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The metabolism of biphenyl structures in lignin by the soil bacterium (*Pseudomonas paucimobilis* SYK-6)

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In the soil bacterium (*Pseudomonas paucimobilis* SYK-6), the metabolism of DDVA (biphenyl structure of lignin) and syringic acid (characteristic aromatic ring in hardwood lignin) proceeds via a common intermediate, 3-methylgallic acid. Protocatechuate is also an intermediate in the metabolism of vanillate and *p*-hydroxybenzoic acid. 3-Methylgallic acid and protocatechuate are the final aromatic intermediates in lignin microbial degradation and these compounds are substrates of protocatechuate-4,5-dioxygenase, which is a key enzyme in obtaining metabolic energy from various structures of lignin in this bacterium.

Lignin metabolism; Biphenyl structure; Syringic acid; Aromatic ring fission; Protocatechuate-4,5-dioxygenase; (*Pseudomonas paucimobilis* SYK-6)

1. INTRODUCTION

Lignins are the most abundant aromatic material in plants and are constructed with various intermonomer linkages between phenylpropanes having guaiacyl, syringyl, *p*-hydroxyphenyl and biphenyl nuclei. Investigations using lignin model compounds have contributed to the elucidation of the specific reactions of lignin metabolism by microorganism [1,2] and have shown that the metabolism of vanillate (guaiacyl nucleus) in bacteria normally proceeds via a demethylation step to protocatechuate followed by aromatic ring fission [3]. But the metabolism of syringic acid (syringic nucleus) and DDVA (biphenyl nucleus) to the ring fission step have not yet been revealed.

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Abbreviations: DDVA, 2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl; OHDDVA, 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl; DDV, 2,2′-dihydroxy-3,3′-dimethoxy-5,5′-diformylbiphenyl

In a previous study [4], we isolated the bacterium (*Pseudomonas paucimobilis* SYK-6) which is able to grow on DDVA, syringic acid, vanillate and other lignin dimeric model compounds as a sole carbon source. We have also isolated the genes coding vanillate degradation enzymes as a 10.5 kbp *Eco*RI DNA fragment from *P. paucimobilis* SYK-6 chromosomal DNA. In this study, the mechanism of DDVA (biphenyl nucleus) and syringic acid degradation by the soil bacterium is discussed.

2. MATERIALS AND METHODS

2.1. *Substrates and authentic compounds*

DDVA (**I**) was prepared by oxidation from DDV [5,6]. MS (TMSi derivative; (**I**)) *m/z*: 622(M), 607, 445. OHDDVA (**II**) was synthesized from DDV by the methods of Lange [7]. MS (TMSi derivative: (**II**)) *m/z*: 680(M), 665, 503. 5-Carboxyvanillate (**III**) was synthesized by the method of Profft and Krauso [8]. MS (TMSi derivative: (**III**)) *m/z*: 428(M), 413, 215. 3-Methylgallic acid (**IV**) was synthesized by the methods of Scheliner [9]. MS (TMSi derivative: (**IV**)) *m/z*: 400(M), 385, 223. Vanillate (**VI**), syringic acid (**VIII**), protocatechuate (**V**) and *p*-hydroxybenzoic acid (**VII**) were purchased from Tokyo Kasei Co.

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2.2. *Bacterial strain and culture condition*

Pseudomonas paucimobilis SYK-6 isolated by Kuwana and others [10], grew well in the medium containing DDVA (**I**) as a sole carbon source. The organism was cultivated at 30°C in L broth for 15 h with agitation and then the cells were collected by centrifugation at 3000 rpm and suspended in W-medium [11]. The lignin model compound was added to the cell suspension (final concentration: 0.2%) and incubated at 30°C with agitation.

2.3. *Identification of metabolites by GC-MS analysis*

Metabolites were extracted with ethylacetate from the culture medium acidified to pH 2.0 with 1 N HCl. The extractives were converted to TMSi-derivatives with BFA (Tokyo Kasei Co.). GC-MS analysis of these metabolites was carried out by comparison with authentic compounds and was recorded with a Shimazu GC-MS QP-1000 (EI, 70 eV; Column, OV-17 on chromosorb W, 1 m × 0.26 cm glass column; column temperature, 170°C–270°C, 5°C/min).

2.4. *Preparation of cell free extracts and enzyme assay*

From the cells grown in the medium containing DDVA (**I**) as a sole carbon source, the cell free extracts were prepared by the methods of Nakazawa and Hayashi [12]. The activity of protocatechuate-4,5-dioxygenase was determined by measuring the rate of increase of α-hydroxy-γ-carboxymuconic semialdehyde (λ_{max} = 410 nm in alkaline solution) according to the method of Ono et al. [14].

