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Tetrahydrofolate-dependent vanillate and syringate *O*-demethylation links tightly to one-carbon metabolic pathway associated with amino acid synthesis and DNA methylation in the lignin metabolism of *Sphingomonas paucimobilis* SYK-6

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Abstract *Sphingomonas paucimobilis* SYK-6 strain can degrade various lignin-related compounds. In the lignin metabolic pathway of this bacterium, vanillate and syringate are demethylated by the tetrahydrofolate (THF)-dependent *O*-demethylation system, which requires the enzymatic function of LigH. Upstream of the *ligH* gene is the 5,10-methylene-THF reductase gene. Its gene product was essential for one-carbon metabolism involved in the amino acid synthesis and DNA methylation in all organisms. When the *metF* gene was inactivated in the genome of SYK-6, the resultant mutant, DLmetF, could not grow on vanillate and syringate as a sole carbon source. Furthermore, DLmetF showed significant accumulation of methyl-THF as a result of vanillate and syringate *O*-demethylation. We report here that THF-dependent vanillate and syringate *O*-demethylation links tightly to the one-carbon metabolic pathway that is associated with amino acid synthesis and DNA methylation, and the methyl group is the sole one-carbon source in *S. paucimobilis* SYK-6.

Key words Lignin biodegradation · Tetrahydrofolate · One-carbon metabolism

Introduction

Lignin is the most abundant aromatic compound in the biosphere, and its degradation is a significant step in the global carbon cycle. *Sphingomonas paucimobilis* SYK-6, a bacterium that can grow on 5,5′-dehydrodivanillic acid (2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl) (DDVA) as a sole carbon source, was isolated from pulp-bleaching wastewater in Japan. This bacterium can also grow on several dimeric model compounds of lignin such as β-aryl ether, phenylcoumarane, pinoresinol, and diarylpropane. The metabolic pathway of DDVA and other dimeric model compounds of lignin in this bacterium was previously reported.[1,2] The ability of *S. paucimobilis* SYK-6 to grow on several low-molecular-weight lignin compounds as a sole source of carbon and energy indicates that SYK-6 strain produces unique and specific enzymes, such as *O*-demethylases, β-etherases, and ring fission dioxygenases. We have identified several genes and characterized the function of these genes involved in this pathway.[3–14] In the lignin metabolic pathway of *S. paucimobilis* SYK-6, the methylether cleavage (*O*-demethylation) step is essential for the substrate of enzymes such as LigZ[4] and LigAB.[9,10] *S. paucimobilis* SYK-6 has *O*-demethylation systems for three substrates [5,5′-dehydrodivanillic acid DDVA, syringate, vanillate] in the metabolic pathway (Fig. 1). DDVA-specific *O*-demethylation is an oxygenative system catalyzed by the enzyme encoded by the *ligX* gene.[12] On the other hand, vanillate- and syringate-specific *O*-demethylation is a tetrahydrofolate (THF)-dependent system required the enzyme function encoded by the *ligH* gene.[3]

The role of THF in the biologic steps is the one-carbon (C1) carrier for amino acid (e.g., methionine, serine) synthesis, DNA methylation, acetyl-coenzyme A (CoA) synthesis, and nucleic acid synthesis. Generally, the THF-dependent C1 metabolic pathway consists of four enzymes: formyl-THF synthetase (EC 6.3.4.3); 5,10-methylene-THF dehydrogenase (EC 1.5.1.5); 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9); and 5,10-methylene-THF reductase (MetF; EC 1.5.1.20)[15–17] (see Fig. 6).

In the present study, we revealed the role of THF-dependent *O*-demethylation of vanillate and syringate in lignin metabolism of *S. paucimobilis* SYK-6. In the lignin-degrading bacterium *S. paucimobilis* SYK-6, THF-dependent *O*-demethylation is tightly linked to the C1 metabolic pathway, especially MetF. The methyl group in the chemical structure of lignin is essential for C1 anabolic

