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Reversal of the β-Oxidation Cycle in *Saccharomyces cerevisiae* for Production of Fuels and Chemicals

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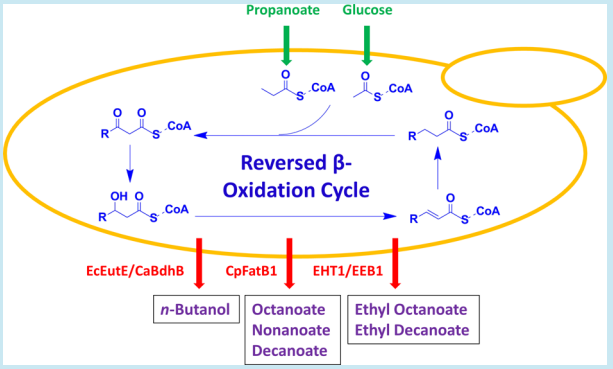
Supporting Information

ABSTRACT: Functionally reversing the β-oxidation cycle represents an efficient and versatile strategy for synthesis of a wide variety of fuels and chemicals. However, due to the compartmentalization of cellular metabolisms, reversing the β-oxidation cycle in eukaryotic systems remains elusive. Here, we report the first successful reversal of the β-oxidation cycle in *Saccharomyces cerevisiae*, an important cell factory for large-scale production of fuels and chemicals. After extensive gene cloning and enzyme activity assays, a reversed β-oxidation pathway was functionally constructed in the yeast cytosol, which led to the synthesis of *n*-butanol, medium-chain fatty acids (MCFAs), and medium-chain fatty acid ethyl esters (MCFAEEs). The resultant recombinant strain provides a new broadly applicable platform for synthesis of fuels and chemicals in *S. cerevisiae*.



KEYWORDS: *β-oxidation, synthetic biology, advanced biofuels, fatty acid, yeast*

Biological conversion of plant-derived lignocellulosic materials into biofuels has been intensively investigated due to increasing concerns on energy security, sustainability, and global climate change.[1−4] Although commercial production of bioethanol has been achieved, ethanol has its intrinsic shortcomings such as low energy density, high corrosivity, and hydroscopicity. Thus, increasing effort has been devoted to produce advanced biofuels, such as *n*-butanol, long-chain alcohols, fatty acid ethyl esters (FAEEs or biodiesels), and alkanes, which have similar properties to current transportation fuels.[5−7] Notably, most of the "drop-in" fuels are derived from fatty acids (fatty acyl-CoAs or fatty acyl-ACPs), which can be produced by the endogenous fatty acid biosynthetic pathways (FABs).[6] However, classic FABs suffer from high energy input and complicated regulation, which significantly hinders efficient and cost-effective production of advanced biofuels. Generally, FABs use malonyl-CoA, which is activated from acetyl-CoA at the cost of ATP consumption as the starter unit, ACP as the acyl activation group, and NADPH as the reducing force.[8−10] Interestingly, the fatty acid degradation pathway, i.e. so-called β-oxidation cycle, which happens in the opposite direction as that of FAB, proceeds in a much more efficient manner, as no ATP consuming reaction is involved and CoA and NADH are used as cofactors.[11,12]

To overcome the major limitation of FABs, a recent report demonstrated that the fatty acid degradation enzymes were functional in both degradative and synthetic directions and the *Escherichia coli* β-oxidation cycle could be functionally reversed to synthesize a series of advanced fuels and chemicals, including short-, medium-, and long- chain alcohols and fatty acids.[13,14] Compared with the current engineering strategy, there are several advantages of this novel platform for advanced biofuel production, such as the high efficiency by avoiding the energy consuming and rate-limiting step to generate malonyl-CoA and the flexibility to produce alcohols and fatty acids with different carbon chain lengths. Although *E. coli* is currently the most popular host for metabolic engineering and synthetic biology studies, *Saccharomyces cerevisiae* has several advantages for industrial applications, such as the high tolerance to lignocellulose hydrolysate inhibitors and toxic products, the capability of high density fermentation, and the absence of phage contamination.[1,2,15−19] Although the reversal of β-oxidation for efficient fuel and chemical synthesis was first demonstrated in *E. coli*,[13,14] it might be extended to this eukaryotic system as well, owing to the ubiquitous nature of the β-oxidation process.[11,12] Therefore, we aimed to develop a new platform, based on the reversed β-oxidation pathway, to synthesize a wide variety of fuels and chemicals efficiently and cost-effectively in *S. cerevisiae*, including but not limited to fatty acids, fatty alcohols, FAEEs, alkanes, and even waxes (Figure 1).

Although the biochemistry is rather similar, the β-oxidation process in eukaryotes is more complicated than that in prokaryotes, mainly due to the compartmentalization of cellular metabolisms. Generally, eukaryotic β-oxidation of fatty acids

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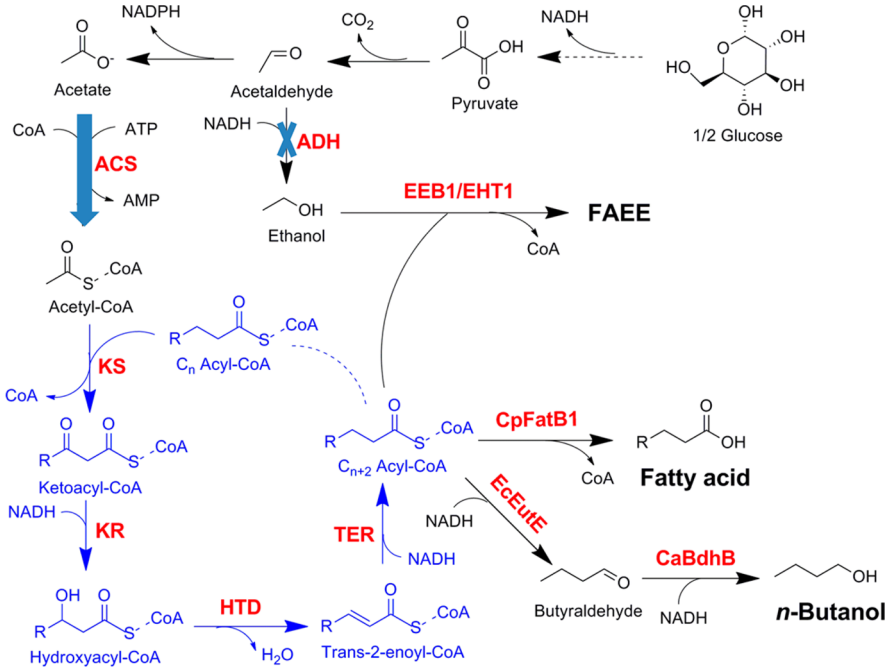

