Communication to the Editor

Biotechnology and Bioengineering

Direct Biosynthesis of Adipic Acid From a Synthetic Pathway in Recombinant *Escherichia Coli*

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ABSTRACT: The C6 dicarboxylic acid, adipic acid, is an important platform chemical in industry. Biobased production of adipic acid is a promising alternative to the current petrochemical route. Here, we report biosynthesis of adipic acid using an artificial pathway inspired by the reversal of beta-oxidation of dicarboxylic acids. The biosynthetic pathway comprises condensation of acetyl-CoA and succinyl-CoA to form the C6 backbone and subsequent reduction, dehydration, hydrogenation, and release of adipic acid from its thioester. The pathway was first tested in vitro with reconstituted pathway enzymes and then functionally introduced into *Escherichia coli* for the biosynthesis and excretion of adipic acid into the culture medium. The production titer was increased by approximately 20-fold through the combination of recruiting enzymes that were more suitable to catalyze the synthetic reactions and increasing availability of the condensation substrates. This work demonstrates direct biosynthesis of adipic acid via non-natural synthetic pathway, which may enable its renewable production.

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KEYWORDS: adipic acid; biosynthesis; beta-oxidation; *Escherichia coli*; synthetic biology

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With increasing concerns about fossil oil shortage, more attention has been focused on the sustainable production of chemicals from renewable sources (Jang et al., 2012). Recently a variety of valuable chemicals such as alkanes and 1,4-butanediol have been produced in microorganisms engineered with synthetic pathways (Choi and Lee, 2013; Schirmer et al., 2010; Yim et al., 2011). Adipic acid is one of the most important bulk chemicals and primarily used as the precursor of polyamide nylon-6,6 (Polen et al., 2013). Industrial production of adipic acid relies mainly on chemical oxidation of a mixture of cyclohexanone and cyclohexanol, which are derived from the fossil fuel component, benzene (Jang et al., 2012; Polen et al., 2013). For bio-based production of adipic acid from renewable sources, many attempts have been made. Earlier a combined biological and chemical synthesis route was developed in which glucose was fermented to produce *cis*,*cis*-muconic acid by metabolically engineered *E. coli* and *cis*,*cis*-muconic acid was further converted into adipic acid *via* chemical hydrogenation (Draths and Frost, 1994; Niu et al., 2002). A recent patent by Verdezyne, Inc. (Carlsbad, CA) reported production of adipic acid and other long-chain dicarboxylic acids through ω-oxidation of fatty acids in engineered yeasts (Picataggio and Beardslee, 2012). In a recent patent application by DSM, an alpha-keto acid pathway was proposed for adipic acid biosynthesis in *E. coli*, where alpha-ketopimelic acid was converted into adipic acid via decarboxylation and oxidation. Production titers up to 33 mg L^{-1} were achieved depending on the catalyzing enzymes employed and the carbon sources used (Raemakers-Franken et al., 2012). In another interesting patent by Genomatica, Inc. (San Diego, CA), various biochemical routes have been proposed for the biosynthesis of adipic acid, 6-aminocaproic acid, and caprolactam (Burgard et al., 2011). One of the adipic acid biosynthetic routes employs the reversal of adipic acid degradation pathway, which is analogous to the reversal of the β-oxidation

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cycle for the biosynthesis of fatty acids (Dellomonaco et al., 2011). However, details on the assembly and characterization of the biosynthetic pathway in engineered microorganisms have not been reported. In this short communication, we demonstrate the production of adipic acid directly from glucose in engineered *E. coli*.

