APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2002, p. 4416–4424 Vol. 68, No. 9
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.9.4416–4424.2002
Copyright © 2002, American Society for Microbiology. All Rights Reserved.

# Cloning and Characterization of the Ferulic Acid Catabolic Genes of *Sphingomonas paucimobilis* SYK-6

Eiji Masai,[1]* Kyo Harada,[1] Xue Peng,[1] Hirotaka Kitayama,[1] Yoshihiro Katayama,[2] and Masao Fukuda[1]

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

Received 18 March 2002/Accepted 11 June 2002

***Sphingomonas paucimobilis*** **SYK-6 degrades ferulic acid to vanillin, and it is further metabolized through the protocatechuate 4,5-cleavage pathway. We obtained a Tn*5* mutant of SYK-6, FA2, which was able to grow on vanillic acid but not on ferulic acid. A cosmid which complemented the growth deficiency of FA2 on ferulic acid was isolated. The 5.2-kb *Bam*HI-*Eco*RI fragment in this cosmid conferred the transformation activity of ferulic acid to vanillin on *Escherichia coli* host cells. A sequencing analysis revealed the genes *ferB* and *ferA* in this fragment; these genes consist of 852- and 2,127-bp open reading frames, respectively. The deduced amino acid sequence of *ferB* showed 40 to 48% identity with that of the feruloyl-coenzyme A (CoA) hydratase/lyase genes of *Pseudomonas* and *Amycolatopsis* ferulic acid degraders. On the other hand, the deduced amino acid sequence of *ferA* showed no significant similarity to the feruloyl-CoA synthetase genes of other ferulic acid degraders. However, the deduced amino acid sequence of *ferA* did show 31% identity with pimeloyl-CoA synthetase of *Pseudomonas mendocina* 35, which has been classified as a new superfamily of acyl-CoA synthetase (ADP forming) with succinyl-CoA synthetase (L. B. Sánchez, M. Y. Galperin, and M. Müller, J. Biol. Chem. 275: 5794–5803, 2000). On the basis of the enzyme activity of *E. coli* carrying each of these genes, *ferA* and *ferB* were shown to encode a feruloyl-CoA synthetase and feruloyl-CoA hydratase/lyase, respectively. *p*-coumaric acid, caffeic acid, and sinapinic acid were converted to their corresponding benzaldehyde derivatives by the cell extract containing FerA and FerB, thereby indicating their broad substrate specificities. We found a *ferB* homolog, *ferB2*, upstream of a 5-carboxyvanillic acid decarboxylase gene (*ligW*) involved in the degradation of 5,5′-dehydrodivanillic acid. The deduced amino acid sequence of *ferB2* showed 49% identity with *ferB*, and its gene product showed feruloyl-CoA hydratase/lyase activity with a substrate specificity similar to that of FerB. Insertional inactivation of each *fer* gene in *S. paucimobilis* SYK-6 suggested that the *ferA* gene is essential and that *ferB* and *ferB2* genes are involved in ferulic acid degradation.**

Lignin is the most abundant aromatic substance in the biosphere, and therefore its utilization could potentially be advantageous in many areas. One present method for making use of lignin is to convert it into useful chemical materials by the implementation of microbial lignin degradation systems. It is believed that lignin degradation is initiated by white rot fungi, which secrete extracellular degradation enzymes such as lignin peroxidase, manganese peroxidase, and laccase (8). The resulting low-molecular-weight lignin is further degraded and mineralized by bacteria (34). Bacterial lignin degradation systems consist of many unique and specific enzymes with the ability to catalyze the production of various useful compounds (12). Due their productivity, bacterial enzyme systems are expected to serve as useful tools for the conversion of lignin into intermediate metabolites.

*Sphingomonas paucimobilis* SYK-6 has the ability to degrade a wide variety of dimeric lignin compounds, including β-aryl ether, biphenyl, phenylcoumarane, diarylpropane, and pinoresinol (11, 12). We have already characterized the enzyme genes involved in the degradation of β-aryl ether and biphenyl (12, 20, 21). These dimeric lignin compounds are degraded to vanillic acid or syringic acid, and they are further degraded through the protocatechuate 4,5-cleavage pathway (9, 13, 14, 17).

In this study we focused on the degradation of ferulic acid, which is the precursor of lignin biosynthesis. Cinnamic acid derivatives, including ferulic acid and *p*-coumaric acid, play important roles in the cross-linking of the cell walls of various grasses and represent up to 1.5% of the weight of their cell walls. Cinnamic acid derivatives are therefore expected to become popular as an abundant class of bioresources.

Two types of ferulic acid side chain cleavage have been reported for a number of microorganisms (24). One type is catalyzed by nonoxidative decarboxylase, which eliminates one carbon from the ferulic acid side chain, resulting in the formation of 4-hydroxy-3-methoxystyrene. The other type is characterized by the elimination of two carbons from the ferulic acid side chain, and this latter type can produce vanillin, a valuable flavor compound. For this reason the enzymes and the genes involved in the transformation of ferulic acid to vanillin have been the focus of much attention. Two enzymes involved in the side chain cleavage reaction have been reported for *Pseudomonas fluorescens* AN103 (7), *Pseudomonas* sp. strain HR199 (18, 23), *Pseudomonas putida* WCS358 (33), and *Amycolatopsis* sp. strain HR167 (1). The feruloyl-coenzyme A (CoA) synthetase catalyzes the transfer of CoA to the carboxyl group of ferulic acid, which then forms feruloyl-CoA in the presence of

* Corresponding author. Mailing address: Department of Bioengineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2188, Japan. Phone: 81-258-47-9428. Fax: 81-258-47-9450. E-mail: emasai@vos.nagaokaut.ac.jp.

