Modular pathway engineering for the microbial production of branched-chain fatty alcohols

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Abstract
The intrinsic structural properties of branched long-chain fatty alcohols (BLFLs) in the range of C12 to C18 make them more suitable as diesel fuel replacements and for other industrial applications than their straight-chain counterparts. While microbial production of straight long-chain fatty alcohols has been achieved, biosynthesis of BLFLs has never been reported. In this work, we engineered four different biosynthetic pathways in Escherichia coli to produce BLFLs. We then employed a modular engineering approach to optimize the supply of α-keto acid precursors and produced either odd-chain or even-chain BLFLs with high selectivity, reaching 70 and 75% of total fatty alcohols, respectively. The acyl-ACP and alcohol-producing modules were also extensively optimized to balance enzyme expression level and ratio, resulting in a 6.5-fold improvement in BLFL titers. The best performing strain overexpressed 14 genes from 6 engineered operons and produced 350 mg/L of BLFLs in fed-batch fermenter. The modular engineering strategy successfully facilitated microbial production of BLFLs and allowed us to quickly optimize new BLFL pathway with high titers and product specificity. More generally, this work provides pathways and knowledge for the production of BLFLs and BLFL-related, industry-relevant chemicals in high titers and yields.

Keywords: Branched long-chain fatty alcohols, Branched-chain fatty acids, Advanced biofuels, Modular pathway engineering

Background
Finite energy resources and increased environmental concerns demand the development of sustainable and renewable approaches to the production of fuels, chemicals, and materials. Engineering microbial metabolic pathways to synthesize desired products is an attractive approach. With recent advances in genetic techniques and metabolic engineering methodologies, various engineered microbes have been developed to produce an array of chemicals derived from inexpensive and renewable substrates [1, 2]. Target molecules are typically designed to replace or mimic those obtained from petroleum or other non-renewable sources [3, 4]. Among these chemicals, long-chain fatty alcohols (LCFLs) in the range of C12–C18 have numerous applications as fuels, emollients, plasticizers, thickeners, and detergents [5–7]. At the industrial scale, LCFLs are currently produced either through hydrogenation of pretreated natural fats and oils (oleochemicals) or hydroformylation of petrochemicals (e.g. crude oil, natural gas) [8–10]. Because both processes require harsh reaction conditions and release harmful byproducts to the environment [11], microbial production of fatty alcohols from renewable sugars is a promising alternative.

Long-chain fatty alcohols have been recently biosynthesized from engineered microbes through fatty acid biosynthetic pathway [5, 10–15]. The majority of previously biosynthesized LCFLs contain straight aliphatic chains. Straight LCFLs have relatively high freezing points and viscosities compared to their branched-chain isomers, thus limiting their low-temperature operability—an

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conversion of BCFAs to BLFLs has not been explored. Engineering a protein lipoylation pathway and an α-keto acid synthesis module that converts glucose to α-keto acids, an acyl-ACP generation module that converts α-keto acids to branched-chain acyl-ACPs, and an alcohol formation module that converts branched-chain acyl-ACPs to final branched-chain products. To begin, we experimentally determined the most efficient enzyme combinations to convert branched-chain acyl-ACPs to BLFLs using an engineered branched-chain-acyl-ACP-producing basal strain (BC33). This initial pathway identification allowed us to ascertain whether BLFL production was even feasible, given the available enzymes. Next, we optimized each module separately and later the entire pathway collectively to produce even-chain-iso and odd-chain-iso fatty alcohols from glucose. Our modular approach enabled us to isolate each module for testing and optimization, without confounding the system by simultaneously engineering the other modules.

Methods
Materials and media
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 the other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Minimal medium (M9 medium supplemented with 75 mM MOPS at pH 7.4, 2 mM MgSO4, 1 mg/L thiamine, 50 μg/mL lipoic acid, 10 μM FeSO4, 0.1 mM CaCl2, and micronutrients, including 3 μM (NH4)6Mo7O24, 0.4 mM boric acid, 30 μM CoCl2, 15 μM CuSO4, 80 μM MnCl2, and 10 μM ZnSO4) containing 2% glucose and 0.5% yeast extract as carbon sources was used for cell growth and fatty acid production.