Notably, adipic acid is a natural product that has been found in the juice of sugar beet and identified as a metabolic intermediate in the biodegradation pathways of cyclic alkanes, cyclic alcohols, and long chain aliphatic dicarboxylic acids (Cerdan et al., 1988; Cheng et al., 2002; Polen et al., 2013). In addition, adipic acid can be activated to adipyl-CoA and further degraded to acetyl-CoA and succinyl-CoA in a process similar to that of β-oxidation of fatty acids in organisms such as the filamentous fungus *Penicillium chrysogenum* (Thykaer et al., 2002). As the biosynthetic pathway for adipic acid remains unclear in the native producer sugar beet, we attempted to construct a synthetic pathway for the production of adipic acid by reversal of its degradation pathway (Fig. 1). The proposed pathway consists of six enzymatic steps that begin with the condensation of acetyl-CoA and succinyl-CoA to form the C6 backbone 3-oxoadipyl-CoA, and proceed via 3-hydroxyadipyl-CoA, 2,3-dehydroadipyl-CoA, and adipyl-CoA to adipic acid.

First we sought to identify the candidate enzymes that might catalyze the conversion of acetyl-CoA and succinyl-CoA into adipic acid. The first step, condensation of acetyl-CoA and succinyl-CoA, is catalyzed by a 3-oxoadipyl-CoA thiolase. The *E. coli paaJ* gene product, β-ketoadipyl-CoA thiolase was selected because the enzyme has been shown to cleave 3-oxoadipyl-CoA into acetyl-CoA and succinyl-CoA and the enzyme reaction could be reversible (Nogales et al., 2007; Teufel et al., 2010). 3-Oxoadipyl-CoA reduction, 3-hydroxyadipyl-CoA dehydration, and 2,3-dehydroadipyl-CoA hydrogenation constitute the second to fourth steps of the synthetic pathway. Previously, 3-hydroxybutyryl-CoA dehydrogenase (Hbd) and crotonase (Crt) from *Clostridium acetobutylicum* and *trans*-enoyl-CoA reductase (Ter) from *Euglena gracilis* were reported to have broad substrate specificities ranging from C4 to C6 substrates. For example, the three-enzyme combination was able to convert acetoacetyl-CoA into butyryl-CoA, 3-ketovaleryl-CoA into valeryl-CoA, and 3-oxohexanoyl-CoA into hexanoyl-CoA as well (Dekishima et al., 2011; Tseng and Prather, 2012). As 3-oxoadipyl-CoA is analogous to 3-oxohexanoyl-CoA, the

