDEKYPDRPM.LFE  91
LigG      GESLKESMVILRYLEQRYPEPAVAHPD  91
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Figure 3 Alignment of N-terminal sequences of the various glutathione *S*-transferases. The sequences were obtained from the SWISS-PROT databases. Alpha, human GST A1–1 [51] (P08263); mu, rat GST M3–3 [15] (P04905); pi, pig GST P1–1 [8] (P80031); sigma, squid GST [16] (P46088); insect, theta GST of *Lucilia cuprina* [61] (P42860); plant, theta GST from *Arabidopsis thaliana* [47] (P46422); E. coli, *E. coli* GST [37] (P39100); DM11, dichloromethane dehalogenase (DcmA) of *Methylophilus* sp DM11 [2] (P43387); PcpC, tetrahydroquinone reductase of *Sphingomonas chlorophenolica* [41] (Q03520); LigF, β-etherase [33] (P30347); LigE, β-etherase [32] (P27457); LigG, GST homolog. The residues in α-helices and β-strands in GSTs of which three-dimensional structures were solved are underlined. Boldface roman type represents the conserved residues. Residues on a dark background are suggested to interact directly with the sulfur atom of the GSH thiol group.

DDVA is initially O-demethylated by strain SYK-6 to generate 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl (OH-DDVA) (Figure 1). OH-DDVA is cleaved by the extradiol dioxygenase LigZ, and transformed into the *meta*-cleavage compound (compound III in Figure 1) [42]. Specific hydrolase LigY hydrolyzed the *meta*-cleavage compound, and 5-carboxyvanillate was generated [43]. The O-demethylation step of DDVA is not clear at present; however, a specific enzyme for DDVA which differs from that for vanillate and syringate was suggested to be involved in this reaction (see the section 'O-demethylation').

A 15-kb *Eco*RI fragment carrying the *ligZ* gene was isolated by complementing the growth deficiency of a mutant on OH-DDVA [42]. The *ligZ* gene which encodes 334 amino acids was expressed in *E. coli*. LigZ catalyzed the *meta*-cleavage of OH-DDVA, which yielded the yellow *meta*-cleavage compound (λ_{max}, 455 nm at pH 12) (compound III). The substrate specificity of LigZ was examined by measuring oxygen consumption. The activity was observed only toward OH-DDVA and 2,2′,3,3′-tetrahydroxy-5,5′-dicarboxybiphenyl (DDPA), and no activity was found toward other lignin-related diol compounds such as PCA, 3-*O*-methylgallate and gallate, and aromatic diol compounds including 2,3-dihydroxybiphenyl, 3-methylcatechol and catechol. LigZ activity was lost upon addition of EDTA and it was recovered by addition of Fe^{2+} to the reaction mixture. This suggests that LigZ contains Fe^{2+} in the catalytic center.

The deduced amino acid sequence of the *ligZ* gene product exhibits only partial similarity with the sequence of the class III (or type II [10]) extradiol dioxygenase [52] including the β-subunit of PCA 4,5-dioxygenase (LigB) of *S. paucimobilis* SYK-6 [40] (21% identity) and a subunit of 2′-aminobiphenyl-2,3-diol dioxygenase (CarBb) of *Pseudomonas* sp CA10 [48] (21% identity). However, LigZ possesses an evolutionary relationship with that class of enzyme. According to Spence *et al* [52] and Eltis and Bolin [10], the extradiol dioxygenases are divided into three classes based on their amino acid sequence similarity. The three-dimensional structure of the class II enzyme, 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) was determined by Senda *et al* [49] and Han *et al* [11]. Class II enzymes are composed of two domains having approximately the same folding pattern. Class I comprises single-domain enzymes including the BphC2 and BphC3 of *Rhodococcus globerulus* P6 [1]. Thus, class II enzymes are thought to have evolved from a class I enzyme through gene duplication. The class III enzymes have little similarity to the enzymes of class I and class II. This suggested little evolutionary relationship between class III and class I and II enzymes.

The LigAB purified to homogeneity from cell extracts of *E. coli* carrying *ligAB* was subjected to gel filtration chromatography, which indicated that the molecular mass of LigAB is 96 kDa, suggesting that it is an $\alpha_2\beta_2$ tetramer (unpublished data). Sugimoto *et al* determined the three-dimensional structure of LigAB of strain SYK-6 [53]. Interestingly, the three-dimensional structure of LigAB is completely different from the class II enzyme, BphC of *Pseudomonas* sp KKS102 [49] indicating no evolutionary relationship between them. LigB contains the active site having a non-heme iron coordinated by His12, His61, Glu242, and a water molecule. Figure 4 shows the alignment of the class III extradiol dioxygenases. His12, His61 and Glu242 (numbering refers to the LigB sequence) are well conserved in all the sequences. On the other hand, LigZ exhibits only a little sequence similarity with class III enzymes as a whole; however, the similarity between amino acid positions 202–254 of LigZ and positions 147–195 of LigB is significantly high (Figure 5). Thus, LigZ has some evolutionary relationship with class III enzymes, although it is unique to them. It will be necessary to determine the three-dimensional structure of LigZ to understand the structural and functional relationship between LigZ and class III enzymes.

