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Beta-ketoadipic acid and muconolactone production from a lignin-related aromatic compound through the protocatechuate 3,4-metabolic pathway

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In this work, the effects of PcaJ (beta-ketoadipate:succinyl-coenzyme A transferase)- and PcaD (beta-ketoadipate enol-lactone hydrolase)-inactivation on protocatechuic acid metabolism in *Pseudomonas putida* KT2440 were evaluated. Beta-ketoadipic acid was produced from protocatechuic acid by the inactivation of PcaJ as expected; however, a portion of the produced beta-ketoadipic acid was converted to levulinic acid through a purification step consisting of extraction from the culture and recrystallization. On the other hand, muconolactone was purified from the culture of the PcaD-inactivated mutant of KT2440, although beta-ketoadipate enol-lactone was supposed to be produced because it is the substrate of PcaD. Under aerobic conditions, it has been reported that lignin-related aromatics are metabolized through PCA 2,3- or 3,4- or 4,5-ring cleavage pathways, and muconolactone is an intermediate observed in the metabolism of catechol, not protocatechuic acid. Our results will provide a prospective route to produce muconolactone with a high yield through the protocatechuate-3,4-metabolic pathway.

[**Key words:** Beta-ketoadipic acid; Muconolactone; Protocatechuic acid; Lignin-related aromatics; Bioreactor]

Global attention to the production of eco-friendly materials is growing rapidly due to environmental problems such as global warming, acid rain and soil deterioration. Thus, sustainable biomaterials production is now developing throughout the world to mitigate such environmental problems by substituting biomaterials from reproducible plant biomass for variety of chemicals that have been produced from fossil fuels (1,2). Chemicals useful in the polymer industry, such as lactate, succinate, propanediol, and polyhydroxyalkanoate, have been reported to be producible from holocellulose-derived sugars (3–6); however, the number of reports on the production of such useful bio-chemicals from either lignin or lignin-related aromatic compounds is small. Lignin is a major component of plant cell walls, which comprise 90% of dried plant weight. The major use of lignin has been as a heating source in the pulping process; it has not been applied in other ways because, although it makes up 15–35% of plant cell walls and is the most abundant aromatic substance in nature, it is consisted of extremely complicated structure. Lignin can be decomposed and solubilized by thermochemical treatments such as alkaline hydrolysis; however, the structures of the solubilized compounds are diverse, not homogenous, and are hard to efficiently funnel to certain compounds.

Useful chemicals such as muconic acid (7,8), polyhydroxyalkanoate (9), guaiacol (10), 3-carboxymuconate (11), and 2-pyrone dicarboxylate (12) are producible from lignin-related aromatic compounds as well as from sugars although there are only a small number of reports regarding the bio-production of useful chemicals from lignin. Thus, investigating further approaches to the bio-production of chemicals from lignin-related aromatics is also crucial for the development of sustainable materials production.

Thus, in this work, we first focused on beta-ketoadipic acid (KA) production from lignin-related aromatics. KA is an intermediate in the beta-ketoadipate pathway. It is located in the downstream part of the metabolic pathways of aromatic compounds, and a variety of aromatics are metabolized to a central metabolic pathway through KA. KA has a structure similar to that of adipic acid (1,4-butanedicarboxylic acid), which has a carboxyl group at both ends and is being produced at a rate of 2.8 Mt/year (13) as a raw material to synthesize polyamides and polyesters. In addition, KA holds a carbonyl group at the beta position that generally shows high reactivity due to nucleophilicity. Moreover, it has been suggested that adipic acid could be produced from KA thorough several petrochemical reaction steps (14). Since the chemical characteristics of KA are considered to have the potential to be used as a polymer feedstock, we aimed to produce KA from lignin-related aromatic compounds in this study.

Second, we discussed here a new prospective route for producing a lactone compound through the protocatechuate (PCA) 3,4-

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metabolic pathway, which is a major metabolic pathway for aerobic lignin-related aromatics degradation. Lactone compounds, e.g., 3-carboxymuconolactone (CML), muconolactone (ML), and beta-ketoadipate enol-latone (KEL), are intermediates observed in the metabolism of lignin-related aromatics (15). It has been reported that CML is producible from lignin-related aromatics (16); however, no previous published reports have shown that either ML or KEL was produced from lignin or its derivatives thorough PCA-3,4-cleavage pathway, although it has been reported that mandelate could be converted to ML (17). ML, an isomer of KEL, has a gamma-crotonolactone backbone that has been used as a pharmaceutical reagent (18) and in polymer syntheses (19). Thus, ML and KEL may be considered chemicals that will be useful in sustainable materials production.