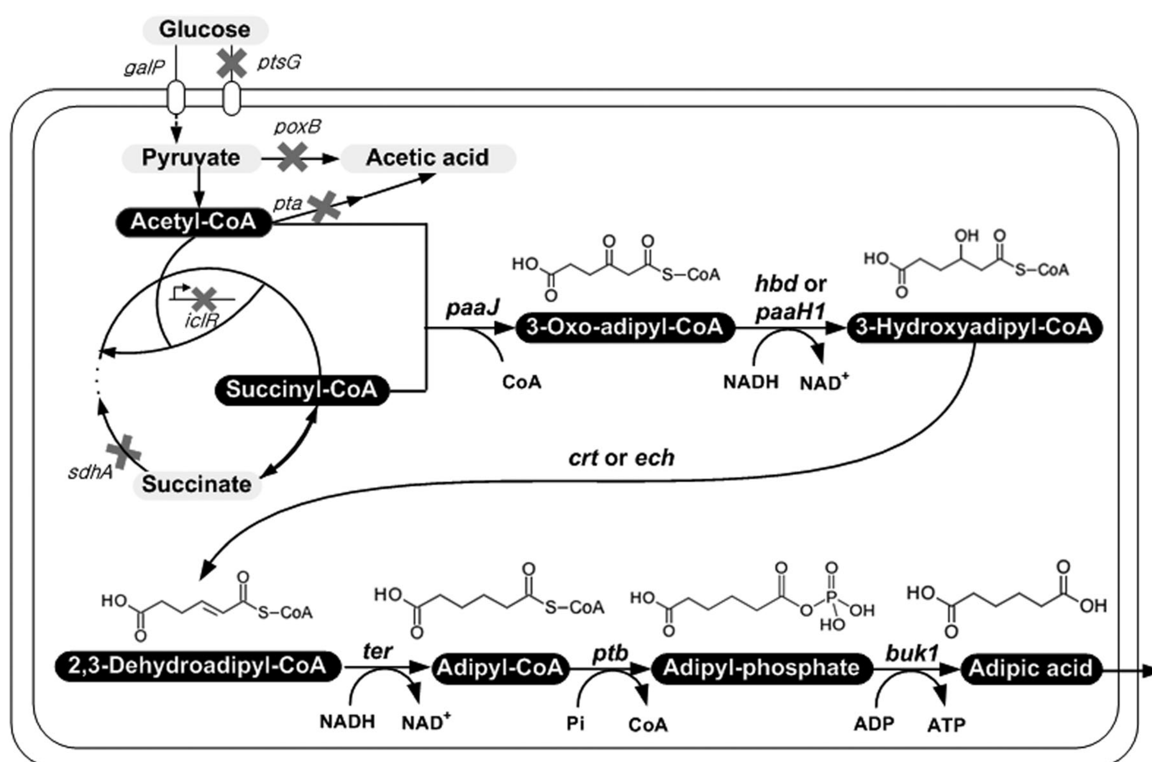


Figure 1. A synthetic pathway for the biosynthesis of adipic acid both in vitro and in vivo. The compounds with black background and white font are the key metabolites of the pathway. In vitro biosynthesis occurs with acetyl-CoA and succinyl-CoA as substrates in reconstituted enzymatic reactions. Intracellular pools of acetyl-CoA and succinyl-CoA can also be driven by the pathway enzymes heterologously expressed in organisms such as *Escherichia coli* for in vivo biosynthesis. Enzymes encoded by the genes shown are: *buk1*, butyryl kinase; *crt*, crotonase; *ech*, putative enoyl-CoA hydratase; *galP*, D-galactose transporter; *hbd*, 3-hydroxybutyryl-CoA dehydrogenase; *iclR*, DNA-binding transcriptional repressor; *paaH1*, 3-hydroxy-acyl-CoA reductase; *paaJ*, β-ketoadipyl-CoA thiolase; *poxB*, pyruvate dehydrogenase; *pta*, phosphotransacetylase; *ptb*, phosphate butyryltransferase; *ptsG*, fused glucose-specific phosphotransferase system enzymes: IIB component/IIC component; *sdhA*, succinate dehydrogenase flavoprotein subunit; *ter*, *trans*-enoyl-CoA reductase. The gray Xs indicate knockout of genes in *E. coli* QZ1111 (Liu et al., 2012).

three-enzyme combination might be able to catalyze the conversion of 3-oxoadipyl-CoA into adipyl-CoA and thus the three enzymes were recruited. The last two enzymatic steps are designed to convert adipyl-CoA into adipic acid via adipyl-phosphate. It is known that combination of phosphate butyryltransferase (Ptb) and butyryl kinase (Buk1) of *C. acetobutylicum* converted butyryl-CoA into butyryl-phosphate and finally butyrate. The butyryl kinase has been shown to exhibit broad substrate specificity toward C2–C6 substrates (Hartmanis, 1987). Assuming that the two enzymes are able to convert adipyl-CoA into adipic acid, they were selected for further test.

To validate feasibility of the synthetic pathway in vitro, each of the aforementioned enzymes (PaaJ, Hbd, Crt, Ter, Ptb, and Buk1) was expressed as the N-terminal fusion protein with hexahistidine and purified by Ni-affinity column chromatography. The starting substrates, acetyl-CoA and succinyl-CoA were then incubated with PaaJ, Hbd, Crt, and Ter with appropriate cofactors in a stepwise fashion, and the resulting products were analyzed by mass spectrometry (Fig. S1). Compounds with identical molecular weights to those of the metabolic intermediates of the synthetic pathway were detected in the respective reaction solutions (Table I), suggesting that the combination of PaaJ, Hbd, Crt, and Ter were able to produce adipyl-CoA. When Ptb and Buk1 were additionally included in the incubation, quadrupole-time-of-flight mass spectrometry (Q-TOF-MS) analysis of the reaction solution identified a compound with major ion fragments characteristic of authentic adipic acid standard, indicating the appearance of our target product in the enzymatic reaction (Fig. 2). Collectively, the recruited enzymes, PaaJ, Hbd, Crt, Ter, Ptb, and Buk1, performed the respective catalytic reactions and the six-enzyme combination was able to produce adipic acid from acetyl-CoA and succinyl-CoA.

