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Cloning and Sequencing of the Gene for a *Pseudomonas paucimobilis* Enzyme That Cleaves β-Aryl Ether

EIJI MASAI,[1*] YOSHIHIRO KATAYAMA,[1,2] SHINYA KAWAI,[1] SEIJI NISHIKAWA,[1]
MAKARI YAMASAKI,[3] AND NORIYUKI MOROHOSHI[1]

Laboratory of Wood Chemistry, Faculty of Agriculture, Tokyo Noko University, Fuchu, Tokyo 183,[1] Cooperative Research Center, Tokyo Noko University, Koganei,[2] and The University of Tokyo, Bunkyo-ku,[3] Tokyo, Japan

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We isolated *Pseudomonas paucimobilis* SYK-6, which was able to degrade various dimeric lignin compounds (Y. Katayama, S. Nishikawa, M. Nakamura, K. Yano, M. Yamasaki, N. Morohoshi, and T. Haraguchi, Mokuzai Gakkaishi 33:77–79, 1987). This metabolic process is a distinct characteristic of this bacterium, which is equipped with an enzymatic modification system for various dimeric lignin compounds involved in the tricarboxylic acid cycle. Cleavage of the β-aryl ether linkage is essential in this process, because this linkage is the most abundant (approximately 50%) in lignin. Here, we report the isolation and characterization of the β-etherase gene, which contains an open reading frame of 843 bp and which we call *ligE*. This gene was expressed in *Escherichia coli*, and the enzyme had the same kinetic properties as the *P. paucimobilis* SYK-6 enzyme.

Lignin is the most abundant aromatic material in the biosphere. It is a polymer constructed with phenylpropanoid units linked through a variety of nonhydrolyzable C—C and C—O—C bonds and is resistant to degradation by microorganisms. Several bacterial strains, however, can degrade model lignin compounds. Elucidation of lignin biodegradation is important because of its ecological significance. In a previous study, we isolated *Pseudomonas paucimobilis* SYK-6, which was able to grow on various dimeric lignin compounds, including arylglycerol-β-aryl ether, biphenyl, phenylcoumarane, pinoresinol, and diarylpropane, as the sole carbon source (6). The metabolism of various types of model lignin compounds in *P. paucimobilis* SYK-6 has been reported (Fig. 1) (4, 5). Protocatechuate 4,5-dioxygenase is a key enzyme in the process used to obtain metabolic energy from various structures of lignin (5). We isolated the protocatechuate 4,5-dioxygenase gene from this bacterium and determined its nucleotide sequence (12, 13). Cleavage of β-aryl ether is the most important process in lignin degradation by this bacterium because it is the most abundant linkage (approximately 50%). Detection of β-etherase activity in a cellular membrane fraction of *P. paucimobilis* SYK-6 was reported earlier (10); this enzyme catalyzed the unique and reductive cleavage of arylglycerol-β-aryl ether (C α carbonyl type) (compound I).

Here, we report the isolation and characterization of the β-etherase gene by a sensitive fluorescence assay. This is the first report of the cloning of a bacterial gene responsible for specific lignin degradation and offers an understanding of the major lignin degradation process in the biosphere at the molecular level.

MATERIALS AND METHODS

Model compounds. The chemical structures of the model compounds used in this study are shown in Fig. 1 and 2B. The synthesis of compounds I, IV, V (10), and 2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl (5) and the isolation of β-hydroxypropiovanillone (compound II) (10) were reported previously. Vanillin (compound III) was purchased from Tokyo Kasei Co.

Bacterial strains, plasmids, and genomic DNA library. *P. paucimobilis* IAM12578 (nalidixic acid resistant) was used as a host strain and had no β-aryl ether cleavage activity. *Escherichia coli* HB101, JM109, and MV1190 were used as host strains. A library of partially *Sal*I-digested chromosomal DNA from *P. paucimobilis* SYK-6 was constructed in cosmid vector pVK100 (kanamycin resistant and tetracycline resistant) (11). Helper plasmid pRK2013 was used as in the previous study (11). pUC19 and pUC18 were used as subcloning vehicles.