Plasmids and strains
Plasmids and oligonucleotides used in this study are listed in Table 1. Genes encoding S. elongatus AAR (aar), M. marinum CAR (car), and B. subtilis Sfp (sfp) were codon-optimized for E. coli expression and synthesized.
by Integrated DNA Technologies (Coralville, IA, USA). Genes encoding ACR (maqu2507) and Maqu2220 (maqu2220) were amplified by PCR from templates provided by Dr. Brett M. Barney (University of Minnesota, MN) and Dr. Jay D. Keasling (Joint BioEnergy Institute, CA). To create all plasmids (Table 1), the corresponding genes were assembled into the backbones of BioBrick plasmids [35], using either restriction sites or Golden-Gate DNA assembly method [36].

*Escherichia coli* DH10B was used for cloning purposes. *E. coli* strain CL111 [37] (a gift from Dr. Cronan’s Lab, University of Illinois at Urbana-Champaign) was used for production purposes. Strain BC30 was created by integrating the *bkd* operon (*lpdV*, *bkdAA*, *bkdAB*, and *bkdB*) at the *fadE* locus in the genome under the control of a *PlacUV5* promoter, using a previously described technique [38]. Plasmid pSa-PrPlaFabH-ScFabH was transformed into BC30 strain, and the *ScFabH* was knocked out by P1 transduction, creating strain BC33 [37]. Strains BO33A-J were created by transforming the corresponding plasmids (Table 2) into BC33 competent cells, respectively. Strain BC43 was created by switching native *leuABCD* promoter *P* _leuLp_ and *P* _leuLp2_ to *PlacUV5* promoter, using a previously described CRISPR-Cas9 gene replacement method [39]. Similarly, to create strain BC63, the *fadR-lplA* operon under the control of a *PlacUV5* promoter was integrated to the *ldhA* locus of BC43 strain by the same CRISPR-Cas9 gene replacement method. Strains BO63V and BO63L were created by transforming the corresponding plasmids into strain BC63.

**Cell culturing and α-keto acids supplementation**

Cells were pre-cultivated in LB medium with proper antibiotics. Overnight cultures were inoculated 2% v/v into M9 minimal medium (described in “Materials and media” section) with corresponding antibiotics for adaptation. Overnight cultures in minimal medium were then used to inoculate 5 mL of the same fresh minimal medium, with an initial OD _600_ of 0.08. When OD _600_ reached 0.8, cells were induced with proper inducers (1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), 0.4% arabinose and/or 200 nM anhydrotetracycline (aTc), or otherwise specified). For α-keto acid supplementation experiments, one of the α-keto acids (3-methyl-2-oxobutyric acid, 3-methyl-2-oxopentanoic acid, or 4-methyl-2-oxopentanoic acid) was added at OD _600_ = 0.8 to a final concentration of 1 g/L. Cells were harvested 3 days after induction.

**Fermentation**

Fed-batch fermentation was carried out using a New Brunswick Bioflo 110 fermenter with a pH meter, a dissolved oxygen electrode, and a temperature electrode. M9 medium (described in “Materials and media” section, 500 mL) was inoculated with an overnight culture
of strain BO63L to an initial OD$_{600}$ to 0.08, along with appropriate antibiotics and 0.001% Antifoam 204. The fermentation was initiated with the following settings: Temperature was set to 30 °C, pH was controlled at 7.4 by automatic feeding of 6 N ammonium hydroxide, the airflow rate was kept at 1.5 L/min, and the average stirring rate was maintained at 500 rpm. Gene expression was induced at OD$_{600}$ = 10 by addition of 1 mM IPTG, 0.4% arabinose, and 0.22 μM aC (final concentration). A glucose stock solution (400 g/L glucose and 12 g/L MgSO$_4$) was intermittently pulsed into the bioreactor to re-supply glucose, and a yeast extract solution (20%) was intermittently pulsed into the bioreactor to re-supply amino acids. Broth samples (~ 3 mL) were collected at a series of time points to measure cell density and alcohol titer.