5-Carboxyvanillate accumulated in the reaction mixture of OH-DDVA containing a cell extract of *E. coli* having a 15-kb *Eco*RI fragment carrying the *ligZ* gene. This result indicated that a gene for the *meta*-cleavage derivative hydrolase which is responsible for the step following ring cleavage by LigZ is included in this fragment. The hydrolase gene (*ligY*) is located approximately 1.4 kb downstream from *ligZ*, and consists of a 996-bp open reading frame (ORF) [43]. The nucleotide sequence and the deduced amino acid sequence of the *ligY* gene had no similar sequence in the database. In order to verify the hydrolysis catalyzed by LigY, the incorporation of ^{18}O from $H_2{}^{18}O$ into the substrate was examined [43]. The reaction mixture of LigZ and LigY was incubated with OH-DDVA in the presence of $H_2{}^{18}O$ and the product was methylated and analyzed by GC-MS. The mass spectrum of the methylester derivative of the product exhibited a molecular ion peak at *m/z* 256 corresponding to 5-carboxyvanillate methylester containing ^{18}O. This indicates that LigY catalyzed hydrolysis of the *meta*-cleavage product of OH-DDVA. Interestingly, LigY was able to hydrolyze the *meta*-cleavage product of OH-DDVA efficiently only when the LigY was added coincidently with LigZ in the reaction mixture containing OH-DDVA. The coexistence of LigZ seems to be essential for LigY activity. An intriguing possibility is that LigZ is physically associated with LigY. The close physical association between two enzymes has been reported on *meta*-cleavage pathway enzymes [12,44]. Harayama *et al* [12] suggested that 4-oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase in the catechol *meta*-cleavage pathway encoded on the TOL plasmid formed a physical complex *in vivo*. The product of the decarboxylase was an enol form compound, 2-hydroxypent-2,4-dienoate. The hydratase acts on only this enol form; however, it is spontaneously transformed into a keto form, 2-oxopent-4-enoate. It was suggested that physical association of the decarboxylase and the hydratase assures efficient transformation of the intermediate. In the case of OH-DDVA degradation, the substrate specificity of LigY may be restricted to the enol form of the *meta*-cleavage derivative of OH-DDVA, which may be spontaneously transformed into the keto form. Physical association may be necessary for efficient hydrolysis of the enol form of the *meta*-cleavage derivative to 5-carboxyvanillate by LigY.

In an earlier study, we detected 3-*O*-methylgallate as an intermediate metabolite during 5-carboxyvanillate degradation by strain SYK-6 [20]. We also detected vanillate

```
                1         10        20        30        40        50        60        70        80        90
LigB/SYK-6      MARVTTGITSSHIPALGAAIQTGTSDNDYWGPVFKGYQPIRDWIKQPGNMPDVVILVYNDHASAFDMNIIPTFAIGCAETFKPADEGWGP   90
CarBb/CA10      MGKIVAAGGTSHILMSPKG......CEESAARVVNGIAELGRRLKEAR..PDVLVIITSDHMFNINLSMQPRFVVGIADSYTPM..GDMD   80
PhnC/RP007      MAKIVGGFMMFHDPLIPATPTA..PPAAQREICMHAYAIIVERLRALQ..VDTVVVIADDHYTLNGPYCIPMAMIGIGDIE.....GPYE   81
MpcI/JMP22      ..MPIQLECLSHTPLHGY....VDPAPEVVAEVERVQAAARDRVRAFD..PELVVVFAPDHFNGFFYDVMPPFCIGAAATAI....GDFK   78
MhpB/EC         ..MHAYLHCLSHSPLVGY....VDPAQEVLDEVNGVIASARERIAAFS..PELVVLFAPDHYNGFFYDVMPPFCLGVGATAI....GDFG   78
HppB/PWD1       ..MKQALLCMSHSPLLHH....LDPPADVKASVEAAFDQARAFVHNFD..PDVIVNFGPDHYNGFFYDLMPPFCIGYKAKGS....GDYD   78
EdoD/I1         ..MTLALVCTSHSPLLEFN..N..PPPEVRTEVDRAFAQARQFIEEYD..PDLVVSFAPDHYNGFFYKLMPSFCIGFEASGV....GDFG   78

                91       100       110       120       130       140       150       160       170
LigB/SYK-6      RPVPDVKGHPDLAWHIAQSL....ILDEFDMTIMNQMDVDHGCTVPLSMIFGEPEEWPCKVIPFPVNVVTYPPPSGKRCFALGDSIRAAV  176
CarBb/CA10      IPRDLVPGSREVGRAIALQA....DEDGFDLCQAEEYSLDHGIMIPILFMG....MKEIPVVPVIVNINTDPIPSARRCVALAESIRQAI  162
PhnC/Rp007      PWLGIPRAQIENNRPLAHHIMQYGLEYGIDWAVSKSLVLDHSATVPIHYAVRP..VKGMRAIPVYLNTGIEPFITSWRAHEIGRVIGDAV  169
MpcI/JMP22      SLAGKLPVPADLALSLAESV....MAADIDVALSHRMQVDHGCADALAALTGS..LHRYPVIPVFINSVAPPMATLRRARLLGDAVGRFL  162
MhpB/EC         SAAGELPVPVELAEACAHAV....MKSGIDLAVSYCMQVDHGFAQPLEFLLGG..LDKVPVLPVFNKAVATPLPGFQRTHMLGETIGRFT  162
HppB/PWD1       SFAGELNVPEAMAEDLAQFV....MDQGLDIAISRQMEVDHGAVQPMEIIYGD..VASKPLIPVFVNSVARPFVKVARVRKFGEAVGAYF  162
EdoD/I1         SSAGRLDVPSALAEQMAQSV....LDQGVDIAVSLEMEVDHGAVQPLEILLGG..IATKPVIPIFVNSVAAPFAPMKRIRLLGEAVGTFL  162

                180       190       200                                                 210       220
LigB/SYK-6      ES.FPEDLNVHVWGTGGMSHQLQGPRAG.....................................................LINKEFDLNFIDKLI.SD  220
CarBb/CA10      EKRTPDGCRVAVVGAGGLSHWLCVPRHG.....................................................EVSEKFDHMVMDELVRGN  208
PhnC/Rp007      ANWQ.GDERVAIYGTGGLSHWPGMAEMG.....................................................AINEGWDRKIMKLVAQGD  214
MpcI/JMP22      SR...AGKRVLVVGSGGISHEPPVPELAGASEEVAERLIAGRN..PSPESAARQARTVAAAKSFVAGDSHLHPLNPEWDRAFLSLLASGE  247
MhpB/EC         ST...LNKRVLFLGSGGLSHQPPVPELAKADAHMRDRLLGSGKDLPASERELRQQRVISAAEKFVEDQRTLHPLNPIWDNQFMTLLEQGR  249
HppB/PWD1       KN...SDKKVLFIGSGGLSHDPPVPQIATADEAQRKMLTDGRNPTP.QARAARQQRVIDTAVKFAADEADIMDLNPEWDRGFLDVCASGR  248
EdoD/I1         KN...FDGNVLLLGSGGLSHDPPVPRLEAATGEQRKMLLGCNHPTA.AARAVRQQRVIDTAKAFTRGEAGIMDLAPEWDRQLLDILASGE  248

                230       240       250       260       270       280       290       300
LigB/SYK-6      PEELSKMPHIQYLRESGSEGVELVMWLIMRGALPEKVRDLYTFYHIPASNTALGAMILQPEETAGTPLEPRKVMSGHSLAQA          302
CarBb/CA10      AEKLVAMGNEAIIDQGGNAGVEILTWIMAAVASEASS.............GEKVFYEAMTQWFTGIGGMEFHVK........          269
PhnC/Rp007      VESLIALSDEEILRDGGNGGIEIKNWICAMGALGACR.............GEVIAYEPVAEWVCGCGYMEMKVA........          275
MpcI/JMP22      LTAVDGMTNDAITRDGGKSAHEIRTWVAAFGALAAYG.PYR.........ASLDFYRAIPEWIAGFATMHAEPAAV......          313
MhpB/EC         IQELDAVSNEELSAIAGKSTHEIKTWVAAFAAISAFG.NWR.........SEGRYYRPIPEWIAGFGSLSARTEN.......          314
HppB/PWD1       IEDFDRYTADDMDAVAGHSSHEVRNWVAAYSALRACG.EYE.........IAYEFYRPIKEYISGFAVTTAILRDI......          314
EdoD/I1         LERLDAWTAKEMAATAGNSAHEVRTWVAAHNALKAAAGQYT.........VTSEYYRPIPEYIAGFAVTTAVPVHKTAGLR.          320
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Figure 4 Amino acid sequence alignment of the class III extradiol dioxygenases. The class III [52] extradiol dioxygenase had been classified as type II by Eltis and Bolin [10]. The sequences were obtained from the DDBJ amino acid sequence database (DAD) or DDBJ nucleotide sequence database (DDBJ). LigB/SYK-6, β-subunit of PCA 4,5-dioxygenase [40] (M34835–2); CarBb/CA10, β-subunit of 2′-aminobiphenyl-2,3-diol dioxygenase of *Pseudomonas* sp CA10 [48] (D89064–4); PhnC/RP007, extradiol dioxygenase of *Burkholderia* sp strain RP007 [25] (AF061751); MpcI, catechol 2,3-dioxygenase of *Ralstonia eutropha (Alcaligenes eutrophus)* JMP222 [17] (X52414–1); MhpB/Ec, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase of *E. coli* K-12 [52] (AE000142–2); HppB/PWD1, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase of *Rhodococcus globerulus* PWD1 [3] (U89712–3); EdoD/I1, extradiol dioxygenase of *Rhodococcus* sp I1 [24] (AJ006125–1). Sugimoto *et al* [53] determined the three-dimensional structure of PCA 4,5-dioxygenase (LigAB). According to their results, the β-subunit (LigB) contains the active site containing non-heme iron coordinated by His12, His61, Glu242, and a water molecule. These amino acid residues (highlighted by a black background) are completely conserved among the class III enzymes listed above. Boldface roman type indicates the conserved residues among all the sequences.

