Engineering *Escherichia coli* to produce branched-chain fatty acids in high percentages

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Abstract

Branched-chain fatty acids (BCFAs) are key precursors of branched-chain fuels, which have cold-flow properties superior to straight chain fuels. BCFA production in Gram-negative bacterial hosts is inherently challenging because it competes directly with essential and efficient straight-chain fatty acid (SCFA) biosynthesis. Previously, *Escherichia coli* strains engineered for BCFA production also co-produced a large percentage of SCFA, complicating efficient isolation of BCFA. Here, we identified a key bottleneck in BCFA production: incomplete lipoylation of 2-oxoacid dehydrogenases. We engineered two protein lipoylation pathways that not only restored 2-oxoacid dehydrogenase lipoylation, but also increased BCFA production dramatically. *E. coli* expressing an optimized lipoylation pathway produced 276 mg/L BCFA, comprising 85% of the total free fatty acids (FFAs). Furthermore, we fine-tuned BCFA branch positions, yielding strains specifically producing ante-iso or odd-chain iso BCFA as 77% of total FFA, separately. When coupled with an engineered branched-chain amino acid pathway to enrich the branched-chain α -ketoacid pool, BCFA can be produced from glucose at 181 mg/L and 72% of total FFA. While *E. coli* can metabolize BCFAs, we demonstrated that they are not incorporated into the cell membrane, allowing our system to produce a high percentage of BCFA without affecting membrane fluidity. Overall, this work establishes a platform for high percentage BCFA production, providing the basis for efficient and specific production of a variety of branched-chain hydrocarbons in engineered bacterial hosts.

Key Words: branched-chain fatty acid, biofuels, protein lipoylation, cold flow

1. Introduction

Engineering microbes to synthesize advanced biofuels with tunable physical and combustion properties represents a major advance toward economical production of renewable biofuels. Extensive research has focused on engineering the fatty acid biosynthetic pathway for biofuel production (Handke, et al., 2011; Yu et al. 2011; Zhang et al., 2012; Fujita, et al., 2007; Xu et al., 2014; Steen et al., 2010). Fatty acids can be converted, *in vivo*, to advanced biofuels and valuable chemicals such as alkanes, alcohols, and fatty acid ethyl esters (Rude et al., 2011; Rui et al., 2014; Steen et al., 2010; Feng et al., 2015; Schirmer et al., 2010; Zhang et al., 2012; Peralta-Yahya et al., 2012). Most bacteria utilize the multienzyme fatty acid synthase II (FASII) platform for fatty acid biosynthesis, which has been extensively characterized, making it an ideal engineering candidate. A variety of strategies have been developed to increase titers and yields of free fatty acids (FFAs) in engineered microbes, including redirection of central metabolism towards FFA (Lin et al., 2013; Bhan et al., 2013), storage of FFAs in triacylglycerols (Lin et al., 2013), thioesterase engineering (Jing et al., 2011; Torella et al., 2013; Lennen et al., 2010; Leber et al., 2014; Wu and San., 2014; Li et al., 2012), pathway enzyme optimization (Choi and Da Silva, 2014; Wu and San, 2014), reversal of β -oxidation (Dellomonaco et al., 2011), dynamic regulation of intermediate enzymes (Xu et al., 2014; Zhang et al., 2012; Liu et al., 2015), and regulation of cell-to-cell variation (Xiao et al., 2016).

While the majority of previous engineering efforts have focused on microbial production of straight-chain fatty acids (SCFAs) and their derivatives, branched-chain fuels offer improved properties such as lower freezing point, better cold flow, and lower cloud point (Knothe and Dunn 2009; Knothe 2009; Knothe 2005), which are vital to practical

76 biofuel use. For example, the freezing point standard for grade #2 diesel is -30°C. In order to
77 reach the freezing point standard from a mixture of straight and branched methyl laurate
78 ($C_{11}H_{23}COOCH_3$), the mixture would require nearly 100% ante-iso methyl laurate, which
79 has a freezing point of -31°C, and negligible straight-chain methyl laurate (freezing point of
80 5.2°C) (Knothe and Dunn 2009; US Department of Energy 2014; Center for Disease Control
81 and Prevention 1995). In the case of jet fuel, branched-chain hydrocarbons comprise at least
82 30% of JP-4 military grade jet fuel, whereas straight-chain hydrocarbons must constitute
83 less than 20%, depending on the crude oil source and refining method (US Department of
84 Energy, 2014). Thus, there is much impetus to produce a high percentage of BCFAs for
85 optimal fatty acid-derived biofuel quality.

86 Several groups have reported BCFA production in engineered *E. coli*. In all cases, a
87 heterologous branched-chain α -ketoacid dehydrogenase complex (BKD, consisting of LpdV,
88 BkdAA, BkdAB, and BkdB) was expressed to convert branched-chain α -ketoacids into
89 branched-chain α -ketoacyl-CoA starters (BCCSs) and a branched-chain-specific FabH (β -
90 Ketoacyl-(acyl-carrier-protein) Synthase III) was used to incorporate BCCSs to enter fatty
91 acid elongation (Figure 1) (Howard et al., 2013; Haushalter et al., 2014; Jiang W. et al.,
92 2015). In the initial report of this pathway, BCFAs were identified by mass spectrometry, but
93 were produced in very low quantities compared to co-produced SCFAs (Howard et al.,
94 2013). The authors also remarked that expression of the BCFA pathway in *E. coli* reduced
95 overall cell fitness, indicating an uncharacterized problem associated with either the
96 enzymes involved or BCFAs as a product. In subsequent work, the 2-methyl-butryl-CoA
97 biosynthetic pathway was engineered to increase flux to BCCSs and FabH expression was
98 dynamically regulated, ultimately generating 140 mg/L ante-iso BCFA, with BCFA reaching

20% of total FFA (Hauschalter et al., 2014). In separate work, the *E. coli* native FabH (_{Ec}FabH) was found to compete with the branched-chain-specific FabH, limiting BCFA production. Deletion of _{Ec}FabH from an engineered strain expressing the *Staphylococcus aureus* FabH (_{sa}FabH) further improved BCFA titer and percentage, ultimately generating 52% BCFA at 120 mg/L (Jiang W. et al., 2015). These approaches have demonstrated the capability to produce BCFA in *E. coli* by enforcing branch-chain incorporation at the BKD- and FabH-catalyzed steps.