Figure 1. Overview of the synthesis of advanced biofuels by reversing the β-oxidation cycle in *Saccharomyces cerevisiae*. Reversed β-oxidation pathways were constructed via constitutive overexpression of the β-oxidation structural genes in the cytosol of yeast. To increase the cytosolic acetyl-CoA level, competing pathways for acetyl-CoA biosynthesis were inactivated and an acetyl-CoA synthetase mutant (ACS) free of feedback inhibition was overexpressed. KS, β-ketoacyl-CoA synthase; KR, β-ketoacyl-CoA reductase; HTD, β-hydroxyacyl-CoA dehydratase; TER, *trans*-2-enoyl-CoA reductase; CpFatB1, thioesterase from *Cuphea palustris*; EcEutE, CoA-acylating aldehyde dehydrogenase from *Escherichia coli*; CaBdhB, butanol dehydrogenase from *Clostridium acetobutylium*; EEB1/EHT1, acyl-CoA:ethanol *O*-acyltransferases; ADH, alcohol dehydrogenase; FAEE, fatty acid ethyl ester.

happens in peroxisomes and/or mitochondria.[20] In *S. cerevisiae*, peroxisomes are the only cellular compartment responsible for the complete degradation of medium- and long- chain fatty acids.[11,12] Due to the dependence on fatty acids for optimal activities and the lack of precursors and cofactors for biosynthesis, peroxisomes may be not a good location to reverse the β-oxidation cycle. Thus, we chose to construct the reversed β-oxidation pathway in the cytosol. To identify functional pathway enzymes, a number of β-oxidation enzymes were cloned from various species and expressed in the cytosol of *S. cerevisiae*. As proof of concept, *n*-butanol was chosen as a reporter molecule because its synthesis requires the reversal of only one β-oxidation cycle. The ability to reverse the β-oxidation cycle for multiple times was then demonstrated by the synthesis of medium-chain fatty acids (MCFAs) and medium-chain fatty acid ethyl esters (MCFAEEs) in *S. cerevisiae*. To the best of our knowledge, this is the first report on the reversal of the β-oxidation cycle in a eukaryote.

RESULTS AND DISCUSSION

Cloning of Functional β-Oxidation Enzymes Expressed in the Cytosol of *S. cerevisiae*. To construct a reversed β-oxidation cycle in *S. cerevisiae*, a number of genes encoding the β-oxidation pathway from various species were cloned. The corresponding enzymes included: (1) *E. coli* and yeast β-oxidation enzymes;[11,12,21,22] (2) proteins sharing high homology with the *E. coli* reversed β-oxidation pathway enzymes;[13] (3) putative enzymes that are predicted to have the desired functions or similar activities. To facilitate gene cloning and pathway assembly, six helper plasmids (pH1, pH2, pH3, pH4, pH5, pH6) with the whole promoter or terminator as the homology region between two adjacent cassettes were constructed. As shown in Supporting Information Figure S1 and Table 1, besides the enhanced green fluorescent protein (eGFP) cassette (promoter-eGFP-terminator), there was an additional promoter sequence at the 3′ end or terminator sequence at the 5′ end in each helper plasmid, serving as the homologous region for pathway assembly. Each eGFP sequence was flanked by a unique 5′ *Bam*HI site and 3′ *Xho*I site, which allowed the cloning of desired genes by replacing the eGFP sequence. In addition, unique restriction enzyme sites were also introduced to flank both ends of each cassette, which conferred the flexibility to remove the whole cassette by restriction digestion. The whole expression cassettes together with the downstream homologous sequences were PCR amplified and cotransformed into *S. cerevisiae* to assemble the whole pathway via the DNA assembler method (Supporting Information Figure S1).[23]

Due to the availability of the commercial CoA substrates, enzyme activity assays were performed using C_4−CoAs, including acetoacetyl-CoA, β-hydroxybutyryl-CoA, and crotonoyl-CoA (*trans*-2-butenoyl-CoA). Considering the broad specificity of the β-oxidation enzymes, the enzyme assays using C_4−CoAs should be sufficient to represent the β-oxidation activities.[24] Since β-oxidation enzymes were localized either in the mitochondria and/or the peroxisomes in eukaryotes, the targeting sequences were predicted using the online tools (MITOPROT[25] for the mitochondrial targeting sequences and PeroxisomeDB 2.0[26] for the peroxisomal targeting sequences) and removed during cloning process in order to express the proteins in the cytosol of *S. cerevisiae*.

The β-ketoacyl-CoA synthase (KS) activity was carried out in the thiolysis direction using acetoacetyl-CoA as the substrate. Several KS candidate genes were cloned from both *S. cerevisiae*

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Table 1. List of Plasmids Constructed in This Study[a]

