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Rapid Communication Open Access

Stable Chiral Carboxymuconolactone Production from a Lignin-Related Aromatic Compound, Protocatechuic Acid

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Rapid Communication

Carboxymuconolactone, an intermediate in protocatechuic acid metabolism, has been reported in two forms from protocatechuic acid: 4-carboxymuconolactone in *Pseudomonas putida* and other prokaryotes, and 3-carboxymuconolatone in *Neurospora crassa*. In this study, we examined the production of both forms from protocatechuic acid with protocatechuate-3,4-dioxygenase and 3-carboxymuconate cycloisomerase from either *P. putida* or *N. crassa*. We show here that a chiral carboxymuconolactone, *4R*-3-carboxymuconolactone, could be produced, although 4-carboxymuconolactone was unstable and thus hard to produce.

The production of biofuels and chemicals from lignocellulosic biomass is part of a biorefinery concept to develop a sustainable and eco-friendly production-based society. Although cellulose and hemicellulose have been recognized as useful organic substances for bioproduction, another component, lignin, has not been focused on as a feedstock due to its heterogeneity and recalcitrance. Recently, however, there have been reports of the production of chemicals from lignin-derived aromatics, such as PHA [1,2], muconic acid [3,4], ketoadipic acid [5], and pyruvic acid [6]. We have been focusing on the production of lactone compounds from lignin-related aromatics, e.g., carboxymuconolactone (CML), muconolactone (ML), and beta-ketoadipate enol-lactone (KEL) [5], that are intermediates observed in the metabolism of lignin-related aromatics [7], since a gamma-crotonolactone backbone has been used as a pharmaceutical reagent [8] and in polymer syntheses [9]. Thus CML, ML, and KEL would be considered useful chemicals for the production of sustainable materials. In this study we aimed to develop a CML-producing bacterial strain by modifying the aromatic metabolic pathway of *Pseudomonas putida*, since it can funnel a variety of lignin-related aromatics, including ferulic acid, *p*-coumaric acid, vanillic acid, *p*-hydroxybenzoic acid, and others to protocatechuic acid (PCA) and further metabolize to succinyl-CoA and acetyl-CoA through the PCA-3,4-ring cleavage pathway (Figure 1) [10]. CML is an intermediate in PCA metabolism. Two CMLs in the metabolic pathways of microbes have been reported. One is 3-CML in eukaryotic PCA metabolism [11,12], and the other is 4-CML in prokaryotic metabolism (Figure 1). As an important phenomenon, we found previously that biologically produced 3-carboxymuconic acid through the prokaryotic PCA metabolic pathway was converted to 3-CML, not 4-CML, through the acidification treatment of 3-carboxymuconic acid. This phenomenon suggested that 3-CML would be more stable than 4-CML. We therefore first prepared a 3-CML–producing bacterial strain.

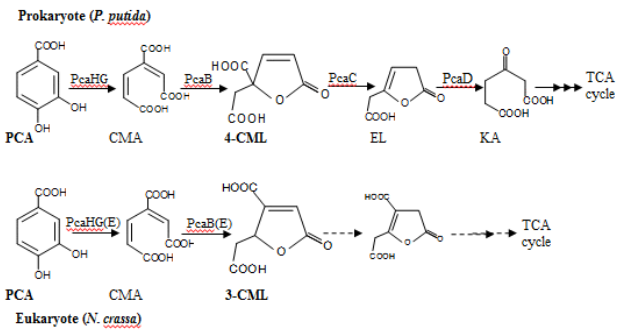

Figure 1: The metabolic pathway of protocatechuic acid in prokaryote (*P. putida*) and eukaryote (*N. crassa*). PCA, protocatechuic acid; CMA, beta-carboxy-*cis,cis*-muconic acid; 4-CML, gamma-carboxymuconolactone; 3-CML, beta-carboxymuconolactone; EL, beta-ketoadipate enol-lactone; KA, beta-ketoadipic acid; PcaHG and PcaHG(E), PCA-3,4-dioxygenase; PcaB and PcaB(E), CMA cycloisomerase; PcaC, gamma-carboxymuconolactone decarboxylase; PcaD, beta-ketoadipate enol-lactone hydrolase. The metabolic route for 3-CML are undefined (dashed arrows).

