ARTICLE

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Engineering of *Saccharomyces cerevisiae* for the Synthesis of Short Chain Fatty Acids

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ABSTRACT: Carbon feedstocks from fossilized sources are being rapidly depleted due to rising demand for industrial and commercial applications. Many petroleum-derived chemicals can be directly or functionally substituted with chemicals derived from renewable feedstocks. Several short chain organic acids may fulfill this role using their functional groups as a target for chemical catalysis. *Saccharomyces cerevisiae* was engineered to produce short chain carboxylic acids (C_6 to C_{10}) from glucose using the heterologous *Homo sapiens* type I fatty acid synthase (hFAS). This synthase was activated by phosphopantetheine transfereases AcpS and Sfp from *Escherichia coli* and *Bacillus subtilis*, respectively, both in vitro and in vivo. hFAS was produced in the holo-form and produced carboxylic acids in vitro, confirmed by NADPH and ADIFAB assays. Overexpression of hFAS in a yeast *FAS2* knockout strain, deficient in de novo fatty acid synthesis, demonstrated the full functional replacement of the native fungal FAS by hFAS. Two active heterologous short chain thioesterases (TEs) from *Cuphea palustris* (CpFatB1) and *Rattus norvegicus* (TEII) were evaluated for short chain fatty acid (SCFA) synthesis in vitro and in vivo. Three hFAS mutants were constructed: a mutant deficient in the native TE domain, a mutant with a linked CpFatB1 TE and a mutant with a linked TEII TE. Using the native yeast fatty acid synthase for growth, the overexpression of the hFAS mutants and the short-chain TEs (linked or plasmid-based) increased in vivo caprylic acid and total SCFA production up to 64-fold (63 mg/L) and 52-fold (68 mg/L), respectively, over the native yeast levels. Combined over-expression of the phosphopantetheine transferase with the hFAS mutant resulted in C_8 titers of up to 82 mg/L and total SCFA titers of up to 111 mg/L.

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KEYWORDS: *Saccharomyces cerevisiae*; short chain fatty acids; *Homo sapiens* fatty acid synthase; thioesterase; biorenewable chemicals

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Introduction

The production of chemicals in the United States is a $700 billion industry (annually), and continues to grow at an increasing rate each year (American Chemistry Council (ACC), 2012). There are over 70,000 different chemical products that are used in industries ranging from personal care to agriculture, manufacturing, and construction (ACC, 2012). Unfortunately, this vital industry is not self-sustaining. Carbon feedstocks from fossilized sources are being rapidly depleted due to rising demand for industrial and commercial applications (Carlson, 2011). With escalation and instability in supply costs, growing environmental concerns, competing policy agendas, and increasing foreign dependency, the need to shift to a renewable-based industry is increasingly important. For the production of industrial chemicals, the successful transition from a non-renewable petroleum feedstock to a renewable bio-based feedstock is desirable.

Many petroleum-derived chemicals can be directly or functionally substituted with chemicals from renewable feedstocks (Nikolau et al., 2008; Werpy and Peterson, 2004). Among these compounds, several organic acids may fulfill a role as platform molecules using their functional groups as a target for chemical catalysis. Due to the relatively simplistic and well-studied fatty acid biosynthesis pathway, free fatty acids (FFAs) are of particular interest (Lennen and Pfleger, 2012; Nikolau et al., 2008). FFAs can be reduced and decarboxylated to yield α-olefins, a top 30 industrial organic chemical by weight produced in the United States (McCoy et al., 2007). FFAs also serve as precursors to soaps, surfactants, and lubricants and can be derived into fatty alcohols, fatty acid ethyl esters, or fatty acid methyl esters (Lennen and Pfleger, 2012; Peralta-Yahya et al., 2012; Polyolefin Comonomers—World Markets, 1999). There have been numerous studies focusing on the overproduction of FFAs; most current work has used *Escherichia coli* as a host (Jang et al., 2012; Lennen and Pfleger, 2012; Lennen et al., 2010; Lu et al., 2008; Zhang et al., 2011). Liu et al. (2012) was able to produce 4.8 g/L extracellular fatty acid (primarily C_{16}), Zhang et al. (2011) achieved upwards of 2 g/L (primarily C_{14}) and Lennen et al. (2010) reported nearly

600 mg/L C_{12} using a plant thioesterase (BTE). Jing et al. (2011) screened 31 heterologous acyl–acyl carrier protein (ACP) TEs expressed in *E. coli* and reported the ability to produce FFAs ranging from C_4 to C_{16} in length. Additionally, Jiang et al. (2011) and Liu et al. (2006) have reported the overproduction of butyric acid, up to 86.9 g/L, using *Clostridium tyrobutyricum* as a host.