plasmids	constructs
p425	pRS425, multicopy plasmid with *LEU2* marker
p426	pRS426, multicopy plasmid with *URA3* marker
pH1	p425-*Stu*I-GPM1p-*Bam*HI-eGFP-*Xho*I-ADH1t-*Avr*II
pH2	p425-*Avr*II-ADH1t-GPDp-*Bam*HI-eGFP-*Xho*I-CYC1t-*Sbf*I-ENO2p
pH3	p425-*Sbf*I-ENO2p-*Bam*HI-eGFP-*Xho*I-PGK1t-*Not*I-TPI1p
pH4	p425-*Not*I-TPI1p-*Bam*HI-eGFP-*Xho*I-TPI1t-*Sac*II-TEF1p
pH5	p425-*Sac*II-TEF1p-*Bam*HI-eGFP-*Xho*I-TEF1t-*Xma*I
pH6	pR25-TEF1t-PGK1p-*Bam*HI-eGFP-*Hind*III-HXT7t
CaThl	pH1-CaThl
ERG10	pH1-ERG10
cytoFox3	pH1-cytoFox3
EcAtoB	pH1-EcAtoB
EcYqeF	pH1-EcYqeF
EcFadA	pH1-EcFadA
cytoFOX2	pH2-cytoFOX2
EcFadB	pH2-EcFadB
cytoTdeFOX2	pH2-cytoTdeFOX2
cytoCgFOX2	pH2-cytoCgFOX2
cytoNdFOX2	pH2-cytoNdFOX2
cytoVpFOX2	pH2-cytoVpFOX2
cytoNcFOX2	pH2-cytoNcFOX2
cytoKlFOX2	pH2-cytoKlFOX2
cytoTpFOX2	pH2-cytoTpFOX2
cytoZrFOX2	pH2-cytoZrFOX2
cytoAgFOX2	pH2-cytoAgFOX2
cytoPpFOX2	pH2-cytoPpFOX2
cytoPfFOX2	pH2-cytoPfFOX2
cytoHpFOX2	pH2-cytoHpFOX2
cytoCpFOX2	pH2-cytoCpFOX2
cytoPgFOX2	pH2-cytoPgFOX2
cytoCdFOX2	pH2-cytoCdFOX2
cytoCtrFOX2	pH2-cytoCtrFOX2
cytoSsFOX2	pH2-cytoSsFOX2
cytoLeFOX2	pH2-cytoLeFOX2
cytoCteFOX2	pH2-cytoCteFOX2
cytoYlFOX2	pH2-cytoYlFOX2
CaHbd	pH2-CaHbd
cytoYlKR	pH2-cytoYALI0C08811g
CbCrt	pH6-CbCrt
cytoYlHTD	pH6-cytoYALI0B10406g
cytoETR1	pH3-cytoETR1
ZTA1	pH3-ZTA1
cytoSPS19	pH3-cytoSPS19
YNL134C	pH3-YNL134C
YLR460C	pH3-YLR460C
YCR102C	pH3-YCR102C
EcYdiO	pH3-EcYdiO
EcFabI	pH3-EcFabI
EgTer	pH3-EgTer
TdTer	pH3-TdTer
EcEutE	pH4-EcEutE
CaBdhB	pH5-CaBdhB
CpFatB1	pH5-CpFatB1
EEB1	pH5-EEB1
EHT1	pH5-EHT1
rP31	p426-ERG10-cytoYlKR-cytoYlHTD-TdTer-EcEutE-CaBdhB
rP32	p426-cytoFOX3-cytoYlKR-cytoYlHTD-TdTer-EcEutE-CaBdhB
rP34	p426-ERG10-cytoYlKR-cytoYlHTD-cytoETR1-EcEutE-CaBdhB
rP35	p426-cytoFOX3-cytoYlKR-cytoYlHTD-cytoETR1-EcEutE-CaBdhB
rP37	p426-ERG10-eGFP-cytoYlHTD-TdTer-EcEutE-CaBdhB
rP38	p426-cytoFOX3-eGFP-cytoYlHTD-TdTer-EcEutE-CaBdhB

[a]Ag, *Ashbya gossypii*; Ca, *Clostridium acetobutylicum*; Cb, *Clostridium beijerinckii*; Cd, *Candida dubliniensis*; Cg, *Candida glabrata*; Cp, *Candida parapsilosis*; Cte, *Candida tenuis*; Ctr, *Candida tropicalis*; Ec, *Escherichia coli*; Eg, *Euglena gracilis*; Hp, *Hansenula polymorpha*; Kl, *Kluyveromyces lactis*; Le, *Lodderomyces elongisporus*; Nc, *Naumovozyma castellii*; Nd, *Naumovozyma dairenensis*; Pf, *Pichia farinose*; Pg, *Pichia guilliermondii*; Pp, *Pichia pastoris*; Ss, *Scheffersomyces stipites*; Td, *Treponema denticola*; Tde, *Torulaspora delbrueckii*; Tp, *Tetrapisispora phaffii*; Vp, *Vanderwaltozyma polyspora*; Yl, *Yarrowia lipolytica*; Zr, *Zygosaccharomyces rouxii*.

and *E. coli* (Table 1). The thiolase from *Clostridium acetobutylicum* (CaThl), which is involved in the fermentative production of *n*-butanol,[27] was used as the positive control. Both acetyl-transferase (ERG10, EcAtoB, and EcYqeF) and acyl-transferase (cytoFOX3 and EcFadA) were included for analysis, which showed short-chain specificity[28] and broad specificity,[22] respectively. As shown in Figure 2A, ERG10 showed the highest activity toward acetoacetyl-CoA among all enzymes tested, which was consistent with a previous report that ERG10 was optimal for *n*-butanol production in yeast.[29] Unfortunately, none of the enzymes cloned from *E. coli* were functional. By removing the peroxisomal targeting sequences, FOX3 was relocalized to the cytosol and functionally expressed. Although the activity of cytoFOX3 was lower than that of ERG10, this β-oxidation enzyme was expected to exhibit broad specificity toward different chain length substrates.[11,12] Therefore, cytoFOX3 was chosen to construct the reversed β-oxidation pathways.

The second and third steps of β-oxidation were carried out by a multifunctional enzyme, FadB in *E. coli*[21] and FOX2 in *S. cerevisiae*.[22] Unfortunately, neither of them could be functionally expressed in the cytosol of *S. cerevisiae* (Figure 2B). Therefore, more FOX2 homologues were identified by BLAST search and cloned (Table 1). Unfortunately, although more than 20 candidate enzymes from different species were tested, none was found to possess either KR or HTD activities, toward acetoacetyl-CoA and crotonoyl-CoA, respectively. The difference of the protein folding environments between cytosol and peroxisomes might prevent all the FOX2 homologues from being functionally expressed in the cytosol of *S. cerevisiae*. Different from *S. cerevisiae*, some oleaginous yeasts were found to have another set of β-oxidation system in the mitochondria in addition to the classic system in the peroxisomes.[30] Therefore, the mitochondrial β-oxidation enzymes were cloned from *Y. lipolytica*. The β-hydroxybutyryl-CoA dehydrogenase from *C. acetobutylium* (CaHbd) and crotonase from *C. beijerinckii* (CbCrt)[27,31] were included as positive controls. There are two separate enzymes in the mitochondrial β-oxidation system to possess KR and HTD activities, encoded by *YALI0C08811* and *YALI0B10406*, respectively.[30] This is different from the peroxisomal system, in which a multifunctional enzyme can catalyze the second and third steps of β-