Cloning experiments. Recombinant plasmids in the genomic library were transferred to *P. paucimobilis* IAM12578 by the triparental mating method (1). Transconjugants of *P. paucimobilis* IAM12578 were inoculated on LB agar plates containing nalidixic acid (25 mg/liter) and kanamycin (25 mg/liter). Drug-resistant colonies were inoculated into a 96-well microplate in LB containing fluorescent β-etherase assay substrate V (150 μM) and kanamycin (25 mg/liter). The plasmid DNA (pBE10) from positive clones was isolated by the alkaline sodium dodecyl sulfate (SDS) lysis method (9). A subcloning experiment was carried out by cloning the partial *Sal*I (Takara Shuzo) digest of pBE10 into pUC19, and β-etherase-positive clones (containing pUBE13 and pUBE34) were isolated.

Preparation of cell extract. *E. coli* HB101 containing pBE10 was cultured in LB containing kanamycin (25 mg/liter). When the optical density at 550 nm (OD_{550}) of the culture reached 1.0, it was centrifuged. *E. coli* MV1190 containing pUBE13 or pUBE34 was cultured in LB containing ampicillin (50 mg/liter). When the OD_{550} of the culture reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 3 h, the culture was centrifuged. Cells from 100 ml of culture were homogenized with a mortar and pestle with aluminum oxide on ice. After 2 ml of 50 mM KH_2PO_4–NaOH buffer (pH 7.4) was added, the mixture was centrifuged at 15,000 rpm for 5 min to obtain a cell extract. Preparation of the cell extract of *P. paucimobilis* SYK-6 was described earlier (10).

Assay for β-etherase. The enzyme activity of the cell

* Corresponding author.