```
                1         10        20        30        40                                                  50
LigB            MARVTTGITSSHIPALGAAIQTGTSDN...DYWGPVFKGYQPIRDWIKQPGN.................................MPDVV   54
CarBb           MGKIVAAGGTSHILMSPKG......CE...ESAARVVNGIAELGRRLKEAR.....................................PDVL   46
LigZ            MAEIVLGMWTSHGPTLSTTPEQWTLRVSADLKRKHPFRGEEYSFEELVSCAAARSWRRPVPFPSASDARPGAKVPSPTWPTFAAAKIDVA   90
                *  :     :**                    *       :                                                  **

                60        70        80        90        100       110       120
LigB            ILVYNDHASAFDMNIIPTFAIGCAETF..KPADEGWGPRPVPDVKGHPDLAWHIAQSLI....LDEFDMTIMNQ...MDVDHG.......  128
CarBb           VIITSDHMFNINLSMQPRFVVGIADSY..TPM..GDMDIPRDLVPGSREVGRAIALQAD....EDGFDLCQAEE...YSLDHG.......  118
LigZ            VIMGNDQRELFLEDVTPAITVYLGETIWDQPATPEQAAR...MPPGIHEAEWGIARPSAGTIPASPSSACMCAKRWCRRLRSGGLQDTAR  177
                :::   *:    :   : * : :   ::    *              *   :    **                    :     :  *

                130       140       150       160       170       180       190       200
LigB            CTVPLSMIF......GEPEEWPCKVIP.FPVNVVT.YPP..PSGKRCFALGDSIRAAVES.FPEDLNVHVWGTGGMSHQLQGPRAGLINK  207
CarBb           IMIPILFMG.........MKEIPVVP.VIVNINT.DPI..PSARRCVALAESIRQAIEKRTPDGCRVAVVGAGGLSHWLCVPRHGEVSE  194
LigZ            ARRPLVERRAHSLGFIYRQIIRDQVVPNLPIIINTFFPPNQPTARRCFELGRAVGKAIRS.WKEDLPVAVFGSGGMSHFV.....IDE  259
                   *:                    *:*   : : *   *    *: :**  *  ::  *:      :    * * *:**:** :       : :

                210       220       230       240       250       260       270       280       290
LigB            EFDLNFIDKLI.SDPEELSKMPHIQYLRESGSEGVELVMWLIMRGALPEKVRDLYTFYH.IPASNTALGAMILQPEETAGTPLEPRKVMS  295
CarBb           KFDHMVMDELVRGNAEKLVAMGNEAIIDQGGNAGVEILTWIMAAVASEASS.............GEKVFYEAMTQWFTGIGGMEFHVK.  269
LigZ            DFDRMFFEALRNRDAETLCAIEDKHL..QSGT..SELKTWIAAAGALFDTD............LKGDVVGYEPCYRSEAGTGTPANGFVAW  333
                 **   :: *   : * *  :       : *    *:  *:    *                                :*       *

                300
LigB            GHSLAQA  302
CarBb           .......  269
LigZ            Q......  334
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Figure 5 Amino acid sequence alignment of LigZ with class III extradiol dioxygenases. LigZ exhibited partial similarity with class III extradiol dioxygenases. Amino acid residues that are highlighted by a black background are involved in the non-heme iron coordination. Asterisks and semicolons indicate the identical and similar amino acid residues, respectively.

among the metabolites of 5-carboxyvanillate [39]. The metabolism of 5-carboxyvanillate seems to proceed to not only 3-*O*-methylgallate but also vanillate (Figure 1).