Investigations using yeast and, specifically, *Saccharomyces cerevisiae* for fatty acid production are far more limited. *S. cerevisiae* has recently been engineered for increased production of long chain fatty acids, a 1.92-fold increase in $C_{16:1}$ and 1.77-fold increase in $C_{18:1}$, by disrupting citrate turnover and overexpressing a heterologous ATP-citrate lyase (Tang et al., 2013). Oleaginous yeast are known to produce extremely high levels of lipid bodies, for example, upwards of 72% (w/w) in *Rhodotorula glutinis* (Ratledge, 1991). Recently, *S. cerevisiae* was transformed into an oleaginous yeast by overexpression of the yeast diacylglycerol acyltransferase Dga1p (Kamisaka et al., 2013). Furthermore, the yeast species *Candida krusei* was engineered to improve acid tolerance, a potential challenge when using microorganisms to produce short chain fatty acids (SCFAs) (Wei et al., 2008). However, to our knowledge, no studies have been published focusing on the production of FFAs between C_6 and C_{10} in length in *S. cerevisiae*. Although *S. cerevisiae* does not naturally produce fatty acids in large quantities, its robustness, pH tolerance, simple nutrient requirements, fully sequenced genome and molecular tools, and long history as an industrial workhorse make it an excellent candidate biocatalyst for such purposes.

Short chain FFAs, fatty acids that are less than 10 carbons long, are of particular interest due to their high demand in polyolefin production. Over 50% of all α-olefin consumption is for the production of polyolefins, which are predominantly produced from C_6 to C_8 linear alpha olefins (Lappin and Sauer, 1989). Nearly 65% of all polyolefins are produced from hexene (35%) and octene (30%) monomers (Polyolefin Comonomers—World Markets, 1999). In this study, we demonstrate the ability to overproduce caprylic acid (C_8 FFA), a precursor to octene.

Fatty acids are key components of the cell and essential for cell growth and function. They are major constituents of cellular membranes, store metabolic energy, harbor signaling functions, and are involved in post-translational protein modifications (Tehlivets et al., 2007). In yeast, these fatty acids are primarily C_{16} and C_{18} in length and are produced by the large 2.6 MDa type I cytosolic fatty acid synthase (FAS) (Leibundgut et al., 2008; Tehlivets et al., 2007). This FAS consists of two subunits, encoded by *FAS1* (β-subunit) and *FAS2* (α-subunit) that organize as a barrel-shaped $\alpha_6\beta_6$ complex. Type I FASs are generally considered to be kinetically more efficient, with their coordinated synthesis more readily controlled than dissociated type II FASs (Schweizer and Hofmann, 2004). The yeast FAS, while very efficient in producing fatty acids, is not optimal for the production of SCFAs. The FAS structure, solved with 4 Å resolution, does not allow either an ACP (approximately 9 kDa) or a phosphopantetheine transferase (PPT) (approximately 13 kDa) to passively diffuse into the fatty acid elongation chambers due to their large size (Lomakin et al., 2007; Mootz et al., 2001; White et al., 2005). Short chain TEs are typically larger than 9 kDa (Buchbinder et al., 1995; Dehesh et al., 1996), making it unlikely that passive diffusion of a heterologous TE will suffice. Therefore, to produce SCFAs with yeast FAS, the malonyl–palmitoyl transferase (MPT) domain would need to be mutated for early cleavage of the elongating fatty acid. Given the complex and highly organized structure of the yeast FAS, the successful mutation of the MPT domain, while maintaining the native FAS structure and function, would be challenging. Introduction of a heterologous FAS in conjunction with a short chain TE is a promising alternative for creating SCFAs.