Having proving feasibility of the synthetic pathway in vitro, we then tested whether the pathway enzymes could be used for adipic acid biosynthesis under intracellular conditions in an engineered microorganism. The host strain chosen initially was *E. coli* K12 MG1655. The genes for adipic acid synthesis were expressed from two compatible plasmids: a pTrc99A derivative expressing PaaJ and Ter, and a pZS*27mCherry derivative expressing Hbd, Crt, Ptb, and Buk1 (Table SI). The resulting strain, MG1655/pTrc-ter-paaJ/pZS*27ptb-buk1-hbd-crt (named strain AA1), was cultivated in minimal R/2 medium supplemented with 10 g/L of glucose under aerobic condition at 30°C for 120 h. Upon centrifugation, the cell-free supernatant was extracted with ethyl acetate and derivatized to form trimethylsilyl derivatives for gas chromatography–mass spectrometry (GC–MS) analysis (Fig. S2). To make an external standard curve for adipic acid quantification, authentic adipic acid was dissolved in ethyl acetate, derivatized, and analyzed with GC–MS in the same manner (Fig. S3). It was found that strain AA1 excreted $31 \pm 2\ \mu g/L$ of adipic acid into the culture medium, suggesting trace amount of adipic acid production (Fig. 3A).

Table I. Mass spectrometry data of intermediates of the adipic acid biosynthetic pathway.

Enzyme	Product	MH^+ (m/z) Calculated	Measured
PaaJ	3-Oxoadipyl-CoA	910.1491	910.1433
Hbd	3-Hydroxyadipyl-CoA	912.1647	912.1145
Crt	2,3-Dehydroadipyl-CoA	894.1542	894.0992
Ter	Adipyl-CoA	896.1698	896.1744

In an effort to increase biosynthesis of adipic acid, several genes of the synthetic pathway were replaced with their respective analogues. Replacement of Ter, which is responsible for the hydrogenation step, with butyryl-CoA dehydrogenase (Bcd) of *C. acetobutylicum* abolished the ability to produce adipic acid, indicating that Bcd might not drive the reaction. According to Machado et al. (2012), the 3-hydroxyacyl-CoA reductase (PaaH1) of *Ralstonia eutropha* outperforms Hbd in the reduction of 3-ketohexanoyl-CoA. We expected that PaaH1 might be more active in the reduction of 3-ketoadipyl-CoA, which is an analogue of 3-ketohexanoyl-CoA. Hence, PaaH1 was used to replace Hbd and the replacement resulted in a moderate increase in adipic acid production, although the increase was not statistically significant (strain AA3 vs. AA1; Fig. 3A). We searched for candidate enoyl-CoA hydratases that dehydrate 3-hydroxyadipyl-CoA. One such enzyme is the putative enoyl-CoA hydratase (ECH) encoded by h16_A3307 gene of from *R. eutropha* H16. Although ECH remains yet to be biochemically characterized, the enzyme was used to replace Crt in strain AA3. Strikingly, the production level of the resulting strain AA4 was dramatically elevated to $\sim 120\ \mu g/L$.