**Quantification of the fatty alcohols**

For the quantification of alcohols, 1 mL of cell culture was acidified with 100 μL of concentrated HCl (12 N). Alcohols were extracted twice with 0.5 mL ethyl acetate, and the organic layers were isolated. Next, 200 μL of the organic layer from each sample was transferred to one 2 mL clear glass GC vial (Agilent Technologies, Santa Clara, CA), mixed with 200 μL of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% v/v chlorotrimethylsilane, and incubated at 60 °C for 2 h. Alcohol derivatives were quantified using a GC–MS (Hewlett-Packard model 7890 A, Agilent Technologies) equipped with a 30 m DB5-MS column (J&W Scientific) and a mass spectrometer (5975C, Agilent Technologies) or a FID (Agilent Technologies) detector. For each sample, the column was equilibrated at 80 °C, followed by a ramp to 300 °C at 20 °C/min, and was then held at 300 °C for 3 min. Individual alcohol peaks were identified by comparing their retention time to that of a standard (a mixture of 1-tetradecanol, 1-hexadecanol, and 1-octadecanol, prepared and derivatized identically to samples) and by comparing their mass spectra to the National Institute of Standards and Technology (NIST) Mass Spectral Library. Concentrations of each alcohol were determined by comparing the area of each sample peak to a standard curve generated by standards eluted using the same method. Product
titer for each strain was measured in biological triplicate (starting from three different colonies) and average values are reported.

**Results**

**Engineering alcohol formation modules in BCFA-producing strains**

To create the branched-chain-acyl-ACP-producing basal strain BC33, the *E. coli* fabH was first replaced by *Staphylococcus aureus* fabH (SafabH), a modification which was previously demonstrated to enhance branched-chain fatty acid production [28]. The acyl-CoA dehydrogenase gene (*fadE*) was next replaced by *Bacillus subtilis* bkd, functionally inhibiting β-oxidation (AfadE) and allowing the activation of branched-chain α-keto acids to branched-chain acyl-CoAs that can enter the FASII system (fadE::bkd) (Tables 1, 2). Next, three alcohol formation modules were separately constructed in strain BC33 (Table 2), resulting in alcohol-producing strains BO33A-C. Strain BO33A utilizes the *Synechococcus elongatus* acyl-ACP reductase (AAR, encoded by *aar*, Fig. 1) to convert acyl-ACPs to fatty aldehydes [13]. Strain BO33B first generates FFAs via expression of the *E. coli* cytosolic thioesterase (TesA), which are then converted to fatty aldehydes by the *Mycobacterium marinum* carboxylic acid reductase (CAR, encoded by *car*) with *Bacillus subtilis* Sfp (encoded by *sfp*) coexpression [5] (Fig. 1). Strain BO33C also expresses cytosolic TesA to generate FFAs and overexpresses an *E. coli* acyl-CoA synthetase (FadD) to activate FFAs to fatty acyl-CoAs, which

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**Table 2 Strains used in this research**

