Orthogonal Fatty Acid Biosynthetic Pathway Improves Fatty Acid Ethyl Ester Production in *Saccharomyces cerevisiae*

Dawn T. Eriksen, Mohammad HamediRad, Yongbo Yuan, and Huimin Zhao

**ABSTRACT:** Fatty acid ethyl esters (FAEEs) are a form of biodiesel that can be microbiologically produced via a transesterification reaction of fatty acids with ethanol. The titer of microbially produced FAEEs can be greatly reduced by unbalanced metabolism and an insufficient supply of fatty acids, resulting in a commercially inviable process. Here, we report on a pathway engineering strategy in *Saccharomyces cerevisiae* for enhancing the titer of microbially produced FAEEs by providing the cells with an orthogonal route for fatty acid synthesis. The fatty acids generated from this heterologous pathway would supply the FAEE production, safeguarding endogenous fatty acids for cellular metabolism and growth. We investigated the heterologous expression of a Type-I fatty acid synthase (FAS) from *Brevibacterium ammoniagenes* coupled with WS/DGAT, the wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase that catalyzes the transesterification reaction with ethanol. Strains harboring the orthologous fatty acid synthesis yielded a 6.3-fold increase in FAEE titer compared to strains without the heterologous FAS. Variations in fatty acid chain length and degree of saturation can affect the quality of the biodiesel; therefore, we also investigated the diversity of the fatty acid production profile of FAS enzymes from other *Actinomyces* organisms.

**KEYWORDS:** fatty acid ethyl esters (FAEEs), fatty acid synthase, orthogonal fatty acid synthesis, pathway and metabolic engineering, biodiesel, advanced biofuels

**F**atty acids are one catalytic conversion away from chemicals that can replace petroleum-derived diesel molecules and have therefore become a major focus in advanced biofuel research. Fatty acid ethyl esters (FAEEs), a form of biodiesel, are a specific advanced biofuel of interest. Replacing currently used petroleum-derived diesel with biodiesel could reduce emissions of contaminants such as carbon monoxide, sulfur, aromatic hydrocarbons, and soot particles and provide a more sustainable energy future than petroleum products. Longer chain hydrocarbons, such as fatty acids and FAEEs, are already one of the most widely used renewable fuels in Europe and have the potential to meet commodity/specialty chemical demands in various markets.

Fatty acid biosynthesis is an iterative process that requires the coordination of multiple reactions to elongate the carbon chain (Figure 1a). Two classes of fatty acid synthases (FASs) catalyze these reactions, Type-I and Type-II, which are categorized based on their respective enzyme architectures. The Type-I FAS category consists of large multifunctional enzymes, wherein nearly all of the reactions involved in the fatty acid synthesis are combined into a single, globular enzyme. Typically, this family of enzyme is found in euukaryotes, with an interesting prokaryotic exception of the mycolic-acid-producing subgroup of *Actinomyces*. The Type-II FAS family, generally observed in prokaryotes, is comprised of distinct individualized enzymes that complete each condensation and reduction separately. Each enzyme of the Type-II system requires an independent expression mechanism or operon structures.

The fatty acids produced by the Type-I or Type-II FAS enzymes can be converted to FAEEs through an esterification reaction with ethanol, performed by a bacterial wax ester synthase (WS). Initial microdiesel studies on FAEE production in microorganisms using the WS were performed in *Escherichia coli*. However, *S. cerevisiae* is an intuitive chassis for biodiesel production due to its endogenous ability to synthesize ethanol. Some of the original work on FAEE production in *S. cerevisiae* investigated five different wax ester synthases and identified the WS2 from *Marinobacter hydrocarbonoclasticus* as the most active enzyme. This enzyme underwent additional enzymatic studies.

