Metabolic Engineering of *Escherichia coli* for Efficient Production of 2-Pyrone-4,6-dicarboxylic Acid from Glucose

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ABSTRACT: 2-Pyrone-4,6-dicarboxylic acid (PDC) is a pseudoaromatic dicarboxylic acid and is a promising biobased building block chemical that can be used to make diverse polyesters with novel functionalities. In this study, *Escherichia coli* was metabolically engineered to produce PDC from glucose. First, an efficient biosynthetic pathway for PDC production from glucose was suggested by *in silico* metabolic flux simulation. This best pathway employs a single-step biosynthetic route to protocatechuic acid (PCA), a metabolic precursor for PDC biosynthesis. On the basis of the selected PDC biosynthetic pathway, a shikimate dehydrogenase (encoded by *aroE*)-deficient *E. coli* strain was engineered by introducing heterologous genes of different microbial origin encoding enzymes responsible for converting 3-dehydroshikimate (DHS) to PDC, which allowed *de novo* biosynthesis of PDC from glucose. Next, production of PDC was further improved by applying stepwise rational metabolic engineering strategies. These include elimination of feedback inhibition on 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (encoded by *aroG*) by overexpressing a feedback-resistant variant, enhancement of the precursor phosphoenolpyruvate supply by changing the native promoter of the *ppsA* gene with the strong *trc* promoter, and reducing accumulation of the major byproduct DHS by overexpression of a DHS importer (encoded by *shIA*). Furthermore, cofactor (NADP⁺/NADPH) utilization was manipulated through genetic modifications of the *E. coli* soluble pyridine nucleotide transhydrogenase (encoded by *sthA*), and the resultant impact on PDC production was investigated. Fed-batch fermentation of the final engineered *E. coli* strain allowed production of 16.72 g/L of PDC from glucose with the yield and productivity of 0.201 g/g and 0.172 g/L/h, respectively, representing the highest PDC production performance indices reported to date.

KEYWORDS: 2-pyrone-4,6-dicarboxylic acid, protocatechuic acid, biomonomer, metabolic engineering, *Escherichia coli*

To the best of our knowledge, synthesis of PDC by means of chemical methods has never been reported. Over the past decades, a wide range of chemicals and materials have been produced from renewable resources by metabolically engineered microorganisms.⁷,⁸ Production of aromatic compounds including aromatic amino acids has been well documented.⁷,⁸ However, microbial production of PDC has rarely been reported except for the following two studies. In the first study, whole-cell biotransformation of protocatechuc acid (PCA) into PDC was achieved in a recombinant *Pseudomonas putida* strain by introducing PCA 4,5-dioxygenase (encoded by *ligA* and *ligB*) and 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS) dehydrogenase (encoded by *ligC*) from *S. paucimobilis* SYK-6.⁹ In the second study more recently reported, de

Received: July 3, 2018
Published: August 10, 2018

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new PDC biosynthesis from glucose was realized by constructing a PDC biosynthetic pathway that stems from the shikimate (SHK) pathway. However, the PDC titer obtained was rather low (350 mg/L). The authors in this
report simultaneously adopted two different pathway routes to obtain the precursor PCA, which might be less than optimal due to the potential carbon flux loss at multiple nodes. Additionally, all the genes in the above study were overexpressed using the strong T7 promoter from plasmid, which is not necessarily optimal for the production of metabolites including PDC.1,11,12

To develop a bacterial strain capable of efficiently producing PDC from simple carbon sources, we aimed at establishing a synthetic pathway allowing efficient carbon flux throughout the pathway from glucose to PDC (Figure 1) aided by in silico metabolic flux simulation studies. After successful de novo biosynthesis of PDC from glucose by introducing the necessary and screened heterologous enzymes, PDC production was further improved through elimination of feedback repression on the key enzyme in the aromatic pathway, increase of precursors availability and importer engineering for byproduct reduction. In addition, the redox cofactor (NADPH) regeneration for optimal PDC biosynthesis was considered, which revealed an important role of redox cofactor balance on PDC production. Ultimately, fed-batch culture of the best-performing engineered strain was performed to demonstrate its potential for the production of PDC from glucose.

RESULTS AND DISCUSSION

Comparison of Different PDC Biosynthetic Pathways via in Silico Flux Response Analysis. The biosynthetic pathway from glucose to PDC comprises two parts (Figure 1). At first, carbon flow is directed from glucose to PCA, the first heterologous metabolite in the PDC biosynthetic pathway. Next, PCA is converted to PDC by an aromatic ring cleavage operon. In E. coli, PCA can be produced through two different routes (Figure 2A). One route is from 3-dehydroshikimate (DHS) to PCA via a single heterologous enzymatic reaction catalyzed by DHS dehydratase, which is designated as the single-step route in this study. Several previous studies have employed this single-step PCA route for building synthetic metabolic pathways leading to production of various PCA-derived chemicals, including adipic acid, cis,cis-muconic acid and catechol.14–16 To promote the availability of DHS to be converted into PCA using this single-step route, formation of SHK from DHS was prevented by the inactivation of SHK dehydrogenase.17 The other route to PCA synthesis is from 4-hydroxybenzoate (4HBA), which is derived from the end product of SHK pathway and converted into PCA by 4HBA hydroxylase. Combing the conversions of DHS to 4HBA and 4HBA to PCA, it takes totally six enzymatic steps to obtain PCA from DHS through 4HBA, which is designated as the six-step route. Recently, this six-step PCA route has been utilized in a number of studies for constructing novel biosynthesis pathways for value-added aromatic products.17,18

Despite both the PCA-supplying routes described above have been reported and used for production of various aromatic chemicals of interest, a systematic comparison between the two has not been made. In order to select more efficient biosynthetic pathway for PDC production, we first performed in silico flux analyses on both the genome-scale metabolic networks employing the single-step or six-step pathway for PDC production from glucose in E. coli. The simulation results indicated that the single-step PDC pathway was more efficient for PDC production than the six-step pathway, as reflected by the higher specific PDC production rate (Figure 2B). The theoretical maximum PDC yields obtainable by employing the single-step pathway and the six-step pathway were calculated to be 0.906 and 0.738 g/g glucose, respectively, suggesting again that the single-step pathway is more efficient for PDC production. It was hypothesized that the six-step pathway is less efficient due to the requirement for cofactors (e.g., 1 molecule of ATP and 2 molecules of NADPH) (Figure 2A). To verify this assumption, another simulation was performed for the six-step pathway where cofactor requirement was artificially removed (Figure 2A). This artificial six-step pathway without cofactor requirement showed increased specific PDC production rate (Figure 2B), which proved our hypothesis. Based on these results, the single-step pathway was selected for constructing the PDC biosynthetic pathway from glucose in E. coli.