Despite these efforts, the ratio of BCFA to SCFA produced in these studies remained low; fuels derived from these FFAs would not have acceptable freezing points or cold flow properties. BCFA and SCFA have similar physico-chemical properties, thus making it difficult to separate the microbially produced BCFAs from SCFAs by economically viable means. Here, we sought to identify the bottleneck limiting BCFA production, thereby increase BCFA titer and, more critically, the BCFA percentage of total FFA.

Previous work demonstrated that _{sa}FabH is almost exclusively specific for BCCSs, with limited reactivity towards acetyl-CoA (Qiu, et al., 2005). However, engineered strains carrying _{sa}FabH as the sole FabH produced a significant amount of SCFA even after supplementation with 1 g/L branched-chain α -ketoacid (Jiang W. et al., 2015), suggesting that the bottleneck is likely the low activity of BKD, the enzyme which catalyzes the conversion of branched-chain α -ketoacids to BCCSs. BKD, a multienzyme complex in the 2-oxoacid dehydrogenase (OADH) family, is composed of two E1 subunits (encoded by *bkdAA* and *bkdAB*), one E2 subunit (lipoamide acyltransferase, encoded by *bkdB*), and one E3 subunit (dihydrolipoamide dehydrogenase, encoded by *lpdV*) (Aevarsson et al., 1999). The E2 subunit requires lipoylation at specific lysine residues for proper dehydrogenase

activity (Aevvarsson et al., 1999; Wang et al., 1993). Wild-type *E. coli* has two OADHs, pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGD), both of which require E2 subunit lipoylation for enzyme activity (Hassan and Cronan, 2011; Smith and Neidhardt, 1983). Further, the H subunit of the glycine cleavage system is also lipoylated in *E. coli* (Okamura-Ikeda et al., 2010). Both PDH and OGD catalyze reactions vital for cell growth and normal metabolism. If the native lipoylation pathways are insufficient for full lipoylation of both native and heterologous enzymes, it is possible that BKD overexpression may compete with PDH and OGD for protein lipoylation. In this work, we engineered two protein lipoylation pathways to address key problems in BCFA production.

2. Materials and Methods

2.1 Materials

Phusion DNA polymerase was purchased from New England Biolabs (Beverly, MA, USA). Restriction enzymes, T4 ligase, gel purification kits, and plasmid miniprep kits were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). BCFA standards (Bacterial Acid Methyl Ester Mix), SCFA standards (GLC-20 and GLC-30), and all other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2 Plasmids and strains

Plasmids used in this study are listed in Table 1. All plasmids were constructed using standard restriction digestion cloning based on the Biobrick platform or Gibson assembly, as described previously (Lee, et al., 2011; Gibson et al., 2009).

E. coli DH10B was used for cloning. Strains DH1(*AfadE*), CL111 (Lai and Cronan,

2003) (a gift from Dr. John Cronan's Lab, University of Illinois at Urbana-Champaign), and their derivatives were used for fatty acid production. All *ΔfabH* strains were generated from strain CL111 using a protocol similar to previous work (Jiang W. et al., 2015). Specifically, CL111 was first transformed with the plasmids containing BCFA-producing pathway. The straight-chain-specific *fabH* was then eliminated by phage P1 transduction with lysates grown on strain CAG12094 as previously described (Lai and Cronan, 2003). The transduction mixtures were plated on LB-agar medium supplemented with appropriate antibiotics (tetracycline, 15 mg/L; kanamycin, 50 mg/L; ampicillin, 100 mg/L; chloramphenicol, 30 mg/L). Colonies were screened for sensitivity to a mixture of spectinomycin and streptomycin (17.5 mg/L each). Deletion of *fabH* was confirmed by colony PCR. Correct colonies were then screened for BCFA production following methods described in Section 2.3.

The *bkd* operon (*lpdV*, *bkdAA*, *bkdAB*, and *bkdB*) under the control of a P_{LacUV5} promoter was integrated at the *fadE* locus according to a previously described technique (Datsenko and Wanner, 2000). The *fadR-lplA* operon under the control of a P_{LacUV5} promoter was integrated to the *ldhA* locus using a previously described CRISPR-Cas9 gene replacement method (Jiang Y. et al. 2015).

2.3 Cell culturing and free fatty acid (FFA) production

Cells were pre-cultivated in LB medium with relevant antibiotics: kanamycin (50 mg/L), ampicillin (100 mg/L), and chloramphenicol (30 mg/L). Overnight cultures were inoculated 2% v/v into modified M9 minimal medium (containing 75 mM MOPS at pH 7.4, 2 mM $MgSO_4$, 1 mg/L thiamine, 10 μM $FeSO_4$, 0.1 mM $CaCl_2$ and micronutrients including 3 μM $(NH_4)_6Mo_7O_{24}$, 0.4 mM boric acid, 30 μM $CoCl_2$, 15 μM $CuSO_4$, 80 μM $MnCl_2$, and 10 μM

168 ZnSO₄) with 2% glucose, 0.5% yeast extract, and corresponding antibiotics for adaptation.
169 Lipoic acid was added (50 mg/L) to corresponding BCFA-producing cultures as described in
170 the Results section. Overnight cultures in minimal medium were then used to inoculate 5
171 mL of the same fresh minimal medium with an initial OD₆₀₀ of 0.08. Cells were induced
172 with relevant inducers (1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), 0.4%
173 arabinose) when OD₆₀₀ reached 0.8-1.0. Branched-chain α-ketoacid precursors were added
174 at the moment of induction (1 g/L) when indicated in the Results Section. Cells were
175 harvested 48 hours after induction and analyzed for FA production.