Ongoing work for improving FAEE production in *S. cerevisiae* has focused primarily on host engineering strategies. Several key enzymes were overexpressed to improve the availability of important components in the pathway, such as malonyl-

**Received:** September 21, 2014
coenzyme A (CoA), cytosolic acyl-CoA, and nicotinamide adenine dinucleotide phosphate (NADPH). \(^8,11,12\) Flux through the pathway was improved by increasing chromosomal integrations of the \(ws2\) gene and overexpression of a phosphoketolase pathway, which redirected the carbon flux toward the fatty acid precursors. \(^11,13\) Regulation of the fatty acid synthesis pathway has also been engineered to improve FAEE production. Post-translational regulation was relieved by engineering a mutant acetyl-CoA carboxylase 1 (ACC1). \(^13\) Transcriptional regulation of the \(FAS1\) and \(FAS2\) genes was overcome by replacing the endogenous promoters with the \(P_{TFF1}\) constitutive promoter. \(^15\)

In these studies, endogenous fatty acids were diverted from growth and normal cellular metabolism in order to generate the FAEEs. Synthetic biology can offer an orthologous approach to endogenous fatty acid production by heterologously overexpressing enzymes in a parallel fatty acid synthesis pathway (Figure 1b). This strategy would provide additional fatty acids to supplement growth and to supply the fatty acids needed for FAEE synthesis. By increasing the fatty acid pool with a heterologous enzyme pathway, the flux directed away from endogenous cellular metabolism would be reduced. In addition, this approach could bypass endogenous regulation. Fatty acid synthesis is energy-intensive, and cells have evolved highly complex regulation mechanisms for control. Through the heterologous expression of a fatty acid synthesis pathway, regulation would not play a major role in limiting the fatty acid flux. \(^16\)-\(^18\)

Host engineering strategies have been successful in improving FAEE production, \(^8,11,15,19\) however, the complex and energy-intensive biosynthesis of fatty acids continues to be a limiting factor. Microbial cells have evolved complex schemes to optimize growth and survival that are not consistent with FAEE production. More diversified tactics are needed to overcome these limitations. This is the first time that a strategy has been investigated to entirely bypass endogenous fatty acid regulation and supply a completely separate fatty acid pool for FAEE production, without depleting fatty acids slated for growth and metabolism.

In this study, we attempted to overexpress \textit{Actinomyces} bacterial Type-I FAS enzymes to provide a heterologous biosynthetic route for fatty acid synthesis. The FAS from \textit{Brevibacterium ammoniagenes}, FAS-B (bafas), and its respective phosphopantetheine transferase (\(ppt1\)) gene \(^20,23\) were coexpressed with a fatty acid acyl-CoA ligase (\(faa1\)) and the wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase (\(ws2\)). This new orthogonal fatty acid synthesis route increased FAEE production by over 6-fold compared to FAEE production that was dependent on endogenous fatty acid production. The major FAEEs produced were chains of 16 and 18 carbons in length (C16 and C18). This study also investigated the fatty acid production profile of four other

---

**Figure 1. (a) Basic pathway for fatty acid synthesis.** Different organisms may have varied enzyme architecture, but the basic catalysis is conserved. The first step is the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC). This forms malonyl-CoA, which is subsequently converted by a malonyl CoA:ACP transacylase (MCAT) to malonyl-ACP. The growing acyl chain is then covalently attached to the acyl-carrier protein (ACP) through a thioester linkage on the terminal sulfhydryl of the phosphopantetheine prosthetic group. This terminal thiol forms the tether to which the growing carbon chain is elongated. The first elongation step to enter into the iterative cycle involves a ketoacyl synthase (KS). The intermediate is then reduced by ketoacyl ACP reductases (KR) to form a \(\beta\)-hydroxy intermediate. A dehydratase (DH) then dehydrates the \(\beta\)-hydroxy intermediate to a trans-2-enoyl-ACP before further reduction by an enoyl ACP reductase (ER). Another ketoacyl synthase (KS) will continue the elongation. The final step and release mechanism can vary between organisms, dependent on either thioesters (TE) or acyltransferases (AT) to discharge the carbon chain from the iterative cycle to yield the free fatty acid. (b) Description of the heterologous FAEE pathway. The orthologous fatty acid synthesis pathway constructed in this study is represented by red arrows; the heterologous expression of the Type-I FAS is a key component. This heterologous expression provides a parallel route to produce fatty acids, resulting in reduced bottlenecks of the fatty acid synthesis, bypassing pathway constructed in this study is represented by red arrows; the heterologous expression of the Type-I FAS is a key component. This heterologous expression provides a parallel route to produce fatty acids, resulting in reduced bottlenecks of the fatty acid synthesis, bypassing
FAS enzymes to screen for FASs yielding a more varied carbon chain length and degree of saturation.