Biosynthesis of PDC from Glucose. To construct the PDC biosynthetic pathway based on the single-step PCA route, we first focused on constructing the upstream pathway module from glucose to PCA. As described earlier, PCA can be produced from glucose via the single-step conversion of DHS to PCA catalyzed by DHS dehydratase. Naturally occurring and structurally diverse DHS dehydratases have been discovered in different microbial species (Supplementary Table S1),19–23 and employed in many reports for microbial production of PCA and PCA-derived products.10,13,14,16

To identify a suitable DHS dehydratase candidate for efficient production of PCA from glucose, three DHS dehydratases including AroZ of Klebsiella pneumoniae KCTC
2208, AsbF of *Bacillus thuringiensis* ATCC 10792 and QsuB of *Corynebacterium glutamicum* ATCC 13032 were examined for the following reasons. The DHS dehydratase AroZ (encoded by *aroZ*) has been used in an earlier study to construct a cis,cis-muconic acid biosynthetic pathway, which produced as high as 36.8 g/L of cis,cis-muconic acid in fed-batch culture. In a more recent study, the DHS dehydratase AsbF (encoded by *asbF*) was compared with two other DHS dehydratases for providing PCA as a precursor, showing better performance. The DHS dehydratase QsuB (encoded by *qsuB*), which has been expressed in a recombinant plant *Arabidopsis*, engineer the lignin deposition in cell walls. However, the performance of *Chlorobium* *parasitica* ligAB operon for an optimal PCA to PDC conversion compared to T7 promoter. Also, it is noteworthy that intermediary metabolites (e.g., CHMS isoforms) might have also been produced over the course of PCA conversion, as can be seen from the fact that complete PCA conversion did not lead to a PDC yield of 100 mol %. However, the intermediates were not investigated in this study due to analytical difficulties. 

Having constructed both pathway modules from glucose to PCA and from PCA to PDC, the complete biosynthesis pathway from glucose to PDC was then assembled by constructing the plasmid pTacFABC. The *E. coli* GYT0 strain harboring pTacFABC successfully produced 0.97 g/L of PDC in flask culture using glucose as the sole carbon source (Figure 3B). Moreover, no PCA formation was detected in the culture broth, which suggested that the *pmdABC* operon bears sufficient capability for catalyzing the downstream pathway module from PCA to PDC (Figure 3B). The PDC production was also confirmed by GC–MS analysis (Supplementary Figure S2). Since the key enzyme DHS dehydratase, AsbF, was of heterologous origin, it was examined whether an *E. coli*-codon optimized *asbF* gene, designated as *asbF*<sup>opt</sup> (Supplementary Table S2), would enhance PDC production; there was a report showing that a codon-optimized *asbF* gene exhibited higher AsbF activity. The *asbF*<sup>opt</sup> gene obtained by gene synthesis service was cloned in the same configuration as for the *asbF* gene, giving pTrcF<sup>opt</sup> and pTrcF<sup>opt</sup>ABC. In flask culture, however, the *E. coli* GYTO strain harboring pTrcF<sup>opt</sup> and pTrcF<sup>opt</sup>ABC produced 0.32 g/L of PCA (Figure 3A), which was less by 71.9% than that obtained with the wild-type *asbF*; the *E. coli* GYITO strain harboring pTrcF<sup>opt</sup>ABC produced 0.55 g/L of PDC (Figure 3B), less by 43.3% than that obtained with the wild-type *asbF*. The low activity of *asbF*<sup>opt</sup> might be due to the formation of inclusion body, which requires further investigation. Overall, the de novo production of PDC from glucose was successfully accomplished through the use of the *B. thuringiensis* *asbF* gene and *C. testosteroni* *pmdABC* operon based on the single-step pathway.

Redirecting Flux into the Truncated SHK Pathway for Improved PDC Production. After the single-step PDC biosynthetic pathway was functionally established in *E. coli*, we next focused on engineering the cellular targets that could redirect central metabolic flux toward PDC biosynthetic pathway, aiming at higher PDC productivity. As the first step in SHK pathway, condensation of erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP) generates 3-deoxy-D-arabinohexulosonate 7-phosphate (DAHP) by the action of DAHP synthase (Figure 1), which dictates the carbon flux directed into SHK pathway. In *E. coli*, the total activity of DAHP synthase is comprised of three isoenzymes, AorG transformants (Supplementary Figure S1). The *E. coli* W3110 harboring pTacABC achieved complete conversion of PCA under conditions of all the three IPTG concentrations tested. Particularly under the condition of 1 mM IPTG, the highest PDC titer of 2.67 g/L was obtained from 2.50 g/L of PCA supplemented, which corresponded to a conversion yield of 89.4 mol %. In contrast, the *E. coli* BL21(DE3) harboring pETABC achieved only partial conversion of PCA, and the final PDC titers were declined as the IPTG concentrations increased. The highest PDC titer of 2.32 g/L at conversion yield of 78.8 mol % for the *E. coli* BL21(DE3) harboring pETABC was obtained at the IPTG concentration of 0.1 mM. These PCA-feeding experiments indicated that the tac promoter was performing better in driving expression of the *pmdABC* operon for an optimal PCA to PDC conversion compared to T7 promoter. The tac promoter was also confirmed by GC–MS analysis (Supplementary Figure S2).

After the successful validation of upstream pathway conversion from glucose to PCA, we then set out to introduce the downstream pathway module from PCA to PDC. As mentioned, this aromatic ring opening process is catalyzed by PCA 4,5-dioxygenase and CHMS dehydrogenase along with a nonenzymatic, spontaneous conversion step. In previous studies on microbial PDC production, a gene operon containing ligA and ligB genes for PCA 4,5-dioxygenase and ligC for CHMS dehydrogenase from *S. paucimobilis* SYK-6 were employed. In this study, we employed an alternative operon comprising *pmdA*, *pmdB* and *pmdC* genes from *Comamonas testosteroni* ATCC 11996 for converting PCA into PDC. The *pmdA*, *pmdB* and *pmdC* genes are functionally homologous to ligA, ligB and ligC, with amino acid sequence identities of 48.4%, 57.1% and 75.0%, respectively. It was reported that the PCA 4,5-dioxygenase encoded by *pmdABC* shows less promiscuous activity compared with the one reported that the PCA 4,5-dioxygenase encoded by *gysA*<sup>DHS</sup> of *E. coli*, possessing 48.4%, 57.1% and 75.0%, respectively. It was found in the SHK/quinate degradation pathway of *C. glutamicum*, and it has been expressed in a recombinant plant *Arabidopsis* to engineer the lignin deposition in cell walls. However, the potential applicability of QsuB for production of PCA and PDC derivatives in *E. coli* host has not been examined to date.