To produce 3-CML from PCA, *pcaH* and *pcaG*, encoding protocatechuate-3,4-dioxygenase, were amplified from the genomic DNA of *P. putida* KT2440 with a primer set consisting of 5'-GGTGTCAGGCAAAGGTGTTAAGAC-3' and 5'-AGTGGGGTTCTGCTGGTTCGGC-3'. *PcaB* was amplified with a primer set of 5'-CGGAATTCCGGAGGACCATATGCCCCTCCATCATTTGATG-3' and 5'-GCTCTAGAGCCTAATCGTACCAAATGGCAT-3' from the cDNA obtained from *Neurospora crassa* N150 total RNA (Fungal Genetic Stock Center no. 9013, designated *pcaB(E)*). The amplified fragments were inserted into pKT230MC [13] to regulate their

expression under a lactose promoter (pK3CML). *P. putida* PpY1100, an NTG mutant deficient in PCA metabolism [14], was transformed with pK3CML, and the ability of PCA to produce 3-CML in a 10 L–scale jar fermenter was evaluated (Figure 2A). The resulting recombinant strain was inoculated in 10 mL of LB medium containing 50 mg/L Km and shake-cultured at 28°C. The culture was inoculated in 0.2 L of fermentation broth [5] containing 50 mg/L Km and further cultured with shaking. The pre-culture was then inoculated in 5.0 L of fermentation broth in the jar fermenter and run at 28°C at pH 6.5–7.0 with an agitation speed of 700 rpm and an aeration rate of 10 L air/min. When OD_{600} reached approximately 10, 250 mL of 2 M glucose was added, and the addition of 500 mL of 50 g/L PCA solution (pH 8.0) into the culture at a flow rate of 50 mL/h was begun.

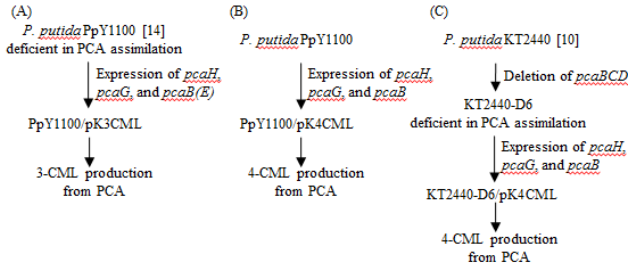

Figure 2: A schematic diagram of the recombinants preparation.

At 26 h after inoculation, the metabolites were extracted from a 1 mL portion of the culture and analyzed with thin-layer chromatography. After a major spot where Rf differed from PCA was detected, 1 L of the culture was centrifuged, and the supernatant was mixed with 1 g of activated charcoal and stirred for 1 h at ambient temperature. The mixture was filtered and the filtrate was acidified to below pH 2 with concentrated HCl, then extracted twice with an equal volume of ethyl acetate. The solvent was evaporated, and the dried substances were dissolved completely in 5 mL of acetone, 5 mL of chloroform was added slowly with stirring, and the solution was kept at 4°C for half a day. The precipitates were collected by filtration, and the resulting substances were dried completely using a vacuum oven (1.70 g). GC-MS analysis of the recrystallized substances showed a major ion peak, and the mass spectrum showed that the molecular ion peak was m/z=402 (Figure 3A). This did not correspond to the molecular weight (330) of trimethylsilylated (TMSi)-3-CML, but it did correspond to TMSi-3-carboxymuconate. It has been reported that the extinction coefficient of 3-carboxymuconoic acid is more than 10-fold that of 3-CML (at 270 nm, pH 7) [11]. The supernatant from the culture that had passed for 26 h after the inoculation and the solution dissolved the recrystallized substances were analyzed with a spectrophotometer, revealing that both solutions showed absorption of the wavelength at 220 nm and no absorption at 270 nm. These data suggested that 3-CML would be produced from PCA by *P. putida* PpY1100/pK3CML; however, the 3-CML would be converted to 3-carboxymuconic acid during the volatilization in GC-MS analysis. To clarify which compound was purified from the culture, the substance was subjected to ^{1}H-, and ^{13}C-NMR spectroscopic analysis was performed (Figures 3B and 3C). Since ^{13}C-NMR showed seven signals (at 36.5, 78.5, 125.9, 157.9, 162.1, 170.4, and 170.8 ppm), and ^{1}H-NMR showed six (at 2.67, 3.10, 5.55, 6.81, 12.5 and 13.0 ppm), the substance produced from PCA was suggested to be 3-CML. Because the 3-CML showed maximal absorption at 215 nm and a negative Cotton effect from CD spectroscopic analysis (Figure 3D), it was apparent that *4R*-3-CML was produced from PCA by *P. putida* PpY1100/pK3CML stably in this study.