176 Cell growth curves were recorded on an Infinite F200PRO (TECAN) plate reader.
177 Overnight LB cultures were used to inoculate modified M9 medium with 2% glucose.
178 Overnight cultures were then used to inoculate 5 mL of the same fresh minimal medium
179 with an initial OD₆₀₀ of 0.08. Upon inoculation, 150 μL of the culture was aliquoted into a
180 96-well plate. The 5 mL culture was used for fatty acid quantification, while the 96-well
181 plate was incubated inside the plate reader with shaking (218.3 rpm, 37 °C) to record cell
182 growth. Relative cell density (in arbitrary units) was measured by monitoring absorption at
183 600 nm. Data were taken every 1500 s until cell cultures reached late stationary phase.

184 **2.4 Quantification of FFAs**

185 Titers of FFAs were quantified using a previously published method (Jiang W. et al.,
186 2015). Briefly, 1 mL of whole culture was acidified with 100 μL of 12 N HCl, then extracted
187 twice with 0.5 mL ethyl acetate, which was spiked with 20 μg/mL of nonadecanoic acid
188 (C19:0) as an internal standard. The organic layer was isolated and FFAs were derivatized to
189 fatty acid methyl esters (FAMES) by adding 90 μL methanol, 10 μL 12N HCl, and 120 μL
190 trimethylsilane-diazomethane. FAMES were analyzed using a gas chromatograph (Hewlett-

Packard model 7890A, Agilent Technologies) equipped with a 30 m DB5-MS column (J&W Scientific) and a mass spectrometer (5975C, Agilent Technologies) or a Flame Ionized Detector (FID) (Agilent Technologies). BCFA species were identified by comparing their retention times to those of standard BCFA methyl esters (Bacterial Acid Methyl Ester Mix, Sigma Aldrich) and by comparing their mass spectra to the Probability Based Matching (PBM) Mass Spectrometry Library. FA concentrations were quantified by comparing the area of each FAME peak to a standard curve generated using standard FAME mixtures with nonadecanoic acid as an internal standard (GLC-20, GLC-30, and Bacterial Acid Methyl Ester Mix, Sigma Aldrich). BCFA titer for each strain was measured in biological triplicate (starting from three different colonies) and average values are reported.

2.5 Membrane lipid analysis

FFAs were first extracted and removed as described in Section 2.4. Membrane lipids were then extracted and analyzed following a published protocol (Folch et al., 1957). Briefly, the aqueous layer containing membrane lipid after FFA removal was lyophilized (Labconco FreeZone 2.5 Liter Benchtop Freeze Dry System, Kansas City, MO) for 2 hours at full vacuum and refrigeration, or until cells were completely desiccated. The pellets were then dissolved in 1 mL chloroform and 1 mL of 15% (v/v) H₂SO₄/methanol and heated at 100°C for 3 hours for transesterification. Reaction mixtures were then cooled on ice for 5 minutes, followed by the addition of 1 mL purified water and vigorous shaking for 5 minutes. The organic phase containing FAMES was then isolated and directly analyzed by GC-FID.

2.6 Growth on FA as sole carbon source

M9 minimal media containing 1 mg/mL palmitic acid, 1 mg/mL 13-methyl-

tetradecanoic acid, or 2% glucose as the sole carbon sources was prepared as previously described (Feng and Cronan, 2009). *E. coli* MG1655 and DH1 *ΔfadE* were first cultivated in LB medium to exponential growth phase. Media was then removed by centrifugation, and cells were rinsed with phosphate buffered saline three times by centrifugation and resuspension. Rinsed cells were next resuspended in M9 minimal medium containing one of the above-described carbon sources at a density of OD₆₀₀=0.6. Cells were then cultivated at 37°C and cell growth was monitored using an Infinite F200PRO Plate Reader (TECAN) for 72 hours.

2.7 Western blotting and protein analysis

Whole culture, volume normalized by optical density at 600 nm, was incubated with loading buffer (2x Laemmli Sample Buffer (Bio-Rad) and β-mercaptoethanol) at 99°C for 10 minutes with vigorous shaking. Then, 15 μl of whole culture lysates was loaded onto two 12% polyacrylamide denaturing gels and separated by electrophoresis. Gels were duplicated and run in parallel. One gel was then stained with Coomassie blue and the other was immunoblotted. For immunoblotting, protein was blotted to a polyvinylidene fluoride (PVDF) membrane using a standard protocol (Gallagher et al., 2008). Lipoyl modified enzymes were detected using a rabbit-anti-lipoyl primary antibody (EMD-Millipore) followed by a goat-anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Stained membranes were imaged using a GE ImageQuant (LAS 4000 mini).