O-demethylation enzymes

DDVA, syringate, and vanillate are transformed into OH-DDVA, 3-*O*-methylgallate and PCA, respectively by O-demethylation enzymes in *S. paucimobilis* SYK-6. In lignin catabolism by strain SYK-6, the O-demethylation processes are essential to generate substrates for the subsequent ring cleavage steps. To address the O-demethylation enzymes, strain SYK-6 mutants deficient in O-demethylation activity were isolated. Two types of mutants were obtained following nitrosoguanidine mutagenesis. The mutant strain NT-1 could not grow on DDVA, but could assimilate syringate, vanillate, and OH-DDVA. Strain DC-49 grew on neither vanillate nor syringate but could degrade PCA, 3-*O*-methylgallate, and DDVA. This fact strongly suggested that strain SYK-6 has at least two O-demethylation enzyme species, one for DDVA and another for vanillate and syringate. A DNA fragment conferring degradation activity on vanillate and syringate to strain DC-49 was isolated from the strain SYK-6 genomic library. This fragment could not complement the growth deficiency of strain NT-1 on DDVA. The nucleotide sequence of the smallest DNA fragment conferring O-demethylation activity on strain DC-49 indicated that an ORF (*ligH*) encoding 557 amino acids is responsible for this activity. The deduced amino acid sequence of *ligH* revealed significant similarity with formyltetrahydrofolate synthetases of *Clostridium thermoaceticum* [26], *C. cylindrosporum* [46], and *C. acidiurici* [60] (approximately 60% identity). The primary structure of LigH suggested that it may require ATP and tetrahydrofolate (THF), however, O-demethylation activity for both vanillate and syringate in the cell extracts of strains SYK-6 and DC-49 carrying *ligH* depended on only THF. An anaerobic O-demethylation enzyme for vanillate in *Acetobacterium dehalogenans* consists of four proteins including two methyltransferases which mediate the methyl transfer from vanillate to the reduced corrinoid protein and from corrinoid protein to THF [22]. THF dependence is a common feature between O-demethylation enzymes of strain SYK-6 and *Acetobacterium*. On the other hand a monooxygenase encoded by *vanA* and *vanB* in *Pseudomonas* sp ATCC 19151 [6] and *Pseudomonas* sp HR199 [45] catalyzed conversion of vanillate into PCA and formaldehyde in the presence of NADH. The O-demethylation enzyme of strain SYK-6 for vanillate and syringate is distinct from the O-demethylation enzymes that have been reported previously. Further research is needed to illustrate the combination of the multiple O-demethylation enzymes in the lignin degradation system in SYK-6.

The protocatechuate 4,5-cleavage pathway

PCA is one of the key intermediates of microbial catabolism of aromatic compounds. It is well known that the aromatic ring opening of PCA is catalyzed by one of the three dioxygenase species: PCA 3,4-dioxygenase (3,4-PCD) [13], PCA 4,5-dioxygenase (4,5-PCD) [40] and PCA 2,3-dioxygenase (2,3-PCD) [63]. The 3,4-PCD is the most extensively characterized enzyme, and the metabolic pathway for the 3,4-PCD product, β-carboxy-*cis*, *cis*-muconate into succinyl CoA and acetyl CoA is called the β-ketoadipate pathway, which has also been adequately characterized [13]. On the other hand, the PCA 4,5-cleavage pathway and the PCA 2,3-cleavage pathway are poorly characterized.

In the case of *S. paucimobilis* SYK-6, PCA is initially transformed to 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS) by 4,5-PCD and finally converted into pyruvate and oxaloacetate via the PCA 4,5-cleavage pathway proposed by Dagley *et al* [23] (Figure 1). The PCA 4,5-cleavage pathway was enzymatically characterized by Maruyama *et al* [27–31] and Dagley *et al* [23,54]. According to their publications, CHMS is nonenzymatically converted into an intramolecular hemiacetal form and then dehydrogenated by CHMS dehydrogenase. The resulting 2-pyrone-4,6-dicarboxylate (PDC) is hydrolyzed by PDC hydrolase to produce 4-oxalomesaconate (OMA) or its tautomer, 4-carboxy-2-hydroxymuconate (CHM). OMA is converted into 4-carboxy-4-hydroxy-2-oxoadipate (CHA) by OMA hydratase. Finally, CHA is cleaved by CHA aldolase to yield pyruvate and oxaloacetate (Figure 1).

The DNA fragment involved in vanillate degradation was isolated by screening the strain SYK-6 gene library in *Pseudomonas putida* PpY 1100. The plasmid pVA01 containing this fragment conferred the ability to grow on vanillate to *P. putida* PpY 1100 [21] (Figure 6a). The cell extracts of *P. putida* PpY 1100 (pVA01) and *E. coli* (pVA01) developed the yellow color of CHMS (λ_{max}, 410 nm at pH 9.5) in the presence of PCA indicating 4,5-PCD activity. The nucleotide sequence of the 1.9-kb *Sal*I fragment indicated that the 4,5-PCD gene consisted of two ORFs of 417 bp (*ligA*) and 906 bp (*ligB*) [40]. At that time (1990), no similar protein was found in the databases; however, currently, a group of enzymes which has similarity with *ligB* is classified in class III extradiol dioxygenases as described in the section 'Biphenyl degradation'.

We found that the *P. putida* PpY 1100 containing a deletion derivative of pVA01 (pVAD4) accumulated PDC from PCA (Figure 6a). This suggested that the region deleted from pVA01 would contain the PDC hydrolase gene and the region remaining in pVAD4 would include the CHMS dehydrogenase gene. The CHMS dehydrogenase gene (*ligC*) was suggested to be located downstream of *ligB* [38]. The nucleotide sequence of this region indicated that the *ligC* gene was truncated at the 3′ end. We were not able to detect the CHMS dehydrogenase activity in *E. coli* containing the expression plasmid carrying this truncated *ligC*. *P. putida* PpY1100 harboring this truncated *ligC* on pVAD4, however, exhibited good CHMS dehydrogenase activity, and was employed to prepare PDC from PCA. This strain converted 86% of PCA into PDC after 20 h at 28°C in 2 L of minimal medium containing 10.4 mmol of PCA [36]. Deletion analysis of the insert fragment of pVA01 limited the location of PDC hydrolase gene to the 1.5-kb *Sph*I fragment [36]. The nucleotide sequence of this fragment revealed an 879-bp ORF (*ligI*) encoding 293 amino acids [36]. The *ligI* gene is located approximately 5.4 kb upstream of *ligA* and it is transcribed divergently from *ligABC*. This indicates that the PCA 4,5-cleavage pathway is composed of at least two distinct operons. Downstream from *ligI*, an incomplete ORF was found