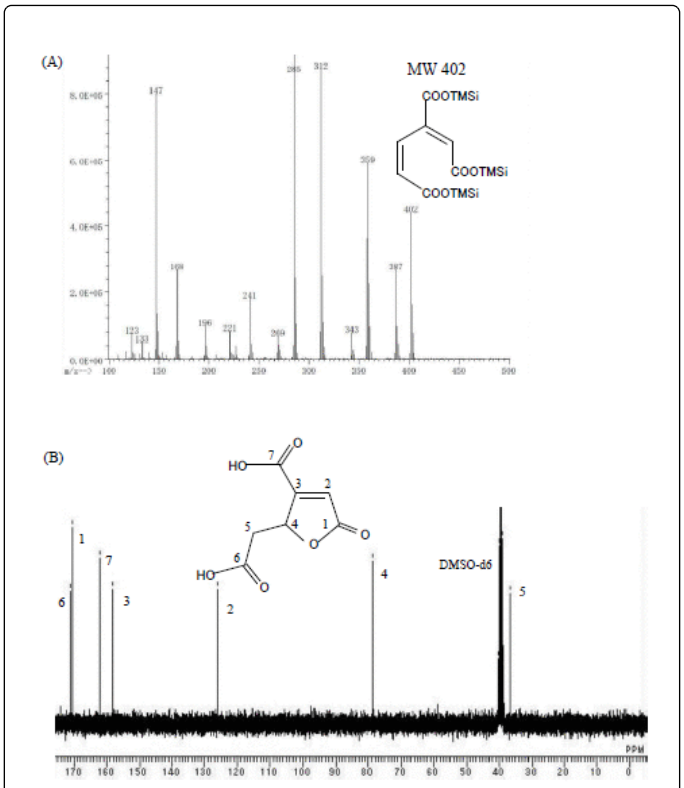

Figure 3 (I): Production of 3-CML from PCA by *P. putida* PpY1100/pK3CML. (A) Mass spectrum of the recrystallized compound from the culture. (B) ^{13}C-NMR spectrum of the recrystallized compound (in DMSO-d6, FT-NMR JMN-AL; JEOL, Tokyo, Japan)

4-CML, the other CML which has been reported as an intermediate in PCA metabolism, is an actual intermediate observed during PCA degradation in *P. putida* [15]. Therefore, we also examined whether 4-CML could be produced in addition to 3-CML. The *pcaB (E)* in pK3CML was replaced by the *pcaB* from KT2440 (designated as pK4CML) to examine whether it was possible to produce 4-CML from PCA by PpY1100/pK4CML (Figure 2B). The *pcaB* was amplified with a primer set consisting of 5'-GATGATGGCGATTGCCTTCC-3' and 5'-GTCTCCTTCAGGCAGTGAAACG-3'. PpY1100/pK4CML was cultured and the metabolites were extracted by the same procedures as described above. However, neither 4-CML, carboxymuconic acid (CMA, Figure 1), nor ketoadipate enol-lactone (EL, Figure 1) was detected through GC-MS analyses. Though PpY1100 is deficient in PCA assimilation, thus far we have not learned in which step the strain is deficient in PCA metabolism. If this strain was deficient only in PcaHG activity but maintained the other activities required for PCA assimilation, 4-CML would not accumulate in the culture. We next prepared the 4-carboxymuconolactone decarboxylase (*pcaC*) mutant of *P. putida* KT2440 (Figure 2C). The DNA region containing 3-carboxymuconate cycloisomerase (*pcaB*), beta-ketoadipate enol-lactone hydrolase (*pcaD*), and 4-carboxymuconolactone decarboxylase (*pcaC*) was amplified by PCR from the genomic DNA of KT2440 with

a primer consisting of 5'-GCTCTAGAGTACCTGCACCATGATGATTAAGGACG-3', and 5'-CCCAAGCTTATCGGTGTAAAGGCCATTCGCG -3'.

The amplified approximately 2.6-kbp fragment was cloned into pK19*mobsacB* [16], and the resulting plasmid was digested with SalI to eliminate the 0.6-kbp portion containing *pcaD* and a portion of *pcaB* and *pcaC* from the plasmid. The resulting 7.8-kbp fragment was ligated with a chloramphenicol acetyl transferase (*cat*) gene from pUC6C [17] to generate pmBD6C6. To replace the *pcaBDC* loci in the genomic DNA of *P. putida* KT2440 with *ΔpcaBDC::cat* on the basis of homologous recombination, pmBD6C6 was introduced into KT2440 cells via the tri-parental mating method, and the mutant was screened as described previously [5].