3. RESULTS

3.1. *DDVA (I) degradation*

The change in UV absorption spectra of a DDVA (**I**)-containing culture during incubation (fig.1A) indicated that DDVA (**I**) was rapidly degraded. This phenomenon was also observed in the case of syringic acid (**VIII**), vanillate (**VI**) and *p*-hydroxybenzoic acid (**VII**). GC-MS analysis of the metabolites in a DDVA-containing culture showed that OHDDVA (**II**) and 5-carboxyvanillate (**III**) were produced as intermediates as shown in fig.2A.

3.2. *5-Carboxyvanillate (III) degradation*

GC-MS analysis of the metabolites in a 5-carboxyvanillate (**III**)-containing culture showed that 3-methylgallic acid (**IV**) was produced as an intermediate as shown in fig.2B. This showed that 5-carboxy vanillate (**III**) had undergone decarboxylation and hydroxylation at the 5 position of its aromatic ring to produce 3-methylgallic acid (**IV**) followed by aromatic ring fission.

3.3. *Syringic acid (VIII), vanillate (VI) and p-hydroxybenzoic acid (VII) degradation*

GC-MS analysis of the metabolites in a syringic acid (**VIII**)-containing culture showed that 3-methylgallic acid (**IV**) was produced as an intermediate. This showed that syringic acid (**VIII**) was degraded via 3-methylgallic acid (**IV**) which was also an intermediate derived from DDVA (**I**)

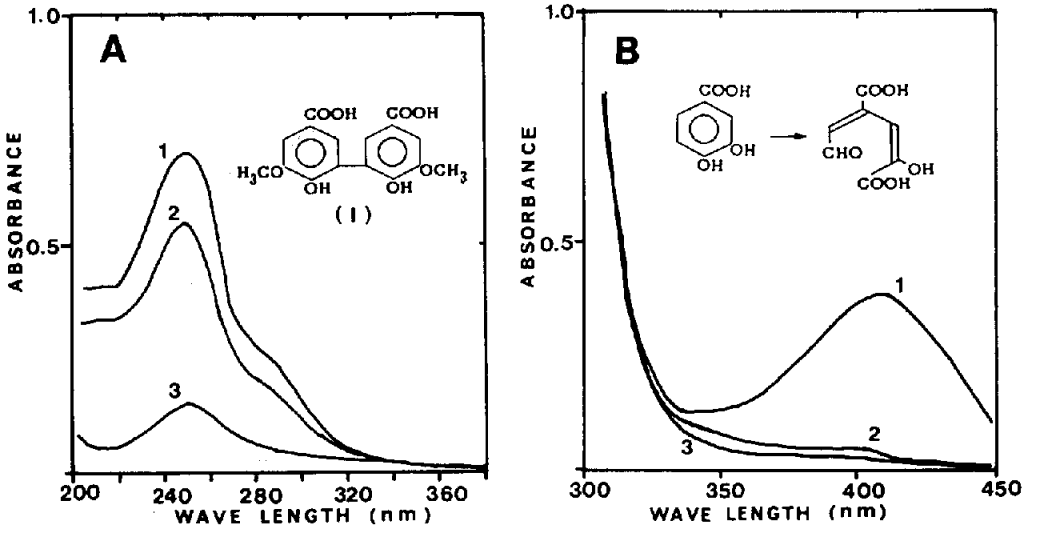

Fig.1. UV absorption spectra of the reaction mixtures. (A) The cell suspension culture containing DDVA (**I**). Incubation time for curve 1 (0 h), 2 (1.5 h), 3 (18 h). (B) The reaction mixture of protocatechuate (**V**) and the cell free extract (reaction time was 2 min). Curves: 1, the extracts from the cells grown in a DDVA (**I**)-containing culture; 2, the extracts from the cells grown in L broth; 3, the boiled extracts from the cells grown in a DDVA (**I**)-containing culture.