3. Results

3.1 Heterologous BKD expression restricts lipoylation of *E. coli* native 2-oxoacid

dehydrogenases

To elucidate the impact of BKD overexpression on cell growth and free fatty acid (FFA) production, we first constructed a FFA-overproducing strain, SC01 (Tables 1, 2), by overexpressing a cytosolic thioesterase (TesA) in a *fadE* deleted *E. coli* DH1 strain. Next the *Bacillus subtilis* BKD was introduced to strain SC01, resulting in strain SC03. BKD overexpression significantly reduced cell growth when compared to strain SC01, $p < 0.01$ (Figure 2A). Expression of a red fluorescent protein (RFP, strain SC02) did not affect cell growth, indicating that the impaired cell growth was likely not caused by metabolic burden from protein expression. When strains SC01 and SC03 were cultivated for FFA production, a significant reduction in total FFA occurred with BKD overexpression, indicating that BKD expression also inhibits total FFA production (Figure 2B).

In our quest to understand the observed BKD toxicity, we examined the highly conserved OADH enzyme class. Previous work determined that overexpression of the PDH E2 subunit in *E. coli* repressed PDH activity, suggesting that *E. coli* has limited protein lipoylation capacity (Machado and Clark, 1992). Expression of heterologous BKD requires lipoylation of its E2 subunit, which might similarly deplete the cellular lipoylation capability, leading to unlipoylated PDH and OGD in addition to under-lipoylated BKD (Figure 3A, Panel ii). Because both PDH and OGD contribute to central metabolism, we hypothesized that BKD overexpression without expanded lipoylation capacity restricts lipoylation of PDH and OGD (Figure 3A, ii), resulting in the observed growth defect and reduced fatty acid production (Figure 2).

To test our hypothesis, whole cell lysates of strains with (SC03) or without BKD (SC01) overexpression were analyzed by SDS-PAGE and Western Blot using an anti-lipoyl-protein

monoclonal antibody (Figure 3B). Without BKD, we observed lipoylated proteins corresponding to the molecular weights of the PDH E2 subunit (62 kDa) and the OGD E2 subunit (44 kDa, lane 1 in Figure 3B). Strain SC03 with BKD overexpression showed a strong lipoyl-protein band corresponding to the size of the BKD E2 subunit (45 kDa, cannot be resolved with the 44 kDa OGD E2), but the previously visible band of lipoylated-PDH E2 disappeared (Lane 2 in figure 3B). Overall, our data revealed that BKD overexpression depletes lipoylation of *E. coli* native OADHs, leading to impaired cell growth (Figure 2A) and reduced FA production (Figure 2B).

3.2 Engineering a protein lipoylation pathway restores OADH lipoylation, allowing BCFA production in high percentages

We hypothesized that the protein lipoylation deficiency caused by BKD overexpression could be overcome by engineering a protein lipoylation pathway. *E. coli* utilizes two pathways for protein lipoylation (Figure 1). The first pathway synthesizes lipoate *de novo*, for which an octanoyl moiety from octanoyl-ACP is transferred to an E2 subunit, followed by insertion of two sulfur atoms into the octanoyl side chain of an octanolyated E2 subunit, forming a five membered ring (Jordan and Cronan, 2003; Zhao, et al., 2003; Cicchillo et al., 2004; Cicchillo et al. 2004). Alternatively, exogenous lipoic acid can be ligated directly to an E2 subunit by lipoyl ligase A (LplA, encoded by *lplA*) (Hermes and Cronan, 2009; Morris et al., 1994; Cronan 2014). To test our hypothesis, we first engineered a strain to utilize exogenous lipoic acid, in order to maximize the availability of octanoyl-ACP for fatty acid elongation. We cloned *lplA* under the control of a P_{BAD} promoter and transformed to our previously-described BCFA-producing strain BC11A (CL111 (*plsX'**fabH*; *aadA*)::Tet^A pSa-P_{EcfabH}-Bs*fabH2*, pB5c-tesA-bkd; Table 2) (Jiang W. et al., 2015), resulting in strain

BC11L. Overexpression of LplA on a plasmid partially improved PDH lipoylation (Lane 3 in Figure 3B). Completing the lipoylation pathway via lipoic acid supplementation further improved PDH E2 and BKD E2 lipoylation (Lane 4 in Figure 3B), demonstrating that the engineered pathway improved lipoylation of not only PDH, but also BKD and ODH.

We next tested whether lipoylation restoration increases BCFA titer and percentage. Without lipoic acid supplementation, strain BC11L produced 49 mg/L BCFA, statistically indistinguishable from its parental strain lacking plasmid LplA (BC11A, Figure 4A). Supplementing lipoic acid in the BC11L culture dramatically improved BCFA production by 4-fold, reaching 200 mg/L and 81% compared to 29% BCFA without plasmid LplA (BC11A, Figure 4A). BCFA production alone in BC11L surpasses total FFA in the parental strain BC11A, suggesting that lipoylation additionally improved cellular capacity for FA production, presumably by improving cell fitness through native OADH lipoylation. Among the produced BCFAs, odd-chain-iso FAs, 11-methyldodecanoic acid (C13 iso) and 13-methyltetradecanoic acid (C15 iso), are the major products, individually representing 34% and 53% of total FFAs, respectively (Figure 4B). Lipoic acid added to a strain lacking plasmid LplA (BC11A) did not affect fatty acid production. Furthermore, leaky expression of LplA (without arabinose, P_{BAD} -*lplA*) restored BCFA production to the same level as with fully induced LplA, consistent with its role as a protein-modifying enzyme rather than a metabolic enzyme (data not shown).

Additionally, when another branched-chain-specific FabH, $_{sa}$ FabH, was used instead of $_{Bs}$ FabH2, BCFA production was similarly improved with LplA overexpression (Strain BC13L; 220 mg/L, 79% BCFA) compared to the parental strain lacking plasmid LplA (Strain BC13A; 126 mg/L, 56% BCFA) (Figure 4C, 4D). Expression of FadR, a global

regulator of fatty acid biosynthesis (Zhang et al., 2012), further increased BCFA production to 276 mg/L and 85% of total FFA (Strain BC13FL, Figure 4C, 4D). Overall, our results demonstrate that engineering the protein lipoylation pathway promotes native OADH and BKD lipoylation, leading to enhanced BCFA production and a significant increase in BCFA percentage.