| Strains Relevant genotype References |
|--------------------------------------|--|
| **Parental strains** |
| CL111      | UB1005, attHKO22::(pIsXyabH, aadA) fabH::kan [37] |
| CL111(Δkan) | UB1005, attHKO22::(pIsXyabH, aadA), Δkan [28] |
| BC30       | CL111(Δkan) fabE::bkdAA-bkdAB-bkdAB [This study] |
| BC33       | CL111(Δkan) fabE::bkdAA-bkdAB-bkdAB pSa·P\textunderscore fadE::bkdH [This study] |
| **Alcohol-producing strains** |
| BO33A      | BC33 pB5k-aar-lplA [This study] |
| BO33B      | BC33 pB5k-sfp-car, pE8c·tesA-lplA [This study] |
| BO33C      | BC33 pB5k-maqu2507-lplA, pE5c·tesA-fadD [This study] |
| BO33D      | BC33 pB5k-maqu2220-lplA [This study] |
| BO33E1     | BC33 pB5k-aar-lplA, pB5k-adhA [This study] |
| BO33E2     | BC33 pB5k-aar-lplA, pB5k-yqhD [This study] |
| BO33E3     | BC33 pB5k-aar-lplA, pB5k-yigB [This study] |
| BO33E4     | BC33 pE2c·aar-lplA-yigB [This study] |
| BO33F      | BC33 pA8c·aar-lplA, pB2k·alsS·ivlCD-yigB [This study] |
| BO33F1     | BC33 pE2c·aar-lplA-yigB [This study] |
| BO33F2     | BC33 pE2c·aar-lplA-yigB, pB2k·alsS·ivlCD [This study] |
| BO33F3     | BC33, pB5c·aar-lplA-yigB, pA6k·alsS·ivlCD [This study] |
| BO33G1     | BC33, pA8c·leu\textsuperscript{amiz}·bcd, pB5k·aar-lplA [This study] |
| BO33G2     | BC33, pE8c·leu\textsuperscript{amiz}·bcd, pB5k·aar-lplA [This study] |
| BO33H1     | BC33 pB5k-maqu2220-lplA, pE8c·adhA [This study] |
| BO33H2     | BC33 pB5k-maqu2220-lplA, pE8c·yahD [This study] |
| BO33H3     | BC33 pB5k-maqu2220-lplA, pE8c·yigB [This study] |
| BO33I1     | BC33, pA6k·alsS·ivlCD, pB5c·maqu2220-lplA [This study] |
| BO33I2     | BC33, pE2k·alsS·ivlCD, pB5c·maqu2220-lplA [This study] |
| BO33J1     | BC33, pA8c·leu\textsuperscript{amiz}·bcd, pB5k·maqu2220-lplA [This study] |
| BO33J2     | BC33, pA8c·lplA, pB5k·maqu2220·leu\textsuperscript{amiz}·bcd [This study] |
| BO43I      | BC43 pB5c-maqu2220-lplA, pE2s·alsS·ivlCD [This study] |
| BO63V      | BC63 pB5k-maqu2220, pE2s·alsS·ivlCD [This study] |
| BO63L      | BC62 pB5k-maqu2220, pE2s·alsS·ivlCD, pA8c·leuABCD [This study] |
are then converted to aldehydes by overexpressing an acyl-CoA reductase from *Marinobacter aquaeolei* VT8 (ACR, encoded by *maqu2507*) [40] (Fig. 1). All strains rely on native *E. coli* alcohol dehydrogenases (ADHs) to reduce aldehydes to alcohols. The native *E. coli* lipoyl ligase (encoded by *lplA*) is also expressed in all strains to improve the lipoylation of 2-oxoacid dehydrogenases including BKD, which requires lipoylation to function [27].

We first sought to test the three alcohol formation modules by assessing their capacities to convert branched-chain acyl-ACPs to BLFLs. Strains BO33A-C were cultivated at previously determined optimal temperatures for each pathway (see “Methods” section) [5, 12, 41] and supplemented with 1 g/L of 4-methyl-2-oxopentanoic acid, the most favorable α-keto acids for BCFA production [28]. Each strain produced some BLFLs, representing the first report of BLFL production in *E. coli*. The CAR pathway (BO33B) and the ACR pathway (BO33C) produced 28 and 18 mg/L BLFLs, respectively. In addition, the chain lengths of the products range from C14 to C18, consistent with the FFA profile of the TesA-overexpressing strain [42]. Meanwhile, the AAR pathway (BO33A) produced the highest BLFL titer among these three strains: 54 mg/L, comprising 84% of the total fatty alcohols (Fig. 2a). The branch and chain-length profile of BLFLs produced in BO33A is consistent with the profile of BCFAs produced by strains with similar genetic backgrounds as previously reported (Fig. 2b) [27], indicating that the AAR pathway has little preference between straight-chain and branched-chain acyl-ACP substrates. Interestingly, the CAR pathway produced twofold more straight-chain fatty alcohols but sixfold less BLFLs than the AAR pathway, indicating that the CAR pathway is primarily straight-chain-specific. While these results serve as a proof-of-principle that BLFL can be produced at high percentages in *E. coli*, the initial low titers indicate that additional engineering efforts are needed to obtain desirable titers and yields of branched-chain products.