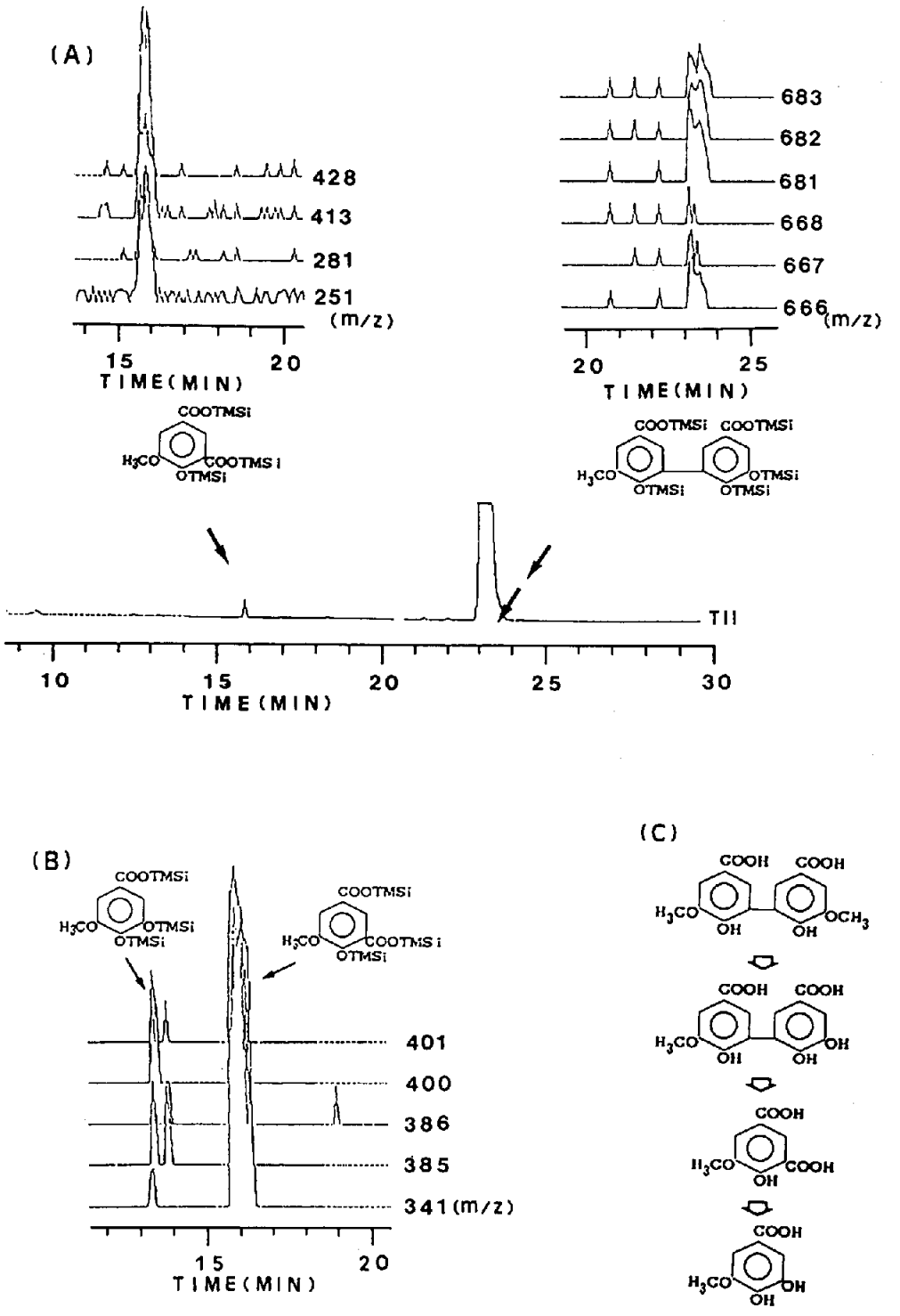

Fig.2. Gas chromatogram and mass chromatogram of degradation products. (A) Degradation products from DDVA (I), (B) degradation products from 5-carboxyvanillate (III), (C) degradation pathway of DDVA (I).

and 5-carboxyvanillate (**III**). GC-MS analysis of the degradation products in vanillate (**VI**) or *p*-hydroxybenzoic acid (**VII**) cultures showed that protocatechuate (**V**) was a common intermediate in their degradation.

3.4. *Protocatechuate-4,5-dioxygenase activity in the cell free extracts*

Fig.1B shows the protocatechuate-4,5-dioxygenase activity in *P. paucimobilis* SYK-6 cells grown with DDVA (**I**) or L broth. The enzyme activity in the cells grown with DDVA (**I**) was remarkably increased. This result showed that the enzyme, which converted protocatechuate (**V**) into α-hydroxy-γ-carboxymuconate semialdehyde, was induced by DDVA (**I**).