3.3 Production of BCFAs with controlled branch position

We next tested whether the chain structure of produced BCFAs can be tailored. Our highest performing strain, BC13FL, was cultured and supplemented with 1 g/L 3-methyl-2-oxobutyric acid, 4-methyl-2-oxopentanoic acid, or 3-methyl-2-oxopentanoic acid. BCFA production strictly mirrored the branch structure of the supplemented α -ketoacid, generating even-chain-iso-FAs, odd-chain-iso-FAs, and odd-chain-anteiso-FAs as the major products, respectively (Figure 5). Under all conditions, BCFA production exceeded 83% of total FFA. Specifically, when supplemented with 4-methyl-2-oxopentanoic acid, 93% of BCFA were odd-chain iso FFA. Likewise, when supplemented with 3-methyl-2-oxopentanoic acid or 3-methyl-2-oxobutyric acid, 93% and 74% of BCFA produced were odd-chain-anteiso or even-chain-iso FFA, respectively. These results demonstrate position of the branch in BCFAs can be controlled by the supply of α -ketoacid precursors.

3.4 Engineering the endogenous lipoylation pathway

Next, we sought to engineer an endogenous pathway for enhanced lipoylation, without the requirement for lipoic acid supplementation. The basic endogenous lipoylation pathway contains a lipoyl(octanoyl) transferase (encoded by *lipB*) and a lipoyl synthase (encoded by *lipA*) (Figure 6A, Martin et al., 2009). When *lipA* and *lipB* were coexpressed under the control of a P_{BAD} promoter with native RBSs (resulting strain BC53A), BCFA production

only reached 22% of that produced by a strain with the engineered exogenous lipoylation pathway (BC13L, with lipoic acid supplementation) (Figure 6B). We then optimized the expression level of LipA and LipB by placing them under the control of strong, synthetic RBSs (BC53B), which increased BCFA production by 32%, compared to BC53A, yet only reached 54% of that produced by BC13L with added lipoic acid. To identify any limitation in the endogenous lipoylation pathway (either LipA or LipB), lipoic acid was supplied to the original LipA-LipB strain (BC53A), which increased BCFA production by 25%. Improved BCFA production after lipoic acid supplementation suggests that LipB remained functional in BC53A because LipB has secondary activity to transfer lipoic acid to E2 domain, Thus, LipA appears to be the rate-limiting step in the engineered endogenous lipoylation pathway (Morris et al., 1995). LipA belongs to the radical S-adenosyl-methionine family that requires proper assembly of the [4Fe-4S] cluster for folding and function. Previous overexpression of LipA in *E. coli* led to its accumulation in inclusion bodies, or soluble yet non-functional enzyme (Ollagnier-de Choudens and Fontecave 1999; Miller et al. 2000; Kriek et al. 2003). To optimize LipA function, we coexpressed the LipA-LipB pathway with seven iron-sulfur-cluster chaperones (*isc* operon, encoded by *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, *fdx*, *iscX*), which improved BCFA production (BC53C) to 75% of that achieved by a strain containing the exogenous lipoylation pathway (Figure 6B). Overall, we show that the engineered endogenous lipoylation pathway improved BCFA production, nearly as much as the exogenous pathway, reducing the need for lipoic acid supplementation to increase BCFA titer and percentage.

3.5 Production of BCFA from glucose

After releasing the lipoylation bottleneck, we attempted to generate BCFA from glucose

by engineering the branched-chain amino acid pathway to enrich for branched-chain α -ketoacid precursors (Figure 1). In order to accommodate additional genes required for the branched-chain amino acid pathway, we integrated several operons into the genome, and strategically selected integration loci to simultaneously eliminate competing pathways. We first integrated the *bkd* operon to the *fadE* locus to reduce potential metabolic burden caused by BKD overexpression and to prevent potential BCFA consumption in β -oxidation. Next, we integrated a synthetic operon containing *fadR-lplA* to the *ldhA* locus to direct flux away from D-lactate production. Meanwhile, the *B. subtilis alsS* and the *E. coli ilvCD* gene clusters were expressed from a low-copy number plasmid (pA6k-*alsS-ilvCD*), whose encoding enzymes convert pyruvate to 3-methyl-2-oxobutyric acid (Figure 1). An engineered *E. coli leuABCD* operon carrying a feedback-resistant *leuA* was expressed together with *tesA* from a high copy number plasmid (pE8c-*tesA-mleuABCD*) to convert 3-methyl-2-oxobutyric acid to 4-methyl-2-oxopentanoic acid (Connor and Liao, 2008). Overall, the resulting strain BC33 overexpressed 15 enzymes and produced 181 mg/L BCFAs from glucose, comprising 71% of total FFA (Figure 7A, 7B). As expected, 13-methyl-tetradecanoic acid (C15 iso) was the major FA species (72% of the BCFA) due to the accumulation of 4-methyl-2-oxopentanoic acid.

3.6 Membrane composition of high percentage BCFA-producing strains

As our engineered strains produced more than 80% BCFAs, it is of great interest to identify whether any of the produced BCFAs were incorporated into the lipid membrane. While some Gram-positive bacteria alter the branched-chain content of their membranes to modulate fluidity, membranes of wild-type *E. coli* are naturally composed strictly of SCFAs. If BCFAs produced from engineered pathways become incorporated into *E. coli* membrane

lipids, they may alter the cell membrane fluidity and present a major cellular stress, as observed with medium chain fatty acid toxicity (Lennen et al., 2011; Sherkhanov et al., 2014).