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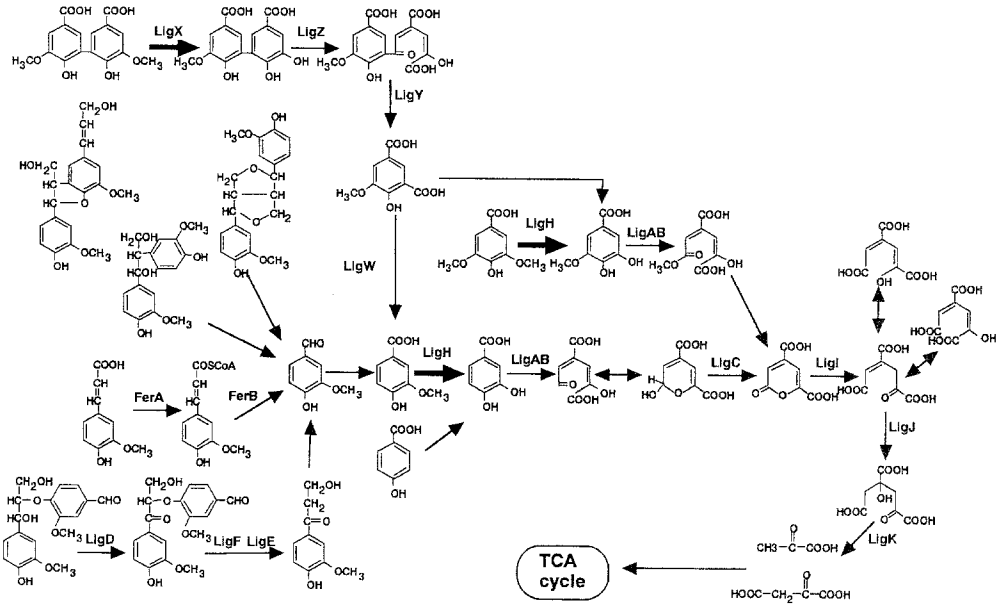

Fig. 1. Lignin metabolic pathway in *Sphingomonas paucimobilis* SYK-6. *LigD*, Cα-dehydrogenase;[6] *LigE*, *LigF*, β-etherase;[7,8] *LigX*, DDVA (2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl) *O*-demethylase;[12] *LigZ*, OH-DDVA (2,2′,3′-trihydroxy-3-methoxy-5,5′-divanillic acid) dioxygenase;[4] *LigY*, OH-DDVA *meta*-cleavage compound hydrolase;[11] *LigW*, 5-carboxy vanillate decarboxylase; *LigH*, enzyme essential for vanillate and syringate *O*-demethylations;[3] *LigAB*, protocatechuate 4,5-dioxygenase;[9] *LigC*, 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase;[14] *LigI*; 2-pyrone-4,6-dicarboxylate hydrolase;[5] *LigJ*, 4-oxalomesaconate hydratase;[13] *LigK*, 4-carboxy-4-hydroxy-2-oxoadipate aldolase; *FerA*, Ferulate:CoA ligase; *FerB*, feruloyl-CoA hydoratase/lyase

reactions, such as amino acid synthesis and DNA methylation in *S. paucimobilis* SYK-6.

Materials and methods

Sphingomonas paucimobilis wild-type strain SYK-6 was isolated for its ability to utilize DDVA as a carbon source, from pulp-bleaching wastewater as described previously.[1] *S. paucimobilis* strains were routinely grown in Luria-Bertani (LB) medium at 28°C. When DDVA and other phenolic compounds were used as a carbon source, each was added to the W medium[18] at a final concentration of 0.2% (w/v). Kanamycin and nalidixic acid were added to the selective medium at final concentrations of 25 mg/l for *S. paucimobilis* strains.

Vanillic acid and syringic acid were purchased from Tokyo Kasei (Tokyo, Japan); and tetrahydrofolic acid, folinic acid, and 5-methyltetrahydrofolic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). All antibiotics were purchased from Wako Pure Chemical Industries (Saitama, Japan). All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, *E. coli* klenow fragment, and a kilosequence kit were obtained from Takara Shuzo (Kyoto, Japan).