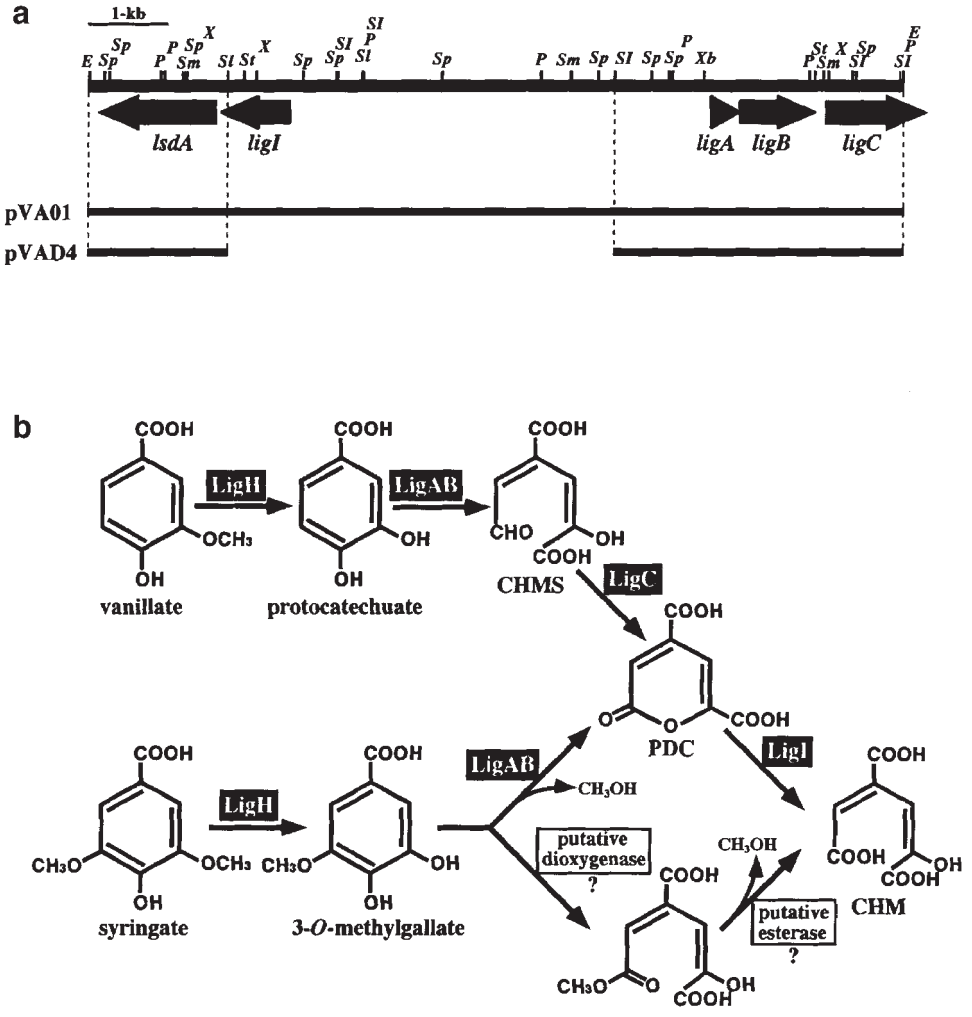

Figure 6 Gene organization of PCA 4,5-cleavage pathway genes (a) and the proposed catabolic pathway for vanillate and syringate by *S. paucimobilis* SYK-6 (b). (a) pVA01, pKT230 with a 10.5-kb *Eco*RI fragment which conferred an ability to grow on vanillate to *P. putida* PpY1100; pVAD4, deletion plasmid of pVA01. *E*, *Eco*RI; *P*, *Pst*I; *Sl*, *Sal*I; *Sm*, *Sma*I; *Sp*, *Sph*I; *St*, *Stu*I; *X*, *Xho*I; *Xb*, *Xba*I. (b) The *ligI* insertional mutant of *S. paucimobilis* SYK-6 (strain DLI) lost the ability to grow on vanillate, indicating that vanillate is degraded via the PCA 4,5-cleavage pathway in SYK-6. Dagley *et al* proposed the alternate catabolic route for 3-*O*-methylgallate catalyzed by a putative dioxygenase and an esterase, which are included in this figure [9,23].

which showed significant similarity to the lignostilbene α,β-dioxygenase (LSD) genes of *Sphingomonas paucimobilis* TMY 1009 [18,19]. LSD has been reported to be a dioxygenase catalyzing the cleavage of the interphenyl double bond of lignostilbenes. On the other hand, no sequences similar to the deduced amino acid sequence of the *ligI* gene were found in a homology search of the databases. This suggests that LigI constitutes a unique class of hydrolases. LigI produced in *E. coli* was purified to near homogeneity, and estimated to be a monomer (32 kDa). PDC hydrolase from *Comomonas testosteroni* [23] and *Pseudomonas ochraceae* [28] was able to catalyze the interconversion between PDC and CHM or OMA. LigI also catalyzed this reaction. A higher affinity with CHM and OMA than PDC and a higher V_{max} for PDC hydrolysis than PDC synthesis were common features of the PDC hydrolases of strain SYK-6 and *P. ochraceae*. Thiol reagents inhibited the LigI activity, suggesting that a cysteine residue is at the catalytic site.