We analyzed the membrane FA composition of engineered strains BC13L (79% BCFA) and BC13A (52% BCFA) (Figure 8A). We first validated our method by measuring the membrane FA profile of wild-type *E. coli*; the obtained membrane FA profile was consistent with previous characterizations (Figure 8A) (Oursel et al., 2007). Next, the membrane profiles of strains BC13L and BC13A were analyzed and were indistinguishable from wild-type *E. coli*, with negligible incorporation of BCFAs into the cell membrane and no major shifts in the content of unsaturated FAs or chain length distribution (Figure 8B). SCFAs in the membrane of strains BC13L and BC13A are presumably synthesized by the weak activity of *s*_aFabH towards acetyl-CoA (Qiu et al., 2005). Therefore, when engineering *E. coli* for BCFA production, it may be necessary to retain some SCFA biosynthetic capacity to support cell survival. On the other hand, the inability to incorporate BCFA into lipid membrane (resulting in membrane FA profiles in Figure 8A different from FFA profiles in Figures 4, 5, and 7) suggests that *E. coli* has tight control over the lipid composition of cell membrane, which is not surprising considering the important physiological role of lipid membrane.

Finally, we attempted to elucidate the mechanism restricting BCFA from membrane incorporation. Extracellular FAs are transported into cells by FadL and are activated either to acyl-CoAs by the acyl-CoA synthetase (FadD), or to acyl-ACPs by the acyl-ACP synthase (AAS, encoded by *aas*) (Parsons and Rock, 2013). Fatty acyl-CoAs have two fates (Figure 8D): they can be incorporated into phospholipids by PlsB/PlsC, or undergo β -

oxidation to be utilized as a carbon source. Acyl-ACPs may also be incorporated into phospholipids by phospholipid acyltransferases PlsB/PlsC (Parsons and Rock, 2013) or can be recycled back to free fatty acid in the presence of cytosolic TesA. If BCFAs are restricted from membrane incorporation at the FadL or FadD steps, they cannot be metabolized as a carbon source. If restriction does not occur here, BCFA membrane incorporation must be regulated downstream of FadD, i.e., PlsB/PlsC (Figure 8D). To elucidate the restriction mechanism, *E. coli* MG1655 was cultivated in media containing 13-methyltetradecanoic acid (C15-iso, the most abundant BCFA species produced in our engineered strains) as the sole carbon source. Interestingly, *E. coli* grew with 13-methyltetradecanoic acid as the sole source of carbon (although at a slower rate than that in palmitic acid, Figure 8C), indicating that BCFAs are substrates of both FadL and FadD (Figure 8D). This finding further allows us to conclude that restriction of BCFAs from membrane incorporation occurs downstream of FadD, likely at the AAS and/or the PlsB/PlsC step.

4. Discussion

There exist biological challenges inherent to the production of BCFA in organisms that rely completely on SCFA for survival. Despite this biological complication, we have demonstrated the capability to engineer *E. coli*, a strictly SCFA-producing organism, to generate a high percentage of BCFA and restrict this BCFA from the cell membrane. Previously, several groups attempted to engineer *E. coli* to produce BCFAs, but the highest BCFA percentage was 52% (Jiang W. et al. 2015), implying a limit to the percentage of BCFA that can be produced in *E. coli*. This work demonstrated that incomplete protein lipoylation restricted previous BCFA production and a high percentage of BCFA (85% of

total FFA) can be produced in *E. coli* by engineering its protein lipoylation pathway. By control of upstream precursors, BCFA chain structure can be strictly controlled, where 93% of all BCFA produced are odd-chain iso, ante-iso (93% of total BCFA), or even-chain iso (70% of BCFA). Furthermore a single BCFA species, 13-methyltetradecanoic acid can be produced as over 57% of total FFAs; alternately, 12-methyltetradecanoic acid can be generated as a primary product comprising 51% of total FFA. This tunability demonstrates a powerful platform for the production of a desired species as demonstrated by the production of 13-methyl-tetradecanoic acid from glucose. Highly selective production of desired BCFAs is essential for practical application of BCFA-derived products.

BCFAs and their derivatives have broad applications. Biodiesel, jet fuel, and gasoline all require significant branched-chain composition. BCFAs with a percentage of 80%, as produced in this study, can be converted to biofuels to meet specific requirements of certain fuels in freezing point and cold flow properties, unreachable by SCFA-derived fuels. Beyond biofuels, 12-methyltetradecanoic acid (C15-anteiso FA or 12-MTA) is utilized as an anti-tumor compound (Wright et al., 2005; Yang et al., 2003). Other BCFA species have potential for use as powerful nutritional supplements which have shown great promise as protective agents against neonatal necrotizing enterofasciitis (Ran-Ressler et al., 2011, 2008; Walker WA, 1999). Thus, our method to produce high percentages of BCFAs may have broader applications, benefiting both bioenergy and pharmaceutical societies.

5. Conclusions

We identified the major bottleneck in BCFA production — depletion of protein lipoylation caused by BKD overexpression. Engineering of protein lipoylation pathways

restored protein lipoylation and increased the biosynthesis of BCFA (Figure 1). Using this approach, we demonstrated the production of BCFAs up to 181 mg/L, and BCFA percentage up to 79% from glucose. Furthermore, the position of branch in FA can be controlled by supply of the α -ketoacid precursor. We also demonstrated that the overproduced BCFAs cannot be incorporated to cell membrane, likely restricted by AAS and/or PlsB/PlsC. Overall, this work provides a platform for the production of BCFA in high percentages that could be used for the microbial production of branched-chain biofuels with tunable chemical structures and combustion properties.

