Title

Enhancement of protocatechuate decarboxylase activity for the effective production of muconate from lignin-related aromatic compounds.

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Abstract

The decarboxylation reaction of protocatechuate has been described as a bottleneck and a rate-limiting step in *cis, cis*-muconate (ccMA) bioproduction from renewable feedstocks such as sugar. Because sugars are already in high demand in the development of many bio-based products, our work focuses on improving protocatechuate decarboxylase (Pdc) activity and ccMA production in particular, from lignin-related aromatic compounds. We previously had transformed an *Escherichia coli* strain using *aroY*, which had been used as a protocatechuate decarboxylase encoding gene from *Klebsiella pneumoniae* subsp. *pneumoniae* A170-40, and inserted other required genes from *Pseudomonas putida* KT2440, to allow the production of ccMA from vanillin. This recombinant strain produced ccMA from vanillin, however the Pdc reaction step remained a bottleneck during incubation. In the current study, we identify a way to increase protocatechuate decarboxylase activity in *E. coli* through enzyme production involving both *aroY* and *kpdB*; the latter which encodes for the B subunit of 4-hydroxybenzoate decarboxylase. This permits expression of Pdc activity at a level approximately 14-fold greater than the strain with *aroY* only. The expression level of AroY increased, apparently as a function of the co-expression of AroY and KpdB. Our results also imply that ccMA may inhibit vanillate demethylation, a reaction step that is rate limiting for efficient ccMA production from lignin-related aromatic compounds, so even though ccMA production may be enhanced, other challenges to overcome vanillate demethylation inhibition still remain.

Keywords

Protocatechuate decarboxylase, *cis, cis*-muconate, lignin, biocatalysis.

1. Introduction

Cis, cis-Muconic acid (ccMA) has potential use as a platform chemical to synthesize a variety of plastics including polyamides (e.g. nylon-6,6), polyurethanes, and polyethylene terephthalate (PET) (Weber *et al.*, 2012; Curran *et al.*, 2013; Polen *et al.*, 2013; Lin *et al.*, 2014). Bio-based muconic acid production has been reported as a \$22 billion market (Myriant Corporation, 2014). *Cis, cis*-Muconic acid can be produced via the ortho-cleavage of catechol, which is one of the intermediates of aromatic compound metabolism. Some bacteria can convert benzoic acid to catechol via benzoate 1,2-dioxygenase and 1,2-dihydroxybenzoate dehydrogenase, and further metabolize catechol via ccMA by the beta-ketoadipate pathway (Harwood and Parales, 1996; Polen *et al.*, 2013). The production of ccMA from benzoic acid by microbial action has also been reported (van Duuren *et al.*, 2011; van Duuren *et al.*, 2012; Polen *et al.*, 2013). Although this represents a bioconversion process, the benzoic acid source has typically been petroleum-based and it is preferable to use a renewable feedstock. It has previously been reported that ccMA can be produced from sugar using an engineered *Escherichia coli* (Draths and Frost, 1994; Lin *et al.*, 2014) and *Saccharomyces cerevisiae* (Weber *et al.*, 2012; Curran *et al.*, 2013). In these reports, the genes for 3-hydroxyshikimate dehydratase, protocatechuic acid (PA, 3,4-dihydroxybenzoate) decarboxylase, and catechol 1,2-dioxygenase were introduced into the microorganisms, and refinements were made to overproduce aromatic amino acids and related compounds in order to increase the supply of precursors for ccMA production. The resulting engineered strains were able to synthesize ccMA from 3-dehydroxyshikimate. Although advancements in the biological production of ccMA from sugars have occurred, two major issues remain. One of which is the activity of protocatechuate decarboxylase (Pdc, EC 4.1.1.63); and in previous studies, it has been demonstrated that low activity levels of Pdc remains a bottleneck for ccMA production from sugars (Draths and Frost, 1994; Weber *et al.*, 2012; Curran *et al.*, 2013). Only a few

publications are listed in the comprehensive enzyme information system (BRENDA; <http://www.brenda-enzymes.org/>) relative to this Pdc activity. Among these reports, He & Wiegel, 1996, noted that Pdc is a bidirectional enzyme, but that at equilibrium carboxylation is favored (He and Wiegel, 1996). Decarboxylation of PA is what is desired relative to the effective production of ccMA from renewable feedstocks so a mechanism or reaction conditions that shifts the equilibrium of the enzymatic action in this direction is desired. Although there are relatively few reports that discuss the isolation of Pdc from bacteria, Pdc derived from *Sedimentibacter (Clostridium) hydroxybenzoicus* has been isolated and reported as a homopentamer (He and Wiegel, 1996). In the previous studies, the *aroY* (accession no. **AB479384**) (Draths and Frost, 1994) or the gene encoding Pdc (**AB364296**) from *Enterobacter cloacae* P241 (Yoshida *et al.*, 2010) has also been used to encode Pdc in ccMA production from glucose. Protocatechuate decarboxylase belongs to the family of hydroxyarylic acid decarboxylases and is composed of multiple subunits encoded by B, C and D genes as typified by 4-hydroxybenzoate decarboxylase which is encoded by *bsdB*, *bsdC* and *bsdD* (Lupa *et al.*, 2005). It has been also reported that these genes form a gene cluster (Lupa *et al.*, 2005).