Cloning and nucleotide sequencing

All of the recombinant DNA methods used to construct the plasmids or to study the cloned fragment have been described previously.[19] The complemental DNA, pDE20 containing 20 kbp *Eco* RI fragment, of strain DC-49 was isolated from the genomic library of *S. paucimobilis* SYK-6 as described previously.[3] The various deletion derivatives of pUB6.5, containing 6.5 kbp *Bam* HI fragment in 20 kbp *Eco* RI fragment of pDE20, were constructed with the restriction endonucleases and exonuclease of the Kilosequence kit. Subcloning was performed with the plasmid pUC119. Nucleotide sequencing was performed by the dideoxy-chain termination method with an Auto Read Sequence Kit and an A.L.F. DNA Sequencer II obtained from Amasham Pharmacia Biotech (Uppsala, Sweden). The nucleotide sequence between the *Bam* HI restriction sites of pUB6.5 was determined. The nucleotide sequence and deduced amino acid sequences were analyzed with GENETYX version 10.1 software (Software Development, Tokyo, Japan), and a similarity search was carried out with the SwissProt database.

In vitro *O*-demethylation and analyticals

S. paucimobilis SYK-6 and the mutants were cultured in LB medium containing nalidixic acid (25 mg/l). The cell-free extracts were prepared as described in our previous study.[3] The complete reaction mixture contained (in a final volume of 1 ml) 100 mM Tris-HCl buffer (pH 7.5), 3.5 mM $MgCl_2$, 5 mM THF, 5 mM ATP, 1 mM substrate (vanillate or syringate), and 1–3 mg of protein from the cell-free extracts. For gas chromatography analysis, the treatment and analysis of the reaction mixture after incubation for 12 h at 28°C were done as described previously.[3] For high-performance liquid

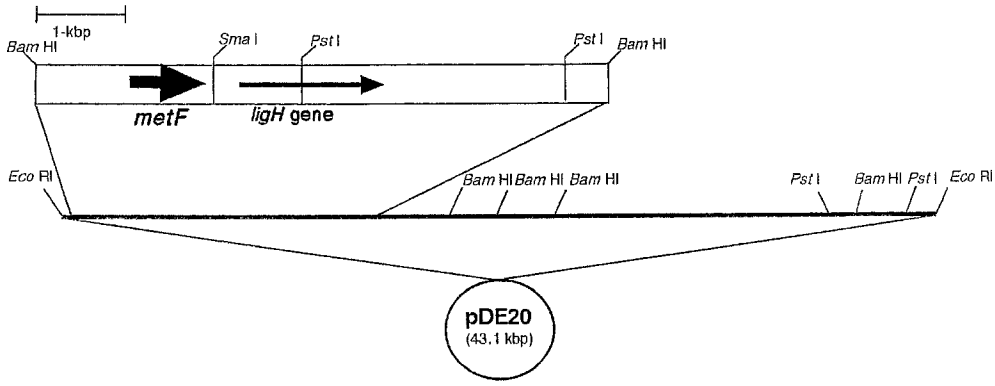

Fig. 2. Physical map of the 20-kbp *Eco* RI fragment and localization of *ligH* and *metF* genes in the 6.5-kbp *Bam* HI fragment

chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) analysis, after incubation for 12 h at 28°C the reaction mixture was freeze-dried (freeze dryer FD-81; Tokyo Rikakikai, Tokyo, Japan). The resulting powder was dissolved in 0.1 ml of the mobile phase directly. The mixture was centrifuged at 10000 *g* for 5 min, and the supernatant was filtered using a 0.45-μm filter (Gelman Sciences Japan). Aliquots of these solutions were taken for analysis by HPLC and LC-MS. Experimental work was carried out using the Mariner Biospectrometry Workstation (Perspective Biosystems). Chromatographic separation was carried out on an ODS-1251-SS (25 cm × 4.6 mm) cartridge column (Senshu Science, Tokyo, Japan) using a mobile phase consisting of 1% acetic acid/acetonitrile (88:12) at a flow rate of 0.5 ml/min. An injection volume of 20 μl was used. A mass spectrometer fitted with a spray voltage of 3.4 kV was used throughout the study.

Preparation of insertional inactivated mutants of *S. paucimobilis* SYK-6

The 5,10-methylenetetrahydrofolate reductase (*metF*) gene-inactivated mutant DLmetF was prepared as follows. The 1.8-kbp DNA fragment carrying *metF* was cloned into pHSG398 (Takara Shuzo, Kyoto, Japan). The 1.3-kbp *Sal* I fragment containing the kanamycin resistance gene from pUC4K (Amersham Pharmacia Biotech UK) was inserted into *Apa* I site (blunted) in the *metF* gene in pHSG398. The resulting plasmid, pHDLmetF, was digested with *Eco* RI and *Bam* HI; and the insert containing the *metF* gene was cloned into pK19*mobsacB*[20] to generate pKDLmetF. The mutant-inactivated *metF* gene of *S. paucimobilis* SYK-6 was prepared as described by Masai et al.[5].