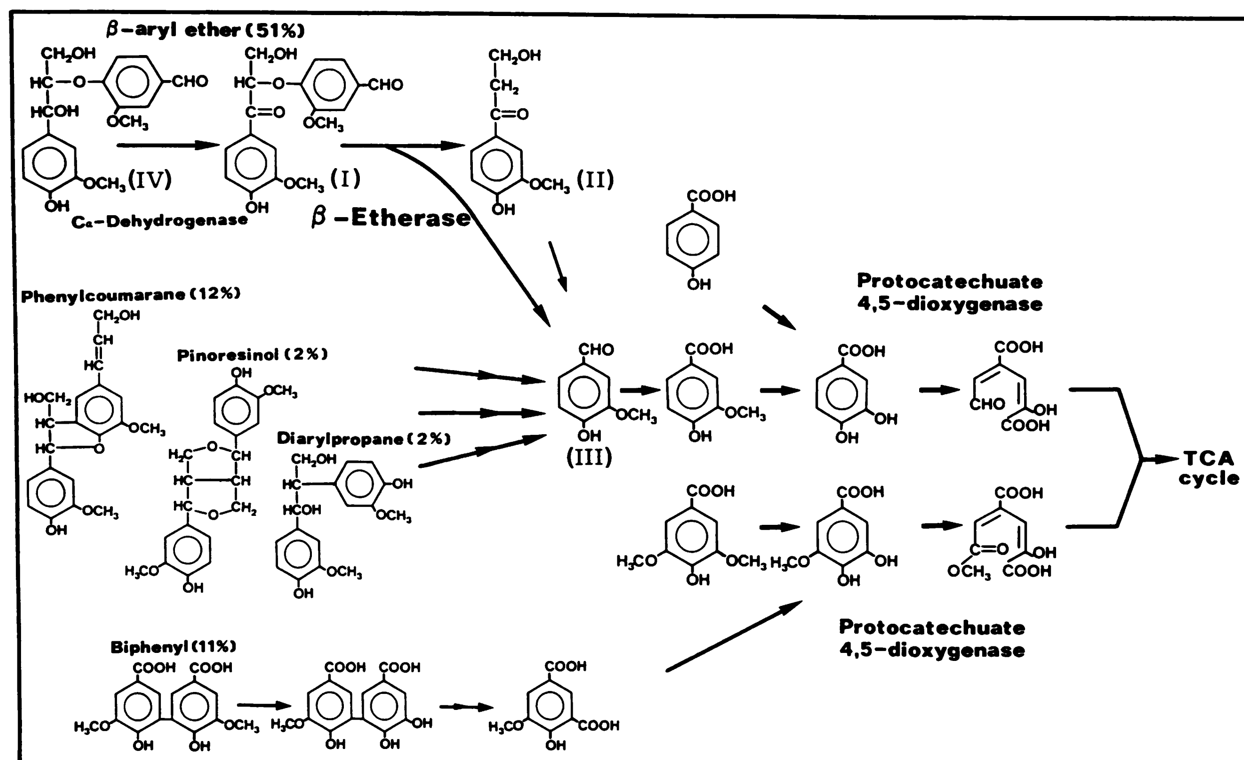

FIG. 1. Degradation pathway of various dimeric lignin compounds by *P. paucimobilis* SYK-6. *P. paucimobilis* SYK-6 could grow on these dimeric lignin compounds as sole carbon sources through the degradation process shown. The percentages are the ratio of each intermonomer linkage in native lignin (2). Roman numerals show the model lignin compounds used in this study. TCA, tricarboxylic acid.

extract was measured in a total volume of 1 ml, including 50 mM KH_2PO_4–NaOH buffer (pH 7.4), 1 mM dithiothreitol, 150 μM assay substrate (compound V dissolved in dimethyl sulfoxide), and enzyme preparation. The enzyme reaction was begun with the addition of substrate, and the reaction mixture was incubated at 28°C. The reaction was terminated by addition of 100 μl of the reaction mixture to 1.4 ml of 100 mM glycine–NaOH buffer (pH 10). The fluorescence of 4-methylumbelliferone released from the substrate was measured with excitation at 360 nm and emission at 450 nm with a fluorometric analyzer (Fluororead model 200; Ajinomoto). For the whole cells, 10 ml of culture at an OD_{550} of 1.0 was centrifuged and suspended in 1 ml of 50 mM KH_2PO_4–NaOH buffer (pH 7.4) containing 150 μM compound V, and the assay was carried out as described above.

Identification of metabolites by GC-MS. The method of identification of metabolites by gas chromatography-mass spectrometry (GC-MS; Shimadzu, QP-1000) was described earlier (5), except that this time an OV-1 capillary column was used.

Nucleotide sequence of the β-etherase gene. A Kilosequence kit (Takara Shuzo) was used to construct various deletion derivatives of pUBE13 and pUBE34. The nucleotide sequences of these deletion derivatives were determined by the dideoxy method (15) with Sequenase 2.0 (U.S. Biochemical Corp.). [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol) was from Amersham.

Amino acid sequence. The cell extract prepared from *E. coli* MV1190 containing pUBE13 was fractionated by salting out with ammonium sulfate. The 30 to 40% fraction was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide gel) (8), and proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore). The area at 32 kDa was cut out, and the amino acid sequence was determined with a 477A gas-phase amino acid sequencer (Applied Biosystems Inc.).

Nucleotide sequence accession number. The sequence reported in this article has been deposited in the GenBank, EMBL, and DDBJ data banks (accession number M69107).

RESULTS

Isolation of the β-etherase gene. About 2,000 transconjugants of *P. paucimobilis* IAM12578 were obtained. To obtain the β-etherase gene, the β-etherase activity of transconjugants was determined in 96-well microplates containing sensitive fluorescence assay substrate V. Three positive colonies producing fluorescence were obtained, and the plasmids were isolated; they all contained the same 24-kb *Sal*I fragment. We designated this plasmid pBE10. About 65% of compound V (150 μM) was cleaved within 10 min by whole cells of *P. paucimobilis* IAM12578 containing pBE10.