The *Homo sapiens* type I FAS is structurally very different from its yeast counterpart, even though the underlying chemistry is conserved between these two systems. The cytosolic human FAS (hFAS) is an α_2 X-shaped homodimer of 540 kDa (Maier et al., 2010). hFAS is not constrained by a scaffolding structure and displays a remarkable degree of flexibility, which allows rotation between the upper and lower halves of the molecule (Brignole et al., 2009). The TE domain, responsible for cleaving the elongating fatty acid, is on the C-terminus and isolated from the core scaffold (Leibundgut et al., 2008). The lack of structural scaffolding, in addition to the large pockets with access to the growing acyl-chain, makes the hFAS an ideal candidate for SCFA production.

In this study, we engineered *S. cerevisiae* to produce SCFAs by introducing the hFAS and heterologous short chain TE genes. Exogenous phosphopantetheine transfereases (PPTs) were expressed to create the active holo-hFAS form, and in vitro and in vivo activity of the hFAS was confirmed. The TE domain was removed from hFAS to allow the premature cleaving of the elongating fatty acids by independent or linked exogeneous short chain TEs and the production of SCFAs was demonstrated. To our knowledge, this is the first report of an active mammalian FAS expressed in yeast and the first report of the in vivo production of SCFAs in *S. cerevisiae*.

Materials and Methods

Yeast and Bacterial Strains

E. coli strain XL1-Blue (Stratagene, Santa Clara, CA) was used for plasmid preparation and storage. *S. cerevisiae* host strain BY4741 (Open Biosystems, Huntsville, AL) was used for in vitro studies. *S. cerevisiae* host strain BJ5464 (Jones, 1991) was used for in vivo studies. *S. cerevisiae* diploid BY4743 (EUROSCARF, Frankfurt, Germany) was sporulated to obtain a *fas2* haploid. Table I provides a description of the strains used and constructed in this study.

Media and Cultivation

Luria–Bertani (LB) medium containing 0.1 mg/mL ampicillin for selection (Sambrook and Russell, 2001) was used for *E. coli* cultivation. *S. cerevisiae* strains were cultivated in

Table I. List of strains.

Strains	Relevant description	Refs./source
XL-1 Blue (*E. coli*)	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacI^{q}ZΔM15 Tn10 (Tet^r)]*	Stratagene
BY4741 (*S. cerevisiae*)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BJ5464 (*S. cerevisiae*)	*MATα ura3-52 his3Δ200 leu2Δ1 trp1 pep4::HIS3prb1Δ1.6R can1 GAL*	Jones (1991)
BY4743	*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 lys2Δ0/LYS2 MET15/met15Δ0 fas2::kanMX4/FAS2*	EUROSCARF
BYΔFAS2	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 fas2::kanMX4*	This study
BYPACPS	BY4741 *ura3*::P_{PGK1}-*ACPS-loxP-HIS3-loxP*	This study
BYPSFP	BY4741 *ura3*::P_{PGK1}-*SFP-loxP-HIS3-loxP*	This study
BYAACPS	BY4741 *ura3*::P_{ADH2}-*ACPS-loxP-HIS3-loxP*	This study
BYASFP	BY4741 *ura3*::P_{ADH2}-*SFP-loxP-HIS3-loxP*	This study
BJAACPS	BJ5464 *ura3*::P_{ADH2}-*ACPS-loxP-TRP1-loxP*	This study
BJASFP	BJ5464 *ura3*::P_{ADH2}-*SFP-loxP-TRP1-loxP*	This study

complex YPD media (10 or 20 g/L dextrose, 20 g/L peptone, 10 g/L yeast extract (BD Biosciences, Sparks, MD)), semi-defined SDC-A media (20 g/L dextrose, 5 g/L casamino acids, 5 g/L ammonium sulfate, 1.7 g/L yeast nitrogen base without amino acids, 100 mg/L adenine sulfate), or synthetic SD minimal medium (20 g/L dextrose, 5 g/L ammonium sulfate, 1.7 g/L yeast nitrogen base without amino acids, and supplementary constituents as needed: 100 mg/L adenine sulfate, 100 mg/L uracil, 100 mg/L L-histidine-HCL, 100 mg/L L-methionine, 150 mg/L L-leucine, 150 mg/L L-lysine-HCL) (Amberg et al., 2005). All plates contained 20 g/L Bacto-agar.