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9. Figure legends

Figure 1. Engineered pathway for the production of high percentage branched-chain fatty acids (BCFAs) in *E. coli*. BCFAs can be produced by expression of a *B. subtilis* branched-chain α -ketoacid dehydrogenase complex (LpdV, BkdAA, BkdAB, and BkdB), a branched-chain α -ketoacyl-CoA-specific FabH (e.g. *sa*FabH) and a cytosolic thioesterase (e.g. TesA). Cellular branched-chain α -ketoacid precursors can be enriched by engineering a branched-chain amino acid biosynthesis pathway (eg. AlsS, IlvCD, and LeuABCD). The percentage of BCFA products can be dramatically enhanced by engineering protein lipoylation, either through an endogenous pathway (overexpression of LipB and LipA) or an exogenous pathway (LplA overexpression in combination with lipoic acid supplement). *E. coli* native pathways are colored grey. Overexpressed enzymes are colored orange. Native *Ec*FabH (boxed with dotted line) was deleted from the engineered strain.

Figure 2: Expression of BKD reduces cell growth and free fatty acid production. (A) Growth of fatty acid-overproducing strains with (SC03) or without (SC01) BKD overexpression ($p < 0.01$, Student's T-test). (B) Overexpression of BKD reduced free fatty acid (FFA) production ($p < 0.01$).

Figure 3: Expression of BKD reduces lipoylation of native 2-oxoacid dehydrogenases (OADHs). (A) Model illustrating the proposed mechanism of BKD toxicity. (i) Native 2-oxoacid dehydrogenases (OADHs, blue) are lipoylated for proper function. (ii) When BKD (yellow) is overexpressed, protein lipoylation capability is depleted, leading to unlipoylated OADHs. (iii) OADHs can be fully lipoylated through the overexpression of LplA, which

ligates lipoic acid to the E2 subunit of each OADH. (B) Top, 12% SDS-PAGE gel stained with Coomassie blue; bottom, Western Blot of the same samples blotted using an anti-lipoyl monoclonal antibody. Conditions for each Western sample are listed beneath each lane.

Figure 4: LplA expression with lipoic acid supplementation increases BCFA production.

(A) FFA production from strains expressing B_s FabH2 as the sole FabH. Strain BC11A was modified to overexpress plasmid *lplA* (BC11L) (B) Fatty acid species produced by each strain, under the same condition as the bar in (A). (C, D) Strains expressing the S_a FabH as the sole FabH. Strain BC13A was engineered to express a plasmid *LplA*, generating strain BC13L. Expression of *FadR* in strain BC13L generates strain BC13FL. All cultures (C,D) were supplemented with 50 mg/L lipoic acid. All cultures (A-D) were supplemented with 1 g/L 4-methyl-2-oxopentanoate.

Figure 5: Production of high percentage even chain iso, odd chain iso, and ante-iso

BCFA species. Strain BC13FL was supplemented with 1 g/L of 4-methyl-2-oxopentanoic acid, 3-methyl-2-oxopentanoic acid, or 3-methyl-2-oxobutyric acid, separately. (A) FFA profiles of the strain under each cultivation condition are shown. (B) Percent of BCFA's corresponding to each branch position with the corresponding α -ketoacid shown in (A).

Figure 6: Engineering the endogenous lipoylation pathway improves BCFA production

(A) Graphical representation of exogenous and endogenous lipoylation pathways in *E. coli*. * indicates a secondary activity of LipB (Morris et al., 1995.) (B) BCFA production from strains carrying an endogenous lipoylation pathway (BC53A, BC53B, and BC53C) are

compared with that carrying an exogenous lipoylation pathway (BC13L). All strains were cultivated in parallel and supplemented with 1 g/L 4-methyl-2-oxopentanoic acid. Relative BCFA titers are shown for each modification noted below each column. Synthetic RBS denotes control of *lipA-lipB* each under strong, synthetic RBSs.

Figure 7: Production of BCFA from glucose. Strain BC33 contains an engineered 4-methyl-2-oxopentanoic acid-producing pathway to generate BCFA from glucose (Figure 1 illustrates pathway) (A) Percent composition of straight chain fatty acid (SCFA) and BCFA of strain BC33 (B) **Free** fatty acid species produced by strain BC33 from glucose.

Figure 8: Membrane lipid composition of BCFA-producing strains and BCFA metabolism. (A) Distribution of membrane fatty acid species of BCFA-producing strains (BC13A, 52% BCFA) and BC13L (79% BCFA) compared to wild type *E. coli* (B) Percent of BCFA produced by each strain analyzed for membrane composition. * BCFA percentage of WT is assumed to be zero, as *E. coli* has not been determined to contain any membrane BCFA (C) WT *E. coli* MG1655 or DH1 Δ *fadE* were grown on either 13-methyl-tetradecanoic acid (isopalmitic acid, C15i), palmitic acid (C16), or 2% glucose in minimal medium. Strains lacking *fadE* and no functional β -oxidation showed no growth on fatty acids. (D) Graphical mechanism illustrating pathways for fatty acids to either undergo membrane incorporation, or to be utilized as a carbon source through β -oxidation.