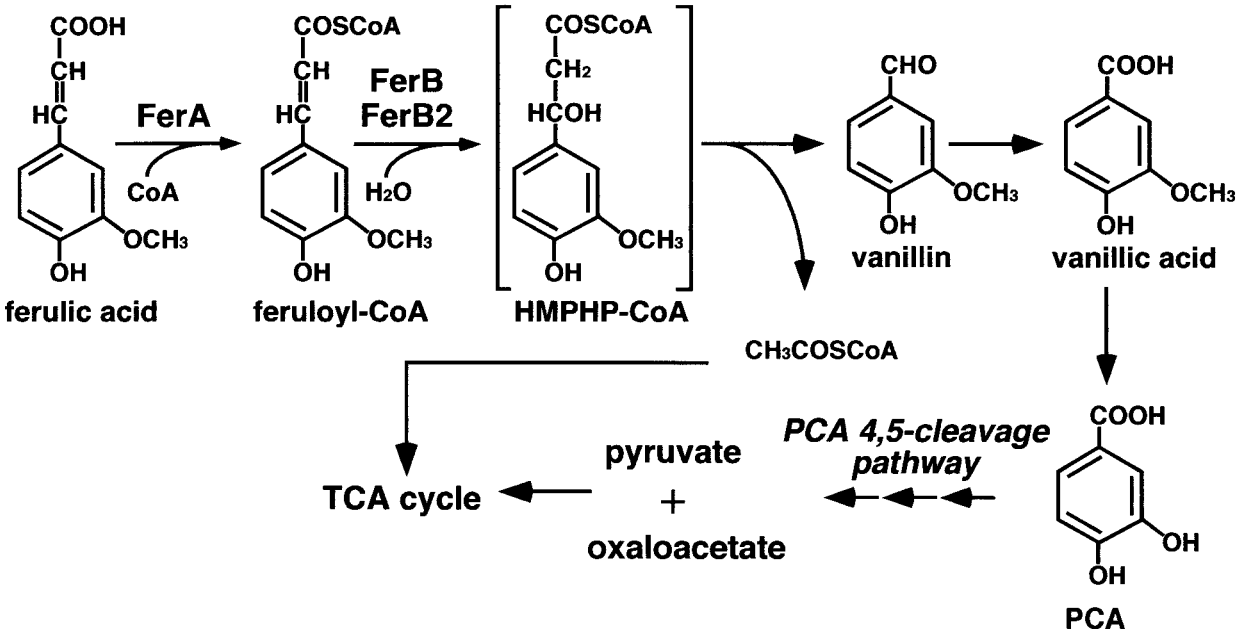


FIG. 1. Ferulic acid catabolic pathway of *S. paucimobilis* SYK-6. Feruloyl-CoA synthetase (FerA) and feruloyl-CoA hydratases/lyases (FerB and FerB2) catalyze the side chain cleavage of ferulic acid to give vanillin and acetyl-CoA. Vanillin is converted to pyruvate and oxaloacetate through the protocatechuate (PCA) 4,5-cleavage pathway.

ATP and $Mg^{2+}$ as cofactors. The resulting feruloyl-CoA is degraded by the feruloyl-CoA hydratase/lyase, which hydrates feruloyl-CoA to form 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl–CoA and cleaves 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl–CoA to produce vanillin and acetyl-CoA (Fig. 1). Although the catabolic pathway of ferulic acid is well characterized, little is known about the diversity of the degradation genes.

In this study we isolated a novel type of the feruloyl-CoA synthetase gene and two feruloyl-CoA hydratase/lyase genes from *S. paucimobilis* SYK-6. The substrate specificities of the gene products and the role of each gene in ferulic acid degradation by SYK-6 were characterized.

(This study was presented in part at the 100th General Meeting of American Society for Microbiology, Los Angeles, Calif., 2000).

## MATERIALS AND METHODS

**Strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. *S. paucimobilis* SYK-6 was grown at 30°C in W minimal medium (20) containing 10 mM vanillic acid or ferulic acid or in Luria-Bertani (LB) medium (Bacto Tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter).

**Chemicals.** Cinnamic acid derivatives, including ferulic acid, caffeic acid, sinapinic acid, cinnamic acid, *p*-coumaric acid, *o*-coumaric acid, *m*-coumaric acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid, were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Benzaldehyde derivatives and other chemicals were purchased from Tokyo Kasei Kogyo Co., Wako Pure Chemical Industries (Osaka, Japan), or Sigma Chemical Company (St. Louis, Mo.)

**Analysis of the metabolites.** *S. paucimobilis* SYK-6 and its disruption mutants were grown to an optical density at 600 nm ($OD_{600}$) of 1.0 in LB medium at 30°C. Cells were washed twice with W medium and suspended to an $OD_{600}$ of 0.2 in 10 ml of the same medium. After the addition of ferulic acid to a final concentration of 10 mM, the mixtures were shaken at 30°C. A portion of the cultures (200 μl) was collected every 3 h from 0 to 48 h and were acidified with hydrochloric acid to a pH value of 1. The metabolites were extracted with 200 μl of ethyl acetate, and then the extract was dried in vacuo and was trimethylsilylated. The resultant samples were analyzed by gas chromatography-mass spectrometry analysis (GC-MS) by using a model 5971A (Hewlett-Packard Co., Palo Alto, Calif.) with an Ultra-2 capillary column (50 m by 0.2 mm; Hewlett-Packard Co.). The analytical conditions for GC-MS were the same as those described previously (14).

**Isolation of a Tn*5* insertion mutant and cloning of the genes.** Tn*5* insertion mutants of *S. paucimobilis* SYK-6 were generated by using pSUP5011, which was transferred from *Escherichia coli* S17-1 to *S. paucimobilis* SYK-6 by conjugation. A mutant which grows on vanillic acid but not on ferulic acid was selected in the following way. Tn*5* insertion mutants of SYK-6 were grown in LB to an $OD_{600}$ of 1.0 at 30°C. Cells were then washed twice with W medium and suspended to an $OD_{600}$ of 0.2 in 10 ml of the same medium. After addition of ferulic acid to a final concentration of 10 mM the culture was shaken at 30°C. To enrich cultures for target mutants unable to grow with ferulic acid by the method of pencillin screening, ampicillin (200 mg/liter) and D-cycloserine (100 mg/liter) were added to the culture when the $OD_{600}$ reached 0.4, and the culture was then incubated for 48 h. The resultant cells were inoculated on W medium containing either ferulic acid or vanillic acid, and then a mutant was selected.

A gene library of SYK-6 constructed with pVK100 as a vector was introduced into a mutant strain by conjugation. The resulting transconjugants were plated on W medium containing 10 mM ferulic acid.

**Preparation of cell extracts.** *E. coli* JM109 harboring each plasmid was grown in 200 ml of LB medium containing 100 mg of ampicillin/liter. When the $OD_{600}$ of the culture reached 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 5 h of incubation, cells were harvested by centrifugation and ruptured by passage though a French pressure cell in 100 mM Tris-HCl buffer (pH 7.5). The cell lysate was centrifuged at 15,000 × *g*, and the supernatant was collected. Streptomycin was added to a final concentration of 1% to the supernatant, and the resultant supernatant was incubated on ice for 10 min and centrifuged at 15,000 × *g* for 15 min to remove nucleic acids. The protein concentration was measured by a protein assay kit with bovine serum albumin as the standard (Bio-Rad, Hercules, Calif.).

**SDS-PAGE and N-terminal amino acid sequencing.** The expression of the enzymes was examined by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE). To determine the N-terminal amino acid sequence the cell extract was subjected to SDS-PAGE and was electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad). The enzyme band was cut out and analyzed on a PPSQ-21 protein sequencer (Shimadzu Co., Kyoto, Japan).

**Enzyme assay.** Substrate was added to a final concentration of 500 μM to 1 ml of 100 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM CoA, 2.5 mM $MgSO_4$, 2.5 mM ATP, and crude extract of *E. coli* JM109 harboring pKHR201 carrying the *ferBA* genes (400 μg of protein). After incubation for 3 min at 25°C, the mixture was extracted and analyzed by GC-MS as described above. The ferulic

TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Reference or source |
|---|---|---|
| Strains | | |
| *S. paucimobilis* | | |
| SYK-6 | Wild type; Nal$^r$ Sm$^r$ | 11 |
| FA2 | A Tn*5* mutant of SYK-6, vanillic acid$^+$ and ferulic acid deficient | This study |
| FAK | Mutant derivative of SYK-6; Km$^r$ gene insertion mutant of *ferA;* Nal$^r$ Sm$^r$ Km$^r$ | This study |
| FBK | Mutant derivative of SYK-6; Km$^r$ gene insertion mutant of *ferB;* Nal$^r$ Sm$^r$ Km$^r$ | This study |
| FB2K | Mutant derivative of SYK-6; Km$^r$ gene insertion mutant of *ferB2;* Nal$^r$ Sm$^r$ Km$^r$ | This study |
| *E. coli* | | |
| JM109 | *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*$^q$ *lacZ*ΔM15] | 35 |
| S17-1 | *recA;* harboring the *tra* genes of plasmid RP4 in the chromosome, *proA thi* | 31 |
| HB101 | *supE44 hsdS20*($r_B^-$ $m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* | 4 |
| Plasmids | | |
| pSUP5011 | pBR325::Tn*5-mob* Ap$^r$ Km$^r$ Cm$^r$ | 31 |
| pVK100 | Broad-host-range cosmid vector, Km$^r$ Tet$^r$ | 5 |
| pRK2013 | Km$^r$ Tra$^+$ Mob$^+$ | 6 |
| pTS1210 | Broad-host-range vector, pSa *ori* pBR *ori,* Km$^r$ Amp$^r$ | T. Nakazawa |
| pTS1210MCS | pTS1210 with multicloning site from pUC19 into *Eco*RI and *Hin*dIII sites | This study |
| pBluescript II KS(+) and SK(+) | Cloning vectors; Ap$^r$ | 30 |
| pUC18 | Cloning vector; Ap$^r$ | 35 |
| pUC4K | Ap$^r$ Km$^r$ | 32 |
| pK19*mobsacB* | *oriT sacB* Km$^r$ | 29 |
| pKYO2 | pVK100 with an approx 20-kb fragment carrying *ferBA* | This study |
| pBAB3 | SK(+) with a 5.2-kb *Bam*HI-*Eco*RI fragment of pKYO2 | This study |
| pBAB19 | SK(+) carrying the same fragment as pBAB3 in the opposite direction | This study |
| pKB4 | KS(+) carrying the same fragment as pBAB3 in the opposite direction | This study |
| pKH128 | Deletion derivative of pBAB3 | This study |
| pKHR201, pKHR128, pKHR126, pKHR213 | Deletion derivatives of pKB4 | This study |
| pKHR201E | pKHR201; a 2.7-kb *Eco*RI fragment in the insert was deleted | This study |
| pAH41 | KS(+) with a 1.2-kb DNA fragment carrying *ferB2* | This study |
| pUCBam | pUC18 with a 5.2-kb *Bam*HI-*Eco*RI fragment from pBAB3 | This study |
| pUCBamKm | pUCBam with insertion of the Km$^r$ gene from pUC4K into an *Apa*I site | This study |
| pSCFA | pK19*mobsacB* with a 6.5-kb *Bam*HI fragment from pUCBamKm | This study |
| pXESK | SK(+) with a 1.0-kb *Xho*I-*Eco*RI fragment from pKHR201 | This study |
| pXESKKm | pXESK with insertion of the Km$^r$ gene from pUC4K into a *Sal*I site | This study |
| pSCFB | pK19*mobsacB* with a 2.3-kb *Kpn*I-*Eco*RI fragment from pXESKKm | This study |
| pKS7E2 | KS(+) with a 7.0-kb *Eco*RI fragment carrying *ligW* and *ferB2* | 22 |
| pKS7E2Km | pKS7E2 with insertion of the Km$^r$ gene from pUC4K into a *Bst*XI site | This study |
| pSCFB2 | pK19*mobsacB* with an 8.3-kb *Eco*RI fragment from pKS7E2Km | This study |
| pBABX | pTS1210MCS with a 4.0-kb *Xho*I-*Eco*RI fragment carrying *ferA* | This study |

acid transformation activity (FerA activity) was estimated by the decrease in the amount of the substrate. The total activity of FerA and FerB was estimated by the increase in the amount of product generated by the successive reactions catalyzed by FerA and FerB.

To examine the metal ion dependency of FerA, EDTA or a metal salt solution containing either $MgSO_4 \cdot 7H_2O$, $Na_2SO_4 \cdot 10H_2O$, $FeSO_4 \cdot 7H_2O$, $Fe_2(SO_4)_3 \cdot nH_2O$, $CuSO_4 \cdot 5H_2O$, $CaSO_4 \cdot 2H_2O$, $CoSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, or $MnSO_4 \cdot 4H_2O$ was added to a final concentration of 5 mM for EDTA or 2.5 mM for the metal salt solution to 1 ml of the reaction mixture. Ferulic acid was added to a final concentration of 500 μM to initiate the reaction, and feruloyl-CoA synthetase activity (FerA activity) was determined by measuring the absorbance at 352 nm derived from the generation of feruloyl-CoA with a DU-7500 spectrophotometer (Beckman, Fullerton, Calif.).

**DNA manipulation and nucleotide sequencing.** DNA manipulations were carried out essentially as described elsewhere (2, 26). A Kilosequence kit (Takara Shuzo Co., Ltd., Kyoto, Japan) was used to construct a series of deletion derivatives. The nucleotide sequences were determined by the dideoxy termination method (28) with an ALFexpress DNA sequencer (Pharmacia Biotech, Milwaukee, Wis.). A Sanger reaction was carried out by using a Thermosequenase fluorescence-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). Sequencing analysis and homology alignment were carried out with the GeneWorks programs (IntelliGenetics, Inc., Mountain View, Calif.). Multiple sequence alignment was produced by using the program CLUSTAL W, and the phylogenetic tree was inferred from the alignments by using the neighbor-joining method (25). Graphics for phylogenetic trees were produced by using the TreeView program (19). The DDBJ databases were used for searching homologous proteins. Southern hybridization analysis was done with the DIG System (Roche Molecular Biochemicals, Mannheim, Germany).

**Insertional inactivation of the ferulic acid catabolic genes.** To disrupt each *ferA*, *ferB*, and *ferB2* in SYK-6, the kanamycin resistance gene from pUC4K was inserted in each of the three genes by the gene replacement technique. The *fer* gene disrupted plasmids by using pK19*mobsacB* as a vector, and pSCFA, pSCFB, and pSCFB2 were introduced into *E. coli* S17-1 and then introduced into SYK-6 by conjugation. The kanamycin-resistant transformants were selected on an LB agar plate containing 50 mg of kanamycin/liter and 25 mg of nalidixic acid/liter. They were cultured for 12 h in LB liquid medium containing 10% sucrose. The candidates for mutants were isolated on an LB agar plate containing 10% sucrose and kanamycin in order to select the cells in which the *sacB*-containing vector portion was deleted by a double crossover.

To examine the disruption of each gene, Southern hybridization analysis was carried out. The total DNA of the candidates for *ferA*, *ferB*, and *ferB2* mutants were digested with *Eco*RI, *Bam*HI and *Eco*RI, and *Sal*I, respectively. The 1.3-kb *Sal*I fragment with kanamycin resistance, the 3.0-kb *Eco*RI fragment carrying *ferA*, the 1.0-kb *Xho*I-*Eco*RI fragment carrying *ferB*, and the 2.6-kb *Sal*I fragment carrying *ferB2* were labeled with the DIG System and were used as probes.

**Nucleotide sequence accession numbers.** The nucleotide sequences of *ferBA* and *ferB2* have been deposited in the DDBJ, EMBL, and GenBank sequence databases under accession number nos. AB072376 and AB072377, respectively.

## RESULTS

**Cloning of the ferulic acid catabolic genes.** To determine the ferulic acid catabolic pathway, *S. paucimobilis* SYK-6 was grown in 10 ml of W medium containing 10 mM ferulic acid for 12 h. The culture was acidified and extracted with ethyl acetate. GC-MS analysis of the trimethylsilyl (TMS) derivative of the metabolite showed the production of a compound with a retention time of 23.4 min (data not shown). Its retention time and the mass spectrum corresponded to those of authentic TMS-vanillic acid. This result suggests that the two carbons of ferulic acid side chain were eliminated by SYK-6.