For expression studies using the *ADH2* promoter, YPD with 10 g/L dextrose was used. For expression studies using the *PGK1* promoter, selective SDC-A medium was used. In these media, glucose is exhausted before 20 h. For the *FAS2* knockout strain, the medium contained 2 mM myristic acid with tween 40 (5 g/L) to supplement growth (Chirala et al., 1987). The fatty acid supplement was heated to 55°C for 30 min to increase solubility and then filter-sterilized.

Yeast strains were grown at 30°C in an air shaker (New Brunswick Scientific, Enfield, CT) at 250 rpm. Cells were inoculated from −80°C stock and grown overnight in 5 mL medium in 16 mm × 125 mm culture tubes. Yeast expression studies used 50 or 200 mL medium and protein purification studies used 5 or 250 mL medium. Cell growth was initiated at an optical density (600 nm) of 0.2 (Shimadzu UV-2450 spectrophotometer; Columbia, MD) and grown for 10 h (P_{PGK1} regulation) or 48 h (P_{ADH2} regulation).

Vector and Strain Construction

Standard molecular biology procedures were carried out as described in Sambrook and Russell (2001). Restriction enzymes, T4 DNA ligase, Taq DNA polymerase, and deoxynucleotides were purchased from New England Biolabs (Ipswich, MA). KOD Hot-start DNA polymerase was obtained from Novagen (San Diego, CA). Oligonucleotide primers were purchased from IDT DNA (San Diego, CA). Vector pCMV-SPORT6-*FASN*, carrying the gene for *H. sapiens* FAS, was obtained from Open Biosystems. Plasmid pKOS12-128a, carrying *sfp* from *B. subtilis*, was obtained from Kosan Biosciences (Kealey et al., 1998). pUC57-CpFatB1, carrying the *CpFatB1* TE gene from *Cuphea palustris* (Dehesh et al., 1996) codon optimized for expression in *E. coli*, was obtained from Dr. Basil Nikolau's lab at Iowa State University. pJLA502-TEII, carrying the *TEII* TE gene from *Rattus norvegicus* (Libertini and Smith, 1978; Naggert et al., 1991), was obtained from Dr. Stuart Smith at the Oakland Children's Hospital.

TRP1 was excised from pXP216 (Fang et al., 2011) using restriction enzymes *Bam*HI and *Kpn*I and inserted into pXP842 (Shen et al., 2012), replacing the *URA3* marker, to create pXP843. *acpS* was amplified from *E. coli* XL1-Blue and inserted into the two 2μ backbone vectors pXP222 and pXP842 (Fang et al., 2011; Shen et al., 2012) to give pXP222-ACPS and pXP842-ACPS. *FAS2* was amplified from *S. cerevisiae* and inserted into the CEN/ARS vector pXP100 (Fang et al., 2011) to give pXP100-FAS2. *FASN* (for hFAS), *sfp*, *TEII*, and *CpFatB1* were amplified and cloned into 2μ backbone vectors (Fang et al., 2011; Shen et al., 2012) to give pXP218-hFAS, pXP841-hFAS, pXP842-hFAS, pXP222-SFP, pXP842-SFP, pXP843-SFP, pXP842-TEII, and pXP842-CpFatB1 (Table II). *FASN*ΔTE was created using PCR primers to remove the C-terminal TE domain. Using PCR, an *Nhe*1 restriction cut site was added in the linker for attachment of the TE sequences to obtain *FASN-TEII* and *FASN-CpFatB1*. The 6x-histidine tags were placed on the C-terminus of *FASN*, *FASN*ΔTE, *FASN-TEII*, *FASN-CpFatB1*, *TEII*, and *CpFatB1* for

Table II. List of plasmids constructed.