**RESULTS AND DISCUSSION**

**Design of an Orthogonal Fatty Acid Biosynthesis Route.** Optimizing a metabolic pathway involves the arduous process of balancing gene expression for each of the enzymes in the pathway.\(^{22,23}\) Therefore, to reduce the need for pathway optimization, our strategy relied on a Type-I FAS. Based on the single expression system controlling every domain of the chain elongation mechanism, pathway optimization is not needed for the chain elongation steps. Expression levels between the elongation enzymes do not need to be balanced and mass transfer of the reactants between the enzymes is not a concern due to the enzyme-bound intermediates being cycled through the catalytic domains. This natural protein scaffold allows for higher local intermediate concentration, which increases the reaction rate, allowing for an assumption that fatty acid synthesis is only limited by the concentration of the substrates.\(^{24}\) Balancing protein expression and mass transfer are two problems that may occur in the heterologous expression of Type-II systems and have been shown to be significant factors in optimizing biosynthetic pathways.\(^{22,23,25}\)

The Type-I FAS *bafas* gene and the *ppt1* gene were used to construct the orthologous fatty acid synthesis pathway. The Type-I FAS chosen for this study, FAS-B, has a high degree of similarity to the two yeast FAS genes, *fas1* and *fas2* with 30% and 46% identity, respectively.\(^{26}\) This multidomain protein was shown to be heterologously expressed in *E. coli*, and the main product produced by this enzyme was palmitic acid.\(^{21}\) Heterologous expression of an engineered version of this enzyme was also investigated in *S. cerevisiae*.\(^{27}\) The PPT1 is responsible for activating the acyl carrier protein of the FAS. Additionally, the free thiol moiety of the PPT1 covalently binds to the elongating fatty acid chain, transferring the chain through the distinct reaction domains of the FAS.\(^{28}\) To investigate whether the heterologous enzymes baFAS and PPT1 could function similarly to the endogenous fatty acid synthase in *S. cerevisiae*, the genes were expressed in a BY4741 strain that was deficient in endogenous fatty acid production. In this Δfas strain, the full *S. cerevisiae fas1* gene was replaced with a KanMX cassette by the WS/DGAT in a transesterification reaction with ethanol to produce FAEEs (Figure 1b).

**Construction of a Heterologous FAEE Pathway.** The heterologous *bafas* and *ppt1* were then coupled with the *faa1* gene from *Saccharomyces cerevisiae* and the *ws2* gene from *Marinobacter hydrocarbonoclasticus*.\(^{8}\) Overexpression of the *faa1* gene is important for accommodating the increased flux of fatty acids and removing dependence on the endogenous FAA1 enzymes. The activated fatty acid acyl-CoAs are used as a cosubstrate by the WS/DGAT in a transesterification reaction with ethanol to produce FAEEs (Figure 1b).