Expression of β-etherase gene in *E. coli* and characterization of the expression product. Whole cells of *E. coli* HB101 containing pBE10 could not cleave fluorescent substrate V effectively. However, the cell extract of this recombinant had the ability to cleave the β-ether linkage of fluorescent substrate V, indicating that this gene was expressed in *E. coli* (Table 1). The kinetic properties of the cell extract prepared from *E. coli* HB101 containing pBE10 were compared with those of *P. paucimobilis* SYK-6. GC-MS analysis of the reaction products of compound I with the cell extract

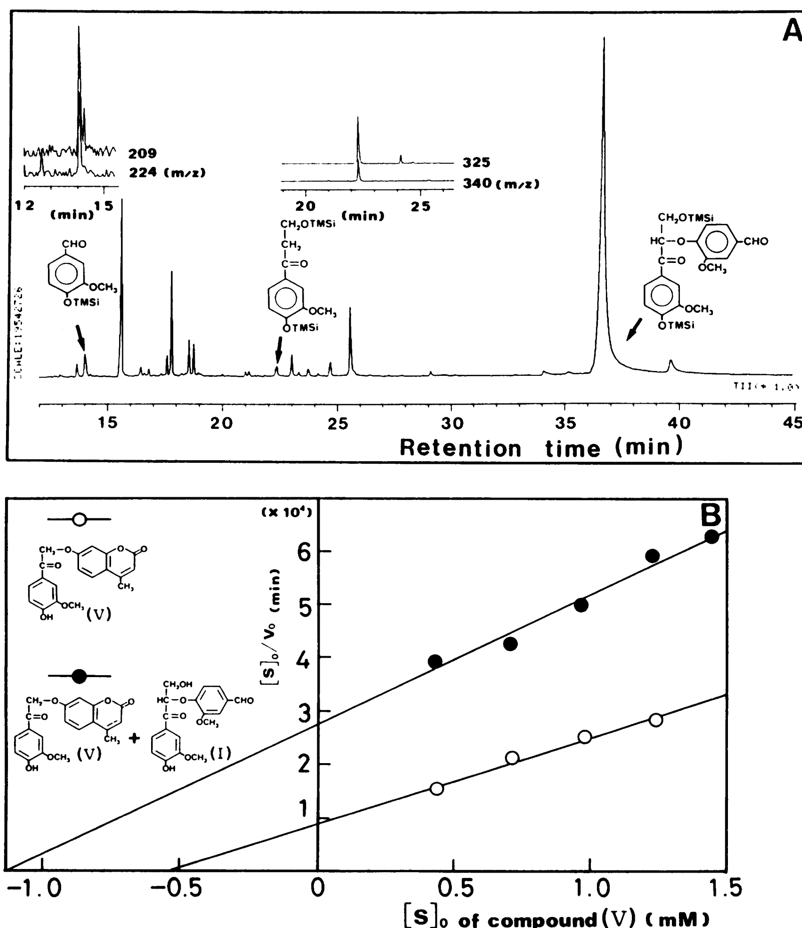

FIG. 2. Kinetic properties of β-etherase expressed in *E. coli* containing pBE10. (A) Gas chromatogram and mass chromatogram of degradation products from compound I by a cell extract prepared from *E. coli* HB101 containing pBE10. (B) Kinetic patterns obtained via plots of $[S]_0/V_0$ versus $[S]_0$ with the cell extract. The reaction mixture contained various concentrations of substrate V and 0.26 mM (●) or 0 mM (○) of compound I. The cleavage of β-ether linkages in the fluorescent substrate V and compound I competitively inhibited each other.

of *E. coli* HB101 containing pBE10 revealed an accumulation of β-hydroxypropiovanillone (compound II) and vanillin (compound III) (Fig. 2A). The kinetic patterns obtained via plots of $[S]_0/V_0$ versus $[S]_0$ with the cell extract showed that cleavage of the β-ether linkage in fluorescent substrate V and compound I competitively inhibited each other (Fig. 2B). Thus, this enzyme from the recombinant had the same kinetic properties as the *P. paucimobilis* SYK-6 enzyme (10).