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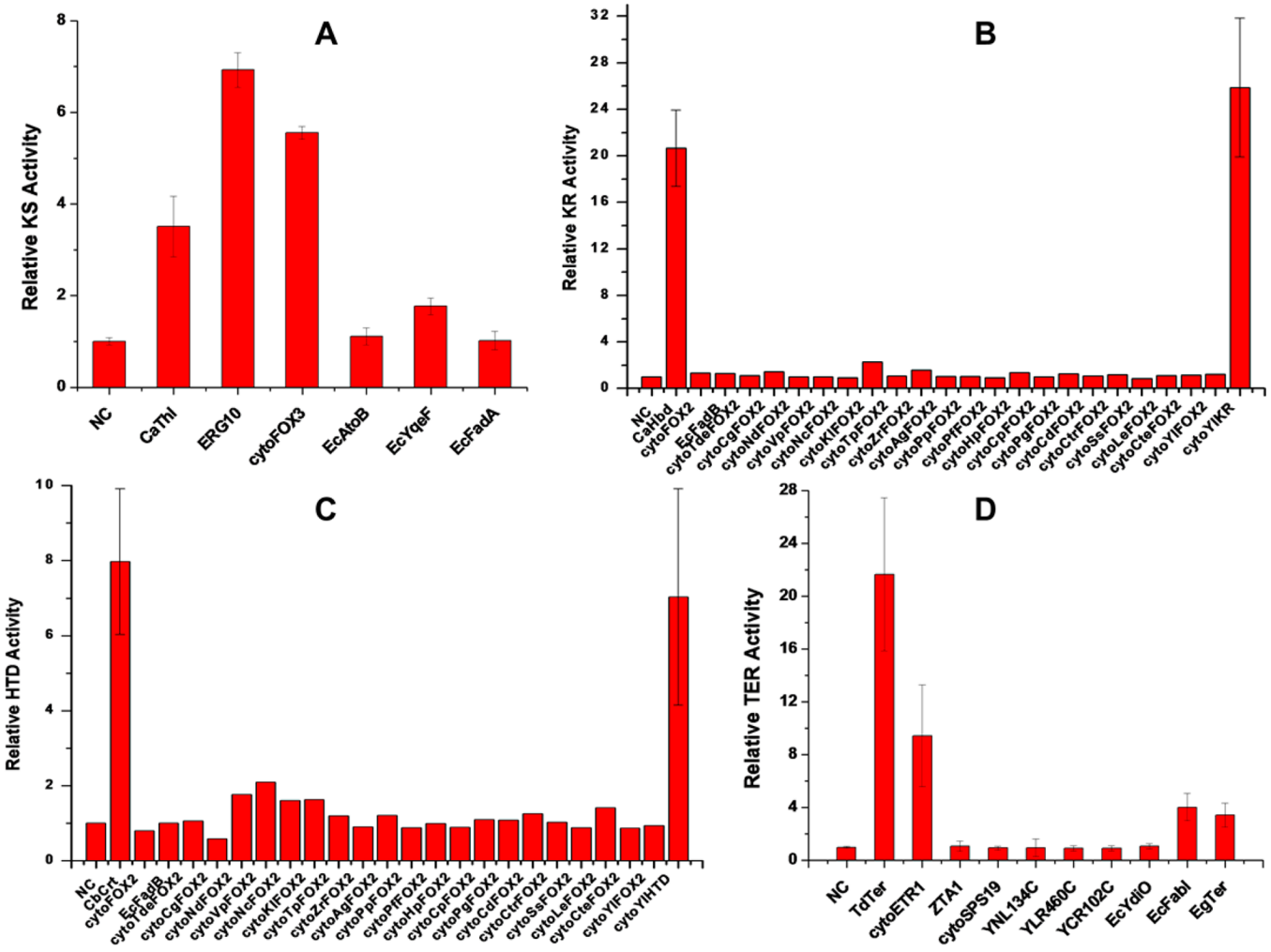

Figure 2. Enzyme activity assays of the reversed β-oxidation pathway enzymes, including KS (A), KR (B), HTD (C), and TER (D). The CoA-dependent *n*-butanol biosynthetic pathway enzymes including CaThl, CaHbd, CbCrt, and TdTer were used as positive controls for KS, KR, HTD, and TER activity assays, respectively.

oxidation. As shown in Figure 2C, both of the mitochondrial β-oxidation enzymes could be functionally expressed in the cytosol of yeast, which showed comparable enzymatic activities with the clostridial equivalents. Thus, these two enzymes were chosen to construct the reversed β-oxidation pathways.

The first step of β-oxidation is the only irreversible step and carried out by fatty acid oxidase with the involvement of an oxygen molecule.[11,12] Instead, *trans*-2-enoyl-CoA reductases (TERs), which are involved in fatty acid biosynthesis (FAB) and only catalyze the reaction in the reduction direction, were included as the last step of the reversed β-oxidation pathways. Several TERs, such as those from *Euglena gracilis* (EgTer) and *Treponema denticola* (TdTer), have been characterized[32−34] and determined to significantly improve the production of *n*-butanol[35,36] and short-chain fatty acids[14] in *E. coli*. Therefore, TERs from *E. coli*, *S. cerevisiae*, *E. gracilis*, and *T. denticola* and some homologues that shared high homology were cloned (Table 1). As shown in Figure 2D, several candidates involved in the biosynthesis of fatty acids, such as the endogenous ETR1 (cytoETR1), FabI from *E. coli* (EcFabI), TER from *E. gracilis* (EgTer), and TER from *T. denticola* (TdTer), were determined to possess the TER activity. Consistent with the previous work,[32,33] TdTer was found to have the highest activity when crotonoyl-CoA was used as the substrate. Therefore, cytoETR1 and TdTer were chosen to construct the reversed β-oxidation pathways.

***n*-Butanol Biosynthesis as Proof-of-concept.** After intensive cloning and screening by enzyme activity assays, several candidates were found to be functionally expressed in the cytosol of yeast with the desired activities, with at least one functional enzyme in each step of the β-oxidation cycle. Therefore, using the helper plasmids constructed and the DNA assembler method,[23,37] a reversed β-oxidation pathway could be readily constructed. As a proof of concept, *n*-butanol biosynthesis that requires only one cycle reversal of β-oxidation was selected. Reversed β-oxidation pathways were constructed using ERG10/cytoFOX3-cytoYlKR-cytoYlHTD-TdTer/cytoETR1, based on the enzyme activity assay results. To synthesize *n*-butanol from butyryl-CoA, the CoA-acylating aldehyde dehydrogenase from *E. coli* (EcEutE)[38,39] and butanol dehydrogenase from *C. acetobutylium* (CaBdhB)[27] were included in the reversed β-oxidation pathways. As shown in Figure 3 and Supporting Information Figure S2, *n*-butanol, although at a very low titer, could be detected using the strains containing the reversed β-oxidation pathways, including rP31, rP32, rP34, and rP35. Currently, rP34 produced the highest amount of *n*-butanol, with a titer as high as 20 mg/L in the wild-type yeast strain. Although the titer of *n*-butanol was still