Results

Identification of ORF (*metF*) forming the gene cluster with the *ligH* gene

The *ligH* gene, which was essential for THF-dependent vanillate and syringate *O*-demethylation during the lignin metabolism of SYK-6, was cloned as the 20-kbp *Eco* RI fragment in the previous study.[3] To clarify the vanillate- and syringate-specific *O*-demethylation system, we analyzed the

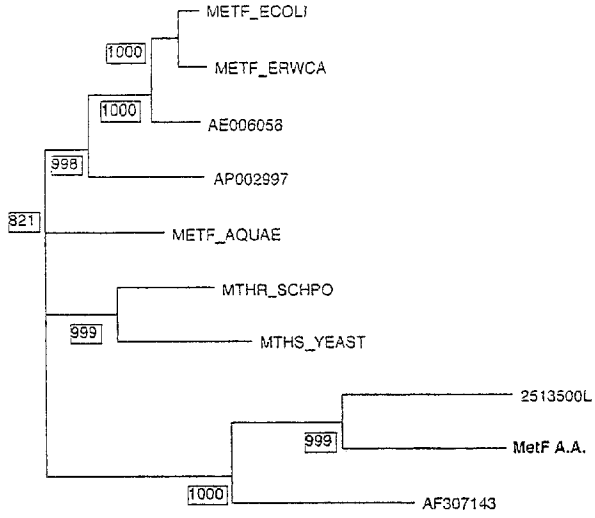

Fig. 3. Phylogenetic tree of 5,10-methylenetetrahydrofolate reductases. The phylogenetic tree has been drawn using the Genetix version 10.1 software. The numbers on some of branches refer to the confidence estimated by bootstrap analysis (100 replications). *2513500L*, MetF protein of *Methylobacterium* sp. strain CM4.[21] *AF307143*, methylenetetrahydrofolate reductase of *Aminobacter* sp. IMB-1.[22] *MTHS_YEAST*, methylenetetrahydrofolate reductase of *Saccharomyces cerevisiae*.[23] *MTHR_SCHPO*, probable methylenetetrahydrofolate reductase 1 of *Schizosaccharomyces pombe* (direct submitted to the EMBL/GenBank/DDBJ databases). *METF_AQUAE*, 5,10-methylenetetrahydrofolate reductase of *Aquifex aeolicales*.[24] *AP002997*, 5,10-methylenetetrahydrofolate reductase of *Mesorhizobium loti* strain MAFF303099.[25] *AE006058*, MetF of *Pasteurella multocida*.[26] METF_ECOLI, 5,10-methylenetetrahydrofolate reductase of *E. coli*.[27] METF_ERWCA, 5,10-methylenetetrahydrofolate reductase of *Erwinia carotovora* (direct submitted to the EMBL/GenBank/DDBJ databases)

gene sequence of the 6.5 kbp *Bam* HI fragment. Computer analysis of the nucleotide sequence indicated a single open reading frame (ORF) in the upstream region of the *ligH* gene (Fig. 2). The deduced gene product of this ORF consists of 237 amino acid residues, and the molecular weight was calculated to be 25651 daltons. The sequence of this ORF had a G + C content of 64%. Computer analysis also showed that 86% of the third bases of codons were G's or C's. The codon usage of this ORF was similar to those in the genes of *S. paucimobilis* SYK-6 (data not shown). A similar search indicated that the deduced amino acid sequence of the ORF was similar to those of some 5,10-methylene tetrahydrofolate reductases (*metF*) (Fig. 3). The phyloge-