Tn*5* mutagenesis with pSUP5011 was used to obtain the insertion mutant of SYK-6, which was unable to degrade ferulic acid. Tn*5* mutants of SYK-6 were subsequently screened for the ability to grow on vanillic acid, but not on ferulic acid, as the sole carbon and energy source. A mutant strain, designated FA2, was obtained. GC-MS analysis indicated that this strain completely lost the ability to degrade ferulic acid. FA2 was used for a complementation test to isolate a wild-type copy of the mutated gene(s). The pVK100 cosmid library carrying the partial *Sal*I-digested fragments of SYK-6 total DNA was introduced from *E. coli* HB101 to FA2 by triparental mating. Four cosmids, each containing approximately 20- to 30-kb DNA fragments, were obtained as they complemented the growth deficiency of FA2 on ferulic acid. A subcloning experiment of a cosmid, pKYO2, indicated that a 5.2-kb *Bam*HI-*Eco*RI fragment included in all the cosmids was responsible for this complementation.

We identified the reaction product of ferulic acid incubated with the cell extract of *E. coli* JM109 harboring pBAB3 carrying the 5.2-kb *Bam*HI-*Eco*RI fragment by GC-MS. A gas chromatogram of the TMS derivatives of the reaction product showed a peak with a retention time of 16.2 min. The retention time and mass spectrum of this compound corresponded to those of a TMS derivative of authentic vanillin (data not shown). Gasson et al. (7) and Overhage et al. (18) have reported that two enzymes, feruloyl-CoA synthetase and feruloyl-CoA hydratase/lyase, are involved in the sequential reactions for the conversion of ferulic acid to vanillin in *P. fluorescens* AN103 and *Pseudomonas* sp. strain HR199, respectively. Feruloyl-CoA synthetase catalyzes the conversion of ferulic acid to feruloyl-CoA, which has an absorption maximum at approximately 350 nm at pH 7.5, in the presence of CoA, ATP, and $Mg^{2+}$. We confirmed the increase in absorbance at 352 nm of the reaction mixture with 100 mM Tris-HCl buffer (pH 7.5) containing ferulic acid and the cell extract of *E. coli* harboring pBAB3 only in the presence of CoA, ATP, and $Mg^{2+}$. These results suggested that the 5.2-kb *Bam*HI-*Eco*RI fragment encodes both feruloyl-CoA synthetase and feruloyl-CoA hydratase/lyase.

**Nucleotide sequence of the feruloyl-CoA synthetase and feruloyl-CoA hydratase/lyase genes.** A series of subclones of the 5.2-kb *Bam*HI-*Eco*RI fragment was generated by using restriction enzymes and exonuclease III. In order to map the region involved in ferulic acid degradation, the ferulic acid transformation activity and vanillin formation ability from ferulic acid were determined in the cell extract of each *E. coli* JM109 harboring a deletion plasmid (Fig. 2A). Deletion of the 1.2-kb region from the 5′ end of the 5.2-kb *Bam*HI-*Eco*RI fragment resulted in a deficiency of vanillin formation ability (pKHR128). However, this mutant retained the ferulic acid transformation activity. Further deletion (pKHR213) resulted in the complete loss of the ferulic acid transformation activity. *E. coli* harboring pBAB19, which carries the same fragment as pBAB3 but in the opposite direction, showed no vanillin formation activity and showed 23% of the ferulic acid transformation activity of the recombinant containing pBAB3. These results suggest that there are feruloyl-CoA synthetase and feruloyl-CoA hydratase/lyase genes carried by pKHR201. The direction of the transcription of these genes is the same as that of the *lac* promoter in pKHR201, and the feruloyl-CoA hydratase/lyase gene is located upstream of the feruloyl-CoA synthetase gene.

The nucleotide sequence of the insert DNA of pKHR201 was determined, and two open reading frames (ORFs) consisting of 852 and 2,127 bp, designated *ferB* and *ferA*, respectively, were found (Fig. 2A). A homology search of the DDBJ database revealed that the deduced amino acid sequence of *ferB* showed 40 to 48% identity with those of the feruloyl-CoA hydratase/lyase genes of the ferulic acid degraders *P. fluorescens* AN103 (ORFA) (7), *Pseudomonas* sp. strain HR199 (Ech) (18), *P. putida* WCS358 (Fca) (33), and *Amycolatopsis* sp. strain HR167 (1). On the other hand, the deduced amino acid sequence of *ferA* showed no significant similarities with those of the feruloyl-CoA synthetase genes of *Pseudomonas* sp. HR199 and *Amycolatopsis* sp. strain HR167. In addition, the FerA amino acid sequence did show 31% identity with pimeloyl-CoA synthetase of *Pseudomonas mendocina* 35, which is involved in biotin synthesis (3).

**Identification of the *ferB* homolog.** Recently we characterized the 5-carboxyvanillic acid decarboxylase gene (*ligW*) of *S. paucimobilis* SYK-6 (22), which is involved in the degradation of a lignin-related biphenyl compound, 5,5′-dehydrodivanillic acid (20, 21). The nucleotide sequencing of the 7.0-kb *Eco*RI fragment of pKS7E2 carrying *ligW* revealed a divergent transcribed ORF of 831 bp, which was located 575 bp upstream of *ligW* (Fig. 2B). The deduced amino acid sequence of this ORF revealed a 49% identity with that of *ferB* and was designated as *ferB2*.

**Expression of *ferA*, *ferB*, and *ferB2* in *E. coli*.** The *ferA* gene expression induced by IPTG in *E. coli* JM109 was examined. A 70-kDa protein was observed by SDS-PAGE of the cell extracts from *E. coli* that harbored pKHR201 carrying *ferBA* and that harbored pKHR126 carrying *ferA* (data not shown). The size of the protein was in good agreement with the value calculated from the deduced amino acid sequence of *ferA* (73,778 Da). N-terminal amino acid sequencing of the 70-kDa protein revealed the sequence TVEAGVRPQAGARDINRL, which corresponded to the deduced N-terminal amino acid sequence of *ferA*, except for the first Met, which appeared to be processed.

The *ferB* and *ferB2* gene expressions induced by IPTG in *E. coli* JM109 were examined with pKHR201 and pKHR201E carrying *ferB* and with pAH41 carrying *ferB2*, respectively. SDS-PAGE showed the expression of 34- and 32-kDa proteins in *E. coli* carrying *ferB* and *ferB2*, respectively (data not shown). These sizes are in good agreement with the value calculated from the deduced amino acid sequences of *ferB* (32,463 Da) and *ferB2* (30,828 Da). N-terminal amino acid sequencing of