All of the above strains relied upon *E. coli* native ADHs to convert fatty aldehydes to alcohols. We next sought to increase BLFL production in strain BO33A by overexpression of ADHs. Three unique ADHs, *adhA* from *Lactococcus lactis* [43], and *yqhD* and *yjgB* [5, 43, 44] from *E. coli*, were overexpressed in strain BO33A, resulting in strains BO33E1, BO33E2, and BO33E3, respectively. When cultivated under the same conditions and with 1 g/L 4-methyl-2-oxopentanoic acid, strains BO33E1 and

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**Fig. 2** Testing three alcohol formation modules. **a** Overall titer distribution and **b** chain-length of three BLFL-producing strains with different alcohol formation modules, BO33A-C (Table 2). *S. elongatus*, *M. marinum*, and *M. aquaeolei* VT8, respectively. **c, d** Effect of alcohol dehydrogenase overexpression on BLFL production. Alcohol profiles of strain BO33A (without adh overexpression) are compared with those from strain BO33E1 (expressing *adhA* from *L. lactis*), BO33E2 (expressing *yqhD* from *E. coli*), and BO33E3 (expressing *yjgB* from *E. coli*). All cultures were supplemented with 1 g/L 4-methyl-2-oxopentanoic acid. Cells were cultivated and induced as described in “Methods” section.
The titer of even-chain-iso BLFLs produced by strain BO33E1 is 50 mg/L total BLFL and 47 mg/L odd-chain-iso fatty alcohol, statistically indistinguishable from the BLFL titer of strain BO33A when supplemented with 3-methyl-2-oxobutyric acid (Fig. 4b; Additional file 1: Figure S2). Strain BO33G1 generated 94% odd-chain-iso fatty alcohol, indicating complete conversion of 3-methyl-2-oxobutyric acid by the modified LeuAmutBCD. Conversely, when leuA\textsuperscript{mut}BCD was expressed under the high copy number plasmid (strain BO33G2), the BLFL titers decreased (p = 0.07) and had large standard variations (Fig. 4b), even when the inducer concentration was reduced. Furthermore, overexpression of leuA\textsuperscript{mut}BCD from the high-copy number plasmid also decreased cell growth rate and cell density (data not shown). These results indicate that expression of the leuA\textsuperscript{mut}BCD operon at too high a level affects cell growth and BLFL production, thus needs to be avoided.

**Balancing gene expression levels in all three modules for enhanced BLFL production from glucose**

With the α-keto acid generation and the alcohol formation modules optimized separately, we next sought to balance the gene expression levels within the completed pathway. We selected the best-performing genetic elements of each individual module to complete the full pathway: the α-keto acid generation module, including alsS and ilvCD, the acyl-ACP synthesis module, including aar and lplA, and the alcohol formation module, including yjgB (Fig. 3a). To perform this optimization,
we altered gene copy numbers to change the expression level of each module. Compatible plasmids with three different copy numbers were used for this purpose: a high copy number (pColE1, 50 copies per cell), a medium copy number (pBBR1, 17–20 copies per cell), and a low copy number (pA15a, 7–10 copies per cell) [35] (Fig. 3b; Additional file 1: Figure S1). Three additional strains BO33E3, BO33F1, and BO33F2 were created (Fig. 3b; Additional file 1: Figure S1). All three strains expressed aar, lplA, and yjgB from the high copy number plasmids under the control of a very tight promoter (P_tet), but produced less BLFL than strains BO33E3 and BO33F, which expressed aar, lplA, and yjgB from the low or the medium copy number plasmid. The low titer is potentially caused by
the insolubility of highly overexpressed AAR [13]. Meanwhile, compared to strain BO33F3 with the low-copy \(\text{alsS}-\text{ilvCD}\) expression, strain BO33F with the medium-copy \(\text{alsS}-\text{ilvCD}\) expression improved BLFL production by 3.2-fold. These results suggest that the optimal \(\text{aar}\) expression level was medium to low, and that \(\text{alsS}-\text{ilvCD}\) required overexpression in a medium to high-copy number plasmid for optimal BLFL production (Fig. 3b; Additional file 1: Figure S1).