To examine the involvement of the PDC hydrolase in vanillate and syringate metabolism in *S. paucimobilis* SYK-6, its *ligI* gene was inactivated by the insertion of a kanamycin resistance gene using the gene replacement technique [36]. The resulting *ligI* insertion mutant strain DLI was not able to grow on vanillate whereas it was able to grow on syringate. This indicated that the *ligI* gene is indispensable for growth on vanillate but not for syringate. Vanillate is degraded through the PCA 4,5-cleavage pathway illustrated in Figure 1 and Figure 6b. In our earlier study, we proposed the possibility that syringate is converted to 3-*O*-methylgallate, which is degraded by LigAB (Figure 6b). The production of 3-*O*-methylgallate from syringate is evident from results obtained with the *ligH* gene [39]. However, 3-*O*-methylgallate would be metabolized mainly through another pathway than the one via PDC, in which both LigAB and LigI are involved. An alternative route for 3-*O*-methylgallate catabolism without going through PDC was suggested for *Pseudomonas putida* TMC [9,23]. In this pathway, a putative dioxygenase is thought to catalyze the cleavage of 3-*O*-methylgallate, and an esterase would release methanol from the ring cleavage product to produce CHM (or OMA). A similar dioxygenase and ester-

ase may be involved in syringate catabolism in strain SYK-6 (Figure 6b). A *ligAB* disruption mutant of strain SYK-6 is expected to address this notion. However, LigAB and LigI would also play some role in syringate catabolism, since LigAB and LigI were induced by syringate (unpublished data).

Perspectives

S. paucimobilis SYK-6 possesses a wide variety of unique catabolic enzymes involved in degradation of typical dimeric lignin compounds and their intermediate metabolites. Structural and functional features of a variety of enzymes and genes involved in lignin catabolism by strain SYK-6 have been elucidated. The strain SYK-6 lignin catabolic genes characterized so far are novel, suggesting unique features of the lignin catabolic system in strain SYK-6. Characterization of the remaining enzyme genes involved in the degradation of β-aryl ether, biphenyl, syringate, and vanillate are in progress. Furthermore, the localization of each lignin degradation gene on the strain SYK-6 chromosome is underway, and is expected to elucidate evolutionary aspects of lignin degradation gene clusters.

Recently, 2-pyrone-4,6-dicarboxylate (PDC) has been focused on as a novel starting material for synthesis of biodegradable polyamide [50]. We have achieved efficient production of PDC from PCA using the recombinant strain *P. putida* PpY 1100 carrying pVAD4, which contains *ligAB* and *ligC*. Furthermore, a strain SYK-6 mutant (strain DLI) in which the PDC hydrolase gene (*ligI*) was inactivated would be a powerful tool for production of PDC from dimeric lignin compounds.

In the future, a combination of the strain SYK-6 specific enzyme systems for dimeric lignin degradation and the microbial, especially fungal, degradation systems for high molecular weight lignin is expected to provide a particularly invaluable process for the efficient utilization of lignin. The well-characterized enzyme system of *S. paucimobilis* SYK-6 for lignin catabolism will serve as a powerful tool for the construction of the sophisticated process.

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References

1 Asturias JA, LD Eltis, M Prucha and KN Timmis. 1994. Analysis of three 2,3-dihydroxybiphenyl 1,2-dioxygenases found in *Rhodococcus globerulus* P6. Identification of a new family of extradiol dioxygenases. J Biol Chem 269: 7807–7815.

2 Bader R and T Leisinger. 1994. Isolation and characterization of the *Methylophilus* sp strain DM11 gene encoding dichloromethane dehalogenase/glutathione *S*-transferase. J Bacteriol 176: 3466–3473.

3 Barnes MR, WA Duetz and PA Williams. 1997. A 3-(3-hydroxyphenyl) propionic acid catabolic pathway in *Rhodococcus globerulus* PWD1: cloning and characterization of the *hpp* operon. J Bacteriol 179: 6145–6153.

4 Blocki FA, LBM Ellis and LP Wackett. 1993. MIF proteins are theta-class glutathione *S*-transferase homologs. Protein Sci 2: 2095–2102.

5 Board PG, M Coggan, MCJ Wilce and MW Parker. 1995. Evidence for an essential serine residue in the active site of the theta class glutathione transferases. Biochem J 311: 247–250.

6 Brunel F and J Davison. 1988. Cloning and sequencing of *Pseudomonas* genes encoding vanillate demethylase. J Bacteriol 170: 4924–4930.

7 Cullen D. 1997. Recent advances on the molecular genetics of ligninolytic fungi. J Biotechnol 53: 273–289.

8 Dirr H, P Reinemer and R Huber. 1994. Refined crystal structure of porcine class Pi glutathione *S*-transferase (pGST P1-1) at 2.1 resolution. J Mol Biol 243: 72–92.

9 Donnelly MI and S Dagley. 1981. Bacterial degradation of 3,4,5-trimethoxycinnamic acid with production of methanol. J Bacteriol 147: 471–476.

10 Eltis LD and JT Bolin. 1996. Evolutionary relationships among extradiol dioxygenases. J Bacteriol 178: 5930–5937.

11 Han S, LD Eltis, KN Timmis, SW Muchmore and JT Bolin. 1995. Crystal structure of the biphenyl-cleaving extradiol dioxygenase from a PCB-degrading pseudomonad. Science 270: 976–980.

12 Harayama S, M Rekik, K-L Ngai and LN Ornston. 1989. Physically associated enzymes produce and metabolize 2-hydroxy-2,4-dienoate, a chemically unstable intermediate formed in catechol metabolism via *meta* cleavage in *Pseudomonas putida*. J Bacteriol 171: 6251–6258.

13 Harwood CS and RE Parales. 1996. The β-ketoadipate pathway and the biology of self-identity. Annu Rev Microbiol 50: 553–590.

14 Itzhaki H and WR Woodson. 1993. Characterization of an ethylene-responsive glutathione *S*-transferase gene cluster in carnation. Plant Mol Biol 22: 43–58.

15 Ji X, P Zhang, RN Armstrong and GL Gilliland. 1992. The three-dimensional structure of a glutathione *S*-transferase from the mu gene class. Structural analysis of the binary complex of isozyme 3–3 and glutathione at 2.2-Å resolution. Biochemistry 31: 10169–10184.

16 Ji X, EC von Rosenvinge, WW Johnson, SL Tomarev, J Piatigorsky, RN Armstrong and GL Gilliland. 1995. Three-dimensional structure, catalytic properties, and evolution of a sigma class glutathione transferase from squid, a progenitor of the lens *S*-crystallins of cephalopods. Biochemistry 34: 5317–5328.

17 Kabisch M and P Fortnagel. 1990. Nucleotide sequence of metapyrocatechase I (catechol 2,3-oxygenase I) gene *mpcI* from *Alcaligenes eutrophus* JMP222. Nucleic Acids Res 18: 3405–3406.