TABLE 1. β-Etherase activity of cell extracts prepared from transformants and *P. paucimobilis* SYK-6[a]

Source of extract	β-Etherase activity (pmol of 4MU/mg of protein/min)[b]
P. paucimobilis SYK-6	2.0
P. paucimobilis IAM12578	0
E. coli HB101	0
E. coli MV1190	0
P. paucimobilis IAM12578(pBE10)	30.0
E. coli HB101(pBE10)	1.6
E. coli MV1190(pUBE13)	2.8
E. coli MV1190(pUBE34)	<0.1

[a] Transformants and host cells were grown in LB, and the culture of *E. coli* MV1190 containing pUBE13 and pUBE34 was added with 1 mM IPTG. *P. paucimobilis* SYK-6 was cultured with 2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl.

[b] 4MU, 4-methylumbelliferone.

Detection of the β-etherase gene product. The 24-kb *Sal*I fragment of pBE10 was partially digested with *Sal*I and subcloned into pUC19. Among the transformants containing these plasmids, *E. coli* MV1190 with a plasmid containing a 1.9-kb *Sal*I fragment exhibited β-etherase activity. The plasmids in which the insert was in the opposite orientation from the *lac* promoter were isolated and designated pUBE13 and pUBE34. The cell extract of *E. coli* MV1190 containing pUBE13 cultured in the presence of IPTG showed high β-etherase activity, while an extract of the same strain containing pUBE34 showed very low levels of β-etherase activity regardless of the presence of IPTG (Table 1). This indicated that the gene on the 1.9-kbp *Sal*I fragment of pUBE13 is in the same orientation as the *lac* promoter and is dependent on the *lac* promoter for expression in *E. coli*. A 32-kDa protein was produced in *E. coli* MV1190 containing pUBE13 cultured in the presence of IPTG (Fig. 3).

Sequencing of the β-etherase gene. The sequence of the *Sma*I-*Sac*I fragment in the 1.9-kbp *Sal*I fragment is shown in Fig. 4. Computer analysis revealed an open reading frame of

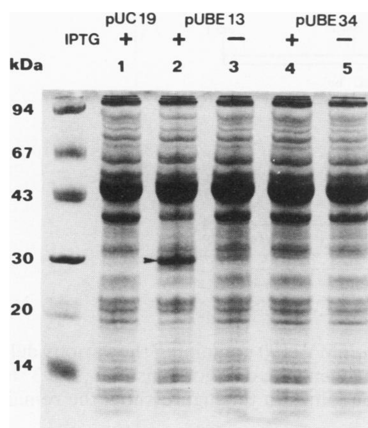

FIG. 3. Detection of the β-etherase protein. Cell extracts of strains grown in the presence (lanes 1, 2, and 4) or absence (lanes 3 and 5) of IPTG were subjected to SDS-PAGE (12% polyacrylamide gel). Lanes: 1, *E. coli* MV1190(pUC19); 2 and 3, *E. coli* MV1190 (pUBE13); 4 and 5, *E. coli* MV1190(pUBE34). The arrowhead to the left of lane 2 indicates the β-etherase protein (32 kDa).

843 bp, with the initiation codon (ATG) at position 42 and the termination codon (TGA) at position 885, encoding 281 amino acids. The molecular weight of the deduced protein was 32,070. This result agreed with the molecular mass of the product from *E. coli* containing pUBE13. This gene had a high G+C content (66%), and the occurrence of G or C in the third base of each codon was extremely high (87%), as is characteristic of *Pseudomonas* genes. There is a sequence similar to a ribosome-binding site (the Shine-Dalgarno sequence [16]) upstream of the initiation codon (GAGG). Neither promoter nor terminator sequences have been observed in the 1.9-kbp *Sal*I fragment.

Deletion analysis of the β-etherase gene and amino acid sequence of β-etherase. To determine whether this predicted open reading frame is needed for β-etherase activity, cell extracts of *E. coli* MV1190 carrying various deletion plasmids were assayed (Fig. 5). The activity was lost in *E. coli* MV1190 carrying deletion plasmids in which the deletion affects the open reading frame. Activity was lost when the 3′ terminus of the β-etherase gene was deleted by 6 bp or the 5′ terminus of this gene was deleted by 33 bp.

The amino acid sequence of the 32-kDa protein was determined, and 10 residues were identified from the N terminus. The result indicated that the amino acid sequence (positions 2 through 11, Ala-Arg-Asn-Asn-Thr-Ile-Thr-Leu-Tyr-Asp) of the 32-kDa protein corresponded to the deduced sequence.