Another issue is the availability of feedstock: With sugars already in high demand for food, and other bio-based products such as biofuels, it is desirable to use a feedstock for the production of ccMA that does not compete with the food chain, and that also is of lower cost than sugar. Protocatechuate, a precursor to ccMA, is also an intermediate in the metabolic pathway for degradation of lignin-related aromatic compounds observed in some species of *Pseudomonas* (Jimenez *et al.*, 2002), *Acinetobacter* (Delneri *et al.*, 1995), and *Sphingobium* (Kamimura *et al.*, 2010). Although this suggests that ccMA could potentially be produced from purified lignin-related compounds such as ferulic acid and vanillin via PA, and catalytic pathways for mixed production of ccMA have been reported (Evtuguin *et al.*, 2009; Ma, 2013), no reports of ccMA production from lignin-derived aromatics via biosynthetic pathways have

101 been previously published. The most important aspect of future work for ccMA production
102 from lignin-related compounds is therefore the development of a way to enhance Pdc
103 production and/or activity. In the current work, we grapple with this issue, focusing on a
104 method to enhance Pdc production, and then evaluating the effectiveness of this method on
105 ccMA production from vanillin, which served as a proxy in this work for other lignin-derived
106 aromatics.

108 2. Materials and methods

109 2.1 Bacterial strains and plasmids

110 *Escherichia coli* strain XL-1 Blue, supplied by National Institute of Genetics in Mishima,
111 Japan, was used as the recombinant host. The plasmids and primers used in this study are listed
112 in Table 1. We used *aroY* from *Klebsiella pneumoniae* subsp *pneumoniae* A170-10 which
113 encodes Pdc. *AroY* (accession no. [AB479384](#)) was amplified from pKD136 which was isolated
114 from the genomic library of *K. pneumoniae* subsp. *pneumoniae* A170-10 ([ATCC25597](#))
115 (Draths and Frost, 1994), and the *kpdB*, *kpdC* and *kpdD* encoding subunits of the putative
116 4-hydroxybenzoate decarboxylase were amplified from the genome of *K. pneumoniae*
117 NBRC14940 ([AB920346](#)). Nucleotide sequencing of the gene harboring plasmid pKD136 was
118 performed to identify any previously unreported subunits and identify any unreported open
119 reading frames (ORF) potentially associated with the *aroY* gene.

120 *Vdh* encoding vanillin dehydrogenase, *vanA* and *vanB* encoding alpha- and beta-subunits
121 respectively of vanillate demethylase, and *catA* encoding catechol dioxygenase were amplified
122 from the genome of *Pseudomonas putida* NBRC100650 (KT2440) ([AE01541](#)). Ampicillin (50
123 µg/ml) or chloramphenicol (10 µg/ml) (or both) were added to the media when these
124 transformants were cultivated.

2.2 *In vivo* assay of the Pdc reaction using whole cell biotransformation

E. coli XL-1blue strain was transformed using the plasmids listed in Table 1 to evaluate the decarboxylation abilities of PA by the whole cell reaction. Each resulting transformant, XL-1Blue/pTS032, XL-1Blue/pTS035, XL-1Blue/pTS039, XL-1Blue/pTS044, XL-1Blue/pTS045, XL-1Blue/pTS046, XL-1Blue/pTS047, XL-1Blue/pTS048, XL-1Blue/pTS051, XL-1Blue/pTS036+pUC118, XL-1Blue/pTS036+pTS039, XL-1Blue/pTS036+pTS044, XL-1Blue/pTS036+pTS045, XL-1Blue/pTS036+pTS046, XL-1Blue/pTS036+pTS047, XL-1Blue/pTS036+pTS048, XL-1Blue/pTS036+pTS051, XL-1Blue/pTS036, XL-1Blue/pTS052 and XL-1Blue/pTS053, was grown in 10 ml of LB medium containing appropriate antibiotics, and shake-incubated (Maniatis *et al.*, 1982). Two hundred microliters of each culture was then used to inoculate to 10 ml of fresh LB medium containing appropriate antibiotics and 1mM isopropyl- β -D-thiogalactopyranoside (IPTG) to induce the expression of the recombinant proteins, and these cultures were then shake-incubated at 30°C until an OD₆₀₀ of approximately 1.5 was achieved. Total cells from each culture were then collected by centrifugation (5 min, 6,000g, 4°C) and the pelleted cells were then washed twice using an ice-cold M9 liquid medium (Maniatis *et al.*, 1982). The pellet was then re-suspended in 1 ml of M9 medium containing 1 mM protocatechuic acid (PA, 95.0%, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the suspension was shake-incubated at 30°C for 3 h.