To compare these three DHS dehydratases, the *aroZ*, *asbF* and *qsuB* genes were cloned into plasmid pTrc99A under *trc* promoter, resulting in plasmids pTrcZ, pTrcF and pTrcB, respectively. These plasmids were transformed individually into *E. coli* GYT0, which is DHS dehydrogenase-deficient strain of *E. coli* W3110 constructed by disrupting the *aroE* gene to block the downstream SHK pathway. Flask cultures showed that the *E. coli* GYT0 strain harboring pTrcF produced the highest titer of PCA (1.14 g/L), which was 4.4-fold and 1.2-fold that of PCA produced by the recombinant GYT0 strains harboring pTrcZ and pTrcB, respectively (Figure 3A). Thus, the DHS dehydratase AsbF was selected for further experiments that include assembling with the downstream pathway module for converting PCA to PDC.

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(accounting for 80%), AroF (15%) and AroH (5%), which are subjected to tight regulation imposed by allosteric control and transcriptional repression. Hence, in order to increase cellular DAHP synthase activity, we overexpressed \textit{aroG} \textsuperscript{fbr} gene encoding a feedback-inhibition resistant mutant of \textit{AroG}, \textsuperscript{20} by generating plasmid pBBR1Gfbr-\textit{fbr}. \textit{E. coli} GYT0 strain harboring pTacFABC and pBBR1Gfbr-\textit{fbr} produced dramatically increased PDC titer (2.07 g/L) (Figure 4), 1.1-fold higher than that obtained by the parental strain in flask culture. When pBBR1Gfbr-\textit{fbr} was transformed into \textit{E. coli} preliminary strain GYT0 harboring pTacFABC, suggesting that carbon flux was successfully redirected toward PDC biosynthesis through the above efforts.

\textit{E. coli} \textit{aroG} \textsuperscript{fbr} gene was thus cloned into plasmid pBBR1Gfbr to generate pBBR1Gfbr-A. When pBBR1Gfbr-\textit{fbr}-A was transformed into \textit{E. coli} GYT0 strain harboring pTacFABC, however, only 1.97 g/L of PDC was produced (Figure 4), which was 4.8% lower than that obtained by GYT0 strain harboring pTacFABC and pBBR1Gfbr-\textit{fbr}. On the other hand, for enrichment of PEP, strategies including amplification of PEP synthetase (encoded by \textit{ppsA}) for improved formation of PEP from pyruvate (PYR), \textsuperscript{31} and inactivation of PYR kinase I (encoded by \textit{pykF}) and PYR kinase II (encoded by \textit{pykA}) for preventing consumption of PEP to PYR in glycolysis, \textsuperscript{32,33} have previously been proven to be effective. On the basis of these validated strategies, we constructed the following engineered strains, GYT1 (carrying \textit{ppsA} overexpression through exchange of its native promoter with the strong \textit{trc} promoter), GYT2 (carrying \textit{pykF} deletion), GYT3 (carrying \textit{pykF} and \textit{pykA} double-deletions) and GYT4 (carrying both \textit{ppsA} overexpression by \textit{trc} promoter exchange and \textit{pykF} deletion). When each of these four strains was transformed with pTacFABC and pBBR1Gfbr-\textit{fbr}-A, and tested in flask cultures, it was found that only GYT1 strain harboring pTacFABC and pBBR1Gfbr-\textit{fbr}-A produced slightly higher PDC titer (2.12 g/L) (Figure 4) than that obtained by the parental strain GYT0 harboring pTacFABC and pBBR1Gfbr-\textit{fbr}-A. All of the other three strains harboring pTacFABC and pBBR1Gfbr-\textit{fbr}-A produced less PDC (Figure 4). These results suggest that the availability of precursors E4P and PEP might not be the limiting factor for PDC production from glucose in our engineered strain under the tested culture condition. Although not performed in this study, \textit{in silico} genome-scale metabolic simulations can be performed for identifying potential bottlenecks and also new engineering targets in the future.

To summarize, among all the gene targets examined above, only \textit{aroG} \textsuperscript{fbr} and \textit{ppsA} overexpression displayed improved PDC production. Thus, we combined these two positive targets by generating GYT1 strain harboring pTacFABC and pBBR1Gfbr-\textit{fbr}-A, which produced 2.28 g/L of PDC in flask culture (Figure 5), showing a cumulatively positive effect on PDC production. Also, this PDC titer represented a 1.4-fold increase over the preliminary strain GYT0 harboring pTacFABC, suggesting that carbon flux was successfully redirected toward PDC biosynthesis through the above efforts.

\textbf{Importer Engineering for Reduced Byproduct DHS Accumulation.} During analysis of the culture supernatants of PDC production strains described above, we observed that concentrations of PCA remained very low (0–10 mg/L) (data not shown), but large amounts of DHS were accumulated. For instance, GYT1 strain harboring pTacFABC and pBBR1Gfbr-\textit{fbr}-A, which produced the highest titer of PDC so far, accumulated 2.52 g/L of DHS (Figure 5).

To avoid carbon loss \textit{via} such a surplus extracellular formation of DHS and, in turn, to reuse it for boosting PDC production, we targeted on a membrane-bound transporter called ShiA in \textit{E. coli}, which reportedly could assimilate extracellular DHS into the cytosol of cells. \textsuperscript{14,15} In the attempt to enhance DHS import and thus its intracellular availability for improved PDC production, we tested \textit{E. coli} native ShiA (EcShiA) as well as a number of ShiA proteins originated from other different microbial species and showing various amino acid sequence similarities relative to EcShiA (Supplementary 

![Figure 4](image-url)  
**Figure 4.** Effects of overexpression of the \textit{aroG} \textsuperscript{fbr}, \textit{tktA} and \textit{ppsA} genes, and deletion of the \textit{pykF} and/or \textit{pykA} genes on PDC production. Symbols are gray box, cell growth (OD\textsubscript{600}); red box, PDC concentration (g/L).