Plasmid	Promoter	Selection marker	Origin
pXP100-FAS2	P_{PGK1}	*LEU2*	CEN/ARS
pXP218-hFAS	P_{PGK1}	*URA3*	2μ
pXP222-ACPS	P_{PGK1}	*LEU2*	2μ
pXP222-SFP	P_{PGK1}	*LEU2*	2μ
pXP841-hFAS	P_{ADH2}	*LEU2*	2μ
pXP841-hFASΔTE	P_{ADH2}	*LEU2*	2μ
pXP842-ACPS	P_{ADH2}	*URA3*	2μ
pXP842-CpFatB1	P_{ADH2}	*URA3*	2μ
pXP842-hFAS	P_{ADH2}	*URA3*	2μ
pXP842-hFAS-CpFatB1	P_{ADH2}	*URA3*	2μ
pXP842-hFAS-TEII	P_{ADH2}	*URA3*	2μ
pXP842-hFASΔTE	P_{ADH2}	*URA3*	2μ
pXP842-SFP	P_{ADH2}	*URA3*	2μ
pXP842-TEII	P_{ADH2}	*URA3*	2μ
pXP843-SFP	P_{ADH2}	*TRP1*	2μ

downstream purification. Details on the vector construction can be found in the Supplemental Material.

All sequences of gene fragments amplified by PCR were verified by DNA sequence analysis (GeneWiz, South Plainfield, NJ). *E. coli* mini-prep DNA was prepared using a Spin Miniprep Kit (Qiagen, Germantown, MD), and plasmid transformation in *E. coli* competent cells was done using a standard heat shock method (Sambrook and Russell, 2001). Plasmid and integrative transformations in *S. cerevisiae* were performed using a high efficiency LiAc method using DMSO (Hoskins, 2012). Isolation of total genomic yeast DNA for integration checking was performed as described by Sambrook and Russell (2001).

acpS and *sfp* under the control of P_{PGK1} or P_{ADH2} were integrated at one copy into the genome of strain BY4741 and BJ5464; creating strains BYAACPS, BYPACPS, BYASFP, BYPSFP, BJAACPS, and BJASFP (Table I). Two-fragment transformation was used as described previously (Fang et al., 2011; Nielsen et al., 2007). Integrations into chromosomal loci were confirmed by PCR analysis using primers that annealed upstream and downstream of the chromosomal site. The *fas2/FAS2 S. cerevisiae* diploid BY4743 was transformed with pXP100-FAS2 and selected on synthetic (SD) plates. Transformants were sporulated to obtain a *fas2* haploid with the genotype *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 fas2::kanMX4*. Details on the strain construction can be found in the Supplemental Material.

Protein Purification

Protease inhibitor cocktail S8830 (Sigma-Aldrich, St. Louis, MO), designed specifically for histidine-tagged proteins, was used for both small (5 mL) and large-scale (250 mL) histidine-tagged purification. All protein concentrations were determined by a Coomassie-Bradford protein assay (Thermo Fisher, San Jose, CA) and all samples were stored at −20°C.

Large-scale (250 mL) cultures were spun down and supernatant removed. For each 1.5 g of wet cells, 1 mL of cell lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, and 20 mM imidazole pH 7.4) was added. Cell pellets were lysed using a French Press Cell Disrupter (Thermo Fisher) at 16,000 psi. Cell suspensions were passed through the French Press four consecutive times to ensure complete disruption. After centrifugation at 20,000 rpm for 20 min at 4°C in an Optima LE-80 k ultracentrifuge (Beckman Coulter, Brea, CA), the supernatant was isolated and passed through a histidine purification column (1 mL of His60 Ni Superflow Resin (Clontech, Mountain View, CA) in a 2 mL capacity gravity flow column (Thermo Fisher)). Purification steps followed those recommended by the manufacturer.

Small-scale (5 mL) cultures were centrifuged and the supernatants removed. Cell pellets were re-suspended in 700 μL of cell lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, and 10 mM imidazole pH 8.0) and vortexed with 0.3 g acid-washed glass beads for 5 min at 4°C. After centrifugation for 10 min at 13,000 rpm in a Microfuge 18 centrifuge (Beckman Coulter) at 4°C, the supernatant was isolated and passed through a 1 mL Ni-NTA spin column (Qiagen). Purification steps followed those recommended by the manufacturer.