2.3 Analytical conditions of metabolites

A 0.12 ml aliquot of each cell culture was centrifuged for 5 min at 16,000g. Benzoic acid (0.1 ml, 10 mM, BA, 99.5%, Wako Pure Chemical Industries, Ltd.) was added to 0.1 ml of the supernatant as an internal standard. The mixture was acidified with HCl, and then extracted twice with ethyl acetate. The ethyl acetate fraction was collected, dried, and the extract

dissolved in a solvent consisting of 10% (v/v) CH₃OH and 1% (v/v) CH₃COOH in H₂O. An 0.1 ml aliquot of the solution was analyzed using high performance liquid chromatography (HPLC, Agilent Technologies Inc., CA, USA) equipped with a ZORBAX Eclipse Plus C18 column (reverse phase, 4.6 mm in diameter, 150 mm in length, 0.5 µm particle size) run at 40°C using a mobile phase gradient (Solvent A: 10% (v/v) CH₃OH and 1% (v/v) CH₃COOH in H₂O. Solvent B: 50% (v/v) CH₃OH and 1% (v/v) CH₃COOH in H₂O introduced 10 min after injection and ramped from 0 to 100% in 10 min). The flow rate of the mobile phase was 1.0 ml/min and the detection wavelength was 280 nm. Vanillin (Vn, 98.0%) was purchased from Nakalai Tesque, Inc. (Kyoto, Japan). Vanillic acid (VA, 95.0%) and catechol (CL, 99.0%) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and *cis, cis*-muconic acid (ccMA) was purchased from Sigma-Aldrich (St Louis, MO).

2.4 *In vitro* assay of Pdc using cell free extracts

The transformants, XL-1Blue/pTS036, XL-1Blue/pTS052 and XL-1/pTS053, were incubated for 20 h at 30°C in shake culture in 5 ml of LB medium containing 10 µg/ml chloramphenicol. One ml of the culture was then used to inoculate 100 ml of LB medium containing the antibiotic and 1 mM IPTG, and incubated in shake culture at 30°C until an OD₆₀₀ of 1.5 was reached. The cells were then collected, and centrifugally washed (6,000g, 5 min, 4°C) twice with 10 mM potassium phosphate buffer (pH 7.0) containing 5 mM dithiothreitol (DTT). The resulting cell pellet was ground with aluminum oxide in an ice-cold mortar and pestle for 10 min and then suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 5 mM DTT. The suspension was centrifuged (16,000g, 10 min, 4°C) and the supernatant was collected into a new ice-cold microtube and held for 1.5 h on ice before measuring the enzymatic decarboxylation activity on PA. During this period, the protein concentration was measured using a Bradford type protein assay reagent, Quick Start protein assay (Bio-Rad

Laboratories, Inc., CA, USA), and heat blocks and reagents for the enzymatic assay were prepared. Potassium phosphate buffer (0.9 ml, 100 mM, pH 7.0) containing 5 mM DTT and 1 mg of protein from the cell free extract was incubated at 30°C for 10 min, and then PA (0.1 ml, 100 mM, pH 7.0) was added to the reaction mixture and mixed by pipetting briefly. Following 2, 4, 6, 8, and 10 min incubations after the addition of PA, a 0.1 ml portion of the reaction mixture was collected and extracted as described above. All samples were run in triplicate. The extract was subjected to HPLC analysis to quantify CL. From this, the initial PA decarboxylation rate (CL production rate, nmol of CL/min/mg of protein) in each cell free extract was calculated.

2.5 Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and liquid chromatography mass spectrometry (LC-MS).

Crude extracts containing 25 µg of protein were subjected to Glycine SDS-PAGE (15% polyacrylamide gel, BIO CRAFT Co., Ltd., Tokyo, Japan). Samples containing 50 µg protein were also subjected to Tricine SDS-PAGE (15-20% gradient polyacrylamide gel, BIO CRAFT Co., Ltd.). The gels were stained with Coomassie Brilliant Blue. Individual bands corresponding to the expected molecular masses of AroY, KpdB and KpdD (calculated from each deduced amino acid sequence) were excised from the polyacrylamide gels, and subjected to liquid chromatography coupled with mass spectrometry (LC-MS) analysis (NanoFrontier LD, Hitachi High Technologies, Tokyo, Japan) after in-gel reduction and alkylation, and trypsin digestion with XL-Tryp kit (APRO Life Science Institute Inc., Tokushima, Japan). The peptides were separated with an Inertsil ODS-3 column (reverse phase, 2.1 mm in diameter, 50 mm in length, 3.0 mm particle size, GL Sciences Inc., Tokyo, Japan) using a mobile phase gradient (Solvent A: 0.1% (v/v) HCOOH in H₂O and Solvent B: 0.1% (v/v) HCOOH in Acetonitrile. Initial composition of the mobile phase was 95% A/5% B. After injection, Solvent B was ramped from 5% to 75% in 15 min linearly, followed by washing with 95%

Solvent B for 10 min). The column oven temperature was 40°C, the flow rate of the mobile phase was 0.2 ml/min, and the spray voltage for ionization of protein was 4.5 KV. Peptide mass fingerprints using Mascot software (Matrix Science Ltd, UK) were done for database searching against the NCBI database.

