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Enhancing muconic acid production from glucose and lignin-derived aromatic compounds via increased protocatechuate decarboxylase activity

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ABSTRACT

The conversion of biomass-derived sugars and aromatic molecules to *cis,cis*-muconic acid (referred to hereafter as muconic acid or muconate) has been of recent interest owing to its facile conversion to adipic acid, an important commodity chemical. Metabolic routes to produce muconate from both sugars and many lignin-derived aromatic compounds require the use of a decarboxylase to convert protocatechuate (PCA, 3,4-dihydroxybenzoate) to catechol (1,2-dihydroxybenzene), two central aromatic intermediates in this pathway. Several studies have identified the PCA decarboxylase as a metabolic bottleneck, causing an accumulation of PCA that subsequently reduces muconate production. A recent study showed that activity of the PCA decarboxylase is enhanced by co-expression of two genetically associated proteins, one of which likely produces a flavin-derived cofactor utilized by the decarboxylase. Using entirely genome-integrated gene expression, we have engineered *Pseudomonas putida* KT2440-derived strains to produce muconate from either aromatic molecules or sugars and demonstrate in both cases that co-expression of these decarboxylase associated proteins reduces PCA accumulation and enhances muconate production relative to strains expressing the PCA decarboxylase alone. In bioreactor experiments, co-expression increased the specific productivity (mg/g cells/h) of muconate from the aromatic lignin monomer *p*-coumarate by 50% and resulted in a titer of >15 g/L. In strains engineered to produce muconate from glucose, co-expression more than tripled the titer, yield, productivity, and specific productivity, with the best strain producing 4.92 ± 0.48 g/L muconate. This study demonstrates that overcoming the PCA decarboxylase bottleneck can increase muconate yields from biomass-derived sugars and aromatic molecules in industrially relevant strains and cultivation conditions.

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1. Introduction

Muconic acid is an intermediate in the β-ketoadipate pathway employed by many microbes for catabolism of aromatic compounds (Harwood and Parales, 1996; Ornston and Stanier, 1966). There is substantial interest in producing muconic acid from biomass, typically motivated by the ability to efficiently convert muconic acid to adipic acid by catalytic hydrogenation under mild conditions (Vardon et al., 2015). Adipic acid is an industrially important dicarboxylic acid that is a precursor to nylon 6,6, among other polymers. It is conventionally produced by nitric acid oxidation of cyclohexanol and cyclohexanone, releasing nitrous acid. With a market volume of 2.6 million tons per year, adipic acid production contributes substantially to the nitrous acid-mediated generation of ozone-producing free radicals, thus prompting substantial efforts to produce it from renewable resources (Deng et al., 2016; Polen et al., 2013; Van de Vyver and Román-Leshkov, 2013). Recently, it has been shown that *trans,trans*-muconic acid can be converted catalytically to diethyl terephthalate, another important commodity polymer precursor (Lu et al., 2015).

Muconic acid can be produced biologically by dioxygenase enzymes that catalyze intradiol ring-cleavage of catechol, a central intermediate in one branch of the β-ketoadipate pathway (Fig. 1) (Xie et al., 2014). However, many aromatic molecules derived from the depolymerization of lignin, which accounts for 15–30% of the dry weight of biomass, are metabolized through a parallel branch of the β-ketoadipate pathway in which protocatechuate (PCA), rather than catechol, serves as the central intermediate. Employing a PCA decarboxylase, which converts PCA to catechol, has enabled the production of muconate from lignin monomers such as *p*-

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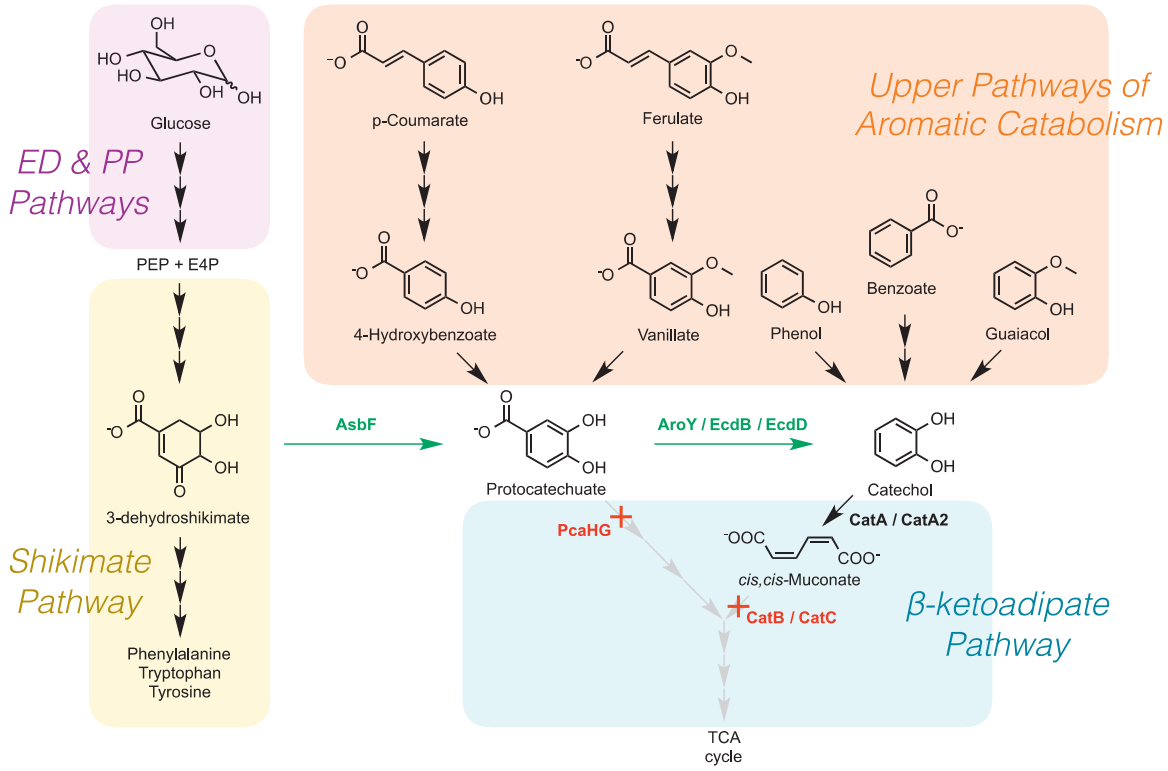