![Figure 5](image-url)  
**Figure 5.** Effect of overexpression of DHS importers from different microbial origins on PDC production. Engineered strains indicated are Control, GYT1 harboring pTacFABC and pBBR1Gfbr-\textit{fbr}; KpShiA, GYT1 harboring pTacFABC and pBBR1Gfbr-\textit{fbr}-KpA; RoShiA, GYT1 harboring pTacFABC and pBBR1Gfbr-\textit{fbr}-RoA; AsShiA, GYT1 harboring pTacFABC and pBBR1Gfbr-\textit{fbr}-AsA; EcShiA, GYT1 harboring pTacFABC and pBBR1Gfbr-\textit{fbr}-EcA; PtcShiA, GYT5 harboring pTacFABC and pBBR1Gfbr-\textit{fbr}; EcShiA + PtcShiA, GYT5 harboring pTacFABC and pBBR1Gfbr-\textit{fbr}-EcA. Symbols are gray box, cell growth (OD\textsubscript{600}); red box, PDC concentration (g/L); yellow box, DHS concentration (g/L).
Table S3). Genes encoding K. pneumonia ShiA (KpShiA), Rhodococcus opacus PD630 ShiA (RoShiA) and Acinetobacter sp. ADP1 ShiA (AsShiA) along with E. coli ShiA, were inserted into plasmid pBBR1Gfr-Ec to generate pBBR1Gfr-Kpa, pBBR1Gfr-Roa, pBBR1Gfr-AsA and pBBR1Gfr-EcA, respectively. Introduction of these four plasmids individually into E. coli GYT1 strain harboring pTacFABC led to distinct profiles of DHS formation and PDC production in flask cultures (Figure 5). Among these four ShiA importers tested, AsShiA and EcShiA showed reduced DHS formation, whereas KpShiA and RoShiA resulted in elevated DHS accumulation (Figure 5). Particularly, GYT1 strain harboring pTacFABC and pBBR1Gfr-EcA produced the least DHS (0.42 g/L), 83.3% lower than that obtained by the parental strain GYT1 harboring pTacFABC and pBBR1Gfr. However, such a considerable decrease in DHS accumulation improved PDC production by only 5.7%, reaching 2.41 g/L. On the other hand, coexpression of AsShiA also lowered DHS formation (by 40.5%) compared to the control strain without expressing AsShiA, probably thanks to its relatively high amino acid sequence similarity to EcShiA (65.3%) (Supplementary Table S3), but the PDC titer was also decreased (1.97 g/L) surprisingly.

Given that overexpression of EcShiA had the best effect on reducing byproduct DHS formation and improving PDC production, we sought to further increase the expression level of EcShiA, in order to further reduce extracellular DHS accumulation thereby increasing PDC production. It was reported that although its chromosomal transcription is constitutive, E. coli shiA gene is repressed under normal growth conditions via a small RNA-mediated translation regulation mechanism, and the small RNA base-pairing interaction region is located upstream of the coding sequence in the mRNA of shiA gene.35 As such, we determined to replace the native promoter of E. coli shiA gene with the strong trc promoter. By doing so, not only could the transcription of shiA gene be strengthened, but also its translation could be deregulated as the small RNA binding region would be eliminated after the promoter exchange. GYT5 strain was thus generated, which carried trc promoter-driven shiA gene in the chromosome. At first, we examined effect of the promoter exchange of shiA alone on reducing DHS formation. As expected, flask culture of GYT5 strain harboring pTacFABC and pBBR1Gfr showed reduced accumulation of DHS (1.86 g/L) by 26.2% compared to the parental strain GYT1 harboring pTacFABC and pBBR1Gfr (Figure 5). However, GYT5 strain harboring pTacFABC and pBBR1Gfr produced less PDC (2.18 g/L). Furthermore, combined effect of the plasmid-borne and chromosomal trc promoter-driven overexpression of E. coli shiA was investigated. Flask culture of GYT5 strain harboring pTacFABC and pBBR1Gfr-EcA showed further reduced accumulation of DHS (0.37 g/L) by 11.9% compared to GYT1 strain harboring pTacFABC and pBBR1Gfr-EcA (Figure 5). But similarly, only 2.16 g/L of PDC was produced, which was less than that by GYT1 strain harboring pTacFABC and pBBR1Gfr-EcA.

Taken together, we successfully reduced accumulation of the major byproduct DHS to a minimum level through overexpression of DHS importer. And GYT1 strain harboring pTacFABC and pBBR1Gfr-EcA, which produced the highest PDC titer (2.41 g/L) along with the second least DHS formation (0.42 g/L), was selected as base strain for further engineering.

Effect of Genetic Manipulations of the Soluble Transhydrogenase (SthA) on PDC Production. In order to further optimize PDC production, it is necessary to consider the cofactor utilization for the following reason. In the last step of PDC pathway (Figure 1), one molecule of the cofactor NADPH+ is converted to NADPH accompanying one molecule of PDC produced; this might interfere with cellular redox cofactor balance. Thus, we targeted on E. coli soluble pyridine nucleotide transhydrogenase (encoded by sthA) as SthA has been reported to be responsible for reoxidation of NADPH to NADP+, particularly under metabolic conditions with excess NADPH formation.36,37

To enhance SthA activity, we decided to overexpress sthA gene through two layers of manipulations. First, we overexpressed it by promoter exchange with the strong trc promoter in GYT1 strain, generating GYT6 strain. Second, based on GYT6, we additionally constructed GYT7 strain, in which three silent point mutations (C15T, T18C, C21T) were introduced into sthA gene in the chromosome, for the following reason. In E. coli, a small regulatory RNA called Spot 42 (encoded by sfp), highly abundant in the presence of glucose, was reported to repress sthA expression by translational regulation through inhibitory base-pairing with the sthA mRNA, and the binding site also localized within the beginning region of the coding sequence of sthA.38 Thus, we attempted to deregulate this repression mechanism by introducing the above three silent point mutations that would affect the complementary base-pairing interaction as seen from the binding free energy increase from −14.52 to −1.57 kcal/mol (Figure 6).

Figure 6. Schematic representation of the chromosomal manipulations of the sthA gene: (1) promoter replacement with the strong trc promoter and (2) further introduction of three silent point mutations (C15T, T18C, C21T) in the coding sequence of sthA, which leads to weakened base-pairing interaction of the sthA mRNA with the small regulatory RNA sfp, as seen from the binding free energy increase from −14.52 to −1.57 kcal/mol.

These two newly constructed strains GYT6 and GYT7 were examined in flask cultures using GYT1 strain as a control. Among these three strains tested, distinct time profiles of cell growth, PDC production and byproducts (i.e., PCA and DHS) formation were observed (Figure 7). Throughout the course of flask culture, GYT6 strain harboring pTacFABC and pBBR1Gfr-EcA produced less PDC than the control strain GYT1 harboring same plasmids (Figure 7B). In the case of GYT7 strain harboring pTacFABC and pBBR1Gfr-EcA, also less amount of PDC was produced than the control as seen from the final titer (2.21 g/L) (Figure 7B). However, at time point of 36 h, GYT7 strain harboring pTacFABC and pBBR1Gfr-EcA produced higher titer of PDC (2.05 g/L)
versus that by the control (1.82 g/L) (Figure 7B), which was accomplished apparently due to the higher maximum PDC productivity of GYT7 strain harboring pTacFABC and pBBR1Gfr-EcA (0.103 g/L/h) over that of the control (0.082 g/L/h). Moreover, it was interestingly noticeable that GYT7 strain harboring pTacFABC and pBBR1Gfr-EcA had higher byproducts formation (Figure 7C and D), particularly DHS, than the control, which indicated a stronger total flux redirected into the aromatic biosynthetic pathway. According to these results, GYT7 strain harboring pTacFABC and pBBR1Gfr-EcA, and GYT1 strain harboring pTacFABC and pBBR1Gfr-EcA, were selected and further evaluated in bioreactor fermentations as discussed below.