Acrylodan-Labeled Intestinal Fatty Acid Binding (ADIFAB) Protein Assay

ADIFAB assay procedures were modified from Richieri et al. (1993, 1999). Lyophilized ADIFAB (0.2 mg) was purchased from FFA Sciences (San Diego, CA) and suspended in storage buffer (50 mM TRIS, 1 mM EDTA, 0.5 mM PMSF, and 0.05% Na Azide pH 8). Five hundred microliters of purified FAS (20–100 μg) in 50 mM Na_2HPO_4, 300 mM NaCl, and 300 mM imidazole (pH 7.4) was combined with acetyl-CoA (50 μM final conc.), malonyl-CoA (500 μM final conc.), and NADPH (150 μM final conc.). The solution was placed on a microfuge tube rotator for 1 h at room temperature. Five hundred microliters of 0.2 μM ADIFAB suspended in measuring buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, and 1 mM Na_2HPO_4 pH 7.4) was added and the 1 mL mixture was incubated for 20 min at 37°C. The solution was transferred to a disposable polysterene cuvette and readings were taken using a Bowman Series 2 luminescence spectrometer at wavelengths 432 and 505 nm (Thermo Fisher). Fatty acid concentration calculations followed those recommended by the manufacturer.

5,5′-Dithio-bis(2-Nitrobenzoic Acid) (DTNB) Assay

DTNB assay procedures were modified from Knudsen et al. (1981) and Kim and Bang (1988). The reduction of DTNB was measured by following an increase in absorbance at 412 nm for 15 min using the UV–Vis spectrophotometer. TE activity was determined by adding purified TE enzyme (20–100 μg) to 1 mL of DTNB assay mixture (working concentrations of 0.4 M tris–HCL buffer pH 7.4, 1 mM EDTA, and 0.2 mM DTNB) in disposable polysterene cuvettes. The mixture was pre-incubated at 37°C for 3 min. The reaction was started by adding the substrate, fatty acid bound to CoA, to a working concentration of 56 μM. Fatty acid substrates included: caprylic acid-CoA (C_8), capric acid-CoA (C_{10}), lauric acid-CoA (C_{12}), and palmitic acid-CoA (C_{16}) (Sigma-Aldrich).

NADPH Absorbance Assay

NADPH assay procedures were modified from Bays et al. (2009). FAS activity was determined by adding purified FAS enzyme to the NADPH activity buffer (0.075 M potassium phosphate buffer pH 6.5, 5 mM DTT, 25 μM acetyl-CoA, and 150 μM NADPH). Enzyme sample (20–100 μg protein) was added to 1 mL of solution and incubated at 37°C for 5 min. The reaction was started by the addition of malonyl-CoA to a working concentration of 250 μM. NADPH conversion to

NADP+ was monitored by loss of absorbance at 340 nm using the UV–Vis spectrophotometer.

Fatty Acid Extraction and Quantification

Fatty acid extraction procedures were modified from Bligh and Dyer (1959). Heptanoic, undecanoic, and nonadecanoic acids were used as internal standards (Sigma-Aldrich). 1.5 mL of a 2:1 methanol:chloroform mixture was added to 0.1 g wet cell mass and vortexed for 10 min with glass beads. Chloroform (500 μL) was added and vortexed for 30 s (2–3×). ddH_20 (500 μL) was added and vortexed for an additional 30 s (2–3×). The lysed cells were then centrifuged for 4 min at 1,000*g*. The lower chloroform layer was recovered and evaporated with nitrogen gas.

Extracellular fatty acids were extracted from 20 mL culture broth by adding 2 mL 1 M HCL and 5 mL of a 1:1 methanol: chloroform mixture. The solution was vortexed for 5 min then centrifuged for 4 min at 1,000*g*. The lower chloroform layer was recovered and evaporated with nitrogen gas.

For methylation, the dried sample was added to 2 mL of 1 N HCL in methanol. This solution was heated at 80°C for 1 h and allowed to cool to room temperature. Two milliliters of 0.9% NaCl was then added with 1.5 mL of hexane and vortexed for 1 min followed by centrifugation for 5 min at 1,000*g*. The upper hexane layer was removed and placed in a GC-MS vial. Additional hexane (1.5 mL) was added to the HCL solution, vortexed for 3 min, and centrifuged for 3 min at 1,000*g*. The hexane layer was removed and combined in the same GC-MS vial. The hexane was then evaporated and the sample stored at 4°C.