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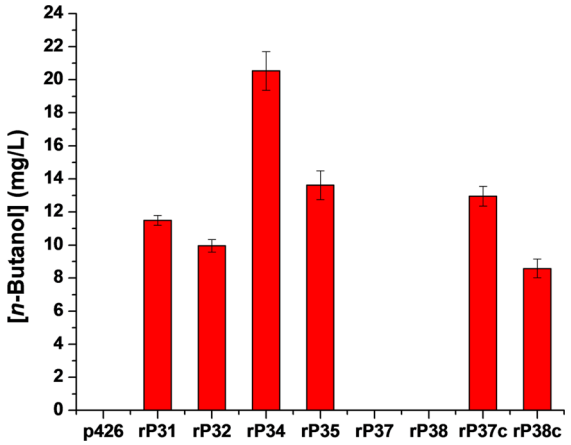

Figure 3. Detection and confirmation of *n*-butanol synthesis by the reversed *β*-oxidation pathways. The production of *n*-butanol could only be detected when a fully functional reversed *β*-oxidation pathway was present (rP31, rP32, rP34, and rP35). The failure of the control strains with either an empty vector (p426) or a nonfunctional pathway (rP37 and rP37) indicated the dependence of *n*-butanol production on the reversed *β*-oxidation pathways in *S. cerevisiae*. The production of *n*-butanol could be complemented by coexpressing cytoYlKR together with the incomplete pathway (rP37c and rP38c).

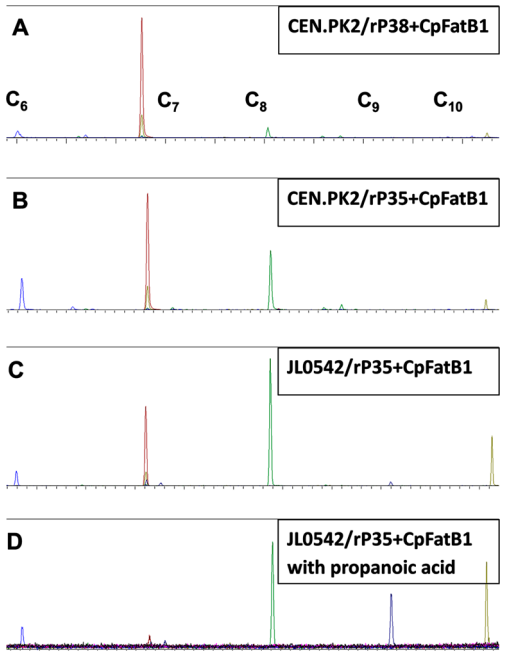

Figure 4. Production of MCFAs by the reversed *β*-oxidation pathway. (A) Extracellular fatty acid profiles of the wild-type strain expressing the thioesterase with an incomplete reversed *β*-oxidation pathway, with 5 mg/L heptanoic acid included as internal standard. (B) Extracellular fatty acid profiles of the wild-type strain expressing the thioesterase with a functional reversed *β*-oxidation pathway, with 5 mg/L heptanoic acid included as an internal standard. (C) Extracellular fatty acid profiles of an acetyl-CoA overproducing strain expressing the thioesterase with a functional reversed *β*-oxidation pathway, with 5 mg/L heptanoic acid included as an internal standard. (D) Production of odd-chain fatty acids with the supplementation of 0.1 g/L propanoic acid. The internal standard (heptanoic acid) was not included to show the full profiles of MCFAs produced by the engineered yeast strain.

rather low compared with that in clostridia[31] and *E. coli*,[13,35,36] it was approximately 10-fold higher than previous reports using the clostridial *n*-butanol fermentative pathway in yeast,[29,40,41] highlighting the high efficiency of the reversed *β*-oxidation pathways.

The assembled pathways containing functional *β*-oxidation enzymes could produce a small amount of *n*-butanol, indicating the successful reversal of the *β*-oxidation cycle. To ensure that the detected *n*-butanol was produced via the reversed *β*-oxidation pathway, pathway deletion and complementation were carried out (Figure 3 and Supporting Information Figure S2). No *n*-butanol production was observed if cytoYlKR was replaced with eGFP (rP37 and rP38). Coexpression of cytoYlKR together with the "incomplete pathways" (rP37c and rP38c) could complement the production of *n*-butanol. In other words, the production of *n*-butanol was dependent on the presence of a functional reversed *β*-oxidation pathway in yeast.

Medium-Chain Fatty Acids (MCFAs) Production. To show the reversal of more than one *β*-oxidation cycle, the production of alcohols with longer carbon chains, such as *n*-hexanol and *n*-octanol, should be achieved. Unfortunately, the CoA acylating aldehyde dehydrogenases or acyl-CoA reductases, which could reduce acyl-CoAs to the corresponding aldehydes, with the desired specificities were not available. On the contrary, thioesterases (TEs), catalyzing the release of free fatty acids from fatty acyl-CoAs, were well characterized and showed broad diversity, ranging from long-chain (C_{14}–C_{18}) and medium-chain (C_8–C_{12}) to short-chain (C_4–C_6) specificity.[42] In the present work, CpFatB1, a plant thioesterase with medium-chain specificity,[43] was included in the reversed *β*-oxidation pathway. Using a modified protocol, all short- and medium- chain fatty acids could be readily separated and detected by GC-MS (Supporting Information Figure S3). In the control strain expressing the thioesterase (CpFatB1) with a nonfunctional reversed *β*-oxidation pathway (rP38), only tiny amount of hexanoic acid (C_6) and octanoic acid (C_8) were detected (Figure 4A and Supporting Information Figure S4). The introduction of a functional reversed *β*-oxidation pathway (rP35) resulted in 6.1-fold improvement of octanoic acid production, with hexanoic acid production increased slightly as well (Figure 4B and Supporting Information Figure S4). Since acetyl-CoA served as both the starter and extender units for the reversed *β*-oxidation pathway, the same plasmids (rP35 and CpFatB1) were introduced into an acetyl-CoA overproducing host (JL0542)[41] constructed in our previous study. The cellular acetyl-CoA level was increased by simultaneous disruption of the competing pathways (GPD1-GPD2 for glycerol biosynthesis and ADH1-ADH4 for ethanol formation) and overexpression of a feedback inhibition insensitive acetyl-CoA synthetase mutant from *Salmonella enterica* (Se*Acs*^{L641P}). In this acetyl-CoA overproducing strain, additional 3.2-fold in octanoic acid production was achieved (Figure 4C and Supporting Information Figure S4). In addition, the enhanced acetyl-CoA supply led to the production of decanoic acid (C_{10}), whose concentration was too low to be detected in the wild-type yeast strain (Figure 4 and Supporting Information Figure S4). On the other hand, these results indicated that acetyl-CoA was the rate-limiting factor for the efficiency of the reversed *β*-oxidation pathway.