The next step was to target the availability of acetyl-CoA and succinyl-CoA, the two condensation substrates for adipic acid biosynthesis in recombinant *E. coli*. According to Bennett et al. (2009), intracellular acetyl-CoA and succinyl-CoA concentrations were determined to be 0.61 and 0.23 mM for the aerobically growing cells of *E. coli*. Analysis of the above adipic acid producers revealed that appreciable levels of acetic acid (1–3 g/L) accumulated in the culture media, while succinic acid did not (data not shown). This implied that intracellular succinyl-CoA other than acetyl-CoA might be a bottleneck that limited adipic acid biosynthesis. Therefore we employed an engineered host, *E. coli* QZ1111 (MG1655 Δ*ptsG* Δ*poxB* Δ*pta* Δ*sdhA* Δ*iclR*) that was previously constructed for aerobic succinic acid production (Liu et al., 2012). Interestingly, recombinant QZ1111 strains over-expressing enzymes of the synthetic pathway produced higher levels of adipic acid than their MG1655 counterparts. The best producer, strain AA7, excreted $639 \pm 34\ \mu g/L$ of adipic acid, which was approximately 20-fold higher than that of strain AA1. In addition, we monitored the profiles of cell growth and extracellular adipic acid levels for strain AA7. As shown in Figure 3B, adipic acid accumulated largely in a cell growth-dependent manner. The long period of lag phase implied that the recombinant cells of *E. coli* may be experiencing a metabolic burden due to overexpression of PaaJ and Ter,