**Bioreactor Fermentations.** Following development of engineered *E. coli* strains for PDC production, bioreactor fermentations were conducted to demonstrate their potentials for scale-up production.

Batch fermentations were first carried out to optimize cultivation conditions and select the best-performing PDC production strain for subsequent fed-batch culture study. As oxygen is required by PmdAB in PDC biosynthesis (Figure 1), we thus tested effects of different dissolved oxygen (DO) levels (25%, 40% and 80% of air saturation) on PDC fermentation, using GYT1 strain harboring pTacFABC and pBBR1Gfr-EcA. Batch fermentation profiles under the three DO conditions are depicted (Supplementary Figure S3) and the results are summarized (Table 1). It was observed that the highest PDC production (4.78 g/L) was obtained under DO level of 40% (Supplementary Figure S3B and Table 1). When DO level was initially set as 25% or 80%, the PDC titer was slightly decreased (Supplementary Figure S3A and S3C, and Table 1).

Using the selected DO setting of 40% of air saturation, GYT7 strain harboring pTacFABC and pBBR1Gfr-EcA was examined in batch fermentation with all other conditions same as for GYT1 strain harboring pTacFABC and pBBR1Gfr-EcA. Unexpectedly, GYT7 strain harboring pTacFABC and pBBR1Gfr-EcA produced only 0.38 g/L of PDC with accumulations of PCA and DHS as high as 4.74 g/L and 9.37 g/L, respectively (Supplementary Figure S4A and Table 1). Out of curiosity, we further examined GYT6 strain harboring pTacFABC and pBBR1Gfr-EcA in batch fermentation under the same conditions, which produced 2.32 g/L of PDC with formations of 1.86 g/L of PCA and 4.57 g/L of DHS (Supplementary Figure S4B and Table 1).

**Table 1. Batch Fermentation Performances of the Engineered Strains for PDC Production**

<table>
<thead>
<tr>
<th>strain</th>
<th>maximum OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>PDC (g/L)</th>
<th>PCA (g/L)</th>
<th>DHS (g/L)</th>
<th>productivity (g/L/h)</th>
<th>yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GYT1/pTacFABC + pBBR1Gfr-EcA</td>
<td>11.90</td>
<td>4.28</td>
<td>0.14</td>
<td>1.71</td>
<td>0.104</td>
<td>0.209</td>
</tr>
<tr>
<td>GYT1/pTacFABC + pBBR1Gfr-EcA</td>
<td>12.45</td>
<td>4.78</td>
<td>0.18</td>
<td>2.79</td>
<td>0.118</td>
<td>0.235</td>
</tr>
<tr>
<td>GYT1/pTacFABC + pBBR1Gfr-EcA</td>
<td>12.25</td>
<td>4.27</td>
<td>0.70</td>
<td>1.70</td>
<td>0.101</td>
<td>0.221</td>
</tr>
<tr>
<td>GYT7/pTacFABC + pBBR1Gfr-EcA</td>
<td>12.85</td>
<td>0.38</td>
<td>4.74</td>
<td>9.37</td>
<td>0.007</td>
<td>0.016</td>
</tr>
<tr>
<td>GYT6/pTacFABC + pBBR1Gfr-EcA</td>
<td>11.90</td>
<td>2.32</td>
<td>1.86</td>
<td>4.57</td>
<td>0.052</td>
<td>0.107</td>
</tr>
</tbody>
</table>

*DO level was set as 25% of air saturation during fermentation. †DO level was set as 40% of air saturation during fermentation. ‡DO level was initially set as 40% of air saturation and then shifted to 80% of upon induction.*

Figure 7. Time profiles of cell growth (A), PDC (B), PCA (C) and DHS (D) of engineered *E. coli* strains in flask cultures. Symbols are blue square, GYT1 harboring pTacFABC and pBBR1Gfr-EcA; red circle, GYT6 harboring pTacFABC and pBBR1Gfr-EcA; green triangle, GYT7 harboring pTacFABC and pBBR1Gfr-EcA.
Comparing the results in bioreactor batch fermentations with those in flask cultures for GYT1, GYT6 and GYT7 strains harboring pTacFABC and pBBR1Gfbr-EcA, it was observed that GYT1 and GYT6 strains harboring pTacFABC and pBBR1Gfbr-EcA had relatively consistent performances in PDC production when they were transferred from flask cultivation to bioreactor cultivation. By contrast, GYT7 strain harboring pTacFABC and pBBR1Gfbr-EcA exhibited a dramatic decay in PDC production capability when moved from flask culture to bioreactor culture. Such a huge change in PDC production performance suggested an intriguing insight into the important role of cellular redox cofactor in affecting PDC production and the flux toward DHS in the context of different cultivation conditions. Yet, further detailed investigation is needed to clarify the underlying mechanisms that have resulted in such outcomes. Although the three silent point mutations of sthA gene in GYT7 strain failed to increase PDC production in this study, this genetic modification strategy might potentially serve as a useful approach to perturb the SthA activity in E. coli when needed.

On the basis of batch fermentation results above, GYT1 strain harboring pTacFABC and pBBR1Gfbr-EcA was selected as the final strain and examined in a fed-batch culture under DO level of 40% of air saturation and with a pH-stat nutrient feeding strategy. The fed-batch fermentation resulted in production of 16.72 g/L of PDC with the productivity of 0.172 g/L/h and the yield of 0.201 g/g glucose (Figure 8). At the same time, 9.76 g/L of DHS was accumulated, while only 0.36 g/L of PCA was formed. It is speculated that the high accumulation of DHS during fed-batch culture might be caused by relatively limited activity of DHS dehydratase and/or decay of DHS importer activity over the course of fermentation. This suggests potential room for further enhancement of PDC production level. Thus, future studies should be focused on improving the activity of DHS dehydratase and/or maintaining of DHS importer activity during large-scale fermentation processes.

### CONCLUSIONS

In this paper, we developed metabolically engineered E. coli platform strains capable of producing PDC using glucose as a carbon source. An efficient metabolic pathway toward PDC was suggested on the basis of in silico flux simulation, and the selected PDC pathway was reconstructed through introduction of screened, efficient heterologous enzymes. Removal of feedback inhibition of a key enzyme, elevated precursor supply and overexpression of DHS importer were important in improving PDC production and reducing byproduct formation. Furthermore, manipulation of the cofactor (NADPH/NADP⁺) utilization through perturbing small regulatory RNA-based soluble pyridine nucleotide transhydrogenase gene expression provided insight into the impact of redox cofactor balancing on PDC production. Finally, fed-batch culture of the best-performing engineered strain produced 16.72 g/L of PDC with an overall productivity of 0.172 g/L/h and yield of 0.201 g/g glucose, which represented the highest titer for PDC production from glucose reported to date. Further improvement in PDC production would be possible by taking advantage of various systems metabolic engineering tools, such as systematic flux optimization, synthetic regulatory sRNA-mediated large-scale gene target identification coupled with high-throughput techniques, as well as bioprocess optimization.