2.6 Biotransformation of vanillin to ccMA via whole cell reaction.

XL-1Blue/pHM002+pTS036, XL-1Blue/pHM002+pTS052 (Table 1) were shake-cultured (20 h, 30°C) in 10 ml of LB medium containing 50 µg/ml ampicillin and 10 µg/ml chloramphenicol. Aliquots (0.2 ml each) were the shake-cultured (30°C, 10 ml fresh LB medium containing 10 µg/ml chloramphenicol and 50 µg/ml ampicillin, and 1 mM IPTG) to induce expression of the recombinant protein. Cells from each culture were collected when an OD₆₀₀ of 1.5 was obtained, and the cells were then centrifugally washed (5 min, 6,000g, 4°C) twice with ice-cold M9 liquid medium. The cell pellet was re-suspended in 1 ml of M9 medium containing 1 mM Vn and the suspension was shake-incubated at 30°C. A portion of the suspension was subjected to HPLC analysis to quantify the metabolites.

3. Results and Discussion

3.1 A new gene enabling enhanced protocatechuate decarboxylase activity in *E. coli*.

Analysis of the nucleotide sequence of the gene, harbored in the plasmid pKD136, revealed an open reading frame (ORF) of approximately 0.2 kb by the 3' downstream region of *aroY* and the gene appeared to be in the same transcription unit with *aroY*. The deduced amino acid sequence for the ORF was not related to other annotated proteins, and did not have homologies with any of the hydroxyarylic acid decarboxylases subunits. To examine the effect of expression of the ORF on Pdc reaction, we co-expressed *aroY* and the ORF in *E. coli*. However the recombinant *E. coli* XL-1Blue/pTS035 did not show a significant increase in

production of Pdc as measured in terms of CL production *in vivo* from PA.

A homology search with the deduced amino acid sequence associated with AroY for the entire *K. pneumoniae* subsp. *pneumoniae* MGH78578 genomic sequence was conducted using metagenomic search tool at the Integrated Microbial Genome system (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>) to examine whether other isozymes for PA decarboxylation may be encoded in the MGH78578 genome. AroY identity associated with three ORFs was as follows: KPN_01161p (99%), KPN_03097p (24%) and KPN_04337p (24%). The KPN_01161p has previously been annotated as a putative carboxylase-related protein associated with AroY at a relatively high confidence level. The KPN_04337p was annotated as a 3-octaprenyl-4-hydroxybenzoate decarboxylase which catalyzes the decarboxylation step in the ubiquinone biosynthesis pathway. Among the three homologous regions, we focused our interest on the KPN_03097 locus. The deduced amino acid sequence for AroY had a 24% identity with that of KPN_03097p which has been annotated as a putative 3-polyprenyl-4-hydroxybenzoate decarboxylase and related decarboxylase. The deduced amino acid sequence for KPN_03097p had a 100% identity with KpdC, a subunit of a heterooligomeric 4-hydroxybenzoate decarboxylase from *K. pneumoniae* subsp. *pneumoniae* NCTC418 (**AAAY57855**); 71% with VdhC, a subunit of vanillate decarboxylase from *Streptomyces* sp. D7 (**AAD28782**); and 63% with BsdC, a 4-hydroxybenzoate decarboxylase subunit C from *Bacillus subtilis* subsp. *subtilis* 168 (**CAB12158**) (Lupa *et al.*, 2005). Since there are no promoter-like sequences between KPN_03098 and KPN_03096 loci, it was predicted that the KPN_03097 locus would be located on the same transcription unit with KPN_03098 and KPN_03096 loci, with the deduced amino acid sequence of these later two having 100% identity with those of KpdB (**AAAY57854**) and KpdD (**AAAY57854**), respectively. Thus we designated these genes as *kpdB* (for KPN_03098 locus), *kpdC* (for KPN_03097 locus) and *kpdD* (for KPN_03096 locus). The DNA fragment containing

kpdBCD genes was amplified from the genomic DNA of *K. pneumoniae* subsp. *pneumoniae* NBRC14190 by PCR and cloned. The nucleotide sequence of the amplified DNA showed 99% identity with that of the MGH78578 strain. The *E. coli* recombinant strain XL-1Blue/pTS039 expressing KpdBCD was capable of PA decarboxylation as reported previously (Lupa *et al.*, 2005), however, no apparent increase in decarboxylation was observed in our work as monitored by the production of CL (Fig. 1). In addition, the results of the *in vivo* Pdc assay with the deletion clones of pTS039 (Table 1) indicated that KpdBCD is a heterooligomeric enzyme composed of the three (B, C, D) different subunits (Fig. 1). We confirmed that a KpdBCD-expressing *E. coli* acquired PA decarboxylating ability upon transformation, however, KpdBCD was unable to decarboxylate PA more effectively than AroY alone.