Table 1. *S. cerevisiae* Strains Used in This Study

<table>
<thead>
<tr>
<th>genetic background</th>
<th>plasmid</th>
<th>strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>pRS426-empty plasmid</td>
<td>DE09</td>
</tr>
<tr>
<td>BY4741</td>
<td>pRS426-TPI baFAS&amp;PPT1</td>
<td>DE10</td>
</tr>
<tr>
<td>BY4741 Δfas1</td>
<td>pRS426-TPI baFAS&amp;PPT1</td>
<td>DE11</td>
</tr>
<tr>
<td>BY4741</td>
<td>pRS426-TPI baFAS&amp;PPT1</td>
<td>DE12</td>
</tr>
<tr>
<td>BY4741</td>
<td>pRS426-WS2</td>
<td>DE13</td>
</tr>
<tr>
<td>BY4741</td>
<td>pRS426-FBDP</td>
<td>DE14</td>
</tr>
<tr>
<td>BY4741 Δfas1</td>
<td>pRS426-FBDP</td>
<td>DE15</td>
</tr>
<tr>
<td>BY4741</td>
<td>pRS426-baFAS</td>
<td>DE16</td>
</tr>
<tr>
<td>BY4741</td>
<td>pRS426-gtFAS</td>
<td>DE17</td>
</tr>
<tr>
<td>BY4741</td>
<td>pRS426-msFAS</td>
<td>DE18</td>
</tr>
<tr>
<td>BY4741</td>
<td>pRS426-refFAS</td>
<td>DE19</td>
</tr>
<tr>
<td>BY4741 Δfas1</td>
<td>pRS426-baFAS</td>
<td>DE20</td>
</tr>
<tr>
<td>BY4741 Δfas1</td>
<td>pRS426-refFAS</td>
<td>DE21</td>
</tr>
<tr>
<td>BY4741 Δfas1</td>
<td>pRS426-msFAS</td>
<td>DE22</td>
</tr>
<tr>
<td>BY4741 Δfas1</td>
<td>pRS426-gtFAS</td>
<td>DE23</td>
</tr>
</tbody>
</table>

**Figure 2.** Comparison of free fatty acid (FFA) production of the heterologous and endogenous fatty acid synthesis system. Endogenous fatty acid synthesis was measured in strain DE10, while the heterologous FAS system was expressed in strain DE11, which lacks endogenous fatty acid synthesis capabilities. Palmitic acid production increased 2.75-fold in the heterologous FAS system. A one-tailed, two-sample unequal-variance t-test was applied to determine whether FFA production was statistically greater in the heterologous system. **p** indicates *p* < 0.01 for biological triplicates.

**Table 1.** *S. cerevisiae* Strains Used in This Study

**Figure 2.** Comparison of free fatty acid (FFA) production of the heterologous and endogenous fatty acid synthesis system. Endogenous fatty acid synthesis was measured in strain DE10, while the heterologous FAS system was expressed in strain DE11, which lacks endogenous fatty acid synthesis capabilities. Palmitic acid production increased 2.75-fold in the heterologous FAS system. A one-tailed, two-sample unequal-variance t-test was applied to determine whether FFA production was statistically greater in the heterologous system. **p** indicates *p* < 0.01 for biological triplicates.

**Construction of a Heterologous FAEE Pathway.** The heterologous *bafas* and *ppt1* were then coupled with the *faa1* gene from *Saccharomyces cerevisiae* and the *ws2* gene from *Marinobacter hydrocarbonoclasticus*.\(^{8}\) Overexpression of the *faa1* gene is important for accommodating the increased flux of fatty acids and removing dependence on the endogenous FAA1 enzymes. The activated fatty acid acyl-CoAs are used as a cosubstrate by the WS/DGAT in a transesterification reaction with ethanol to produce FAEEs (Figure 1b).

**Table 1.** *S. cerevisiae* Strains Used in This Study

**Figure 2.** Comparison of free fatty acid (FFA) production of the heterologous and endogenous fatty acid synthesis system. Endogenous fatty acid synthesis was measured in strain DE10, while the heterologous FAS system was expressed in strain DE11, which lacks endogenous fatty acid synthesis capabilities. Palmitic acid production increased 2.75-fold in the heterologous FAS system. A one-tailed, two-sample unequal-variance t-test was applied to determine whether FFA production was statistically greater in the heterologous system. **p** indicates *p* < 0.01 for biological triplicates.
increased by 7.3-fold \((p < 0.01)\). Oleic acid ethyl ester production was increased by 12-fold compared to DE13 \((p < 0.01)\). Total FAEE production was improved by 6.3-fold in DE14 compared to DE13 \((p < 0.01)\). Strain DE13 produced 1670 \(\mu g\) FAEE/g CDW compared to the 10,498 \(\mu g\) FAEE/g CDW produced by strain DE14. The margin of error was high, a factor which has also been observed in other fatty acid ethyl ester quantification studies using plasmids.\(^{11}\) It is hypothesized that the variation is due to the 2-micron plasmids, which introduce different copy numbers of plasmids into each clone.