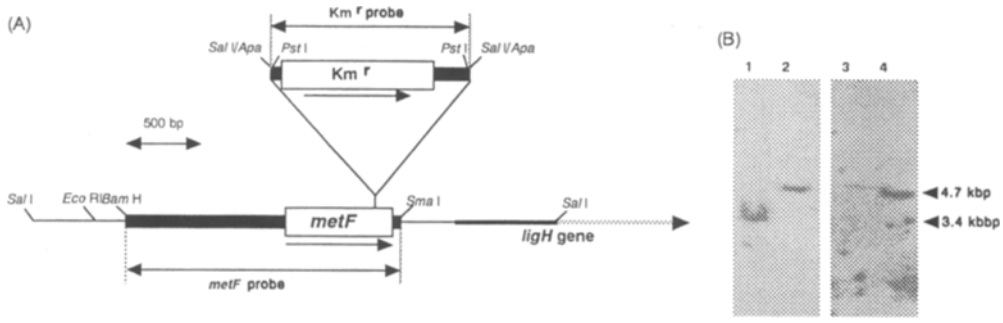

Fig. 4. Insertional inactivation of the *metF* gene in *S. paucimobilis* SYK-6. **A** Insertional inactivation of the *metF* gene by the Km^r gene. **B** Southern hybridization analysis of the *metF*-inactivated mutant DLmetF. Lanes *1* and *3*, total DNA of SYK-6 digested with *Sal* I; lanes *2* and *4*, total DNA of DLmetF digested with *Sal* I. The 3.0-kbp *Bam* HI-*Sma* I fragment carrying *metF* (lanes *1* and *2*) and the 1.3-kbp *Pst* I fragment of kanamycin resistance gene (lanes *3* and *4*) were used as probes

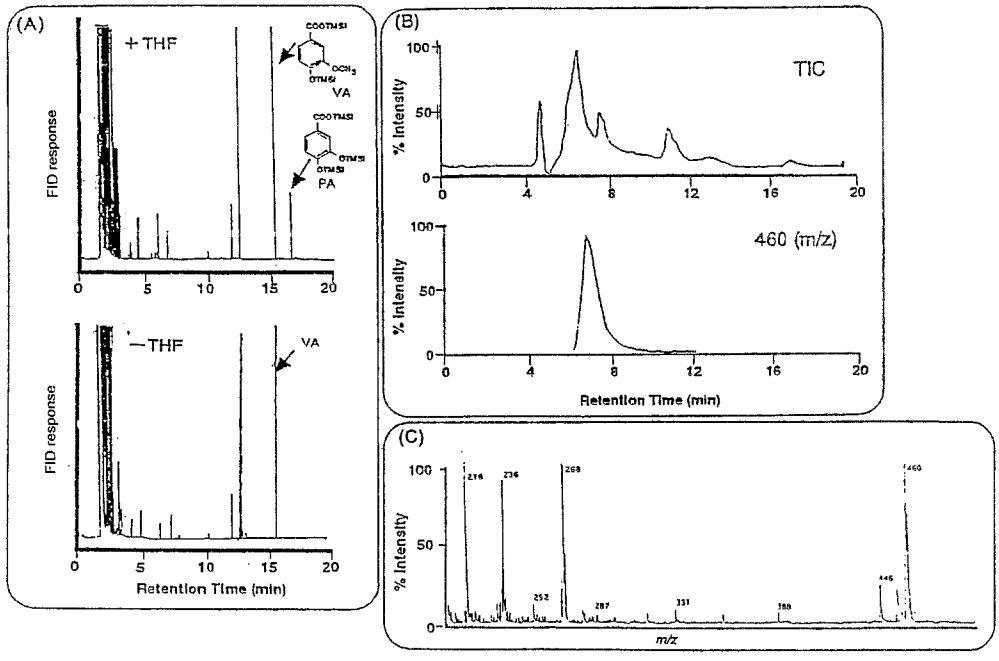

Fig. 5. Gas chromatographic and liquid chromatography-mass spectrometry (LC-MS) analysis of vanillate *O*-demethylation by the cell-free extracts of *metF*-inactivated mutant DLmetF. **A** Gas chromatographic analysis with or without the tetrahydrofolate (*THF*). **B** Total ion chromatogram and APCI mass chromatogram of $[M + H^+]$ ion at *m/z* 460. **C** Fragmentation spectrum of the compound has an *m/z* of $[M + H^+]$ at 460

netic tree is shown in Fig. 3. This ORF was closest to MetF of *Methylobacterium* sp. strain CM4[21] and showed 26.4% identity. It was highly homologous to heterologous microorganisms. Although no ORF was located in the downstream region of the *ligH* gene, the ORF-encoded 5,10-methylenetetrahydrofolate reductase (MetF) existed in the upstream region of the *ligH* gene and formed a gene cluster.