18 Kamoda S and Y Saburi. 1993. Cloning, expression, and sequence analysis of a lignostilbene-α,β-dioxygenase gene from *Pseudomonas paucimobilis* TMY 1009. Biosci Biotechnol Biochem 57: 926–930.

19 Kamoda S and Y Saburi. 1995. Cloning of a lignostilbene-α,β-dioxygenase isozyme gene from *Pseudomonas paucimobilis* TMY 1009. Biosci Biotechnol Biochem 59: 1866–1868.

20 Katayama Y, S Nishikawa, A Murayama, M Yamasaki, N Morohoshi and T Haraguchi. 1988. The metabolism of biphenyl structures in lignin by the soil bacterium (*Pseudomonas paucimobilis*SYK-6). FEBS Lett 233: 129–133.

21 Katayama Y, S Nishikawa, M Nakamura, K Yano, M Yamasaki, N Morohoshi and T Haraguchi. 1987. Cloning and expression of *Pseudomonas paucimobilis* SYK-6 genes involved in the degradation of vanillate and protocatechuate in *P. putida*. Mokuzai Gakkaishi 33: 77–79.

22 Kaufmann F, G Wohlfarth and G Diekert. 1998. *O*-demethylase from *Acetobacterium dehalogenans*. Substrate specificity and function of the participating proteins. Eur J Biochem 253: 706–711.

23 Kersten PJ, S Dagley, JW Whittaker, DM Arciero and JD Lipscomb. 1982. 2-pyrone-4,6-dicarboxylate, a catabolite of gallates in *Pseudomonas* species. J Bacteriol. 152: 1154–1162.

24 Kulakov LA, VA Delcroix, MJ Larkin, VN Ksenzenko and AN Kulakova. 1998. Cloning of new *Rhodococcus* extradiol dioxygenase genes and study of their distribution in different *Rhodococcus* strains. Microbiology 144: 955–963.

25 Laurie AD and G Lloyd-Jones. 1999. The *phn* genes of *Burkholderia* sp strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. J Bacteriol 181: 531–540.