DISCUSSION

We isolated *P. paucimobilis* SYK-6, which is able to degrade various dimeric lignin compounds by the metabolic pathway shown in Fig. 1. This metabolic process is a distinct characteristic of this bacterium, which is equipped with an

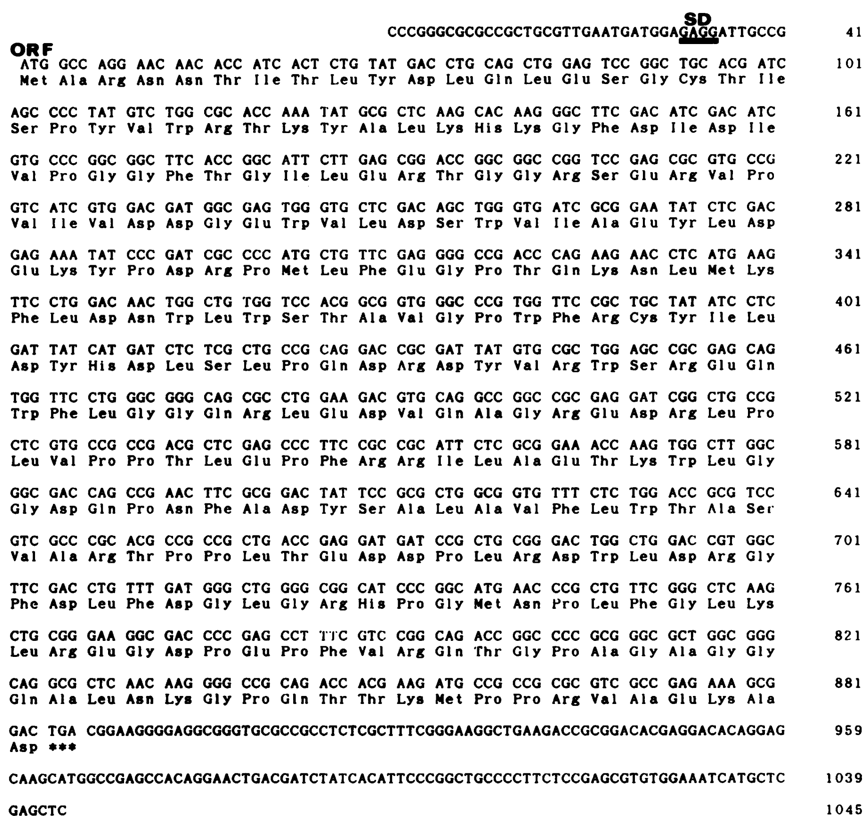

FIG. 4. Nucleotide sequence of the *Sma*I-*Sac*I fragment carrying the β-etherase gene in the 1.9-kbp *Sal*I fragment. The open reading frame (ORF) of the β-etherase gene had the initiation codon (ATG) at position 42 and termination codon (TGA) at position 885. The Shine-Dalgano (SD) sequence is indicated by a thick line under the sequence.

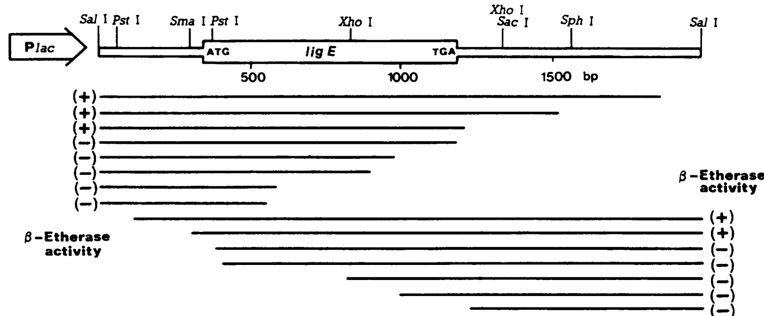

FIG. 5. Deletion analysis of the β-etherase gene (*ligE*). The β-etherase activity of *E. coli* MV1190 containing the various deletion plasmids derived from pUBE13 and pUBE34 was assayed. There is no activity in the transformants carrying deletion plasmids; the 3' terminus of the *ligE* gene is deleted by 6 bp, and the 5' terminus of the *ligE* gene is deleted by 33 bp. The lines in this figure show the remaining region of the 1.9-kbp *Sal*I fragment in the deletion plasmids.

enzymatic modification system for various dimeric lignin compounds involved in the tricarboxylic acid cycle. Analysis of the structures and functions of the genes responsible for the metabolic process will contribute to an understanding of the complete lignin degradation process in the biosphere.