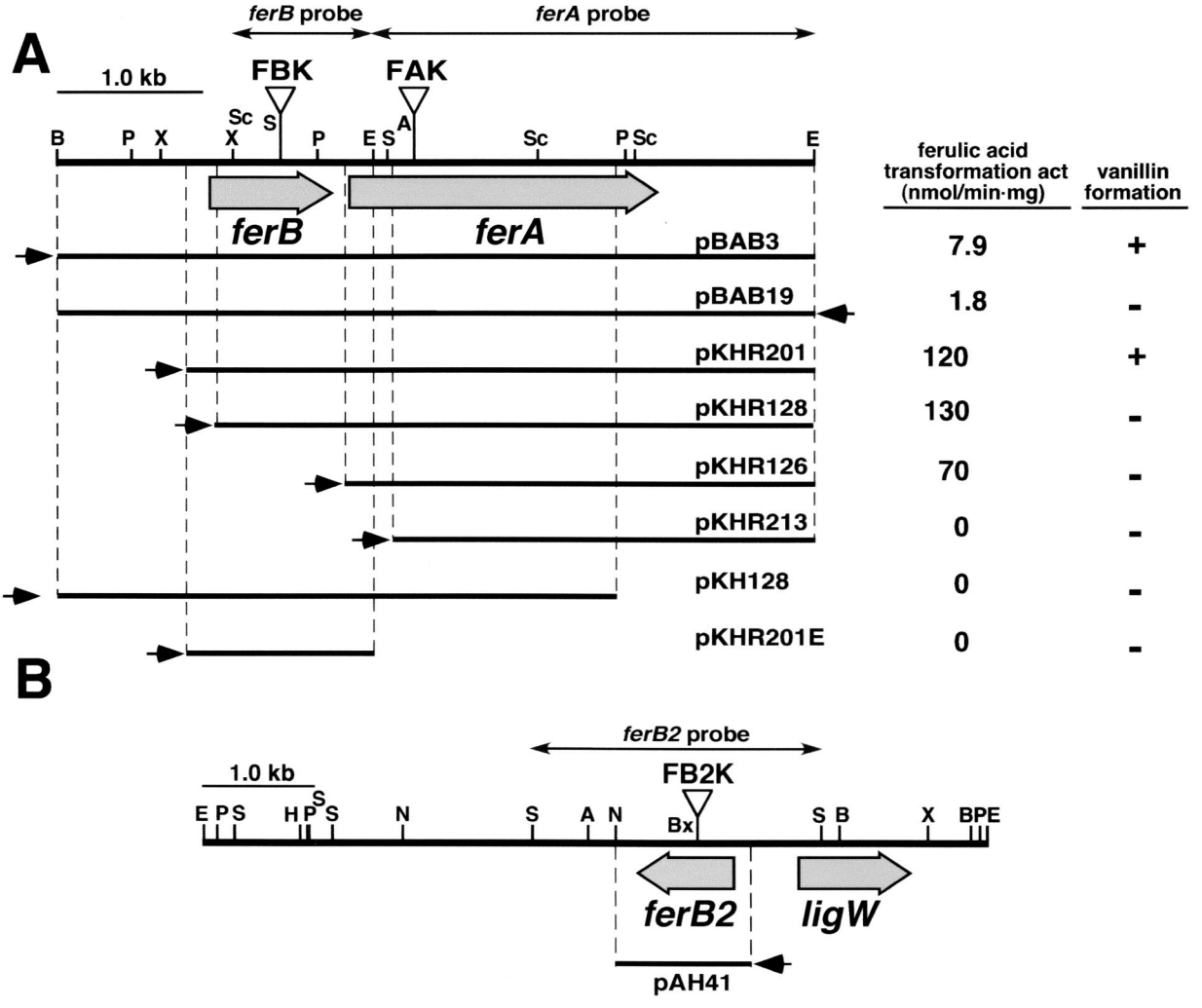


FIG. 2. Deletion analysis of the 5.2-kb *Bam*HI-*Eco*RI fragment carrying *ferBA* (A) and the restriction map of the 7.0-kb *Eco*RI fragment carrying *ferB2* and *ligW* (B). The ferulic acid transformation activity and the ability to generate vanillin from ferulic acid of *E. coli* JM109 harboring each plasmid are presented on the right-hand side of panel A. Small arrows indicate the direction of transcription from the *lac* promoter. Triangles indicate the positions of the $Km^r$ gene insertion of the *ferA* mutant (FAK), *ferB* mutant (FBK), and *ferB2* mutant (FB2K). Double-headed arrows indicate the probes used for Southern hybridization analysis of the *fer* gene mutants. The following genes (and their products) were used: *ferA*, feruloyl-CoA synthetase; *ferB* and *ferB2*, feruloyl-CoA hydratase/lyase; *ligW*, 5-carboxyvanillic acid decarboxylase. Abbreviations: A, *Apa*I; B, *Bam*HI; Bx, *Bst*XI; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; X, *Xho*I.

these proteins was carried out. Only the sequence for a 32-kDa protein expressed in *E. coli* harboring pAH41 was determined: SDELTXETVXXTLDDGIA. This sequence corresponded to the deduced amino acid sequence of *ferB2*. The first Met of FerB2 also appeared to be processed.

**Substrate specificities of FerA, FerB, and FerB2.** The feruloyl-CoA synthetase activity of *E. coli* harboring pKHR126 was detected only in the presence of 0.2 mM CoA, 2.5 mM $Mg^{2+}$, and 2.5 mM ATP. Replacement of $Mg^{2+}$ in the reaction mixture with 2.5 mM of $Mn^{2+}$ or $Co^{2+}$ also showed 94 or 39% of activity obtained with 2.5 mM $Mg^{2+}$, respectively. However, FerA had no activity in the presence of $Cu^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Zn^{2+}$, and $Ca^{2+}$.

To determine the substrate specificity of FerA and FerB, 0.5 mM cinnamic acid derivatives were incubated with the cell extract of *E. coli* JM109 harboring pKHR201 carrying *ferBA* in the presence of CoA, ATP, and $Mg^{2+}$. The amount of the substrate remained, and the corresponding benzaldehyde formed was estimated by GC-MS. FerA transformed ferulic acid, *p*-coumaric acid, caffeic acid, and sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) at a similar rate, and 4-methoxycinnamic acid was also transformed at half the rate of ferulic acid transformation (Table 2). Formation of the FerB reaction products, 4-hydroxybenzaldehyde, protocatechualdehyde, and syringaldehyde, was confirmed in the reaction mixtures from *p*-coumaric acid, caffeic acid, and sinapinic acid, respectively. The formation rates of these benzaldehydes from cinnamic acid derivatives were at almost the same level (approximately 20 nmol/min·mg of protein). However, the formation of 4-methoxybenzaldehyde from 4-methoxycinnamic acid was not observed. The substrate specificity of FerB2 was also determined by the incubation of cinnamic acid derivatives with the cell extracts of *E. coli* harboring pAH41 carrying *ferB2* and *E. coli* containing pKHR126 carrying *ferA*. Formation of van-

TABLE 2. Substrate specificity of FerA[a]

| Substrate | Transformation activity ± SD (nmol/min·mg) |
|---|---|
| Ferulic acid | 120 ± 14 |
| Caffeic acid | 99 ± 17 |
| Sinapinic acid | 130 ± 25 |
| Cinnamic acid | 0 |
| *p*-Coumaric acid | 110 ± 23 |
| *o*-Coumaric acid | 0 |
| *m*-Coumaric acid | 0 |
| 3-Methoxycinnamic acid | 0 |
| 4-Methoxycinnamic acid | 58 ± 6 |

[a] Cinnamic acid derivatives (0.5 mM) were incubated with the cell extract of *E. coli* JM109 harboring pKHR201 carrying *ferBA* in the presence of 0.2 mM CoA, 2.5 mM ATP, and 2.5 mM $Mg^{2+}$. *E. coli* JM109 harboring pBluescript II KS(+) showed no transformation activity toward the substrates tested.

illin, *p*-hydroxybenzaldehyde, protocatechualdehyde, and syringaldehyde from the corresponding cinnamic acid derivatives were found, thus suggesting a substrate specificity of FerB2 similar to that of FerB.