The ability of the reversed *β*-oxidation pathway to accept odd-chain acyl-CoAs as substrates was demonstrated by supplementing propanoic acid to the medium. In *S. cerevisiae*, ACSs, encoded by *ACS1* and *ACS2*, contributed to the activation of propanoate to form propionyl-CoA,[44] which

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might be incorporated into the reversed β-oxidation cycle as well. As shown in Figure 4D, besides octanoic acid and decanoic acid, nonanoic acid (C_9) was also detected with propanoic acid supplementation. Interestingly, the production of heptanoic aicd (C_7) was nearly undetectable, probably due to the substrate specificity of the thioesterase used. Our current work demonstrated that the β-oxidation cycle was reversed at least 4 times to synthesize decanoyl-CoA and the reversed pathway could accept both even- and odd- chain substrates for carbon chain initiation and elongation.

Increased Production of Medium-Chain Fatty Acid Ethyl Esters (MCFAEEs). During sugar fermentation, *S. cerevisiae* produces a broad range of aroma-active substances, including ethyl octanoate and ethyl decanoate. The formation of MCFAEEs is catalyzed by acyl-CoA/ethanol *O*-acyltransferases, encoded by *EEB1* and *EHT1*.[45,46] Previous work indicated that the supply of MCFAs precursors is the rate-limiting factor for MCFAEEs biosynthesis.[45,46] Thus, it was assumed that the production of MCFAEEs would be increased if the β-oxidation cycle could be functionally reversed to provide more acyl-CoAs precursors. As expected, increased production of ethyl octanoate and ethyl decanoate was observed in the strain with a functional reversed β-oxidation pathway. The detection and quantification of ethyl octanoate and ethyl decanoate were achieved by GC-MS (Supporting Information Figure S5). As shown in Figure 5, the production

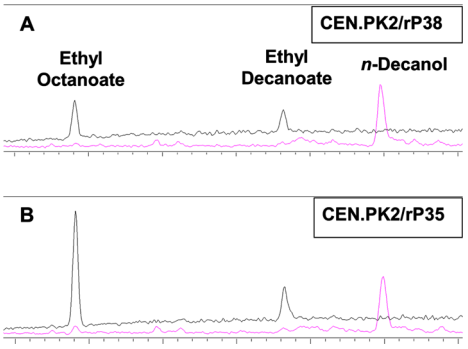

Figure 5. Increased production of MCFAEEs by the reversed β-oxidation pathway. Production of ethyl octanoate and ethyl decanoate was compared between the strains expressing either an incomplete (A) or a functional (B) reversed β-oxidation pathway. *n*-Decanol (1 mg/L) was included as an internal standard.

of ethyl octanoate and ethyl decanoate was increased by 2.8- and 2.1-fold, respectively. Interestingly, although coexpressing *EEB1* or *EHT1* with the reversed β-oxidation pathway did not further increase the production of MCFAEEs (Supporting Information Figure S6), the ratio of ethyl octanoate and ethyl decanoate was changed from 2:1 to around 1:1. These results might be caused by the dual functions of EEB1 and EHT1, with both acyltransferase (FAEE synthesis) and esterase activity (FAEE hydrolysis).[46] In addition, EEB1 and EHT1 showed higher esterase activity toward octanoate ester than decanoate ester,[46] whose overexpression might result in the hydrolysis of ethyl octanoate at a higher rate than that of ethyl decanoate. The production of FAEEs with alkyl chains longer than 10 was not detected under all conditions, probably due to the specificity of the acyltransferases in *S. cerevisiae* (EEB1, EHT1, and other unknown ones). These results also demonstrated that the β-oxidation cycle was reversed by at least four turns.

The reversal of β-oxidation was confirmed by the production of *n*-butanol, MCFAs, and MCFAEEs in *S. cerevisiae*. The yeast β-oxidation enzymes show broad specificity toward short-, medium-, and long- chain substrates,[11,12] which represents a promising versatile platform for synthesis of a wide variety of fuel and chemical molecules with different chain lengths. Meanwhile, we also encountered the challenges to control the chain lengths of the final products. Currently, the chain lengths of the products were mainly controlled by the specificity of the terminal enzymes, such as the thioesterases and acyltransferases. Future work will be focused on protein engineering strategies to design the terminal enzymes with the desired product specificity.[47,48]

The present study demonstrated that the β-oxidation cycle could be reversed by 4 times to synthesize decanoyl-CoA derived molecules, such as decanoic acid and ethyl decanoate. FAEEs with alkyl chain longer than 10 were not detected and no significant difference in the production of dodecanoic acid (C_{12}), tetradecanoic acid (C_{14}), and hexadecanoic acid (C_{16}) was observed between the strains with or without the reversed β-oxidation pathway (data not shown). These findings could result from the substrate specificities of the terminal enzymes used in this study and/or the efficiency of the β-oxidation cycle to be reversed by more than 4 times. The CoA acylating aldehyde dehydrogenase (EcEutE) showed short-chain specificity and only ethanol and *n*-butanol could be synthesized. The thioesterase (CpFatB1) showed medium-chain specificity and C_6−C_{10} fatty acids could be produced. There are no acyltransferases in *S. cerevisiae* that were reported to be active toward the acyl chains longer than 10. In previous work, both system-level engineering approach and synthetic biology approach were applied to construct the functionally reversed β-oxidation pathways in *E. coli*. In the system-level engineering approach, several global transcriptional regulators were manipulated to enable the constitutive expression of all β-oxidation enzymes under anaerobic conditions with the absence of fatty acids and presence of glucose. The resultant strain could fully reverse the β-oxidation cycle to produce C_{16} and C_{18} fatty acids at a titer of higher than 6 g/L.[13] In the synthetic biology approach, all the structural genes of the β-oxidation cycle were overexpressed, without any manipulation on the transcriptional factors. Interestingly, overexpression of the structural genes only enabled the reversal of the β-oxidation cycle up to 5 turns to produce dodecanoic acid (C_{12}), whose titer was lower than 5 mg/L.[14] The decreased number of reversed turns and the dramatically decreased titer for long-chain products indicated some limitations using the synthetic biology approach. Since the synthetic biology approach was used in the present study, we might encounter the same problems as the *E. coli* system. Alternatively, a similar system-level engineering approach can be applied to this eukaryotic system by manipulating the equivalent global regulators.