Disruption of the *metF* gene in *S. paucimobilis* SYK-6

The *metF* gene was disrupted to determine its role in the metabolism of vanillate and syringate in *S. paucimobilis* SYK-6. Gene disruption was carried out using the *metF* disruption plasmid pKDLmetF. The *metF* insertional mutation was confirmed by Southern hybridization analysis (Fig. 4). The resulting mutant strain, DLmetF, completely lost the ability to grow on both vanillate and syringate. When the whole cells of DLmetF, pregrown in LB medium, were incubated in W medium containing 0.2% vanillate or syringate, DLmetF could convert vanillate to protocatechuate and syringate to 3-*O*-methylgallate. This indicated that the gene product of *metF* had an important role in vanillate and syringate utilization as a sole source of carbon.

O-Demethylation activity in the cell-free extracts of the *metF*-inactivated mutant DLmetF

Figure 5 shows a gas chromatogram and APCI-MS spectrum of the reaction products of vanillate *O*-demethylation in the cell-free extracts of DLmetF strain. The cell-free extracts of DLmetF retained vanillate and syringate *O*-demethylation activity. A peak at *m/z* 460 corresponding to $[M + H]^+$ was observed. The spectrum was compatible with the authentic sample, 5-methyl-THF. DLmetF strain accumulated a significant amount of methyl-THF as a result of the *O*-demethylation of those compounds. In the cell-free extracts of wild-type strain SYK-6, such accumulation was not detected in THF-dependent *O*-demethylation (data not

shown). These results indicated that *metF* gene product catalyzed conversion of the methyl-THF produced by the THF-dependent *O*-demethylation activity of SYK-6.

Discussion

Sphingomons paucimobilis SYK-6 has two kinds of system that depend on the substrate specificity in the single cell. One is a DDVA-specific oxygenative *O*-demethylation system, and the other is a vanillate- and syringate-specific THF-dependent *O*-demethylation system.[3,12] As described above, THF is the C1 carrier and plays an important role in biological steps. In the present study, we investigated the role of THF-dependent *O*-demethylation in the lignin metabolic pathway of *S. paucimobilis* SYK-6.

In a previous study, we identified the *ligH* gene as being essential for vanillate and syringate *O*-demethylation.[3] In the upstream region of *ligH*, there was an ORF whose deduced amino acid sequence showed similarity to the 5,10-methylene tetrahydrofolate reductases (MetF) of *Methylobacterium* sp. strain CM4[21] and *Aminobacter* sp. IMB-1.[22] In these bacteria, the methyl group of chloromethane was transferred to tetrahydrofolate. The resulting compound, methyl-THF, was converted to methylene-THF by the MetF protein, and the C1 compounds were utilized as cell carbon throughout the THF-dependent C1 metabolic pathway.[21,22]

The THF-dependent vanillate and syringate *O*-demethylation of SYK-6 required the enzymatic function of LigH, and diol compounds (protocatechuate from vanillate and 3-*O*-methylgallate from syringate) and methyl-THF were produced.[3] We identified the *metF* gene in the upstream region of the *ligH* gene. It was thought that there was a close relation between these gene products because they formed a gene cluster. We inactivated the *metF* gene in the genome of SYK-6 strain. The *metF*-inactivated mutant, DLmetF strain, could not grow on vanillate or syringate as the sole source of carbon. It was shown that MetF had an important role in vanillate and syringate utilization in the SYK-6 strain. The cell-free extracts of this *metF*-inactivated mutant retained vanillate and syringate *O*-demethylation activity, and significant methyl-THF accumulation was detected as a result of the *O*-demethylation of vanillate and syringate (Fig. 5). MetF probably catalyzed the conversion of methyl-THF to methylene-THF in *S. paucimobilis* SYK-6. Hence, the methyl group of lignin is important and the sole C1 source for the SYK-6 strain to synthesize the essential compounds through the C1 metabolic pathway (Fig. 6).

In the lignin metabolic pathway of *S. paucimobilis* SYK-6, the THF-dependent *O*-demethylation system is tightly linked to the THF-dependent C1 metabolic pathway essential for anabolic reaction such as amino acid synthesis and DNA methylation. It is the system used for recovery of the C1 compound derived from the methyl group of vanillate and syringate. The lignin-degrading bacterium *S. paucimobilis* SYK-6 has an excellent system for utilizing carbon derived not only from the benzene ring but also the methyl group that exists abundantly in the chemical structure of lignin.