**Characterization of the insertion mutants of the *ferA*, *ferB*, and *ferB2* genes.** In order to examine the actual roles of the *ferA*, *ferB*, and *ferB2* genes in the ferulic acid catabolism of *S. paucimobilis* SYK-6, these genes were inactivated by the insertion of kanamycin resistance genes by using the gene replacement technique based on homologous recombination (Fig. 2). The plasmids pSCFA, pSCFB, and pSCFB2, which contain the disrupted *ferA*, *ferB*, and *ferB2* genes, respectively, were constructed (Table 1) and were introduced into SYK-6. The disruption of each gene was confirmed by Southern hybridization analysis by using each *fer* gene (Fig. 2) and the kanamycin resistance gene as probes (data not shown). The resulting *ferA* mutant (FAK) and *ferB* mutant (FBK) were unable to grow on ferulic acid, although they were able to grow on vanillic acid. However, it was found that FBK also lost the transformation activity of ferulic acid. This may have been due to a polar effect caused by the insertion of a kanamycin resistance gene in *ferB*. Introduction of pBABX carrying *ferA* in pTS1210MCS into FAK and FBK restored the growth of both strains on ferulic acid. These results indicate that the *ferA* gene is essential to the growth of *S. paucimobilis* SYK-6 on ferulic acid. In contrast, the *ferB* gene appears not to be essential to such growth. On the other hand, the disruption of the *ferB2* gene did not affect the growth of the mutant (FB2K) on ferulic acid.

## DISCUSSION

For some *Pseudomonas* strains and a gram-positive *Amycolatopsis* strain, ferulic acid has been shown to degrade to vanillin by the consecutive enzyme reactions catalyzed by feruloyl-CoA synthetase and feruloyl-CoA hydratase/lyase (1, 7, 18, 33). *S. paucimobilis* SYK-6 has similar yet different enzyme systems for the degradation of ferulic acid. We found the *ferBA* genes, which restored the growth deficiency of the Tn*5* mutant of SYK-6, FA2, on ferulic acid; we also found the *ferB* homolog, *ferB2*, which was located upstream of *ligW*. The gene organization of *ferB* and *ferA* and the short intergenic distance (120 bp) suggest that these two genes constitute an operon. This gene organization is the same as that of *Amycolatopsis* sp. strain HR167 (1). The organization of these genes differs from that of *Pseudomonas* sp. strain HR199. The feruloyl-CoA synthetase gene (*fcs*) and feruloyl-CoA hydratase/lyase gene (*ech*) are separately localized in HR199 (18). However, *ferB2* was located on a different locus in SYK-6. We determined the nucleotide sequence of the 7.0-kb *Eco*RI fragment carrying *ferB2* and *ligW* in order to demonstrate the existence of another feruloyl-CoA synthetase gene; however, no enzyme gene related to acyl-CoA synthetase was found (data not shown).

Three findings led to the conclusion that the *ferA* gene encodes feruloyl-CoA synthetase. (i) The reaction product of ferulic acid catalyzed by FerA showed a specific absorption at 352 nm (pH 7.5), which is a spectral feature of feruloyl-CoA (7). When the reaction was carried out in 100 mM Tris-HCl buffer (pH 8.5), the reaction mixture became yellowish in color and revealed an absorption at 400 nm (data not shown). This result is in good agreement with the properties of feruloyl-CoA (15). (ii) The feruloyl-CoA synthetase activity was observed only when CoA, ATP, and $Mg^{2+}$ were present. (iii) The deduced amino acid sequence of *ferA* was similar to that of an acyl-CoA synthetase gene, the pimeloyl-CoA synthetase gene (*pauA*) of *P. mendocina* 35 (3). The successful cloning of the feruloyl-CoA synthetase gene (*fcs*) was reported for *Pseudomonas* sp. strain HR199 (18) and *Amycolatopsis* sp. strain HR167 (1). The deduced amino acid sequence of the *fcs* genes showed similarity with those of the AMP-forming acyl-CoA synthetase genes. Interestingly, the deduced amino acid sequence of *ferA* did not show significant similarity to those of *fcs* and other AMP-forming acyl-CoA synthetase genes. Recently, Sánchez et al. (27) proposed a new superfamily of nucleoside diphosphate (NDP)-forming acyl-CoA synthetases. This superfamily contains pimeloyl-CoA synthetase, acetyl-CoA synthetase (ADP forming), succinyl-CoA synthetase, (both ADP and GDP forming), malyl-CoA synthetase, and ATP citrate lyase. BLAST searches revealed that the N-terminal (amino acid positions 1 to 532) and C-terminal (amino acid positions 457 to 709) amino acid sequences of FerA had 29% identity in a 457-amino-acid overlap and 37% identity in a 222-amino-acid overlap with the α-subunit (AcdA) and β-subunit (AcdB), respectively, of *Pyrococcus furiosus* acetyl-CoA synthetase (ADP forming) (16). These results suggest that FerA is an ADP-forming acyl-CoA synthetase and that the origin of *ferA* differs completely from that of the *fcs* genes of HR199 and HR167. In the case of NDP-forming acyl-CoA synthetases, a His residue of the enzyme is phosphorylated by nucleoside triphosphates to release NDP. This phosphoryl group transfers to the substrate, and then the phosphoryl group is replaced by CoA. On the basis of the alignment of the amino acid sequence of these enzymes with that of the α-subunit of succinyl-CoA synthetase of *E. coli*, which has been biochemically characterized, the His residue to be phosphorylated in the reaction was implicated. The amino acid sequence alignment of FerA with ADP-forming acyl-CoA synthetases suggested that His270 of FerA was phosphorylated during the reaction.

For the following reasons it was concluded that the *ferB* and *ferB2* genes encode feruloyl-CoA hydratase/lyase. (i) Vanillin was produced from ferulic acid by the addition of FerB or FerB2 to the reaction mixture containing FerA. (ii) The deduced amino acid sequences of *ferB* and *ferB2* were similar to those of the feruloyl-CoA hydratase/lyase genes of *P. fluore-*