The high efficiency of the reversed β-oxidation pathway was characterized by its energetic benefits of the malonyl-CoA independence and the use of CoA and NADH instead of ACP and NADPH as cofactors.[13] However, compared with the high efficiency of the reversed β-oxidation pathway in *E. coli*, this system seemed to not work well in yeast, as shown by the relatively low *n*-butanol and fatty acid productivity. One explanation for this observation is the difference in acetyl-CoA biosynthesis. In *E. coli*, acetyl-CoA is steadily synthesized from pyruvate by either pyruvate dehydrogenase (PDH) under aerobic conditions[49] and pyruvate-formate lyase (PFL) under

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anaerobic conditions.[50] While in *S. cerevisiae*, acetyl-CoA is mainly generated in the mitochondria, and the reversed β-oxidation pathway enzymes are localized in the cytosol. *S. cerevisiae* lacks the machinery to export the mitochondrial acetyl-CoA to the cytosol,[51] making the situation even worse. Thus, due to the compartmentalization of acetyl-CoA metabolism, there is not enough driving force for the reversed β-oxidation pathway. In the cytosol of *S. cerevisiae*, acetyl-CoA is generated via the PDH-bypass pathway, from pyruvate to acetaldehyde and then to acetate, which is activated to acetyl-CoA by ACS at the cost of two ATP molecules.[52] The activation of acetate is rate-limiting, due to the low activity of ACS and high energy input. Several metabolic engineering strategies have been carried out to boost the availability of acetyl-CoA in yeast, such as the use of an ACS mutant with higher activity[40,53−55] and the introduction of heterologous acetyl-CoA biosynthetic pathways with less energy input.[56,57] Notably, these strategies have been applied to improve the production of isoprenoids,[53,54] polyhydroxybutyrate,[55,56] *n*-butanol,[40] and fatty acids[57] in *S. cerevisiae*. In the acetyl-CoA overproducing host, the production of octanoic acid was increased by 3.2-fold and the synthesis of decanoic acid was detected, indicating that the cellular acetyl-CoA level was indeed the rate-limiting factor of the reversed β-oxidation pathway. Therefore, the construction of an acetyl-CoA overproducing yeast may significantly enhance the efficiency of the reversed β-oxidation pathway for advanced biofuel production, not only increasing the titer but also diversifying the chain lengths of the final products. Considering the compartmentalization of acetyl-CoA metabolism, an alternative strategy is to relocate the reversed β-oxidation pathway to the same compartment as the precursor metabolite. By fusion with mitochondrial targeting sequences at N-termini, the engineered enzymes can be imported to the mitochondria.[58,59] Since the major β-oxidation enzymes used in the present study originated from the mitochondria of *Y. lipolytica*, they should be functional in the mitochondria of *S. cerevisiae*.

Conclusions. To construct a functional reversed β-oxidation pathway, approximately 40 enzyme homologues were cloned from various species and at least one functional enzyme was obtained for each catalytic step. The production of *n*-butanol, MCFAs, and MCFAEEs indicated that the β-oxidation cycle was functionally reversed. Future work will focus on metabolic engineering strategies to increase the intracellular acetyl-CoA level to push the β-oxidation cycle to be reversed more efficiently.

METHODS

Strains, Media, and Cultivation Conditions. *E. coli* strain DH5α (Cell Media Facility, University of Illinois at Urbana−Champaign) was used to maintain and amplify plasmids. *S. cerevisiae* CEN.PK2−1C (*MATa ura3−52 trp1−289 leu2−3,112 his3Δ1 MAL2−8^C SUC2*, EUROSCARF) was used as the host for gene cloning and *n*-butanol, MCFAs, and MCFAEEs fermentation. Yeast strains were cultivated in complex medium consisting of 2% peptone and 1% yeast extract supplemented with 2% glucose (YPD). Recombinant strains were grown on synthetic complete medium consisting of 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 0.07% amino acid drop out mix without leucine and/or uracil (MP Biomedicals, Solon, OH), supplemented with 2% glucose (SCD-Leu, SCD-Ura, or SCD-Leu-Ura). *E. coli* strains were cultured at 37 °C in Luria−Bertani broth containing 100 μg/mL ampicillin. *S. cerevisiae* strains were cultured at 30 °C and 250 rpm for aerobic growth, and 30 °C and 100 rpm in unbaffled shaker flasks for oxygen limited fermentation. All restriction enzymes, Q5 High Fidelity DNA polymerase, and the *E. coli*−*S. cerevisiae* shuttle vectors, including pRS425 and pRS426, were purchased from New England Biolabs (Ipswich, MA). All chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

DNA Manipulation. The yeast homologous recombination based DNA assembler method[23,37] was used to construct the helper plasmids, clone candidate genes and assemble the reversed β-oxidation pathways (Table 1). Briefly, polymerase chain reaction (PCR) was used to generate DNA fragments with homology arms at both ends, which were purified with a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and cotransformed along with the linearized backbone into *S. cerevisiae*. All genetic elements including promoters, coding sequences, and terminators were PCR-amplified from their corresponding genomic DNAs. Wizard Genomic DNA Purification Kit (Promega, Madison, WI) was used to extract the genomic DNAs from both *E. coli* and various yeast species, according to the manufacturer's protocol. The sequence encoding the medium-chain thioesterase from *Cuphea palustris* (CpFatB1) was synthesized by two gBlock fragments (Supporting Information Table S2) and cloned into pH5 via the DNA assembler method. To confirm the correct clones, yeast plasmids were isolated using a Zymoprep Yeast Plasmid Miniprep II Kit (Zymo Research, Irvine, CA) and retransformed into *E. coli* DH5α competent cells. Plasmids were isolated using a QIAprep Spin Miniprep Kit (Qiagen) and confirmed by DNA sequencing. Yeast strains were transformed using the LiAc/SS carrier DNA/PEG[60] method, and transformants were selected on either SCD-Leu, SCD-Ura, or SCD-Leu-Ura plates.