An amino acid sequence comparison using the CLUSTAL W program (Thompson *et al.*, 1994) demonstrated a 27% identity between the deduced amino acid sequences of AroY and that of KpdC (a subunit of heterooligomeric 4-hydroxybenzoate decarboxylase). We hypothesized that AroY might interact with KpdB and KpdD as a heterooligomeric enzyme to decarboxylate PA efficiently, and thus we examined the expression of *aroY* together with *kpdB* and *kpdD*. As measured by the *in vivo* Pdc assay, the decarboxylation of PA to produce CL was enhanced by the simultaneous co-expression of both *kpdB* and *kpdD* with *aroY* (Fig. 1, pTS036+pTS051) despite the fact that the expression of *kpdB* (Fig. 1, pTS046), *kpdC* (Fig. 1, pTS048) and both expressed together (Fig. 1, pTS051) did not result in PA decarboxylation in our *in vivo* assay when tested in the absence of *aroY*. We further examined if other deletion clones of pTS039 affected PA decarboxylation when AroY was present, to reveal that the essential gene for PA decarboxylation enhancement was *kpdB* (Fig. 1, pTS036+pTS046). We also observed that additional expression of *kpdD* with both of *aroY* and *kpdB* was able to slightly enhance decarboxylation (Fig. 1, pTS036+pTS051). It is important to consider and

note that pTS036 is a pMCL200 derivative plasmid originating from p15A. However, both pTS048 and pTS051 are pUC118 derivatives and their origins are from ColE1. As these two types of plasmids are compatible, the host cells can maintain both. However in this case, the differences in the copy numbers of both plasmids may affect the results. To determine which gene set is better for ccMA bioproduction, we prepared two plasmids, pTS052 and pTS053 (Table 1 and Fig. 2), the former one contained *aroY* and *kpdB* and the latter contained *aroY*, *kpdB* and *kpdD* in a same transcriptional unit. As these plasmids had the same genetic background as pTS036, the effects of KpdB and KpdD on Pdc activity could be compared appropriately. We transformed the *E. coli* XL-1Blue strain with pTS036, pTS052 and pTS053 respectively, for comparison of both enzymatic activity and expression of AroY. Pdc enzymatic activity depended on the addition of DTT and we optimized its concentration at 5mM in the reaction mixture. Using the methods above, the CL concentration increased linearly over the monitoring period, thus we measured and calculated CL production rate as a function of Pdc enzymatic activity. Pdc enzymatic activity in the cell free extract of XL-1Blue/pTS052 was approximately 14-fold greater than that of XL-1Blue/pTS036. Also, the cell free extract of XL-Blue/pTS053 strain had 7-fold greater Pdc enzymatic activity than the XL-1Blue/pTS036 strain. The values could potentially change with changes in reaction conditions including temperature, pH and salt concentration, but enzymatic activity also followed a similar trend in the *in vivo* assay (Fig. 2). As both abilities in PA uptake and CL export of the host cell affect the CL production rates in the *in vivo* assay, differences in the increase rates between the *in vivo* assay and the *in vitro* assay would be observed.

SDS-PAGE analysis of the cell free extract from XL-1Blue/pTS052 showed the apparent expression of two proteins; one with a molecular mass of about 54 kDa, which was consistent with that from the deduced AroY amino acid sequence, and the other about 22 kDa, which was consistent with that from the deduced KpdB amino acid sequence. Protein mass