**Cloning and Characterization of Four Type-I FAS Genes.** Structural features of the fatty acids used in FAEE production can greatly influence the biodiesel’s physical properties, such as ignition quality, heat of combustion, cold flow, oxidative stability, exhaust emissions, viscosity, and lubricity.\(^{29,30}\) Specifically, cold flow and viscosity are affected by the degrees of saturation. In addition, cetane number (CN), a measure of how quickly the fuel begins to autoignite, is affected by carbon chain length.\(^{30,31}\) The most common fatty acid composition of biodiesel currently used in engines includes mainly C14, C16, C18, C16:1, and C18:2.\(^{32}\) Defining and controlling the fatty acid profile of FAEE production can greatly improve biodiesel properties. Each specific Type-I FAS produces fatty acids of different chain lengths, which is an inherent characteristic of the enzyme.\(^{7}\) Recent research suggests the KS region could be responsible for C16/C18 ratio.\(^{33}\) Therefore, identifying other FASs with varied chain length and degrees of saturation would be beneficial for the ability to engineer and domain-swap the FASs.

Four different FAS genes from *Actinomyces* were investigated for heterologous fatty acid synthesis. Each FAS gene was coexpressed with its respective *ppt1*. The activity of each protein was confirmed through complementation of fatty acid synthesis in the *S. cerevisiae* Δfas strain (Supporting Information Figure S1). The heterologous production of free fatty acids from these enzymes was quantified and compared (Figure 4). Each protein produced similar titers of C16–C18 free fatty acids. No short chain fatty acids (C6–C8) were observed, and no carbon chains longer than C18 were observed (data not shown). The fatty acids produced were within the typical and common chain length used in current biodiesel
Table 2. Plasmids Used in This Study

<table>
<thead>
<tr>
<th>name</th>
<th>genes</th>
<th>promoters/terminator</th>
<th>marker</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS426</td>
<td>Marinobacter hydrocarbonoclasticus ws2</td>
<td>PGK1/PGKt</td>
<td>URA</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pRS426-WS2</td>
<td>Brevibacterium ammoniagenes fas and ppt1</td>
<td>TPI/TPI, TEF1/TEF</td>
<td>URA</td>
<td>this study</td>
</tr>
<tr>
<td>pRS426-baFAS</td>
<td>Brevibacterium ammoniagenes fas and ppt1</td>
<td>HXT5/TPI, TEF1/TEF</td>
<td>URA</td>
<td>this study</td>
</tr>
<tr>
<td>pRS426-mFAS</td>
<td>Mycobacterium smegmatis fas and ppt1</td>
<td>HXT5/TPI, TEF1/TEF</td>
<td>URA</td>
<td>this study</td>
</tr>
<tr>
<td>pRS426-refAS</td>
<td>Rhodococcus erythropolis fas and ppt1</td>
<td>HXT5/TPI, TEF1/TEF</td>
<td>URA</td>
<td>this study</td>
</tr>
<tr>
<td>pRS426-gtFAS</td>
<td>Gordonia terrae fas and ppt1</td>
<td>HXT5/TPI, TEF1/TEF</td>
<td>URA</td>
<td>this study</td>
</tr>
<tr>
<td>pRS425-scFAA1</td>
<td>Saccharomyces cerevisiae faa1</td>
<td>PYK1/ADH1</td>
<td>LEU</td>
<td>this study</td>
</tr>
<tr>
<td>pRS416-Helper</td>
<td>ppt1, faa1, ws2</td>
<td>TEF1/TEF, PYK1/ADH1, PGK1/PGK1</td>
<td>URA</td>
<td>this study</td>
</tr>
<tr>
<td>pRS426-FBDF</td>
<td>bafas, ppt1, faa1, ws2</td>
<td>HXT5/TPI, TEF1/TEF, PYK1/ADH1, PGK1/PGK1</td>
<td>URA</td>
<td>this study</td>
</tr>
</tbody>
</table>