**Fig. 4** Optimizing the 4-methyl-2-oxopentanoic acid generation module to produce odd-chain-iso fatty alcohols. 

- **a** Biosynthetic pathways for the production of odd-chain-iso fatty alcohols.
- **b** Titters of engineered odd-chain-iso BLFL-producing strains. Strains BO33G1-2 containing \(\text{leuAmutBCD}\) in plasmids with different copy numbers were cultivated as described in “Methods”, and supplemented with 1 g/L 3-methyl-2-oxobutyric acid. Strain BO33G2 was induced with either 0.1 or 0.4% arabinose.

**BLFL production from glucose using the acyl-ACP reductase Maqu2220**

During the course of this work, Haushalter et al. published the production of straight LCFLs in high titers by overexpression of a marine acyl-ACP reductase, Maqu2220 from *M. aquaeolei* VT8 [48]. Maqu2220 was first characterized as a fatty aldehyde reductase, reducing fatty aldehydes to fatty alcohols [49]. However, it was later confirmed to have acyl-ACP reductase activity and
is capable of converting acyl-ACPs or acyl-CoAs directly to fatty alcohols [50], thus significantly simplifying the fatty alcohol pathway. We tested the efficiency of BLFL production via Maqu2220 by replacing the S. elongatus aar in strain BO33A with maqu2220, resulting in strain BO33D. When supplemented with 1 g/L of 4-methyl-2-oxopentanoic acid, BO33D produced 198 mg/L (89%) BC alcohols, 3.7-fold higher than that of strain BO33A (Fig. 5). Even compared with the previous best-performing strain BO33E3, the BLFL titer of strain BO33D enhanced by 1.5-fold. Most BLFLs (> 99%) were identified in the cell pellet, consistent with previous study [51]. Moreover, overexpression of the ADHs yjgB, yqhD, or adhA did not enhance BLFL titer (Fig. 5), further confirming the previous hypothesis that Maqu2220 contains a catalytic domain with ADH activity.

After confirming the capability of Maqu2220 to convert branched-chain acyl-ACPs to BLFLs, we sought to use Maqu2220 to produce BLFLs from glucose by applying the knowledge learned from optimizing the α-keto acid synthesis module. After constructing and validating strains, we found that the optimal expression level of each module mimicked those characterized using the aar pathway. First, the high-copy number-expression (pColE1) of the alsS-ilvCD operon increased even-chain-iso fatty alcohol production by 9.2-fold over the low-copy number-expression (pA15a) (as seen by comparing strains BO33I1 with BO33I2 in Fig. 6a). Second, when supplemented with 3-methyl-2-oxobutyric acid, the low-copy number-expression of the leuABCD operon produced 2.8-fold more odd-chain-iso fatty alcohols than the medium-copy number-expression (as seen for strains OB33J1 and OB33J2 in Fig. 6a).

Next, we attempted to combine all three modules to produce BLFLs from glucose, which requires overexpression of 14 genes from 6 synthetic operons: P_{tet}-alsS-ilvCD, P_{BAD}-leuA\textsuperscript{mut}BCD, P_{lacUV5}-lpdV_{\text{br}}bkdAA_{\text{br}}bkdAB_{\text{br}}bkdB, P_{lacUV5}-iplA, P_{ecRbbH}\textsuperscript{S}_{\text{fabH}}, and P_{lacUV5}-maqu2220. To reduce the burden imposed by multiple plasmids while maintaining a sufficient enzyme expression level, we tried to integrate some operons into the genome. Based on the findings above, we first created strain BC43 with a single copy leuA\textsuperscript{mut}BCD from strain BC33. Using the Type II CRISPR-Cas9 system, we replaced the native promoters of the genomic leuABCD operon with the strong IPTG-inducible P_{lacUV5} promoter, and replaced the RBS of leuA (including all regulatory sites) with the previously-used strong synthetic RBS. Simultaneously, we replaced the leuA-coding gene with the feedback-resistant leuA\textsuperscript{mut}.