301 spectrometry analysis of the 54 kDa protein band revealed 19 peptides associated with AroY
302 having 55% sequence coverage. In the 22 kDa band, 11 peptides associated with KpdB were
303 identified with 70% sequence coverage (Table 2). Co-expression of KpdB increased AroY
304 expression (Fig. 3A, lane 2), but co-expression of KpdB with AroY and KpdB reduced the
305 expression levels of both AroY and KpdB (Fig. 3A, lane 3). However, the level of AroY
306 expressed appeared to be slightly higher than that observed in the cell free extract expressed
307 AroY only (Fig. 3A, lane 1). Because the expected molecular mass of KpdB is about 9.2 kDa
308 and Glycine SDS-PAGE is not suitable for evaluation of such low molecular mass proteins,
309 Tricine SDS-PAGE was also conducted to observe whether significant expression of KpdB
310 would occur. Although increased expression was not observed at the molecular mass expected
311 from the deduced KpdB amino acid sequence (Fig. 3B, lane 3), we expected KpdB would be
312 observed in the range of 9 kDa to 10 kDa. Protein mass spectrometry analysis revealed 5
313 peptides of KpdB with 62% sequence coverage in the protein band at the molecular mass of
314 KpdB (Table 2) indicating that KpdB was expressed. These results indicate that KpdB
315 expression was associated with a lower CL production rate (Fig. 2). KpdB may therefore,
316 have a role in improving the stability and solubility of AroY in the intracellular fraction of the
317 *E. coli* recombinant strain, however, KpdB may inhibit either or both AroY and KpdB in
318 aspects such as expression and stability of transcripts and proteins in the *E. coli* recombinant
319 strain. This later aspect is demonstrated by the failure of the co-expressed KpdB and KpdB to
320 increase expression of AroY (Fig. 3A, lane 3). Recently it has been reported that PA
321 decarboxylation via AroY was stimulated by the co-expression of both B and C subunits of a
322 family of hydroxyarylic acid decarboxylases in *Sacharomyces cerevisiae* (Weber *et al.*,
323 2012). However our results, using *E. coli* as the host cell, indicate that the expression of KpdB
324 is the sole essential factor required for enhancement of Pdc enzymatic activity for ccMA
325 bioproduction (Fig. 2 and 3). The polycistronic localization of *kpdB* with *aroY* and *kpdB* (Fig.

2, pTS053) or the higher copy number (expression level) of *kpdD* relative to *aroY* may affect the levels in transcription and translation of those genes because PA decarboxylation was highest when *kpdD* was expressed in another transcription unit which was different from *aroY* (Fig. 1, pTS036+pTS051). In this work, we confirmed the significant increase of Pdc enzymatic activity by the co-expression of KpdB with AroY, however, it remains to understand whether KpdD expression is effective on PA decarboxylation.

3.2 Enhanced ccMA production from vanillin in a whole cell system with enhanced expression of Pdc.

Using the newly modified strain with co-expressed AroY and KpnB to enhance Pdc activity, we examined ccMA production from Vn with *E. coli* recombinants. XL-1Blue/pHM002+pTS036 accumulated VA and PA temporarily during the incubation (Fig. 4A). We speculated that PA accumulation was caused by the low decarboxylation activity of AroY in this strain. The strain with enhanced Pdc expression, XL-1Blue/pHM002+pTS052, did not accumulate PA during the incubation period and ccMA production was observed to be enhanced significantly (Fig. 4B). One of the major issues for ccMA production, PA accumulation associated with low Pdc activity, was therefore solved using the modified pathway developed in this work. However, demethylation of VA could potentially be a new bottleneck in ccMA production from lignin-waste materials since we observed VA accumulation in the case of both XL-1Blue/pHM002+pTS036 and XL-1Blue/pHM002+pTS052 (Fig. 4B). Although temporary VA accumulation was observed during ccMA production, the accumulating VA was converted slowly to ccMA via PA decarboxylation by further incubation. We speculated that the VA accumulation was caused by PA accumulation associated with low decarboxylation activity, so we expected the accumulation would be resolved by the expression of enhanced Pdc. But this was not observed

(Fig. 4B). Therefore, we hypothesized that ccMA may inhibit VA demethylase, To confirm this hypothesis we added ccMA (0.5 mM) together with Vn into the culture. However there was no significant decrease in ccMA production compared to that without the addition of ccMA. The host organism, *P. putida*, can take up beta-carboxy-*cis,cis*-muconate into a cell (Meagher *et al.*, 1972; Chua and Hsieh, 1990) but the ability to take up ccMA and whether the cognate transporter is ccMA-inducible has not been demonstrated. We were unable to demonstrate whether ccMA could be taken up and metabolized by *P. putida* in this study, so instead we evaluated CL production from vanillin. If VA accumulation occurred during CL production, our hypothesis that ccMA inhibits VA demethylase would be incorrect. To test this, we prepared an additional plasmid excluding *catA* from pHM002 (designated as pKY003, Table 1) and examined CL production from Vn using the recombinant strains, XL-1Blue/pKY003+pTS036 and XL-1Blue/pKY003+pTS052 and confirmed that no VA accumulation occurred (data not shown). These results suggest that ccMA may cause a feedback inhibition of VA demethylase and that this may prove to be a further bottleneck for ccMA production from vanillin and other lignin-related aromatics.

4. Conclusions

In this work, we focused on sustainable ccMA production from lignin-related aromatic compounds vs. from sugars. We identified a way to enhance Pdc activity through co-expression of *aroY* and the newly identified *kpdB*, and demonstrated an increase in decarboxylation efficiency both *in vitro* and *in vivo*. We further demonstrated that ccMA production from lignin-related compounds could be achieved via the enhanced Pdc pathway; however a new bottleneck was observed in that ccMA appears to inhibit the VA demethylation step in the pathway.