Blends. The degree of saturation of each enzyme varied significantly. Strain DE18, harboring the plasmid that expresses the FAS gene from Mycobacterium smegmatis, yielded fatty acids with no major difference in saturation of C16 and C18 fatty acids. Strain DE16, harboring the plasmid that expresses the FAS gene from B. ammoniagenes, produced markedly more saturated fatty acids than unsaturated ones. Of particular note, strain DE17, harboring the plasmid that expresses the FAS gene from Gordonia terrae, produced twice as much C18:0 as strain DE19, harboring the plasmid that expresses the FAS gene from Rhodococcus erythropolis. C18:1 production was more prominent in DE19 than in DE17.

Conclusion. This work represents the first time that a heterologous FAS pathway has been constructed to overproduce fatty acids for the production of biofuels in S. cerevisiae. This strategy yielded a 6.3-fold improvement in total FAEEs produced. This improvement is based on increased availability of heterologously produced fatty acids providing an orthogonal route for FAEE production, which essentially decoupled FAEE production from endogenous metabolism. However, even though fatty acid synthesis is heterologous and orthogonal, the fact that the precursor of fatty acid synthesis, acetyl-CoA, is produced by the endogenous system should not be ignored. The use of host engineering strategies to improve acetyl-CoA production could further increase the productivity of the pathway. Additionally, combining this synthetic biology approach with the host engineering strategies previously mentioned and elimination of competing fatty acid utilization pathways could synergistically increase the FAEE titer.

The strategies and solutions used in identifying future renewable energy sources will be complex and diverse. In order to construct a broad arsenal of possible routes to success, various strategies must be explored and combined for synergy. We have proposed a new pathway for FAEE production in S. cerevisiae. By means of an orthogonal fatty acid synthesis pathway, additional fatty acids are available to supplement both cellular growth and FAEE synthesis without compromising the fatty acids needed for growth and metabolism. Additionally, the heterologous system was able to overcome endogenous regulation mechanisms of fatty acid synthesis. This novel pathway enabled an increase of FAEE production in S. cerevisiae.

Materials and Methods

Strains and Plasmids. The S. cerevisiae strains and plasmids used in this work are listed in Tables 1 and 2. All of the plasmid constructions were accomplished using DNA assembler. The ws2 gene was codon optimized for S. cerevisiae expression, synthesized by DNA2.0 (Menlo Park, CA), and assembled under the control of the PGK1 promoter/terminator in a pRS426 plasmid (pRS426-WS2). The bafas and ppt1 genes from B. ammoniagenes were cloned from plasmid pGM44 under the control of the HXT5/TPI1 or TPI1/TPI1 promoter/terminator or of the TPI1/TPI1 and TEF1/TEF1 promoter/terminator respectively (Table 2). The HXT5 promoter provided stronger expression of the bafas gene and was needed when expressing the bafas in a strain other than the Δfas strain. 

Escherichia coli 10-β (New England Biolabs, Ipswich, MA) was used for recombinant DNA manipulations. All of the restriction digests were purchased from New England Biolabs.

To construct the full heterologous FAEE pathway, the faa1 gene and ws2 gene were coexpressed on the same plasmid as the bafas and ppt1 genes. Due to the size and GC composition of the fas gene, we tried to avoid excessive PCR amplification of the gene. To increase the homology region for assembly, a helper plasmid was assembled first that included the expression cassettes for the ppt1, ws2, and faa1, generating a 2 kb homology region. The faa1 gene from S. cerevisiae was expressed under the control of the PYK1/ADH1 promoter and terminator. The pRS426-baFAS plasmid was linearized and used as a backbone to insert the helper plasmid expression cassettes in 5 kilobase (kb) fragments with a 2 kb homologous region overlap. Construction of all plasmids was confirmed via restriction digestion and partial DNA sequencing.