Enzyme Activity Assays. To measure the activity of enzymes expressed in yeast, a single colony was inoculated into 5 mL SCD-Leu medium and cultured under aerobic conditions for about 36 h. Then, cells were collected by centrifugation at 4000*g* for 5 min at 4 °C, washed twice with prechilled water, and resuspended in 250 μL Yeast Protein Extraction Reagent (YPER). After incubation at 25 °C with 700 rpm shaking in a thermomixer for 20 min, the supernatant containing all soluble proteins were separated by centrifugation at 14 000*g* for 10 min at 4 °C and ready for enzyme activity assays. All the assays were performed in 96-well microplates at 30 °C and the spectrophotometric changes were monitored using the Biotek Synergy 2 Multi-Mode Microplate Reader (Winooski, VT). The reaction was initiated by the addition of 10−20 μL cell extract, to a total volume of 200 μL. Enzyme activity was calculated using the initial reaction rate and normalized to the total protein concentration determined by the Bradford assay.

The KS activity was measured by monitoring the disappearance of acetoacetyl-CoA, corresponding to the thiolysis direction of the enzymatic reaction, which was monitored by the decrease in absorbance at 303 nm. The reaction mixture contained 100 mM Tris-HCl (pH 8.0), 10 mM $MgSO_4$, 200 μM acetoacetyl-CoA, and 200 μM free CoA.

The KR activity was measured by monitoring the decrease of absorption at 340 nm, corresponding to the consumption of NADH in the reducing direction of the reaction. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.3), 200 μM NADH, and 200 μM acetoacetyl-CoA.

The HTD activity was measured by the decrease of absorption at 263 nm, corresponding to the disruption of the carbon−carbon double bond of crotonoyl-CoA. The assay mixture contained 100 mM Tris-HCl (pH 7.6) and 100 μM crotonoyl-CoA.

The TER activity was measured at 340 nm, corresponding to the consumption of NADH to reduce crotonoyl-CoA. The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.2), 200 μM NADH, and 200 μM crotonoyl-CoA.

_n_-Butanol Fermentation and Detection. A single colony with the reversed β-oxidation pathway from the newly transformed plate was inoculated into 3 mL SCD-Ura medium, and cultured under aerobic conditions for 36 h. Then, 200 μL seed culture was transferred into 10 mL fresh SCD-Ura medium in a 50 mL unbaffled shaker flask at an initial OD_{600} of about 0.05, and cultured under oxygen-limited conditions for *n*-butanol fermentation. Samples were taken every 24 h after inoculation until no further increase in *n*-butanol production was observed.

Samples were centrifuged at 14 000 rpm for 10 min and the resulting supernatant was analyzed by a Shimadzu GCMS-QP2010 Plus GC-MS equipped with an AOC-20i+s autosampler (Shimadzu Inc., Columbia, MD) and a DB-Wax column with a 0.25 μm film thickness, 0.25 mm diameter, and 30 m length (Agilent Inc., Palo Alto, CA). Injection port and interface temperature was set at 250 °C, and the ion source was set to 230 °C. The helium carrier gas was set at a constant flow rate of 2 mL/min. The oven temperature program was set as the following: (a) hold at 50 °C for 3 min, (b) increase at the rate of 15 °C min^{-1} to 120 °C, (c) increase at the rate of 50 °C min^{-1} to 230 °C, (d) and then hold at 230 °C for additional 2.5 min. The mass spectrometer was operated with a solvent cut time of 1.5 min, an event time of 0.2 s, a scan speed of 2500 from the range 30−500 mass to charge (m/z) ratio. Concentrations were determined by the standard curve methods, with 50 mg/L methanol as the internal standard.

MCFAs Fermentation and Detection. A single colony with the reversed β-oxidation pathway and CpFatB1 was inoculated into 3 mL SCD-Leu-Ura medium, and cultured under aerobic conditions for 36 h. Then, 200 μL seed culture was transferred into 20 mL fresh SCD-Leu-Ura medium in a 125 mL baffled shaker flask at an initial OD_{600} of about 0.05, and cultured under aerobic conditions for MCFAs fermentation for 48 h.

Fatty acids were analyzed using a previously described protocol with some modifications.[43] A previous report indicated that the short- and medium-chain fatty acid methyl esters were rather volatile, leading to increased variability of the fatty acid analysis.[43] Thus, *N*-(*t*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) was used to derivatize short- and medium-chain fatty acids in the present study. The fatty acid *t*-butyldimethylsilyl esters were less volatile, which would make the derivatization process more reproducible. Heptanoic acid (5 mg/L) was added as an internal standard. Extracellular fatty acids were extracted from 20 mL culture supernatant by adding 2 mL 1 M HCl and 5 mL of a 2:1 chloroform/methanol mixture. The solution was vortexed and then centrifuged for 5 min at 4000 rpm. The lower chloroform layer was recovered and evaporated in the chemical hood overnight. For derivatization, 100 μL MTBSTFA and 100 μL tetrahydrofuran (THF) were added to the dried sample, which was then incubated at 70 °C for 1 h. The THF layer was diluted 10−100 fold into THF and analyzed using the same GC-MS settings mentioned above, except for the oven temperature program: (a) hold at 50 °C for 3 min, (b) increase at the rate of 5 °C min^{-1} to 120 °C, (c) increase at the rate of 50 °C min^{-1} to 230 °C, and then (d) isothermal heating at 230 °C for additional 3 min.

MCFAEEs Fermentation and Detection. A single colony with the reversed β-oxidation pathway from the fresh plate was inoculated into 3 mL SCD-Ura medium and cultured under aerobic conditions for 36 h. Then, 200 μL seed culture was transferred into 5 mL fresh SCD-Ura medium topped with 10% *n*-dodecane overlay in a 20 mL glass tube at an initial OD_{600} of about 0.05, and cultured under aerobic conditions for MCFAEEs fermentation for 48 h.

The top *n*-dodecane layer was collected and analyzed by GC-MS. *n*-Decanol (1 mg/L) was added as an internal standard. The oven temperature program was set as the following: (a) hold at 50 °C for 2 min isothermal heating, (b) increase at the rate of 50 °C min^{-1} to 230 °C, and then (c) hold at 230 °C for additional 15 min.

Prediction of the Mitochondrial and Peroxisomal Targeting Sequences. The mitochondrial targeting sequences of the candidate proteins were predicted using the MITOPROT online tool (http://ihg.gsf.de/ihg/mitoprot.html).[25] The peroxisomal targeting sequences were predicted using the PTSs predictor from PeroxisomeDB 2.0 (http://www.peroxisomedb.org/Target_signal.php).[26]

■ ASSOCIATED CONTENT

Supporting Information

Additional figures and tables as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

J.L. and H.Z. designed and analyzed experiments, J.L. conducted experiments, and J.L. and H.Z. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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