However, when the resulting strain BC43 was transformed with corresponding plasmids for fermentation, even-chain-iso products predominated over odd-chain-iso products (as seen for strains BO43I in Fig. 6a). This result indicated that the leuA\textsuperscript{mut}BCD requires very fine-tuning and that a single copy of leuA\textsuperscript{mut}BCD was insufficient to convert all the intracellular 3-methyl-2-oxobutyric acid to 4-methyl-2-oxopentanoic acid, further proving that pA15a::P_{BAD}-leuA\textsuperscript{mut}BCD provides optimal expression level for the α-keto acid synthesis module.
Consistency in expression parameters indicates that the optimal levels derived in this work may be applicable in other systems requiring the use of an α-keto acid pathway.

Then, we chose lplA as the gene to be integrated into the host cell’s genome; low expression was previously determined to be sufficient to fully functionalize the BKD E2 subunit [27]. Furthermore, it has been demonstrated that even low-level overexpression of the global regulator of fatty acid metabolism fadR increases fatty acid production [52, 53]. Thus, fadR was cloned to the same operon of lplA under the control of the P_{lacUV5} promoter. The synthetic P_{lacUV5}-fadR-lplA operon was then integrated into the native ldhA (involved in lactate production) site...
Deletion of IdhA was expected to reduce lactate formation during fermentation [54]. Strain BC63 was then engineered to produce BLFL with controlled chain structure. To produce even-chain-iso BLFLs from glucose, we created strain BO63V by co-transforming the plasmids pB5k-maqu2220 and pE2s-alsS-ivlCD into strain BC63 (Table 2, Fig. 6b). When fermented in the absence of α-keto acid, BO63V produced 52.4 mg/L of even-chain-iso fatty alcohols (Fig. 6a; Additional file 1: Figure S4). To produce odd-chain-iso fatty alcohols from glucose, the plasmid pA8c-leuAmutBCD was further transformed to strain BO63V (Fig. 6b). The resulting strain BO63L produced 122 mg/L of odd-chain-iso BLFLs, comprising 70% of the total BLFLs (Fig. 6a; Additional file 1: Figure S4). Although the odd-chain-iso BLFL pathway is less carbon efficient than the even-chain-iso BLFL pathway due to the loss of a CO2 during 4-methyl-2-oxopentanoic acid biosynthesis, the S_FabH has higher specificity towards 3-methylbutyryl-CoA (the precursor for odd-chain-iso products) than 2-methylpropanoyl-CoA (the precursor for even-chain-iso products) [28], thus leading to higher odd-chain-iso BLFL titer. Finally, we assessed the performance of strain BO63L in a 1 L glucose fed-batch fermenter (“Fermentation” section). After 85 h of cultivation, BLFLs accumulated to 350 mg/L (Fig. 7a). The concentration of 14-methylpentadecanol, the most abundant product, reached 217 mg/L (Fig. 7b). Interestingly, straight LCFLs accumulated rapidly after induction, while BLFL titers did not increase until 32 h (Fig. 7a). We suspect that the initial straight-chain alcohol accumulation may result from the leaky expression of Maqu2220 prior to induction. Further optimization of fermentation conditions (such as pH, air flowing rate, stirring rate, etc.) might improve the percentage and yield of BLFL. Overall, these results demonstrate the potential of strain BO63L for high-titer production of BLFLs and suggest that further studies might lead to its use on an industrial scale.

Discussion
Although alcohol-producing pathways have been previously engineered to produce straight LCFLs and short-chain alcohols, little effort has been made to produce BLFLs. In this work, we characterized the capability and substrate specificity of four different alcohol-producing pathways for BLFL production. We demonstrated the highly selective production of two types of BLFLs (odd-chain-iso and even-chain-iso) by engineering the upstream pathways for precursor synthesis. We obtained high BLFL proportions out of total fatty alcohols (strain BO63L, yielded 80%). We also obtained comparable BLFL titers (strain BO63L, 175 mg/L in the absence of any precursor) to BCFA titers, despite the extensive additional engineering required for BLFLs production. The modular engineering strategy allowed us to apply the knowledge learned from the AAR pathway to the new identified Maqu2220 pathway for rapid optimization, quickly yielded strain BO63L that produced 350 mg/L BLFLs in a fed-batch fermenter. Because BLFLs can be directly used in skin-care or sunscreen products, and are good candidates for diesel fuels, our ability to produce specific BLFL species in high percentages may directly benefit the cosmetics and bioenergy fields.