Arylglycerol-β-aryl ether linkage is the most abundant intermonomer linkage between phenylpropanes in lignin (approximately 50%). β-Aryl ether cleavage is the most important process in the biological degradation of lignin. We reported for the first time the detection of β-etherase activity in a cellular membrane fraction of *P. paucimobilis* SYK-6 (10).

The cloning and sequencing of the β-etherase gene of *P. paucimobilis* SYK-6 by a sensitive fluorescence assay is reported here. For the molecular cloning of the gene encoding β-etherase, which is localized in the cellular membrane, we used the β-etherase-negative *P. paucimobilis* IAM12578, which has a cellular membrane similar to that of *P. paucimobilis* SYK-6, as the host cell. We obtained transconjugants of *P. paucimobilis* IAM12578 which had β-etherase activity and isolated pBE10. β-Etherase was effectively expressed in *P. paucimobilis* IAM12578 containing pBE10, and the β-etherase activity in whole cells was seven times as high as the activity in whole cells of *P. paucimobilis* SYK-6. We also detected β-etherase activity in the cell extract of *E. coli* containing pBE10. In a previous report (10), the β-etherase activity of *P. paucimobilis* SYK-6 was detected only in the cell extract treated with the detergent octanoyl-*N*-methylglucamide (MEGA-8). This time, the β-etherase activity in the recombinant was detected without use of a detergent, indicating that the cellular membrane was not essential for an enzymatic function such as tertiary conformation. The β-etherase of *E. coli* HB101 containing pBE10 was compared with the *P. paucimobilis* SYK-6 enzyme. GC-MS analysis and kinetic patterns revealed no difference in the kinetic properties of this enzyme between recombinant and *P. paucimobilis* SYK-6 (Fig. 2).

From the nucleotide sequence, β-etherase was 281 amino acids long and had an M_r of 32,070. We searched the protein data bases (SWISS-PROT) for a sequence homologous with β-etherase but found no significant homologies with previously reported proteins. Though we found that β-etherase was localized in the cellular membrane of *P. paucimobilis* SYK-6, no signal sequence was observed at the N terminus of the deduced amino acid sequence. The hydropathicity plot, determined by the method of Kyte and Doolittle (7) with a window of seven amino acids, shows some of the hydrophobic regions. The amino acid sequence (positions 189 through 201) shows high hydrophobicity. There is a possibility that this region interacts with the cellular membrane of *P. paucimobilis* SYK-6. This is the first successful cloning and sequencing of the gene encoding the specific lignin degradation enzyme β-etherase. Through this gene, we can elucidate the essential degradation process of lignin in the biosphere. We recently found the Cα-dehydrogenase gene in the 24-kbp *Sal*I fragment of pBE10 (data not shown). Cα-dehydrogenase (3, 14) catalyzes the Cα dehydrogenation of arylglycerol-β-aryl ether (Cα alcohol type) (compound IV), and therefore this process produces the specific substrate for β-etherase. Interestingly, the result of subcloning and restriction mapping of the 24-kbp *Sal*I fragment indicated that the Cα-dehydrogenase gene existed in the 3.0-kbp *Sal*I fragment adjacent to the 1.9-kbp *Sal*I fragment encoding β-etherase (data not shown). We called the β-etherase gene and Cα-dehydrogenase gene *ligE* and *ligD*, respectively; *ligA* and *ligB* encode the subunits of protocatechuate 4,5-dioxygenase (13). The functions and regulation of these genes in *P. paucimobilis* SYK-6 are essential for an understanding of the carbon cycle in the biosphere.

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