26 Lovell CR, A Prazybyla and LG Ljungdahl. 1990. Primary structure of thermostable formyltetrahydrofolate synthetase from *Clostridium thermoaceticum*. Biochemistry 29: 5687–5694.
27 Maruyama K. 1979. Isolation and identification of the reaction product of α-hydroxy-γ-carboxymuconic-ε-semialdehyde dehydrogenase. J Biochem 86: 1671–1677.
28 Maruyama K. 1983. Purification and properties of 2-pyrone-4,6-dicarboxylate hydrolase. J Biochem 93: 557–565.
29 Maruyama K. 1985. Purification and properties of γ-oxalmesaconate hydratase from *Pseudomonas ochraceae* grown with phthalate. J Biochem 93: 567–574.
30 Maruyama K. 1990. Purification and properties of 4-hydroxy-4-methyl-2-oxo-glutarate aldolase from *Pseudomonas ochraceae* grown on phthalate. J Biochem 108: 327–333.
31 Maruyama K, N Ariga, M Tsuda and K Deguchi. 1978. Purification and properties of α-hydroxy-γ-carboxymuconic-ε-semialdehyde dehydrogenase. J Biochem 83: 1125–1134.
32 Masai E, Y Katayama, S Kawai, S Nishikawa, M Yamasaki and N Morohoshi. 1991. Cloning and sequencing of the gene for a *Pseudomonas paucimobilis* enzyme that cleaves β-aryl ether. J Bacteriol 173: 7950–7955.
33 Masai E, Y Katayama, S Kubota, S Kawai, M Yamasaki and N Morohoshi. 1993. A bacterial enzyme degrading the model lignin compound β-etherase is a member of the glutathione-*S*-transferase superfamily. FEBS Lett 323: 135–140.
34 Masai E, Y Katayama, S Nishikawa, M Yamasaki, N Morohoshi and T Haraguchi. 1989. Detection and localization of a new enzyme catalyzing the β-aryl ether cleavage in the soil bacterium (*Pseudomonas paucimobilis* SYK-6). FEBS Lett 249: 348–352.
35 Masai E, S Kubota, Y Katayama, S Kawai, M Yamasaki and N Morohoshi. 1993. Characterization of the Cα-dehydrogenase gene involved in the cleavage of β-aryl ether by *Pseudomonas paucimobilis* SYK-6. Biosci Biotech Biochem 57: 1655–1659.
36 Masai E, S Shinohara, H Hara, S Nishikawa, Y Katayama and M Fukuda. 1999. Genetic and biochemical characterization of a 2-pyrone-4,6-dicarboxylic acid hydrolase involved in the protocatechuate 4,5-cleavage pathway of *Sphingomonas paucimobilis* SYK-6. J Bacteriol 181: 55–62.
37 Nishida M, S Harada, S Noguchi, Y Satow, H Inoue and K Takahashi. 1998. Three-dimensional structure of *Escherichia coli* glutathione *S*-transferase complexed with glutathione sulfonate: catalytic roles of Cys10 and His106. J Mol Biol 281: 135–147.
38 Nishikawa S, Y Katayama, M Yamasaki, N Morohoshi and T Haraguchi. 1991. Cloning of the genes involved in protocatechuate *meta*-cleavage pathway. In: Proceedings of the 6th International Symposium on Wood and Pulping Chemistry, 2nd edn, pp 311–313.
39 Nishikawa S, T Sonoki, T Kasahara, T Obi, S Kubota, S Kawai, N Morohoshi and Y Katayama. 1998. Cloning and sequencing of the *Sphingomonas (Pseudomonas) paucimobilis* gene essential for the O demethylation of vanillate and syringate. Appl Environ Microbiol 64: 836–842.
40 Noda Y, S Nishikawa, K Shiozuka, H Kadokura, H Nakajima, K Yoda, Y Katayama, N Morohoshi, T Haraguchi and M Yamasaki. 1990. Molecular cloning of the protocatechuate 4,5-dioxygenase gene of *Pseudomonas paucimobilis*. J Bacteriol 172: 2704–2709.
41 Orser CS, J Dutton, CC Lange, PE Jablonski, L Xun and M Hargis. 1993. Characterization of a *Flavobacterium* glutathione *S*-transferase gene involved reductive dechlorination. J Bacteriol 175: 2640–2644.
42 Peng X, T Egashira, K Hanashiro, E Masai, S Nishikawa, Y Katayama, K Kimbara and M Fukuda. 1998. Cloning of a *Sphingomonas paucimobilis* SYK-6 gene encoding a novel oxygenase that cleaves lignin-related biphenyl and characterization of the enzyme. Appl Environ Microbiol 64: 2520–2527.
43 Peng X, E Masai, Y Katayama and M Fukuda. 1999. Characterization of the *meta*-cleavage compound hydrolase gene involved in degradation of the lignin-related biphenyl structure by *Sphingomonas paucimobilis* SYK-6. Appl Environ Microbiol 65: 2789–2793.
44 Powlowski J, L Sahlman and V Shingler. 1993. Purification and properties of the physically associated *meta*-cleavage pathway enzymes 4-hydroxy-2-ketovalerate aldolase and aldehyde dehydrogenase (acylating) from *Pseudomonas* sp strain CF600. J Bacteriol 175: 377–385.
45 Priefert H, J Rabenhorst and A Steinbuchel. 1997. Molecular characterization of genes of *Pseudomonas* sp strain HR199 involved in bioconversion of vanillin to protocatechuate. J Bacteriol 179: 2595–2607.
46 Rankin CA, GC Haslam and RH Himes. 1993. Sequence and expression of the gene for N10-formyltetrahydrofolate synthetase from *Clostridium cylindrosporum*. Protein Sci 2: 197–205.
47 Reinemer P, L Prade, P Hof, T Neueffind, R Huber, R Zettl, K Palme, J Schell, I Koelln, HD Bartunik and B Bieseler. 1996. Three-dimensional structure of glutathione *S*-transferase from *Arabidopsis thaliana* at 2.2-Å resolution: structural characterization of herbicide-conjugating plant glutathione *S*-transferases and a novel active site architecture. J Mol Biol 255: 289–309.
48 Sato S, N Ouchiyama, T Kimura, H Nojiri, H Yamane and T Omori. 1997. Cloning of genes involved in carbazole degradation of *Pseudomonas* sp strain CA10: nucleotide sequences of genes and characterization of *meta*-cleavage enzymes and hydrolase. J Bacteriol 179: 4841–4849.
49 Senda T, K Sugiyama, H Narita, T Yamamoto, K Kimbara, M Fukuda, M Sato, K Yano and Y Mitsui. 1996. Three-dimensional structures of free form and two substrate complexes of an extradiol ring-cleavage type dioxygenase, the BphC enzyme from *Pseudomonas* sp strain KKS102. J Mol Biol 255: 735–752.
50 Shigehara K, C Seki, N Okamura, M Matsumoto, Y Katayama and S Nishikawa. 1998. Synthesis of polymers with 2H-pyran-2-one-4,6-dicarboxylate (PDC). Nuclei Polymer Preprints, Japan. English edition 47: 415.
51 Sinning I, GJ Kleywegt, SW Cowan, P Reinemer, HW Dirr, R Huber, GL Gilliland, RN Armstrong, X Ji, PG Board, B Olin, B Mannervik and TA Jones. 1993. Structure determination and refinement of human alpha class glutathione transferase A1-1, and a comparison with the Mu and Pi class enzymes. J Mol Biol 232: 192–212.
52 Spence EL, M Kawamukai, J Sanvoisin, H Braven and TD Bugg. 1996. Catechol dioxygenases from *Escherichia coli* (MhpB) and *Alcaligenes eutrophus* (MpcI): sequence analysis and biochemical properties of a third family of extradiol dioxygenases. J Bacteriol 178: 5249–5256.
53 Sugimoto K, T Senda, H Aoshima, E Masai, M Fukuda and Y Mitsui. 1999. Crystal structure of an aromatic-ring-opening dioxygenase LigAB which is a protocatechuate 4,5-dioxygenase. Structure Fold Des 15: 953–965.
54 Tack BF, PJ Chapman and S Dagley. 1972. Purification and properties of 4-hydroxy-4-methyl-2-oxoglutarate aldolase. J Biol Chem 247: 6444–6449.
55 van der Zaal EJ, FNJ Droog, CJM Boot, LAM Hensgens, JHC Hoge, RA Schilperoort and KR Libbenga. 1991. Promoters of auxin-induced genes from tobacco can lead to auxin-inducible and root tip-specific expression. Plant Mol Biol 16: 983–998.
56 Vicña R. 1988. Bacterial degradation of lignin. Enzyme Microb Technol 10: 646–655.
57 Vuilleumier S. 1997. Bacterial glutathione *S*-transferase: what are they good for? J Bacteriol 179: 1431–1441.
58 Vuilleumier S and T Leisinger. 1996. Protein engineering studies of dichloromethane dehalogenase/glutathione *S*-transferase from *Methylophilus* sp strain DM11. Ser12 but not Tyr6 is required for enzyme activity. Eur J Biochem 239: 410–417.
59 Werwath J, HA Arfmann, DH Pieper, KN Timmis and RM Wittich. 1998. Biochemical and genetic characterisation of a gentisate 1,2-dioxygenase from *Sphingomonas* sp strain RW5. J Bacteriol 180: 4171–4176.
60 Whitehead TR and JC Rabinowitz. 1988. Nucleotide sequence of the *Clostridium acidiurici* (‘*Clostridium acidi-urici*’) gene for 10-formyltetrahydrofolate synthetase shows extensive amino acid homology with the trifunctional enzyme C_1-tetrahydrofolate synthase from *Saccharomyces cerevisiae*. J Bacteriol 170: 3255–3261.
61 Wilce MCJ, PG Board, SC Feil and MW Parker. 1995. Crystal structure of a theta-class glutathione transferase. EMBO J 14: 2133–2143.
62 Wilce MCJ and MW Parker. 1994. Structure and function of glutathione *S*-transferases. Biochim Biophys Acta 1205: 1–18.
63 Wolgel SA, JE Dege, PE Perkins-Olson, CH Jaurez-Garcia, RL Crawford, E Munck and JD Lipscomb. 1993. Purification and characterization of protocatechuate 2,3-dioxygenase from *Bacillus macerans*: a new extradiol catecholic dioxygenase. J Bacteriol 175: 4414–4426.
64 Zimmermann W. 1990. Degradation of lignin by bacteria. J Biotechnol 13: 119–130.