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Table 1 Plasmids and oligonucleotide primers used in this study.

Plasmids	Description	Primers	Accession no.
pTS032	1.6 kbp <i>KpnI</i> fragment containing <i>aroY</i> was cloned into pUC118, provided by National Institute of Genetics, Japan.	5'-AGCTGGTACCATTAAAGAGGAGAAATTA ACTA TGACCGCACCGATT CAG-3' 5'-AGCTGGTACCTTATTTT GCGCTACCCTGGTT-3'	<u>AB479384</u>
pTS035	2.4 kbp <i>KpnI</i> fragment containing <i>aroY</i> and 0.8 kbp region located in 3' downstream of <i>aroY</i> containing a small open reading frame was cloned into pUC118.	5'-AGCTGGTACCATTAAAGAGGAGAAATTA ACTA TGACCGCACCGATT CAG-3' 5'-GGGGTACCCTTGC ACTATTTACCCGA-3'	<u>AB479384</u>
pTS039	2.2 kbp <i>EcoRI-XbaI</i> fragment containing <i>kpdB</i> , <i>kpdC</i> , and <i>kpdD</i> from <i>K. pneumoniae</i> NBRC14940 was cloned into pUC118.	5'-TGAATTCATTAAAGAGGAGAAATTA ACTATGA AACTGATTATTGGGATGACG-3' 5'-AGCTTCTAGACGGGCCCCTTTCATTAAC-3'	<u>AB920346</u>
pTS044	2.1 kbp <i>EcoRI-XbaI</i> fragment containing <i>kpdB</i> and <i>kpdC</i> from <i>K. pneumoniae</i> NBRC14940 was cloned into pUC118.	5'-TGAATTCATTAAAGAGGAGAAATTA ACTATGA AACTGATTATTGGGATGACG-3' 5'-AGCTTCTAGATATTTACGGTTGGCCAGCAT-3'	<u>AB920346</u>
pTS045	1.6 kbp <i>EcoRI-XbaI</i> fragment containing <i>kpdC</i> and <i>kpdD</i> from <i>K. pneumoniae</i> NBRC14940 was cloned into pUC118.	5'-AGCTGAATTCATTAAAGAGGAGAAATTA ACTA TGGCTTTTGATGATTTGCG-3' 5'-AGCTTCTAGACGGGCCCCTTTCATTAAC-3'	<u>AB920346</u>
pTS046	0.6 kbp <i>EcoRI-XbaI</i> fragment containing <i>kpdB</i> from <i>K. pneumoniae</i> NBRC14940 was cloned into pUC118.	5'-TGAATTCATTAAAGAGGAGAAATTA ACTATGA AACTGATTATTGGGATGACG-3' 5'-AGCTTCTAGATATTTCGATCTCCTGTGCAAAT-3'	<u>AB920346</u>
pTS047	1.4 kbp <i>EcoRI-XbaI</i> fragment containing <i>kpdC</i> from <i>K. pneumoniae</i> NBRC14940 was cloned into pUC118.	5'-AGCTGAATTCATTAAAGAGGAGAAATTA ACTA TGGCTTTTGATGATTTGCG-3' 5'-AGCTTCTAGATATTTACGGTTGGCCAGCAT-3'	<u>AB920346</u>
pTS048	0.2 kbp <i>EcoRI-XbaI</i> fragment containing <i>kpdD</i> from <i>K. pneumoniae</i> NBRC14940 was cloned into pUC118.	5'-AGCTGAATTCATTAAAGAGGAGAAATTA ACTA TGATTTGTCCACGTTGCG-3' 5'-AGCTTCTAGACGGGCCCCTTTCATTAAC-3'	<u>AB920346</u>
pTS051	0.6 kbp <i>EcoRI-XbaI</i> (blunted) fragment was subcloned into <i>EcoRI</i> site (blunted) of pTS048.		

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Table 1 Plasmids and primers used in this study (continued).