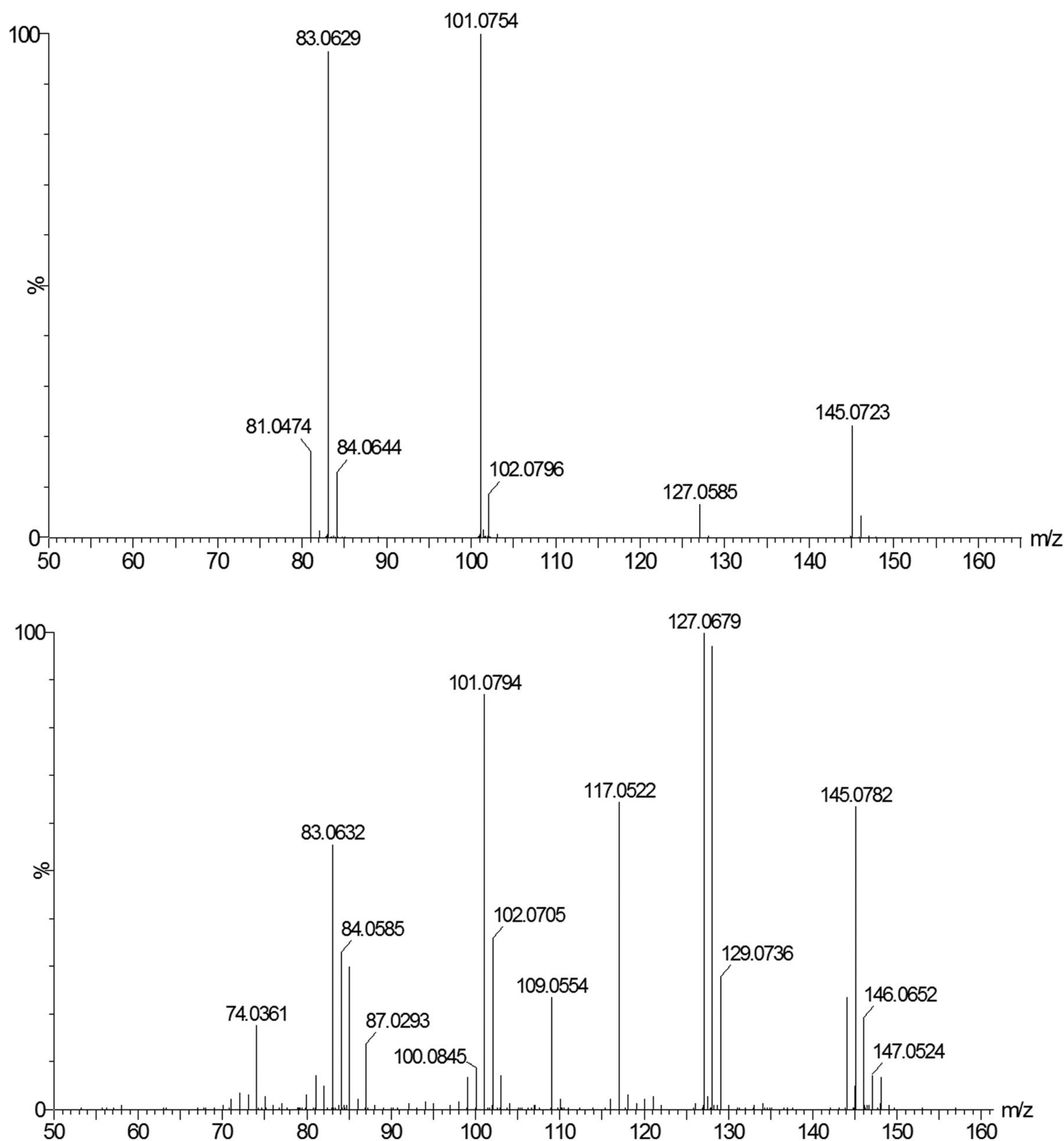
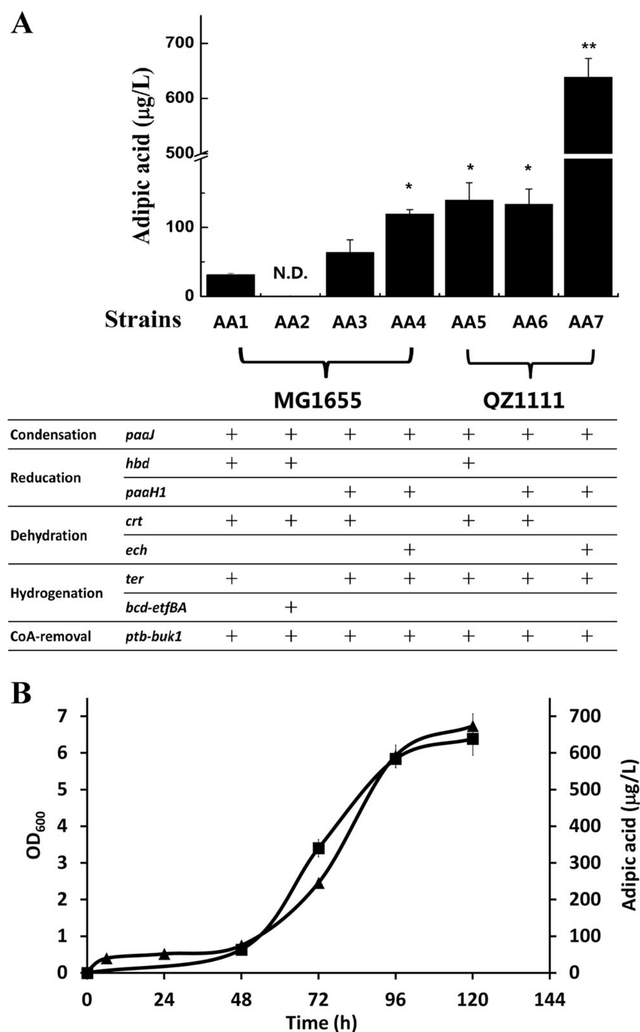


Figure 2. MS/MS spectrum of adipic acid standard (Up) and enzymatic reaction solution (Down). Purified PaaJ, Hbd, Crt, Ter, Ptb, and Buk1 were incubated in a phosphate buffer with 100 μM acetyl-CoA, 100 μM succinyl-CoA, 4 mM NADH, and 2 mM ADP at 30°C for 12 h. The samples were detected by Q-TOF MS, indicating a compound with major ion fragments characteristic of authentic adipic acid standard.

which were driven by the isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible *trc* promoter. Induction of the cells at lower IPTG concentrations shortened the lag period, but did not increase the production of adipic acid (data not shown).