MATERIALS AND METHODS

Preparation of beta-ketoadipate:succinyl-coenzyme A transferase (PcaJ)- and beta-ketoadipate enol lactone hydrolase (PcaD)-inactivated mutants *Pseudomonas putida* KT2440 (NBRC10650) was purchased from the National Bioresource Center (NBRC, Chiba, Japan) and used as a host cell to prepare beta-ketoadipate:succinyl-coenzyme A transferase (EC 2.8.3.6)- and beta-ketoadipate enol-lactone hydrolase (EC 3.1.1.24)-inactivated mutants. An approximately 1.3-kbp DNA fragment including *pcaI* and *pcaJ* was amplified from the genomic DNA of *P. putida* KT2440 by PCR using a primer set consisting of 5′-CAGTCTAGAACCAGGAGCTCGCCTTGATC-3′ and 5′-CAGTCTAGATCACTTGATCAGCGGCACG-3′ and Expand High Fidelity^{PLUS} PCR system (Roche Diagnostics, IN), and cloned with pT7Blue vector (pPcaIJ) in *Escherichia coli* DH5alpha. A 1.0-kbp DNA fragment including a chloramphenicol acetyltransferase gene (*cat*) from pUC6C (20) was inserted into the ApaI site in the *pcaJ* in pPcaIJ. The resulting plasmid was digested with PstI, and the 2.3-kbp fragment was inserted into the PstI site in pK19*mobsacB* (21) (pIJcm19). To replace the native *pcaJ* loci in the genomic DNA of *P. putida* KT2440 to *pcaJ::cat* on the basis of homologous recombination, pIJcm19 was introduced into *P. putida* KT2440 cells via the tri-parental mating method, and kanamycin (Km)-resistant transformants were screened by being spread out on LB agar plates containing 25 mg/L nalidixic acid (Nal) and 25 mg/L Km. The Km-resistant cells were incubated in an LB liquid shake culture containing 10% (w/v) sucrose for 20 h at 28 °C. The shake-culture in 10% sucrose containing LB liquid medium was repeated three times by subculturing a portion of the overnight culture, to induce a double cross over event as described previously (21). The sucrose-resistant cells were spread out on modified LB agar plates (3.3 g/L tryptone, 1.7 g/L yeast extracts, 5 g/L sodium chloride, 15 g/L agar) containing 25 mg/L Nal and 50 mg/L chloramphenicol (Cm). The replacement of *pcaJ* to *pcaJ*:*cat* was confirmed by Southern hybridization. The total DNA was isolated from a Cm-resistant colony that also showed Km-sensitive and sucrose-resistant characteristics, and was digested with SacI. After purification, the digested genomic DNA was subjected to gel electrophoresis and then transferred to a nylon membrane (Hybond-N, GE Healthcare Life Sciences, Tokyo, Japan) using a vacuum-blotter (model 785, Bio-Rad Laboratories, Hercules, CA, USA). The 1.3-kbp PstI fragment from pPcaIJ was used as a probe and labeled using a DIG Labeling Kit (Roche Diagnostics), and then the signals were detected with alkaline phosphate-conjugated anti-DIG antibody. The resulting mutant was also unable to assimilate vanillate (VA) and protocatechuate (PCA), and then designated as *P. putida* KT2440-dJ.

A 0.8-kbp DNA fragment including *pcaD* was amplified from the genomic DNA of *P. putida* KT2440 by PCR using a primer set consisting of 5′-GAAAGCTTCAACGA-GACCGCTGTGGC-3′ and 5′-AATCTAGAGCGTTGTCCTCAGTGAG-3′ and Expand High Fidelity^{PLUS} PCR system, and cloned with pT7Blue vector (pPcaD) in *E. coli* JM109. A 1.0-kbp DNA fragment including a chloramphenicol acetyltransferase gene (*cat*) from pUC6C was inserted into the XmaIII site in the *pcaD* in pPcaD. The resulting plasmid was digested with PstI and EcoRI, and the 1.8-kbp fragment was inserted into the same site in pK19*mobsacB* (pDcm19). To replace the native *pcaD* loci in the genomic DNA of *P. putida* KT2440 to *pcaD::cat* on the basis of homologous recombination, pDcm19 was introduced into *P. putida* KT2440 cells via tri-parental method, and Km-resistant transformants were screened by being spread out on LB agar plates containing 25 mg/L Nal and 25 mg/L Km. The *pcaD*-deficient mutant strain, showing sucrose-resistant, Km-sensitive and Cm-resistant characteristics, was selected in the same way as described above. The replacement of *pcaD* to *pcaD*::*cat* was confirmed by Southern hybridization in which the SphI digested genomic DNA from Cm-resistant colonies and the 0.7-kbp HindIII–BamHI fragment from pPcaD was used. The other procedures were performed as described above. The resulting mutant was also unable to assimilate VA and PCA, and was designated as *P. putida* KT2440-dD.