4. DISCUSSION

In the soil bacterium, *P. paucimobilis* SYK-6, the metabolism of DDVA (**I**) proceeded as shown in fig.3 via two kinds of aromatic ring fissions to the precursors of the citric acid cycle. One was the biphenyl ring fission of OHDDVA (**II**) and the other was the ring fission of 3-methylgallic acid (**IV**). Interestingly, the degradation pathway of DDVA (**I**) and syringic acid (**VIII**) meet at 3-methylgallic acid (**IV**) which undergoes ring fission. On the other hand, the degradation pathway of vanillate (**VI**) and *p*-hydroxybenzoic acid (**VII**) meet at protocatechuate (**V**) as shown in fig.3. From these results, we concluded that 3-methylgallic acid (**IV**) and protocatechuate (**V**) are the final aromatic intermediates derived from lignin biodegradation.

In earlier reports, Zabinsky et al. [13] and Ono et al. [14] showed that protocatechuate (**V**) and 3-methylgallic acid (**IV**) are substrates of protocatechuate-4,5-dioxygenase and are degraded by this enzyme to produce α-hydroxy-γ-carboxymuconate semialdehyde (λ_{max} = 410 nm) and its methyl ester (λ_{max} = 310 nm). In our experiments on protocatechuate (**V**) degradation by cell free extracts, the absorption of λ_{max} = 410 nm was increased markedly (fig.1B) and we could detect the increase of λ_{max} = 310 nm in 3-methylgallic acid (**IV**) degradation (not shown). This enzyme was clearly induced by lignin model compounds in this bacterium. Now further study is in progress to detect the enzyme degrading OHDDVA (**II**).

We can now understand the degradation pathway of every type of aromatic structure in lignin by the soil bacterium. In particular, this is

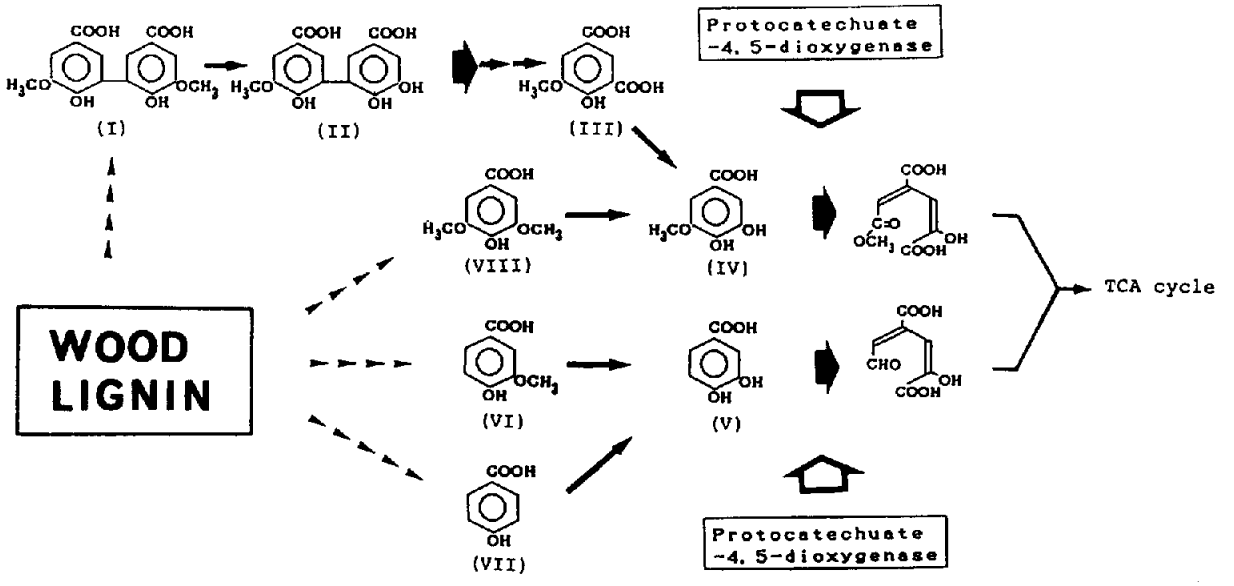

Fig.3. Degradation pathway of lignin related compounds by *Pseudomonas paucimobilis* SYK-6 and the role of protocatechuate-4,5-dioxygenase in this pathway. (→) Degradation reaction by *P. paucimobilis* SYK-6; ( ) ring fission step in this pathway.

the first evidence for the degradation mechanism of the biphenyl structure of the protocatechuate type, and this study showed that protocatechuate-4,5-dioxygenase is an inducible key enzyme to produce the precursors of tricarboxylic acid cycle in microbial degradation process of lignin.

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