Three additional FAS genes were identified through an NCBI Blast search with a protein sequence identity of greater than 50% to the bafas gene. The following organisms were ordered from NRRL: Mycobacterium smegmatis (B-24018), Rhodococcus erythropolis (B-16025), and Gordonia terrae (B-16283). The genomic DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI), and the fas and ppt1 genes were amplified using primers listed in Supporting Information Table S.1. Each gene was amplified in three equal fragments from the genomic DNA and then assembled using DNA assembler. The genes were cloned under the control of the HXT5/TPI1 promoter/terminator and coexpressed with the ppt1 gene in the same plasmid under the TEF1/TEF1 promoter/terminator (Table 2).

All transformations followed the standard lithium acetate transformation protocol for S. cerevisiae as described previously. Transforming DNA into the Δfas strain required a 12-h incubation period after inoculation, due to slow growth rate, and supplementation of the growth medium with exogenous 0.02% palmitic acid with G418 for selection of the fas knockout. The transformants were recovered in YPD media with 0.01% palmitic acid for 5 h. The cells were then transferred to agar plates or liquid media containing 200 μg/mL G418.
(KSE Scientific, Durham, NC) with or without palmitic acid supplementation as needed.

**Media and Culture Conditions.** Seed cultures of strains for FAEE detection were grown in 2% glucose CSM-URA or CSM-LEU (MP Biomedical, Santa Ana, California) to stationary phase at 30 °C with orbital shaking at 250 rpm and then transferred to 125 mL baffled flasks with 25 mL CSM-URA or CSM-LEU at an initial OD₆₀₀ of 0.05 at 30 °C with orbital shaking at 100 rpm. As needed, the flasks received CSM-URA or CSM-LEU treated with 200 μg/mL G418 (KSE Scientific, Durham, NC) for selection of positive clones. Independent colonies were selected to analyze the FAEE and FFA produced. *E. coli* strains were cultured in Luria broth (LB) (Fischer Scientific, Pittsburgh, PA) at 37 °C and 250 rpm, treated with 50 μg/mL ampicillin. Concentrated glycerol (80%) was added to yeast and bacterial cultures, which were immediately flash-frozen and stored as glycerol stocks (15% glycerol) at −80 °C. All of the chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

**Free Fatty Acid Extraction.** Quenching was conducted on the cells to quantify the free fatty acid pool. Cells were flash-frozen in a methanol/dry ice bath. The mixture was centrifuged at 4000 rpm and −20 °C for 5 min before the cell pellet was separated from the media. Cell pellets were then lysed in boiling ethanol in a 100 °C water bath for 10 min. After centrifugation, the supernatant was applied to a freeze-dryer overnight or until dry. The lipids were then extracted twice with 2:1 chloroform/methanol using 50 mM Tris-SO₄, pH 7.5) and 15 units of zymolyase (Zymo Research, Irvine, CA). After incubation at 37 °C and 250 rpm for 30 min, cells were extracted twice with 2:1 chloroform/methanol mixture at 4 °C for 30 min, cells were extracted twice with 2:1 chloroform/methanol using 50 μM myristic acid as an internal standard. After extraction, the fatty acids were allowed to derivatize for 2 h at ambient temperature with 200 μL of 2N TMS-diazomethane, 10 μL methanol and 10 μL HCL. The sample was then transferred to a glass GC vial for analysis.