Even though the production titer of adipic acid was still quite low, in this study we were able to increase its biosynthesis by searching for natural enzymes that were more suitable to catalyze the synthetic reactions and increasing availability of the metabolic precursors. The production of adipic acid may be further elevated by directed evolution of the enzymes that allows superior catalytic activities toward the C6 substrates of each synthetic step. On the other hand, coordinated overexpression of the enzymes should be beneficial to drive biosynthesis of adipic acid and alleviate the metabolic burden and the accumulation of certain metabolic intermediates or side products. In summary, here we demonstrate the feasibility of a synthetic pathway first *in vitro* using reconstituted enzymes and then in vivo for direct production of adipic acid from renewable carbon source. We also show that the production titer of the engineered microorganism was greatly improved by the



		AA1	AA2	AA3	AA4	AA5	AA6	AA7
		MG1655				QZ1111		
Condensation	*paaJ*	+	+	+	+	+	+	+
Reducation	*hbd*	+	+			+		
	paaH1			+	+		+	+
Dehydration	*crt*	+	+	+		+	+	
	ech				+			+
Hydrogenation	*ter*	+		+	+	+	+	+
	bcd-etfBA		+					
CoA-removal	*ptb-buk1*	+	+	+	+	+	+	+

Figure 3. Production of adipic acid in recombinant *E. coli*. (**A**) Extracellular accumulation of adipic acid from strains MG1655 and QZ1111 overexpressing the pathway genes as indicated with +s. The cell cultures were sampled at 120 h for quantification of adipic acid. (**B**) Profiles of cell growth (triangle) and adipic acid accumulation (rectangle) from the strain AA7. The error bars represent the standard deviation of three independent replicates. Statistical analysis was performed with $P < 0.05$ to all samples.

combination of recruiting more suitable enzymes and increasing availability of the metabolic precursors. The synthetic biology and metabolic engineering strategies should be helpful in the development of biobased routes for the production of other non-native bulk chemicals in recombinant microorganisms.

Materials and Methods

DNA Manipulations

E. coli DH5α was used to in gene cloning. *E. coli* BL21(DE3) was used as host to express recombinant enzymes. *E. coli* MG1655 and QZ1111 (Liu et al., 2012) were used to construct recombinant strains for biosynthesis of adipic acid. All strains and plasmids used in this study are listed in Table SI. A description of plasmids construction is provided in the supplemental text. Antibiotics were added at the following concentrations for selection: 100 μg/mL of ampicillin (Ap) and 50 μg/mL of kanamycin (Km).

Preparation of Recombinant Enzymes

E. coli BL21(DE3) was transformed with a pET-28a(+) derivative harboring one of the *paaJ*, *hbd*, *crt*, *ter*, *ptb*, and *buk1* genes that encode enzymes of the synthetic pathway. The recombinant strains were used to inoculate 5 mL Luria-Bertani (LB) medium and cultured overnight at 37°C. This seed culture was then diluted 1:100 into a 250-mL flask containing 50 mL fresh LB medium for protein production. The cultures were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.4–0.6, induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO), and incubated at 30°C overnight. The cells were collected by centrifugation at 8,000*g* for 20 min. The resulting pellets were resuspended with 2 mL of lysis buffer (35 mM Tris–HCl, pH 7.4, 20 mM imidazole, 1 mM dithiothreitol (DTT)) for cell lysis. The lysate was centrifuged at 12,000*g* for 10 min at 4°C and the resulting supernatant passed through a HisTrap™ HP column (1 mL; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The column was washed with 10 mL of the lysis buffer and eluted with 20 mL of the elution buffer (35 mM Tris–HCl, pH 7.4, 200 mM imidazole, 1 mM DTT). The fractions containing the target proteins were concentrated using an Amicon® Ultra-15 centrifugal filter unit with Ultracel-10K membrane (Millipore, Billerica, MA) and change to the stock buffer (35 mM Tris–HCl, pH 7.4, 1 mM DTT). The purified recombinant enzymes were mixed with 50% (v/v) glycerol and stored at −20°C before use.