712 **Table 1: Plasmids used in this study.**

Plasmids	Replication ori	Gene and promoter	Resistance	Reference
pA5k-tesA	p15a	P_{lacUV5} -tesA(<i>E. coli</i>)	Kan ^R	(Jiang W. et al. 2015)
pSa-P_{ecfabH}^{Bs}fabH2	SC101	P_{ecfabH} -fabH2(<i>B. subtilis</i>)	Amp ^R	(Jiang W. et al. 2015)
pSa-P_{ecfabH}^{Sa}fabH	SC101	P_{ecfabH} -fabH(<i>S. aureus</i>)	Amp ^R	(Jiang W. et al. 2015)
pB5c-tesA-bkd	pBBR1	P_{lacUV5} -tesA(<i>E. coli</i>)- lpdV- bkdAA-bkdAB-bkdB (<i>B. subtilis</i>)	Cm ^R	(Jiang W. et al. 2015)
pE8k-lplA	ColE1	P_{BAD} -lplA(<i>E. coli</i>)	Kan ^R	This study
pA5k-fadR-lplA	p15a	P_{lacUV5} -fadR-lplA (<i>E. coli</i>)	Kan ^R	This study
pB5c-rfp	pBBR1	P_{lacUV5} -rfp	Cm ^R	This study
pE8c-tesA-bkd	ColE1	P_{BAD} -tesA(<i>E. coli</i>)- lpdV- bkdAA-bkdAB-bkdB (<i>B. subtilis</i>)	Cm ^R	This study
pA8k-lipA-lipB-fadR	p15a	P_{BAD} -lipA-lipB-fadR (<i>E. coli</i>)	Kan ^R	This study
pA8k-lipA-lipB*	p15a	P_{BAD} -lipA-lipB (<i>E. coli</i>)	Kan ^R	This study
pA58k-lipA-lipB*- isc	p15a	P_{BAD} -lipA-lipB- P_{LacUV5} -iscS- iscU-iscA-hscB-hscA-fdx- iscX	Kan ^R	This study
pE8c-tesA-mleuABCD	ColE1	P_{BAD} -tesA-mleuABCD (<i>E. coli</i>)	Cm ^R	This study
pA6k-alsS-ilvCD (pSA69)	p15a	$P_{Llac0-1}$ - alsS-ilvCD (<i>E. coli</i>)	Kan ^R	(Atsumi et al., 2008)

713 *Indicates LipA-LipB under the control of synthetic RBSs

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Table 2: Strains used throughout the study.

Strains	Relevant genotype	<i>fabH</i> gene	Reference
CL111	UB1005, attHK022::(<i>plsX</i> ⁰ <i>fabH</i> , <i>Spc</i> ^R <i>Str</i> ^R), <i>fabH</i> ::Kan	<i>s_afabH</i>	(Lai et al. 2003)
CAG12094	MG1655, <i>zcb-3059</i> :: <i>Tn10</i>	<i>E_cfabH</i>	(Jiang et al. 2010)
SC01	DH1(<i>ΔfadE</i>) pA5k-tesA	<i>E_cfabH</i>	This study
SC02	DH1(<i>ΔfadE</i>) pB5c-rfp, pA5k-tesA	<i>E_cfabH</i>	This study
SC03	DH1(<i>ΔfadE</i>) pE8c-tesA-bkd	<i>E_cfabH</i>	This study
BC11	CL111 (<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A pSa-P _{ecfabH} -B _s <i>fabH2</i>	<i>B_sfabH2</i>	(Jiang W. et al. 2015)
BC13	CL111 (<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A pSa-P _{ecfabH} -S _a <i>fabH</i>	<i>s_afabH</i>	(Jiang W. et al. 2015)
BC11A	CL111 (<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A pSa-P _{ecfabH} -B _s <i>fabH2</i> , pB5c-tesA-bkd	<i>B_sfabH2</i>	(Jiang W. et al. 2015)
BC11L	CL111 ((<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A , Δ <i>Kan</i> , pSa-P _{ecfabH} - B _s <i>fabH2</i> , pB5c-tesA-bkd, pE8k-lplA	<i>B_sfabH2</i>	This study
BC13A	CL111 (<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A pSa-P _{ecfabH} -S _a <i>fabH</i> , pB5c-tesA-bkd	<i>s_afabH</i>	(Jiang W. et al. 2015)
BC13L	CL111 (<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A , Δ <i>Kan</i> , pSa-P _{ecfabH} - S _a <i>fabH</i> , pB5c-tesA-bkd, pE8k-lplA	<i>s_afabH</i>	This study
BC13FL	CL111 (<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A , Δ <i>Kan</i> , pSa-P _{ecfabH} - S _a <i>fabH</i> , pE8c-tesA-bkd, pA5k-fadR-lplA	<i>s_afabH</i>	This study
BC53A	CL111 (<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A , Δ <i>Kan</i> , pSa-P _{ecfabH} - S _a <i>fabH</i> , pB5c-tesA-bkd, pA8k-lipA-lipB-fadR	<i>s_afabH</i>	This study
BC53B	CL111 (<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A , Δ <i>Kan</i> , pSa-P _{ecfabH} - S _a <i>fabH</i> , pB5c-tesA-bkd, pA8k-lipA-lipB*	<i>s_afabH</i>	This study
BC53C	CL111 (<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A , Δ <i>Kan</i> , pSa-P _{ecfabH} - S _a <i>fabH</i> , pB5c-tesA-bkd, pA58k-lipA-lipB*-isc	<i>s_afabH</i>	This study
BC33	CL111 (<i>plsX'</i> <i>fabH</i> ; <i>aadA</i>)::Tet ^A , Δ <i>Kan</i> , <i>fadE</i> :: <i>bkd</i> , <i>ldhA</i> :: <i>fadR-lplA</i> , pSa-P _{ecfabH} -S _a <i>fabH</i> , pE8c-tesA- mleuABCD, pA6k-alsS-ilvCD	<i>s_afabH</i>	This study

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

B

Lipoic Acid	+	-	+	-	-
Plasmid <i>isc</i> operon	-	-	-	-	+
Synthetic RBS	-	-	-	+	+
Plasmid <i>lipA</i> , <i>lipB</i>	-	+	+	+	+
Plasmid <i>lplA</i>	+	-	-	-	-

Figure 7

Figure 8