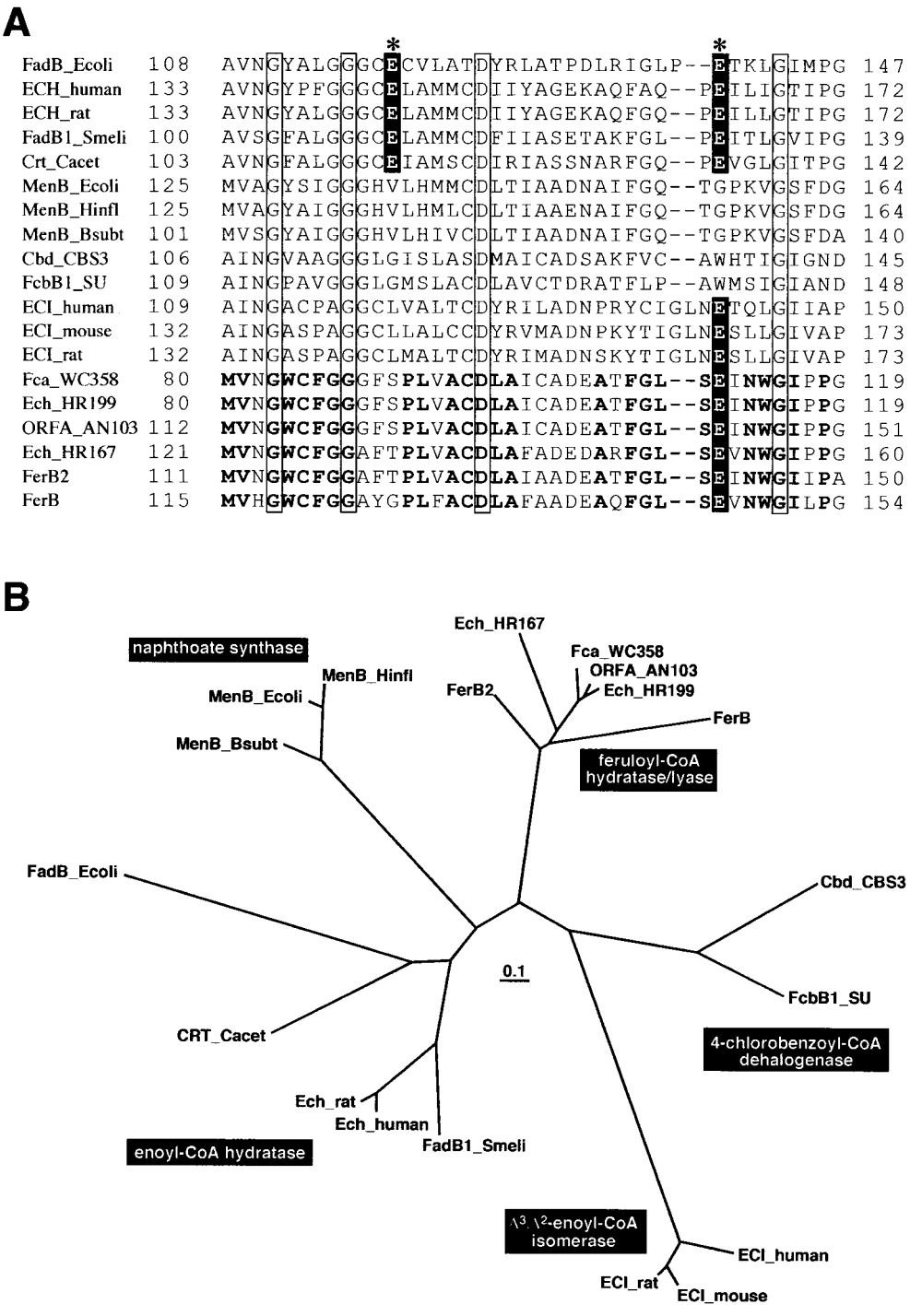


FIG. 3. Comparison of the amino acid sequences of FerB and FerB2 with those of the enoyl-CoA hydratase/isomerase superfamily. (A) Partial amino acid sequence alignment of the region including the catalytic sites of *E. coli* FadB with the corresponding regions of the enoyl-CoA hydratase/isomerase superfamily. The N-terminal domain of FadB (α-subunit of the multienzyme complex of *E. coli*) showed enoyl-CoA hydratase and $\Delta^3,\Delta^2$-enoyl–CoA isomerase activities. Two Glu residues, indicated by asterisks, have been implicated as the catalytic sites for enoyl-CoA hydratase (10). Glu residues aligned with these catalytic Glu are offset by a black background. Amino acids conserved among all of the sequences are shown in squares. Boldface roman type indicates the conserved residues among all of the feruloyl-CoA hydratases/lyases. (B) Phylogenetic tree of FerB and FerB2 with the enoyl-CoA hydratase/isomerase superfamily. The scale corresponds to a genetic distance of 0.1 substitution per position (10% difference). Accession numbers for the sequences are as follows: FadB_Ecoli (to residue 285), P21177; ECH_human, short-chain enoyl-CoA hydratase of human mitochondria (D13900-1); ECH_rat, enoyl-CoA hydratase precursor of rat mitochondria (S06477); FadB1_Smeli, enoyl-CoA hydratase of *Sinorhizobium meliloti* (L39265); Crt_Cacet, 3-hydroxybutyryl–CoA dehydratase of *Clostridium acetobutylicum* ATCC824 (P52046); MenB_Ecoli, naphthoate synthase of *E. coli* (P27290); MenB_Hinfl, naphthoate synthase of *Haemophilus influenzae* Rd (U32777); MenB_Bsubt, naphthoate synthase of *Bacillus subtilis* (F69656); Cbd_CBS3, 4-chlorobenzoate dehalogenase of *Pseudomonas* sp. CBS-3 (A42560); FcbB1_SU, 4-chlorobenzoyl–CoA dehalogenase of *Arthrobacter* sp. SU (M93187); ECI_human, $\Delta^3,\Delta^2$-enoyl–CoA isomerase of human (L24774); ECI_mouse, $\Delta^3,\Delta^2$-enoyl–CoA isomerase of mouse (Z14049); ECI_rat, $\Delta^3,\Delta^2$-enoyl–CoA isomerase of rat (X61184); Fca_WCS358, ferulic acid hydratase of *P. putida* WCS358 (Y14772); Ech_HR199, enoyl-CoA hydratase of *Pseudomonas* sp. HR199 (Y11520); ORFA_AN103, *p*-hydroxycinnamoyl–CoA hydratase/lyase of *P. fluorescens* AN103 (Y13067); Ech_HR167, enoyl-CoA hydratase of *Amycolatopsis* sp. HR167 (AJ290449); FerB2, feruloyl-CoA hydratase/lyase of *S. paucimobilis* SYK-6; FerB, feruloyl-CoA hydratase/lyase of *S. paucimobilis* SYK-6.

*scens* AN103, *P. putida* WCS358, *Pseudomonas* sp. strain HR199, and *Amycolatopsis* sp. strain HR167. Gasson et al. (7) and Overhage et al. (18) reported that ORFA and Ech belong to a superfamily that includes enoyl-CoA hydratase, which is involved in fatty acid β-oxidation; this superfamily also includes a number of other proteins that catalyze, or are assumed to catalyze, the related reactions of CoA thiolesters. The two catalytic Glu residues have been implicated in the α-subunit (FadB) of the multienzyme complex of fatty acid oxidation from *E. coli*, which shows enoyl-CoA hydratase activity (10). The protonated Glu139 of FadB transfers a proton to the α-carbon of 2-*trans*-enoyl-CoA, and the deprotonated Glu119 attracts a proton from water, the oxygen of which launches a nucleophilic attack on the β-carbon of the substrate. Glu119 and Glu139 are well conserved in enoyl-CoA hydratase (Fig. 3A). As indicated by Gasson et al. (7), only a Glu residue corresponding to Glu139 of *E. coli* FadB is conserved among all of the feruloyl-CoA hydratases/lyases, including FerB (Glu146) and FerB2 (Glu142). These Glu residues may involve the transfer of a proton to the substrate in the feruloyl-CoA hydratase/lyase family. The phylogenetic tree of this superfamily indicated that FerB, FerB2, ORFA, Ech, and Fca consist of a subgroup of feruloyl-CoA hydratase/lyase (Fig. 3B). However, both FerB and FerB2 are rather distant from *Pseudomonas* and *Amycolatopsis* feruloyl-CoA hydratases/lyases.