Meanwhile, we partitioned the complete pathway into three modules: a precursor formation module (the α-keto acid synthesis module), an acyl-CoA activation and malonyl-ACP consumption module (the acyl-ACP generation module), and a final product synthesis module (the alcohol formation module). Each module can be separately engineered and tuned for novel chemical synthesis. Furthermore, when the tuned modules are combined, the expression level of each module can be tuned to avoid imbalance and to improve product titer. We demonstrated consistent expression parameters that result in optimal productivities, which can be used for the production of other chemicals. Together with previous successes in modular engineering of the isoprenoid pathway [55], the FFA pathways [56] and the ester pathways [57], we confirm that modular pathway engineering is an effective approach to improve product titer and yield.

The top-performing strain BO63L produced 350 mg/L BLFLs in fed-batch fermenter, still low compared to that of the best straight LCFL-producing strain (3.82 g/L in shake flask) [48]. Our current pathway is not limited by α-keto acid because increasing the supplemented 4-methyl-2-oxopentanoic acid from 1 to 8 g/L did not increase BLFL titer (Additional file 1: Figure S3). Furthermore, conversion of acyl-ACPs to BLFLs is not likely to be the limiting step due to the high conversion efficiency of Maqu2220 [48]. Thus, we believe the current bottleneck lies in the acyl-ACP generation module, which requires further engineering to improve BLFL titer and yield. Previous engineering strategies developed to optimize free fatty acids production could be potentially used to further improve BLFL yield. These strategies include modulation of the fatty acid and the phospholipid biosynthesis pathway [58], implementing synthetic control systems to dynamically regulate pathway gene expression [52, 59–61], and enriching the high-performing subpopulation using PopQC [60].
Conclusions

We have constructed and tested the capability of four different alcohol-producing pathways for BLFL production in engineered *E. coli*. Moreover, by engineering the α-keto acid biosynthetic pathways and balancing the expression levels of three different modules, we achieved BLFL titers of up to 350 mg/L from glucose, and BLFL percentages up to 79%. Overall, this work generates pathways and knowledge for the production of BLFLs in high percentages that will have broader industrial applications than straight LCFLs.

Additional file

Additional file 1: Figure S1. Fatty alcohol profiles of strains involved in Fig. 3b. Figure S2. Fatty alcohol profiles of strains involved in Fig. 4b. Figure S3. Odd-chain-iso fatty alcohol production in strain BO33D with different concentrations of 4-methyl-2-oxopentanoic acid supplementation. The data has been normalized to the odd-chain-iso fatty alcohol production in strain BO33D with 1 g/L 4-methyl-2-oxopentanoic acid supplementation. Figure S4. Fatty alcohol profiles of strains involved in Fig. 6a.
Abbreviations
BLFL: branched-long-chain fatty alcohol; ACP: acyl carrier protein; AAR: acyl-ACP reductase; CAR: carboxylic acid reductase; ACR: acyl-CoA reductase; FFA: free fatty acid; ADH: alcohol dehydrogenase; LCFL: long-chain fatty alcohol; BCFAs: branched-chain fatty acids; BKD: branched-chain α-keto acid dehydrogenase complex; FabH: β-ketoacyl-acyl-carrier protein; IPTG: β-1-thiogalactopyranoside; αTc: anhydrotetracycline; BSTFA: N,O-bis(trimethylsilyl) trifluoroacetamide.

Authors’ contributions
WJ and FZ designed the work. WJ and JQ performed the plasmids and strain constructions, cell culture, fatty acid extraction and analysis. WJ and DL performed the fermenter experiment. WJ, GB and FZ wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests
WJ, GB and FZ filed a patent entitled “Host cells and methods for producing fatty acid-derivatives with high branched-chain percentage”, which is supplemented by this contribution.

Availability of data and materials
All data generated or analysed during this study are included in this published article and its additional files.

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Not applicable.

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