### METHODS

**Bacterial Strains and Media.** The bacterial strains used in this study are listed in Table 2. E. coli DH5α was employed for gene cloning and plasmid propagation, and E. coli W3110 and its derivatives were used for PDC production as host strains. For general cultures during plasmid construction and bacterial genome manipulation, E. coli cells were cultured in Luria–Bertani (LB) broth or on LB plates (1.5%, w/v, agar) with proper antibiotics supplemented as follows: 100 μg/mL of ampicillin, 50 μg/mL of kanamycin, and/or 34 μg/mL of chloramphenical. Bacterial species Comamonas testosteroni ATCC 11996, Bacillus thuringiensis ATCC 10792, Klebsiella pneumonia KCTC 2208, Corynebacterium glutamicum ATCC 13032, Rhodococcus opacus PD630 and Acinetobacter sp. ADP1 served as genetic sources for heterologous enzymes or transporters employed in this study. Cultivation of these wild-type strains was carried out under the conditions specified by the corresponding suppliers.

**Plasmid Construction.** All plasmids used in this study are listed in Table 2. The basic molecular biology experiments including PCR, gel electrophoresis, and bacterial transformation for plasmid and strain construction were conducted according to standard procedures. Commercial kits were used for preparation of bacterial genomic DNA, isolation of plasmid DNA and purification of DNA fragments from agarose gels following the supplier’s instructions (Genotech, Daejeon, South Korea). Services of primer synthesis and DNA sequencing were also provided by Genotech. All the oligonucleotide primers utilized in this work are listed in Supplementary Table S4. To construct pETABC, the pmABC operon from the genome of C. testosteroni ATCC 11996 was amplified using pmABC(pET)-f and pmABC(pET)-r primers, and cloned into pET-22b (+) vector amplified using pET(pmdABC)-f and pET(pmdABC)-r primers by Gibson assembly method. To construct pTacABC, the pmABC operon was amplified using pmABC(pTac)-f and pmABC-
Table 2. Strains and Plasmids Used in This Study

<table>
<thead>
<tr>
<th>name</th>
<th>relevant genotype</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F(^–) endA1 gln V44 thi-1 recA1 relA1 gyrA96 deoR mak5 purB20 g680laczΔ1615 Δ(lacZΔ2YargF)U1469, hsdR17(rK mB)(^–), λ(^–)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F(^–) ompT gal dcm lon hsdR514 (rK mB)(^–) (DE3)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>W3110</td>
<td>Coli genetic stock center strain No. 4744</td>
<td>CGSC⁵⁰</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTrc99A</td>
<td>Ap(^–), trc promoter, pBR322 origin, lacI(^–), 4.2 kb</td>
<td>Lab stock</td>
</tr>
<tr>
<td>pTac1SK</td>
<td>Km(^–), tac promoter, p15A origin, 4.0 kb</td>
<td>Lab stock</td>
</tr>
<tr>
<td>pET-22b(+)</td>
<td>Ap(^–), T7 promoter, pBR322 origin, 5.5 kb</td>
<td>Novagen</td>
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<tr>
<td>pBBR1MCS</td>
<td>Cm(^–), lac, T3 and T7 promoters, 4.7 kb</td>
<td>⁴⁹</td>
</tr>
<tr>
<td>pTrcZ</td>
<td>pTrc99A derivative containing araZ gene from <em>K. pneumonia</em></td>
<td>This study</td>
</tr>
<tr>
<td>pTrcF</td>
<td>pTrc99A derivative containing asbF gene from <em>B. thuringiensis</em></td>
<td>This study</td>
</tr>
<tr>
<td>pTrcF(^\text{OPT})</td>
<td>pTrc99A derivative containing E. coli-codon optimized asbF gene</td>
<td>This study</td>
</tr>
<tr>
<td>pTrcB</td>
<td>pTrc99A derivative containing quab gene from <em>C. glutamicum</em> ATCC13032</td>
<td>This study</td>
</tr>
<tr>
<td>pTacABC</td>
<td>pTac1SK derivative containing pmABC operon from <em>C. testosteroni</em></td>
<td>This study</td>
</tr>
<tr>
<td>pETABC</td>
<td>pET-22b(+) derivative containing pmABC operon from <em>C. testosteroni</em></td>
<td></td>
</tr>
<tr>
<td>pTacFABC</td>
<td>pTacABC derivative containing trc-asbF-rrnBT1T2 cassette</td>
<td>This study</td>
</tr>
<tr>
<td>pTrcFiABC</td>
<td>pTrcABC derivative containing trc-asbF-rrnBT1T2 cassette</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR1G(^{\text{Eco}})</td>
<td>pBRC1cs derivative containing araG(^{\text{Eco}}) gene under the lac promoter</td>
<td>This study</td>
</tr>
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<td>pBBR1G(^{\text{Eco}},-\text{A})</td>
<td>pBBR1G(^{\text{Eco}}) derivative containing RBS-tktA cassette</td>
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<tr>
<td>pBBR1G(^{\text{Eco}},-\text{Eca})</td>
<td>pBBR1G(^{\text{Eco}}) derivative containing RBS-shiA cassette from <em>E. coli</em></td>
<td>This study</td>
</tr>
<tr>
<td>pBBR1G(^{\text{Eco}},-\text{Kpa})</td>
<td>pBBR1G(^{\text{Eco}}) derivative containing RBS-shiA cassette from <em>K. pneumoniae</em></td>
<td>This study</td>
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<td>pBBR1G(^{\text{Eco}},-\text{RoA})</td>
<td>pBBR1G(^{\text{Eco}}) derivative containing RBS-shiA cassette from <em>R. opacus</em> FDS630</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR1G(^{\text{RoA}},-\text{Aa})</td>
<td>pBBR1G(^{\text{RoA}}) derivative containing RBS-shiA cassette from <em>A.phaeomarinus</em> ADP1</td>
<td>This study</td>
</tr>
<tr>
<td>pKD46</td>
<td>Ap(^–), t-Red recombinase under arabinose-inducible arabinBAD promoter, ts origin, 6.3 kb</td>
<td>⁴⁵</td>
</tr>
<tr>
<td>pJW168</td>
<td>Ap(^+), Cre recombinase under IPTG-inducible lacUV5 promoter, ts origin, 5.5 kb</td>
<td>⁴⁶</td>
</tr>
<tr>
<td>pECmulox</td>
<td>Ap(^–), Cm(^–), lux66-cat-lox71, 3.5 kb</td>
<td>⁴⁰</td>
</tr>
<tr>
<td>pMtrc9</td>
<td>Modified pECmulox containing trc promoter downstream of lux66-cat-lox71 cassette</td>
<td>Lab stock</td>
</tr>
</tbody>
</table>

Abbreviations: Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; R, resistance; ts, temperature sensitive. Coli Genetic Stock Center, New Haven, CT.