There are few reports discussing the substrate specificity of the feruloyl-CoA synthetase for various cinnamic acid derivatives. Only one example of this enzyme is available for *P. putida* (36). This enzyme showed activity in the presence of ferulic acid, *p*-coumaric acid, and caffeic acid. In addition to these substrates, sinapinic acid, which had previously not been examined in *P. putida*, was converted to syringaldehyde by FerA and either FerB or FerB2 enzyme mixtures. Furthermore, a decrease in the amount of 4-methoxycinnamic acid by the FerA reaction was observed; however, the FerB reaction product, 4-methoxybenzaldehyde, was not detected. On the other hand, 3-methoxycinnamic acid and cinnamic acid did not serve as the substrate for FerA. These results suggest that certain functional groups, such as hydroxyl and methoxyl, at the *para* position of cinnamic acid derivatives are important for FerA enzyme activity. Viewed from the point of view of utilization of these enzymes for the biochemical conversion of cinnamic acid derivatives, the broad substrate spectra of SYK-6 feruloyl-CoA synthetase and feruloyl-CoA hydratase/lyase are potentially advantageous.

In order to elucidate the roles of these *fer* genes, each *fer* gene was disrupted by insertion of a kanamycin resistance marker gene. This technique revealed that the *ferA* gene was essential to ferulic acid degradation in this particular strain. On the other hand, *ferB* disruption also led to growth deficiency among the cells on ferulic acid. However, this growth deficiency was restored by the introduction of pTS1210MCS carrying the *ferA* gene, thereby indicating that the *ferB* gene was not essential to the growth on ferulic acid. The *ferB2* gene is most likely expressed in the wild-type strain during the degradation of ferulic acid, and its gene product potentially complements feruloyl-CoA hydratase/lyase activity in the *ferB* mutant (FBK).

## ACKNOWLEDGMENTS

We thank T. Nakazawa for providing pTS1210 and S. Kajita for helpful discussion.

## REFERENCES

1. **Achterholt, S., H. Priefert, and A. Steinbuchel.** 2000. Identification of *Amycolatopsis* sp. strain HR167 genes, involved in the bioconversion of ferulic acid to vanillin. Appl. Microbiol. Biotechnol. **54:**799–807.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1990. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
3. **Binieda, A., M. Fuhrmann, B. Lehner, C. Rey-Berthod, S. Frutiger-Hughes, G. Hughes, and N. M. Shaw.** 1999. Purification, characterization, DNA sequence and cloning of a pimeloyl-CoA synthetase from *Pseudomonas mendocina* 35. Biochem. J. **15:**793–801.
4. **Bolivar, F., and K. Backman.** 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. **68:**245–267.
5. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
6. **Figurshi, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
7. **Gasson, M. J., Y. Kitamura, W. R. McLauchlan, A. Narbad, A. J. Parr, E. L. H. Parsons, J. Payne, M. J. C. Rhodes, and N. J. Walton.** 1998. Metabolism of ferulic acid to vanillin. J. Biol. Chem. **273:**4163–4170.
8. **Gold, M. H., and M. Alic.** 1993. Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev. **57:**605–622.
9. **Hara, H., E. Masai, Y. Katayama, and M. Fukuda.** 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.
10. **He, X.-Y., and S.-Y. Yang.** 1997. Glutamate-119 of the large α-subunit is the catalytic base in the hydration of 2-*trans*-enoyl-coenzyme A catalyzed by the multienzyme complex of fatty acid oxidation from *Escherichia coli*. Biochemistry **36:**11044–11049.
11. **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.
12. **Masai, E., Y. Katayama, S. Nishikawa, and M. Fukuda.** 1999. Characterization of *Sphingomonas paucimobilis* SYK-6 genes involved in degradation of lignin-related compounds. J. Ind. Microbiol. Biotechnol. **23:**364–373.
13. **Masai, E., K. Momose, H. Hara, S. Nishikawa, Y. Katayama, and M. Fukuda.** 2000. Genetic and biochemical characterization of 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.
14. **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.
15. **Mitra, A., Y. Kitamura, M. J. Gasson, A. Narbad, A. J. Parr, J. Payne, M. J. C. Rhodes, C. Sewter, and N. J. Walton.** 1999. 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL), an enzyme of phenylpropanoid chain cleavage from *Pseudomonas*. Arch. Biochem. Biophys. **365:**10–16.
16. **Musfeldt, M., M. Selig, and P. Schönheit.** 1999. Acetyl coenzyme A synthetase (ADP forming) from the hyperthermophilic Archaeon *Pyrococcus furiosus*: identification, cloning, separate expression of the encoding genes, *acdAI* and *acdBI*, in *Escherichia coli*, and in vitro reconstitution of the active heterotetrameric enzyme from its recombinant subunits. J. Bacteriol. **181:**5885–5888.
17. **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 genes of *Pseudomonas paucimobilis*. J. Bacteriol. **172:**2704–2709.
18. **Overhage, J., H. Priefert, and A. Steinbüchel.** 1999. Biochemical and genetic analyses of ferulic acid catabolism in *Pseudomonas* sp. strain HR199. Appl. Environ. Microbiol. **65:**4837–4847.
19. **Page, R. D. M.** 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:**357–358.
20. **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.
21. **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.
22. **Peng, X., E. Masai, H. Kitayama, K. Harada, Y. Katayama, and M. Fukuda.** 2002. Characterization of the 5-carboxyvanillate decarboxylase gene and its

role in lignin-related biphenyl catabolism in *Sphingomonas paucimobilis* SYK-6. Appl. Environ. Microbiol. **68:**4407–4415.
23. **Priefert, H., J. Rabenhorst, and A. Steinbüchel.** 1997. Molecular characterization of genes of *Pseudomonas* sp. strain HR199 involved in bioconversion of vanillin to protocatechuate. J. Bacteriol. **179:**2595–2607.
24. **Rosazza, J. P., Z. Huang, L. Dostal, T. Volm, and B. Rousseau.** 1995. Review: biocatalytic transformations of ferulic acid: an abundant aromatic natural product. J. Ind. Microbiol. **15:**457–471.
25. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Sánchez, L. B., M. Y. Galperin, and M. Müller.** 2000. Acetyl-CoA synthetase from the amitochondriate eukaryote *Giardia lamblia* belongs to the newly recognized superfamily of Acyl-CoA synthetase (nucleotide diphosphate-forming). J. Biol. Chem. **275:**5794–5803.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Schäfer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Pühler.** 1994. Small mobilizable multipurpose 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.
30. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. λ ZAP: a bacteriophage lambda expression vector with in vivo excision properties. Nucleic Acids Res. **16:**7583–7600.
31. **Simon, R.** 1984. High frequency mobilization of gram-negative bacterial replicons by the in vitro constructed Tn*5*-mob transposon. Mol. Gen. Genet. **196:**413–420.
32. **Taylor, L. A., and R. E. Rose.** 1988. A correction in the nucleotide sequence of the Tn*903* kanamycin resistance determinant in pUC4K. Nucleic Acids Res. **16:**358.
33. **Venturi, V., F. Zennaro, G. Degrassi, B. C. Okeke, and C. V. Bruschi.** 1998. Genetics of ferulic acid bioconversion to protocatechuic acid in plant-growth-promoting *Pseudomonas putida* WCS358. Microbiology **144:**965–973.
34. **Vicuña, R.** 1988. Bacterial degradation of lignin. Enzyme Microbiol. Technol. **10:**646–655.
35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
36. **Zenk, M. H., B. Ulbrich, J. Busse, and J. Stöckigt.** 1980. Procedure for the enzymatic synthesis and isolation of cinnamoyl-CoA thioesters using a bacterial system. Anal. Biochem. **101:**182–187.