**FAEE Extraction and Thin-Layer Chromatography.** FAEE detection was conducted as previously reported. Cells were harvested and washed with sterile water before being applied to a freeze-dryer overnight. Each sample dried-cell weight was normalized to 25 mg and then rehydrated with digestion buffer (1.2 M glycerol, 100 mM sodium thioglycolate, 50 mM Tris-SO₄, pH 7.5) and 15 units of zymolyase (Zymo Research, Irvine, CA). After incubation at 37 °C and 250 rpm for 30 min, cells were extracted twice with 2:1 chloroform/methanol mixture at 4 °C with continuous vortexing. Lauric acid ethyl ester (25 μg) was used as an internal standard. The lipid mixture was applied to a TLC silica gel 60 F₂₅₄ plate (EMB Millipore, Darmstadt, Germany) with a mobile phase of heptane, 2-propanol, and acetic acid (95:5:1 v/v/v). Lipids were visualized with a 0.05% 2,7-dichlorofluoresceine mixture in ethanol. The FAEEs were isolated by scraping the FAEEs off the TLC plates using a scalpel and then extracted from the TLC powder with a mixture of hexane, methanol, and water (3:2:2 v/v/v). The upper phase was separated and transferred to glass GC-vial inserts for analysis.

**GC-MS Analysis.** The fatty acids were separated and quantified through a GC-MS-QP 2010 Plus (Shimadzu, Kyoto, Japan). The separation was performed by a DB-WAX GC column with the following dimensions: 30 mm × 0.25 mm internal diameter, 0.25-μm film thickness, from Agilent Technologies (Santa Clara, CA). A 1-μL portion was injected via splitless injection at 250 °C at an initial pressure of 98 kPa and a total flow of 14.1 mL/min helium carrier gas. The chromatograph separation initially occurred at a temperature of 40 °C for 1.2 min and then increased at a rate of 30 °C/min to 220 °C and then held for 15 min. The mass transfer line and ion source were at 250 and 200 °C, respectively. The fatty acids were detected with an electron ionization method in scan mode from 50 to 650 m/z.

The FAEEs were quantified using the same GC, column, and injection procedure. The chromatography separation initially occurred at a temperature of 50 °C for 1.5 min and then increased at a rate of 25 °C/min to 180 °C, finally increasing the temperature by 10 °C/min to 250 °C, which held for 3 min. The mass transfer line and ion source were at 250 and 200 °C, respectively. The FAEEs were detected with an electron ionization method in scan mode from 50 to 650 m/z. The identification of both the fatty acids and the FAEEs was achieved by comparing the retention times and mass spectra. All of the data were statistically analyzed using a standard one-tailed, two-sample unequal-variance t-test. Confidence intervals of 95% are designated with a single asterisk, while confidence intervals of more than 99% are designated by a double asterisk.

**ASSOCIATED CONTENT**

Supporting Information

Supplemental experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

Corresponding Author

E-mail: zhao5@illinois.edu. Phone: 217-333-2631. Fax: 217-333-5052.

Present Address

GreenLight Biosciences, Medford, Massachusetts 02155, United States

Author Contributions

D.T. Eriksen and H. Zhao designed the study, analyzed the data, and drafted the manuscript. D.T. Eriksen, M. HamediRad, and Y. Yuan performed the experiments and analyzed the data. All of the authors have read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by the Energy Biosciences Institute at the University of Illinois at Urbana–Champaign. The authors acknowledge Dr. Xueyong Feng, Jonathan Ning, and Jiazheng Lian for troubleshooting help and discussions. Dr. Shubo Shi is acknowledged for his help in establishing protocols. The Metabolomics Center and Graphics Center at the University of Illinois at Urbana–Champaign are acknowledged for initial analytical help and figure design. We express our appreciation to the ARS (NRRL) Collection for providing strains for FAS cloning.

**ABBREVIATIONS**

FAEE, fatty acid ethyl esters; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; ACP, acyl-carrier protein; WS or WS/FAEE, free fatty acid; KanMX, kanamycin-resistance expression cassette
REFERENCES


(20) Stuble, H. P., Meier, S., and Schweizer, E. (1997) Identification, isolation, and biochemical characterization of a phosphopantetheine-