Enzymatic Reactions

Each of the recombinant enzymes was added either individually or in combinations to the reaction solutions at a final concentration of 0.05 mg/mL. To characterize the first condensation step, recombinant PaaJ was incubated in the reaction solution containing 50 mM Tris–HCl (pH 7.0), 100 μM acetyl-CoA (Sigma Catalog #A2056), and 100 μM succinyl-CoA (Sigma Catalog # S1129) at 30°C for 12 h. A control reaction was performed at the same enzymatic reaction conditions except that PaaJ was pre-inactivated at 85°C for 1 h. To characterize the second step of 3-oxoadipyl-CoA reduction, Hbd and 2 mM NADH (Sigma Catalog #43420) were additionally included in the reaction solution. Similarly, Crt and Ter were included in a stepwise manner. The resulting reaction solutions were analyzed by time-of-flight mass spectrometry (TOFMS) with positive ion model (Agilent MS 6230, Palo Alto, CA). To test in vitro biosynthesis of adipic acid, the six enzymes including PaaJ, Hbd, Crt, Ter, Ptb, and Buk1 were added into 50 mM sodium phosphate

buffer (pH 7.0) supplemented with 100 μM acetyl-CoA, 100 μM succinyl-CoA, 4 mM NADH, and 2 mM ADP (Sigma Catalog #A2457). The reaction solution was incubated at 30°C for 12 h and then analyzed by quadrupole-time-of-flight mass spectrometry (Q-TOF MS Premier; Waters, Milford, MA). The collision energy ramp of MS and MS/MS were 4.0 eV and 10∼15 eV, respectively. Under the same conditions, the MS/MS spectrum of the samples was compared to that of adipic acid standard (1 mg/L; Aladdin Catalog #1036766, Shanghai, China).

Adipic Acid Production in Recombinant *E. coli*

E. coli MG1655 and QZ1111 were transformed with two compatible plasmids for the overexpression of the pathway enzymes. One was derived from plasmid pTrc99A that harbors *paaJ* and either *ter* or *bcd-etfBA* genes under the IPTG-inducible *trc* promoter. Another plasmid harbored genes under the control of the $lacI^Q$ promoter, which allowed constitutive expression of *buk1-ptb*, *hbd/paaH1*, and *crt/ech* in combinations. For adipic acid production experiments, recombinant strains were inoculated into 5 mL LB medium and cultured overnight at 37°C. The overnight culture was then transferred into a 250 mL-flask containing 50 mL of R/2 medium supplemented with 10 g/L of glucose to obtain an initial OD_{600} of 0.05. The cells were incubated at 37°C and 220 rpm in a shaking incubator and induced with 50 μM IPTG when OD_{600} reached ∼0.4. After induction, the cells were incubated at 30°C and 220 rpm. The R/2 medium (pH 6.80) was essentially as described earlier (Xia et al., 2010).

Analytical Methods

Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}) using Biophotometer plus (Eppendof, Hamburg, Germany). To determine adipic acid concentrations in the culture media of recombinant *E. coli*, the cell-free supernatant were first acidized with 2 M HCl (1%, v/v) and extracted with 5 volumes of ethyl acetate by vigorous shaking for 10 min (Suh et al., 1997). The extraction was repeated once and the combined organic fractions were transferred to a clean tube. Nitrogen flow was used to evaporate the solvent. The resulting residue was dissolved in 1 volume of ethyl acetate, mixed with half volume of a silylating reagent (Sigma Catalog #15238), and incubated at 60°C for 30 min to form derivatives. The resulting samples were analyzed by gas chromatograph-mass spectrometry using the Thermo Scientific™ TRACE™ 1310 Gas Chromatograph and TSQ8000 system equipped with a TR-5MS GC column (Thermo Catalog #260F142P; Thermo Fisher Scientific Inc., Waltham, MA). The adipic acid standard was dissolved in ethyl acetate, derivatized to form adipic acid, bis (trimethylsilyl) ester, and analyzed by GC–MS in the same manner. Adipic acid, bis(trimethylsilyl) ester had a retention time of 16.72 min and a precursor ion/daughter ion of 111 → 55 (m/z) for quantification, and 172 → 116 (m/z) for qualification.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.