(pTacr)-r primers, and cloned into pTac1SK vector amplified using pTac(pmdABC)-f and pTac(pmdABC)-r primers by Gibson assembly. To construct pTrcZ, the *aroZ* gene from *K. pneumonia* KCTC 2208 was amplified using *aroZ* (pTrc)-f and *aroZ* (pTrc)-r primers, and cloned into pTrc99A vector amplified using pTrc(*aroZ*)-f and pTrc(*aroZ*)-r primers by Gibson assembly. To construct pTrcB, the *gusB* gene from *C. glutamicum* ATCC 13032 was amplified using gusB(f)-f and gusB(r)-r primers, and cloned into pTrc99A vector at SacI and XhoI sites. To construct pTrcF, the *asbF* gene from *B. thuringiensis* ATCC 10792 was amplified using asbF(f)-f and asbF(r)-r primers, and cloned into pTrc99A vector at EcoRI and PstI sites. To construct pTrcF\(^{\text{OPT}}\), an *E. coli* codon-optimized version of *asbF* gene (synthesized by GenScript) was amplified using asbF\(^{\text{OPT}}\)-f and asbF\(^{\text{OPT}}\)-r primers, and cloned into pTrc99A vector at EcoRI and PstI sites. To construct pTacFABC, the *trc-asbF-rrnBT1T2* cassette was amplified from pTrcF using trc-asbF-f and trc-asbF-r primers, and assembled by Gibson assembly with pTacABC linearized by enzyme digest at NheI site. To construct pTacFiABC, the *trc-asbF-rrnBT1T2* cassette was amplified from pTrcF\(^{\text{OPT}}\) using trc-asbF-f and trc-asbF-r primers, and assembled by Gibson assembly with pTacABC linearized by enzyme digest at NheI site. To construct pBBR1G\(^{\text{Eco}},-\text{Eca}\), the RBS-shiA cassette was amplified from *E. coli* using RBS-Eca-f and Eca-r primers, and cloned into pBBR1G\(^{\text{Eco}}\) at HindIII and BamHI sites. To construct pBBR1G\(^{\text{Eco}},-\text{Kpa}\), the RBS-shiA cassette was amplified from *K. pneumoniae* KCTC 2208 using RBS-Kpa-f and RBS-Kpa-r primers, and cloned into pBBR1G\(^{\text{Eco}}\) at HindIII and Spel sites. To construct pBBR1G\(^{\text{Eco}},-\text{RoA}\), the RBS-shiA cassette was amplified from *R. opacus* PD630 using RBS-Roa-f and RBS-Roa-r primers, and cloned into pBBR1G\(^{\text{Eco}}\) at HindIII and BamHI sites. To construct pBBR1G\(^{\text{RoA}},-\text{Aa}\), the RBS-shiA cassette was amplified from *A. phaeomarinus* ADP1 using RBS-Aa-f and RBS-Aa-r primers, and cloned into pBBR1G\(^{\text{RoA}}\) at HindIII and BamHI sites. All the recombinant plasmids were confirmed by colony PCR and DNA sequencing.

**Genome Manipulation.** One-step homologous recombination-based inactivation method⁴⁵ was used for gene deletion and promoter replacement experiments. For deleting *aroE*, the first knockout PCR product was amplified using *aroE-KO-f* and *aroE-KO-r* primers and having 50 bp nucleotide extension homologous to the upstream and downstream regions of *aroE* within the genome. The plasmid pECmulox bearing a *lox71*-chlomphenical resistance gene (*cat*)-lox66 cassette was used as a template for PCR amplifying the first knockout fragment. The homology sequence was elongated to 100 bp via a second PCR using the first PCR product as template and using *aroE-KOEX-f* and *aroE-KOEX-r* primers. For the promoter change of *ppsA*, the pMtrc9 plasmid containing the strong *trc* promoter and the upstream *lox71*-cat-lox66 cassette was employed as a template in the PCR reactions using primer pairs of Ptrc(*ppsA*)-f/Ptrc(*ppsA*)-r and Pptrc(*ppsA*)-EX-f/Ptrc(*ppsA*)-EX-r, instead of pECmulox. PCR products for deletion or promoter replacement of the remaining genes were prepared in the same manner using primers listed in Supplementary Table S4. For construction of the GYT7 strain, the three point mutations (C15T, T18C, C21T) were introduced into the promoter replacement primers. Regardless of gene deletion or promoter replacement, the amplified
knockout PCR products were introduced into E. coli cells expressing the Red recombinase from pKD46 induced by 10 mM L-arabinose. Colonies were selected on LB agar plates supplemented with chloramphenicol, and the successful genome manipulation was confirmed by colony PCR. Subsequently, the helper plasmid pJW168 that expressed the Cre recombinase by 1 mM IPTG was introduced to remove the chloramphenical marker gene. With the temperature-sensitive replication origins, both pKD46 and pJW168 were easily cured through temperature shift between 30 and 42 °C. The excision of the marker gene was further confirmed by performing colony PCR. For promoter replacement, the positive clones were further sequenced to validate the correct manipulation.

In Silico Flux Simulation Experiments. To perform in silico flux response analysis for comparing two biosynthetic pathway routes (single-step and six-step pathways) for PDC, we employed the genome-scale metabolic model EcoMBEL979, which is a modified model of jEjR0430 and comprises 979 metabolic reactions and 814 metabolites. As PDC biosynthetic pathways are not native to E. coli, the heterologous pathway reactions are required to be additionally recruited to the EcoMBEL979 model. To obtain PCA, the following reaction "DHSP + NADPH + O2 + H+ ↔ PCA + NADP+ + H2O" was added to the EcoMBEL979 model for the single-step biosynthetic pathway, whereas the following reaction "4HBA + NADPH + O2 + H+ ↔ PCA + NADP+ + H2O" was added for the six-step biosynthetic pathway. From PCA to PDC, the subsequent reactions were introduced into the EcoMBEL979 model for both pathways: "PCA + O2 ↔ CHMS + H+, "CHMS ↔ CHMS (hemiacetal form)," and "CHMS (hemiacetal form) + NADP+ ↔ PDC + NADPH + H+". Next, we investigated the theoretical impacts of the two different biosynthetic pathways (single-step and six-step) on PDC production, through constraint-based flux simulation with the hypothesis of pseudo steady-state. Cell growth rate was maximized as an objective function as the PDC biosynthetic flux was gradually increased from minimum to maximum values. Over the course of simulation, the glucose uptake rate was set at 10 mM/gDCW/h.