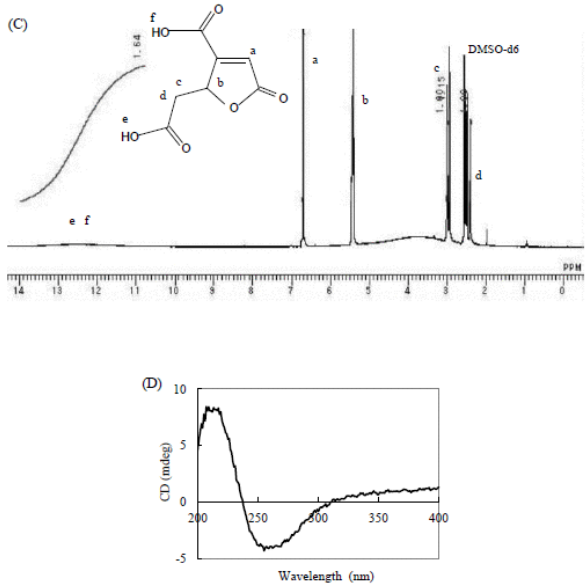

Figure 3 (II): Production of 3-CML from PCA by *P. putida* PpY1100/pK3CML. (C) ^{1}H-NMR spectrum of the recrystallized compound (in DMSO-d6) (D) CD spectrum of 3-CML.

The replacement of *pcaBCD* with *ΔpcaBDC::cat* was confirmed by PCR. The resulting mutant was unable to assimilate PCA and was then designated as *P. putida* KT2440-D6. KT2440-D6/pK4CML was inoculated in 10 mL of LB broth and shake-cultured. PCA (5 g/L in final) was added when the OD_{600} reached 8. The metabolites were extracted from the culture 24 h after the PCA addition and were analyzed by GC-MS. A major peak was observed, and this peak was not observed in the metabolites of KT2440-D6 from PCA. The mass spectrum showed that the molecular ion peak was m/z=286 (Figure 4), which did not correspond to the molecular weight of TMSi-4-CML (m/z=330); rather, m/z=286 corresponds to the molecular weight of TMSi-keto-adipate enol-lactone (TMSi-KEL (enol form)) (Figure 1). Since it has been reported that 4-CML has a labile carboxyl group and its half-life at 30°C and pH 6 is 30 min [18], the 4-CML produced would be quickly converted to KEL through the incubation and extraction. *P. putida* KT2440 possesses a variety of enzymes to metabolize aromatics, and another PcaC (at PP_3645 locus) and PcaD (at PP_3645 locus) are located in the genomic DNA sequence, so these enzymes might degrade 4-CML. However, it has not been clarified whether these two enzymes can metabolize 4-CML to 3-oxoadipic acid. Moreover, KEL would not be accumulated if these two enzymes possessed such metabolic activity. From these results, we concluded that 4-CML produced from PCA by *P. putida* KT2440-D6/pK4CML was unstable and easily converted to KEL through the procedure.

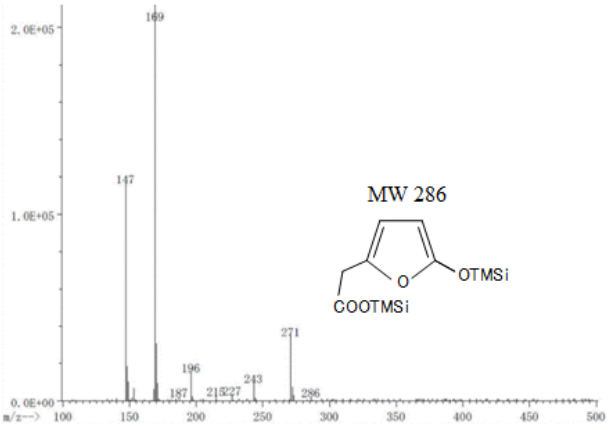

Figure 4: Mass spectrum of the major metabolite from PCA by *P. putida* KT2440-D6/pK4CML.

In this work, we have determined that 4-CML is not stable enough to produce on a mass scale but, in contrast, 3-CML could be produced via microbial fermentation. To the best of our knowledge, this is the first report to produce a carboxymuconolactone from a lignin-related aromatic compound, although both 3- and 4-CML have been identified as metabolites in PCA degradation by prokaryote and eukaryote.

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