Fig. 1. Metabolic pathways for production of muconate from glucose and lignin-derived aromatic compounds. In *P. putida* KT2440, glucose is metabolized through the Entner-Doudoroff (ED) and pentose phosphate (PP) pathways to produce phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), which can be condensed to enter the shikimate pathway for aromatic amino acid biosynthesis. An intermediate in the shikimate pathway, 3-dehydroshikimate, can be converted to PCA by the action of a 3-DHS dehydratase, such as AsbF from *Bacillus cereus* used here. Deletion of the genes encoding the PCA dioxygenase, PcaHG, and integration of genes encoding the PCA decarboxylase AroY from *Enterobacter cloacae* and two associated proteins, EcdB and EcdD, from enables PCA to be converted to catechol rather than entering the β-ketoadipate pathway. Two paralogous dioxygenases, CatA and CatA2, convert catechol to muconate, which accumulates due to deletion of the genes encoding CatB and CatC, two enzymes required for further metabolism of muconate. Lignin-derived aromatic molecules are metabolized through upper pathways to form catechol in the case of phenol or guaiacol while *p*-coumarate, ferulate, 4-hydroxybenzoate, and vanillate are metabolized to form PCA, which can then be converted to catechol by the action of the PCA decarboxylase for subsequent conversion to muconate.

coumarate, ferulate, 4-hydroxybenzoate, and vanillate (Vardon et al., 2015) as well as from glucose via a 3-dehydroshkimate (3-DHS) dehydratase that converts this intermediate in the shikimate pathway for aromatic amino acid biosynthesis to PCA (Curran et al., 2013; Draths and Frost, 1994; Jung et al., 2015; Niu et al., 2002; Weber et al., 2012). An accumulation of PCA has been observed in strains engineered to produce muconate from aromatic molecules and sugars (Curran et al., 2013; Sonoki et al., 2014; Weber et al., 2012) and is an indication of insufficient PCA decarboxylase activity. The accumulation of intermediates not only reduces the yield and productivity of the engineered biocatalyst, but even trace amounts of residual aromatic compounds can significantly affect the separation of muconate from fermentation broth (Vardon et al., 2016).

Sonoki et al. recently described a means of increasing activity of the PCA decarboxylase that may enable those pursuing strategies to produce muconate via PCA to overcome this bottleneck (Sonoki et al., 2014). Most genes encoding decarboxylases in the hydroxyarylic acid decarboxylase family that includes the PCA decarboxylase, AroY, are co-expressed as an operon with two other small genes shown to be important to activity of the decarboxylase (Lupa et al., 2005; 2008; Matsui et al., 2006). These three genes, *BCD*, are typically clustered in an operon and named for the organism in which they are found (i.e. *Klebsiella pneumoniae* decarboxylase: *kpdB*, *kpdC*, and *kpdD*) with the *C* gene encoding the decarboxylase. While AroY from *Klebsiella pneumoniae* exhibits activity when expressed alone in *Escherichia coli*, Sonoki et al. hypothesized that co-expression of KpdB and/or KpdD, might enhance activity of AroY (Sonoki et al., 2014). Weber and colleagues had previously co-expressed KpdB and KpdD with AroY, but did not compare the PCA decarboxylase activity they achieved with the activity of AroY expressed alone so as to be able to interpret the importance of KpdB and KpdD co-expression (Weber et al., 2012). Sonoki and colleagues found that, in an *Escherichia coli* host, plasmid-based co-expression of KpdB and, in some cases KpdD, enhanced PCA decarboxylase activity relative to expression of AroY alone, essentially eliminating this bottleneck and enhancing production of muconate from vanillin, a lignin-monomer model compound (Sonoki et al., 2014). While the function of B and D proteins were previously unknown, it was recently discovered that homologues of the B protein, UbiX from *E. coli* and PAD1 from *Saccharomyces cerevisiae*, synthesize a novel, prenylated flavin cofactor required for the activity of decarboxylases homologous to the hydroxyarylic acid decarboxylases (Lin et al., 2015; Payne et al., 2015; White et al., 2015). It is likely, then, that KpdB also produces this cofactor, which is required for the decarboxylase activity of AroY.

We previously reported the production of muconate from model lignin monomers as well as alkaline pretreated corn stover by an engineered *P. putida* KT2440 strain (Vardon et al., 2015). This strain utilized the AroY PCA decarboxylase from *Enterobacter cloacae*, which enabled muconate production but also exhibited a substantial accumulation of PCA, similar to that observed by others as described above. In the present study, we sought to apply co-expression of the decarboxylase-associated proteins examined by Sonoki et al. to our system, aiming to improve muconate production. Using genome-integrated gene expression in *P. putida* KT2440-based strains, we demonstrate that co-expression of these

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