Cultivation. Shake-flask cultivations were performed in the baffled flasks (300 mL), which contained a working volume of 50 mL MR minimal salts medium (pH 7.0) supplemented with 10 g/L glucose. The MR medium (1 L) contained: 6.67 g KH2PO4, 4 g (NH4)2HPO4, 0.8 g MgSO4·7H2O, 0.8 g citric acid, and 5 mL trace metal solution. Glucose, MgSO4·7H2O, MR salts and trace metal solution were prepared and autoclaved separately. To deal with the auxotroph caused by the disruption of araE gene, the MR medium (1 L) was also supplemented with L-phenylalanine (40 mg), L-tyrosine (40 mg), L-tryptophan (40 mg), 4-hydroxybenzoic acid (10 mg), 4-aminobenzoic acid (10 mg), 2,3-dihydroxybenzoic acid (10 mg) and thiamine hydrochloride (10 mg). Stock solutions of which were separately sterilized through 0.22 μm membrane. To prepare the inoculums for flask cultivation, glycerol stocks of the engineered strains were first inoculated into a 25 mL test tube containing 5 mL of LB medium and cultured at 200 rpm and 37 °C for 12 h. Aliquots of 1 mL of the preculture were used for inoculation. After inoculation, flasks were placed in a rotary incubator at 200 rpm and 37 °C for initial growth. After 6 h of cultivation when cells grew up to the OD600 value of 0.6–0.8, 1 mM IPTG was added for induction, and then cell cultures were immediately transferred to a rotary incubator at 200 rpm and 30 °C for additional 54 h of cultivation. Appropriate antibiotics were added to the medium when necessary. All flask cultivations described in this study were conducted in biological duplicates, and the results were depicted as mean values plus standard deviations in the graph.

Batch reactor fermentations were conducted using a 6.6-L jar fermentor (Bioflo 3000; New Brunswick Scientific Co., Edison, NJ) containing 1.8 L of the MR medium supplemented with 20 g/L of glucose, 3 g/L yeast extract, 40 mg/L of L-phenylalanine, 40 mg/L of L-tyrosine, 40 mg/L of L-tryptophan and 10 mg/L of 4-hydroxybenzoic acid under 30 °C. To prepare seed cultures, 2 mL of overnight LB test tube cultures were transferred into the Erlenmeyer nonbaffled flasks (300 mL) containing 100 mL of the same medium and cultivated in a shaking incubator for 10 h at 200 rpm and 37 °C. A total volume of 200 mL of seed cultures were used to inoculate the fermentor to make a total working volume of 2 L. The fermentation culture pH was maintained at 7.0 with the addition of ammonia solution (28%, v/v). The dissolved oxygen (DO) was maintained at 25%, 40% or 80% of air saturation as indicated by automatically changing the agitation speed from 200 to 1000 rpm and additional supply of pure oxygen when necessary with a constant air flow of 2.0 L/min. Cells were induced with 1 mM IPTG at the OD600 value of 2.0–3.0. All batch fermentations with each of engineered strains were performed twice with reproducible results, and the results of one representative batch fermentation are presented.

Fed-batch fermentation was performed under the same settings except the followings. The initial fermentation medium contained 1.8 L of the MR medium supplemented with 20 g/L of glucose, 3 g/L yeast extract, 100 mg/L of L-phenylalanine, 100 mg/L of L-tyrosine, 100 mg/L of L-tryptophan and 25 mg/L of 4-hydroxybenzoic acid. Cells were induced with 1 mM IPTG at the OD600 value of 4.0–5.0. When glucose was depleted at the end of batch culture, fed-batch mode was initiated by a pH-stat nutrient feeding strategy. The pH-stat loop was whenever the medium pH ≥ 7.02, the nutrient solution was pumped into the fermentor at the rate of 100%. The nutrient solution comprised 700 g/L of glucose, 8 g/L of MgSO4·7H2O, 5 mL trace metal solution, 3 g/L of yeast extract, 100 mg/L of L-phenylalanine, 100 mg/L of L-tyrosine, 100 mg/L of L-tryptophan, 25 mg/L of 4-hydroxybenzoic acid, 1 mM IPTG and appropriate antibiotics. Foam formation was repressed by manually adding Antifoam 289 (Sigma Chemical Co., St. Louis, MO, USA). Fed-batch fermentation was performed three times, and the result of one representative fed-batch culture is presented.

Analytical Procedures. Cell growth was monitored by measuring the optical density at the wavelength of 600 nm (OD600) using the Ultrospec 3100 spectrophotometer (Amersham Biosciences, Uppsala, Sweden). The residual glucose concentration in culture broth was measured using high-performance liquid chromatography (HPLC) (Waters 1515/2414/2707, Waters, Milford, MA). The concentrations of PDC, DHS and PCA were analyzed according to a modified method, which employed a HPLC (1100 Series, Agilent) equipped with a Zorbax SB-Aq column (4.6 × 250 mm, Agilent) operating at 30 °C. The mobile phase consisting of buffer A (25 mM potassium phosphate buffer, pH 2.0) and buffer B (acetonitrile), flowed at 0.8 mL/min according to the following program: 0–1 min, 0% B; 1–8 min, a linear gradient of B from 0% to 70%; 8–11 min, 70% B; 11–18 min, a linear gradient of B from 70% to 0%; 18–20 min, 0% B. The
injection volume of 10 μL was used and photodiode array detector was used to monitor the signal at 280 nm for PDC, DHS and PCA. Samples for HPLC analysis were prepared as follows: cells and debris in the fermentation broth were first pelleted by centrifugation at 13 200 g for 10 min, and then the supernatant was diluted properly and filtered through a 0.22 μm membrane. The quantification was made according to a standard calibration curve established with each authentic compound. To further confirm the production of PDC, culture sample was centrifuged to remove cells and the supernatant wasacidified to pH 1.0 by concentrated HCl. An aliquot (1 mL) of the resulting solution was extracted twice with the same volume of ethyl acetate. The collected extract was vacuum-dried on a rotary evaporator. The residue wasdissolved in pyridine and incubated with bistrimethylsilyl-trifluoroacetamide to prepare trimethylsilyl derivatives, and then subjected to gas chromatography (GC)—mass spectrometry as described previously.9

**ASSOCIATED CONTENT**

## Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00281.

Supporting tables and figures (PDF)

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### Author Contributions

Z.W.L. and S.Y.L. designed the research; Z.W.L. performed the experiments; W.J.K. performed in silico simulation experiments; and Z.W.L. and S.Y.L. wrote the manuscript.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We thank Dr. Masaya Nakamura at Forestry and Forest Products Research Institute (FFPRI) in Japan for generously providing us the authentic PDC compound used as an analytical standard in this study. This work was supported by the Intelligent Synthetic Biology Center through the Global Frontier Project (2011-0031963) and also by the Technology Development Program to Solve Climate Changes on Systems Metabolic Engineering for Biorefineries (NRF-2012M1A2A2026556 and NRF-2012M1A2A2026557) from the Ministry of Science and ICT through the National Research Foundation of Korea.

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