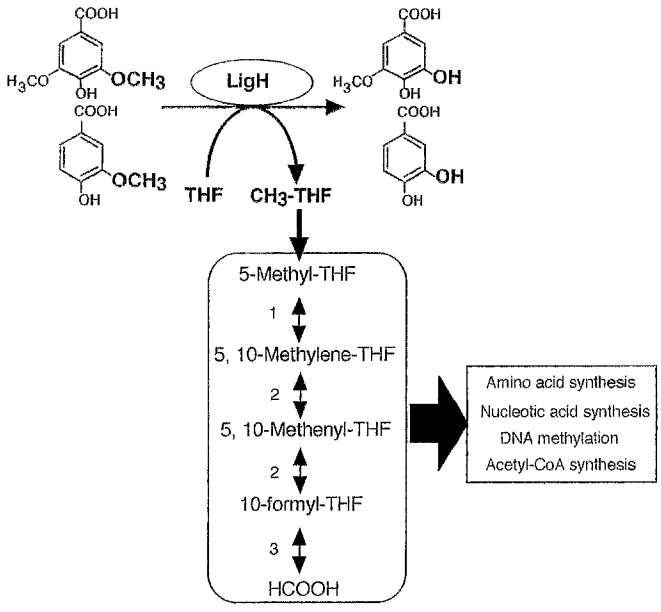

Fig. 6. Proposed model of the link between THF-dependent *O*-demethylation and one-carbon metabolic pathway. Enzymes correspond to the numbered reactions: *1*, 5,10-methylene-THF reductase (EC 1.5.1.20); *2*, 5,10-methylene-THF dehydrogenase (EC 1.5.1.5)/ 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9); *3*, formyl-THF synthetase (EC 6.3.4.3)

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References

1. Katayama Y, Nishikawa S, Murayama A, Yamasaki M, Morohoshi N, Haraguchi T (1988) The metabolism of biphenyl structure in lignin of the soil bacterium (*Pseudomonas paucomobilis* SYK-6). FEBS Lett 233:129–133
2. Masai E, Katayama Y, Nishikawa S, Fukuda M (1999) Characterization of *Sphingomonas paucimobilis* SYK-6 genes involved in degradation of lignin-related compounds. J Ind Microbiol Biotechnol 23:364–373
3. Nishikawa S, Sonoki T, Kasahara T, Obi T, Kubota S, Kawai S, Morohoshi N, Katayama Y (1998) Cloning and sequencing of *Sphingomonas* (*Pseudomonas*) *paucimobilis* gene essential for the *O*-demethylation of vanillate and syringate. Appl Environ Microbiol 64:836–842
4. Peng X, Egashira T, Hanashiro K, Masai E, Nishikawa S, Katayama Y, Kimbara K, Fukuda M (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
5. Masai E, Shinohara S, Hara H, Nishikawa S, Katayama Y, Fukuda M (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
6. Masai E, Kubota S, Katayama Y, Kawai S, Yamasaki M, Morohoshi N (1993) Characterization of the C α-dehydrogenase gene involved in the cleavage of β-aryl ether by *Pseudomonas paucimobilis*. Biotechnol Biophys Biochem 57:1655–1659