Beta-kedoadipic acid production from protocatechuic acid in a mini-jar fermentor culture To make the beta-ketoadipic acid (KA)-producing ability of the cells independent of the induction, the *pcaJ*-inactivated mutant, *P. putida* KT2440-dJ was transformed with pKAD, which harbors the genes encoding vanillate demethylase (*vanA* (modified to produce the oligopeptide-fused VanA) and *vanB*), PCA 3,4-dioxygenase (*pcaH* and *pcaG*), beta-carboxy *cis*,*cis*-muconate cycloisomerase (*pcaB*), gamma-carboxymuconolactone decarboxylase (*pcaC*) and beta-ketoadipate enol-lactone hydrolase (*pcaD*) under the control of a lactose promoter by tri-parental mating method. Since it had been reported that fusion of the 16 amino acids (MTMITPSLHACRSESL) of LacZα to the N terminus of VanA improved the demethylase activity (22), we prepared the modified VanA. The *lac* promoter could be used to express genes in a *P. putida* strain (12,16), and thus we used the same promoter to express the genes listed above in this study. The genes for vanillate demethylase were amplified from the genomic DNA of *P. putida* PpY101 (23) and the rest were from *P. putida* KT2440 by PCR, and cloned into

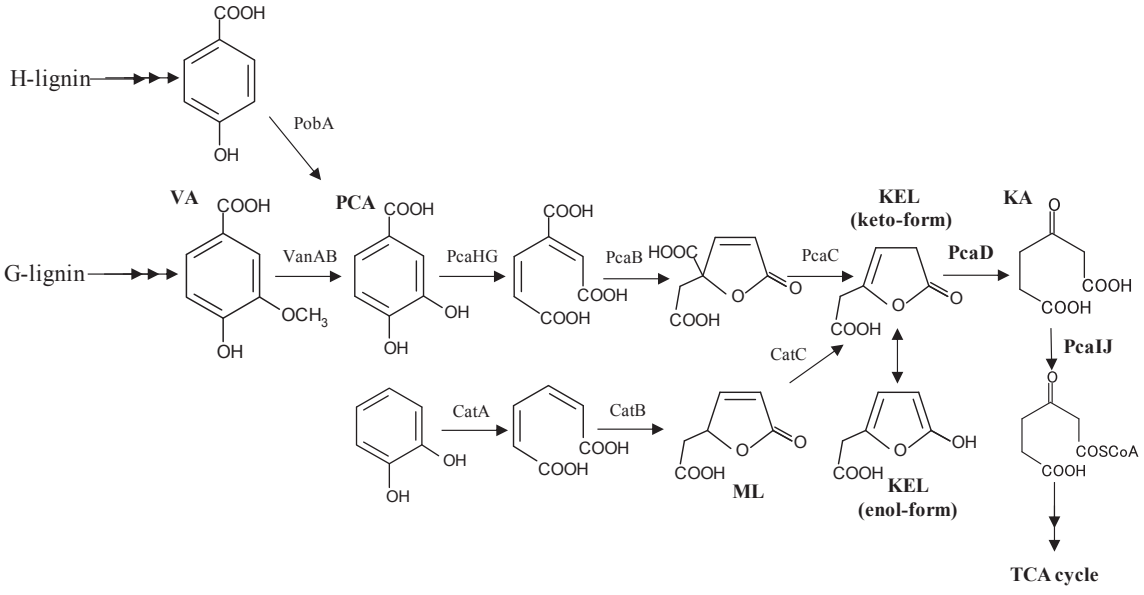

FIG. 1. The metabolic pathway for *p*-hydroxphenyl and guaiacyl lignin-related aromatic compounds in *P. putida* KT2440. H-lignin, *p*-hydroxyphenyl lignin: G-lignin, guaiacyl lignin; HBA, *p*-hydroxybenzoate; VA, vanillate; PCA, protocatechuate; ML, muconolactone; KEL, ketoadipate enol-lactone (either keto- or enol-foam); KA, beta-ketoadipate; PobA, *p*-hydroxybenzoate hydroxylase; VanAB, vanillate demethylase; PcaHG, protocatechuate-3,4-dioxygenase; PcaB, beta-carboxy-*cis*,*cis*-muconate cycloisomerase; PcaC, gamma-carboxymuconolactone decarboxylase; PcaD, beta-ketoadipate enol-lactone hydrolase; CatA, catechol dioxygenase; CatB; *cis*,*cis*-muconate cycloisomerase; CatC; muconolactone delta-isomerase.