Plasmids	Description	Primers	Accession no.
pTS036	1.6 kbp <i>KpnI</i> fragment from pTS032 was subcloned into pMCL210.		
pTS052	0.6 kbp <i>EcoRI-XbaI</i> (blunted) fragment from pTS046 was subcloned into <i>XbaI</i> site (blunted) of pTS036.		
pTS053	0.2 kbp <i>EcoRI-XbaI</i> (blunted) fragment from pTS048 was subcloned into <i>SacI</i> site (blunted) of pTS052.		
pTS001	1.5 kbp <i>SacI-KpnI</i> fragment containing <i>vdh</i> from <i>P. putida</i> KT2440 was cloned into pUC118.	5'-AGCTGAGCTCTTAAAGAGGAGAAATTA ACTAT GTTGCAGGTGCCTTTGCT-3' 5'-AGCTGGTACCCTAGATGGGATAGTGACGCG-3'	<u>NC002947</u>
pKY001	2.0 kbp <i>SacI-SmaI</i> fragment containing <i>vanA</i> and <i>vanB</i> from <i>P. putida</i> KT2440 was cloned into pQE30, purchased from QIAGEN KK, Japan.	5'-ATGCGAGCTCATGTACCCCAAAAACACCT-3' 5'-ATGCCCCGGGTCAGATGTCCAGCACCAGCA-3'	<u>NC002947</u>
pNI001	1.0 kbp <i>KpnI-SmaI</i> fragment containing <i>catA</i> from <i>P. putida</i> KT2440 was cloned into pUC118.	5'-ATGCGGTACCTTAAAGAGGAGAAATTA ACTAT GACCGTGAAAATTTCCCA-3' 5'-AGCTCCCCGGGTCAGCCCTCCTGCAACGCCC-3'	<u>NC002947</u>
pHM002	1.0 kbp <i>KpnI-SmaI</i> fragment from pNI001 was subcloned into <i>KpnI-SmaI</i> sites of pKY003.		
pKY003	1.5 kbp <i>EcoRI-SmaI</i> fragment from pKY001 was subcloned into <i>EcoRI-SacI</i> (blunted) site of pTS001.		

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Figure legends

Fig. 1 Gene constructs used for *in vivo* Pdc assay and PA decarboxylation rates.

Detailed information of the plasmids is shown in Table 1. The values shown are the means of results from triplicate experiments, and error bars on the graph indicate standard deviations. The significance of differences was analyzed by Tukey-Kramer Honestly Significant Difference test at the $P=0.05$ level (Kramer, 1956). Bars with different superscript letters are statistically different from each other ($P<0.05$). Bars with the same superscript letters are not significantly different from each other. Arrows; black, *aroY*; white, *kpdB*; dark gray, *kpdC*; light grey, *kpdD*; dotted; a small ORF located in the 3' downstream region of *aroY*.

Fig. 2 Gene constructs used for *in vivo* Pdc assay and Pdc activities in each cell free extract.

Detailed information of the plasmids is shown in Table 1. The values shown are the means and standard deviations of results from triplicate experiments. The significance of differences was analyzed by Tukey-Kramer Honestly Significant Difference test at the $P=0.05$ level. Different superscript letters are statistically significant different from each other ($P<0.05$).

Fig. 3 SDS-PAGE analysis.

(A) Glycine SDS-PAGE: Each cell free extract containing 25 μ g of crude protein was separated using a 15% polyacrylamide gel (BIO CRAFT Co. Ltd., Tokyo, Japan). Lanes; 1, XL-1Blue/pTS036; 2, XL-1Blue/pTS052; 3, XL-1Blue/pTS053. A protein standard marker (BLUE Star Prestained Protein-Ladder, NIPPON Genetics, Co, Tokyo, Japan) was used to estimate molecular weights of the proteins. (B) Tricine SDS-PAGE: Each cell free

extract containing 40 µg of crude protein was separated using a 15-20% gradient polyacrylamide gel (BIO CRAFT Co. Ltd.). Lanes; 1, XL-1Blue/pTS036; 2, XL-1Blue/pTS052; 3, XL-1Blue/pTS053. A protein standard marker (SIGMA protein marker broad range, Sigma Aldrich, St Louis, MO) was used to estimate molecular mass of the proteins. Arrowheads on the right side show the positions of the bands excised for LC-MS, with each molecular mass of AroY (54013.6 Da), KpdB (21557.6 Da) and KpdD (9225.9 Da), calculated from the amino acid sequence originally deduced.

Fig. 4 Bioconversion of Vn to ccMA by *E. coli* recombinants.

(A) XL-1Blue/pHM002+pTS036 expressing *vdh*, *vanA*, *vanB*, *catA* and *aroY*, (B) XL-1Blue/pHM002+pTS052 expressing *vdh*, *vanA*, *vanB*, *catA*, *aroY* and *kpdB*. Symbols; Vn, closed square; VA, closed circle; PA, open square; ccMA, open circle. The values shown are the means of results from triplicate experiments and error bars on the graph indicate standard deviations. CL concentration was less than 0.005mM during the experiments.

Fig. 1 Sonoki et al.

		<i>In vitro</i> assay CL production rate (nmol of CL/min/mg of protein)	<i>In vivo</i> assay CL production rate (μmol of CL/h)
	0.5 kbp		
pTS036		$8.22^a \pm 0.67$	$0.15^a \pm 0.00$
pTS052		$114.11^b \pm 5.37$	$0.28^b \pm 0.03$
pTS053		$58.29^c \pm 1.61$	$0.19^c \pm 0.01$

Fig. 2 Sonoki et al.

(A)

(B)

Fig. 3 Sonoki et al.

Fig. 4 Sonoki et al.