7. Masai E, Katayama Y, Kawai S, Nishikawa S, Yamasaki M, Morohoshi N (1991) Cloning and sequencing of the gene for *Pseudomonas paucimobilis* enzyme that cleaves β-aryl ether. J Bacteriol 173:7950–7955
8. Masai E, Katayama Y, Kubota S, Kawai S, Yamasaki M, Morohoshi N (1993) A bacterial enzyme degrading the model lignin compound beta-etherase is a member of the glutathione-*S*-transferase superfamily. FEBS Lett 323:135–140
9. Noda Y, Nishikawa S, Kadokura H, Nakajima H, Yoda K, Katayama Y, Morohoshi N, Yamasaki M (1990) Molecular cloning of the protocatechuate 4,5-dioxygenase genes of *Pseudomonas paucimobilis*. J Bacteriol 172:2704–2709
10. Nishikawa S, Katayama Y, Yamasaki M, Morohoshi N, Haraguchi T (1991) Cloning of the genes involved in protocatechuate meta cleavage pathway. In: Proceedings of the 6th International Symposium on Wood and Pulping Chemistry, 2nd ed, pp 311–313
11. Peng X, Egashira T, Masai E, Katayama Y, Fukuda M (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
12. Sonoki T, Obi T, Kubota S, Higashi M, Masai E, Katayama Y (2000) Coexistence of two different *O*-demethylation systems in the lignin metabolism of *Sphingomonas paucimobilis* SYK-6: cloning and sequencing of lignin biphenyl specific *O*-demethylase (LigX). Appl Environ Microbiol 66:2125–2132
13. Hara H, Masai E, Katayama Y, Fukuda M (2000) The 4-oxalomesaconate hydratase gene, involved in the protocatechuate 4,5-cleavage pathway, is essential to vanillate and syringate degradation in *Sphingomonas paucimobilis* SYK-6. J Bacteriol 182: 6950–6957
14. Masai E, Momose K, Hara H, Nishikawa S, Katayama Y, Fukuda M (2000) Genetic and biochemical characterization of the 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase and its role in the protocatechuate 4,5-cleavage pathway in *Sphingomonas paucimobilis* SYK-6. J Bacteriol 182:6651–6658
15. Nagy PL, Marolewski A, Benkovic SJ, Zalkin H (1995) Formyltetrahydrofolate hydrolase, a regulatory enzyme that functions to balance pools of tetrahydrofolate and one-carbon tetrahydrofolate adducts in *Escherichia coli*. J Bacteriol 177:1292–1298
16. Dean RA, Jesse CR (1985) Regulation of expression of the ADE3 gene for yeast C1-tetrahydrofolate synthase, a trifunctional enzyme involved in one-carbon metabolism. J Biol Chem 260: 1248–1256
17. Andrew DH, Douglas AG, Yair SH (2000) Plant one-carbon metabolism and its engineering. Trends Plant Sci 5:206–213
18. Yano K, Nishi T (1980) pKJ1, a naturally occurring conjugative plasmid coding for toluene degradation and resistance to streptomycin and sulfonamides. J Bacteriol 143:552–560
19. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Schafer A, Tauch A, Jager W, Kalinowski J, Thierbach G, Puhler A (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69–73
21. Vannelli T, Messmer M, Studer A, Vuilleumier S, Leisinger T (1999) A corrinoid-dependent catabolic pathway for growth of a *Methylobacterium* strain with chloromethane. Proc Natl Acad Sci USA 96:4615–4620
22. McAnulla C, Woodall CA, McDonald IR, Studer A, Vuilleuminer S, Leisinger T, Murrell JC (2001) Chloromethane utilization gene cluster from *Hyphomicrobium chloromethanicum* strain CM2T and development of functional gene probes to detect halomethane-degrading bacteria. Appl Environ Microbiol 67:307–316
23. Tizon B, Rodriguez-Torres AM, Rodriguez-Belmonte E, Cadahia JL, Cerdan E (1996) Identification of a putative methylene-tetrahydrofolate reductase by sequence analysis of a 6.8kb DNA fragment of yeast chromosome. VII. Yeast 12:1047–1051
24. Deckert G, Warren PV, Gaasterland T, Young WG, Lenox AL, Graham DE, Overbeek R, Snead MA, Keller M, Aujay M, Huber R, Feldman RA, Short JM, Olson GJ, Swanson RV (1998) The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. Nature 392:353–358
25. Kaneko T, Nakamura Y, Sato S, Asamizu E, Kato T, Sasamoto S, Watanabe A, Idesawa K, Ishikawa A, Kawashima K, Kimura T, Kishida Y, Kiyokawa C, Kohara M, Matsumoto M, Matsuno A, Mochizuki Y, Nakayama S, Nakazaki N, Shimpo S, Sugimoto M, Takeuchi C, Yamada M, Tabata S (2000) Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. DNA Res 7:331–338
26. May BJ, Zhang Q, Li L, Paustian ML, Whittam TS, Kapur VS (2001) Complete nucleotide sequence of an avian isolate *Pasteurella multocida*. Proc Natl Acad Sci USA 98:3460–3465
27. Saint-Girons I, Duchange N, Zakin MM, Park I, Margarita D, Ferrara P, Cohen GN (1983) Nucleotide sequence of *metF*, the *E. coli* structural gene for 5,10-methylene tetrahydrofolate reductase and its control region. Nucleic Acids Res 11:6723–6732