Fatty acids were measured at the W.M. Keck Metabolomics Research Laboratory at Iowa State University and the Mass Spectrometry Facility at the University of California Irvine. Fatty acids were detected by gas-chromatography mass spectrometry (GC-MS) and by gas-chromatography gas-chromatography mass spectrometry (GC-GC-MS) for the increased resolution of SCFAs. The GC-MS was a Trace MS+ from Thermo Fisher using a 30 m long × 0.25 mm i.d. DB-5 column from Agilent JW Scientific (Santa Clara, CA). The oven was held at 50°C for 1 min then heated at a rate of 10°C min^{-1} to 290°C and held for an additional minute. The mass spectrometry used electron ionization (70 eV) scanning (1/s) from *m*/*z* 50 to 650. GC-GC-MS used an Agilent LTM (Low Thermal Mass) fast GC system model 6890 with an Agilent GC-MSD model 5975C (Santa Clara, CA). Different independently heated temperature zones for multidimensional chromatography using Agilent Capillary Flow Technology Deans Switches were utilized with fast heating/cooling rates. Fatty acids were separated on a 30 m long × 0.250 mm i.d. fused silica capillary column (HP-5MSI) with Helium carrier gas. The primary oven was held at 100°C for 1 min then heated at a rate of 20°C min^{-1} to 250°C; this temperature was maintained for 5 min. The LTM oven temperature was held at 100°C for 14 min then increased at 200°C min^{-1} to 250°C to flush out undesired sample. The method included a cut step at 9.7 to 12.5 min in the runtime, where activation of a switch valve allowed for unwanted sample to be diverted, amplifying the detection of the desired product.

Relative amounts and distribution of SCFAs were determined by using total ion chromatogram and were normalized to the internal standards. The shorter chain fatty acids (C_6–C_8) were particularly sensitive to evaporation and drying procedures, which contributed to an increase in variability during GC-MS measurements.

Results and Discussion

hFAS Activation by Phosphopantetheinyl Transferases

Fatty acid synthesis is initiated when an acetyl moiety from acetyl coenzyme A (CoA) is transferred to the thiol group of the phosphopantetheine arm of the ACP by the PPT. In the case of yeast FAS, this reaction is carried out by the PPT domain before the folding of the α- and β-chains into a heterododecamer (Lomakin et al., 2007). However, hFAS lacks a PPT domain, thus requiring an external PPT activator. In *H. sapiens*, this functionality is provided by an independently expressed mono-functional enzyme, the holo-ACP synthase (Bunkoczi et al., 2007; Leibundgut et al., 2008; Tehlivets et al., 2007).

To address this we evaluated two PPTs, a group I PPT, ACP synthase (AcpS) from *E. coli*, and a group II PPT, Sfp phosphopantetheinyl transferase from *B. subtilis* (Mootz et al., 2001; Nakano et al., 1992; White et al., 2005). Previously, rat ACP expressed in *E. coli* was shown to be slightly active and increased 5-fold by the co-expression of *acpS*; highlighting the similarities between the type I and type II ACP (Reed et al., 2003; Tropf et al., 1998). Additionally, Sfp has shown the ability to activate heterologous polyketide synthases in yeast and is the closest structurally characterized enzyme to holo-ACP synthase from *H. sapiens* (Bunkoczi et al., 2007; Kealey et al., 1998; Lee et al., 2009). Both PPT genes were cloned under the *PGK1* promoter and integrated at one copy into the *URA3* locus via homologous recombination. The resulting strains are designated, BYPACPS for the *acps* integrant and BYPSFP for the *sfp* integrant (Table I).

To assess the activity of the PPTs to modify hFAS, both were evaluated with the DTNB in vitro assay. The DTNB assay measures the reduction of DTNB to the TNB^{2-} anion in the presence of free thiols. Only the holo-form of hFAS can release coenzyme A from the fatty acid substrate, C_{16}-CoA. Strains BYPACPS and BYPSFP were transformed with vector pXP218-hFAS carrying hFAS. The 6x-histidine tagged hFAS was purified and combined with the DTNB reaction mixture. Interestingly, similar activity profiles were observed, demonstrating that both AcpS and Sfp resulted in holo-hFAS formation (Fig. 1). Negative controls, lacking the PPT or the hFAS showed no activity. The release of free coenzyme A from palmitic-CoA confirms the TE domain of the holo-hFAS to be active. The results were confirmed with the nicotinamide adenine dinucleotide phosphate (NADPH) in vitro assay. Successful oxidation of NADPH to NADP+ confirms the

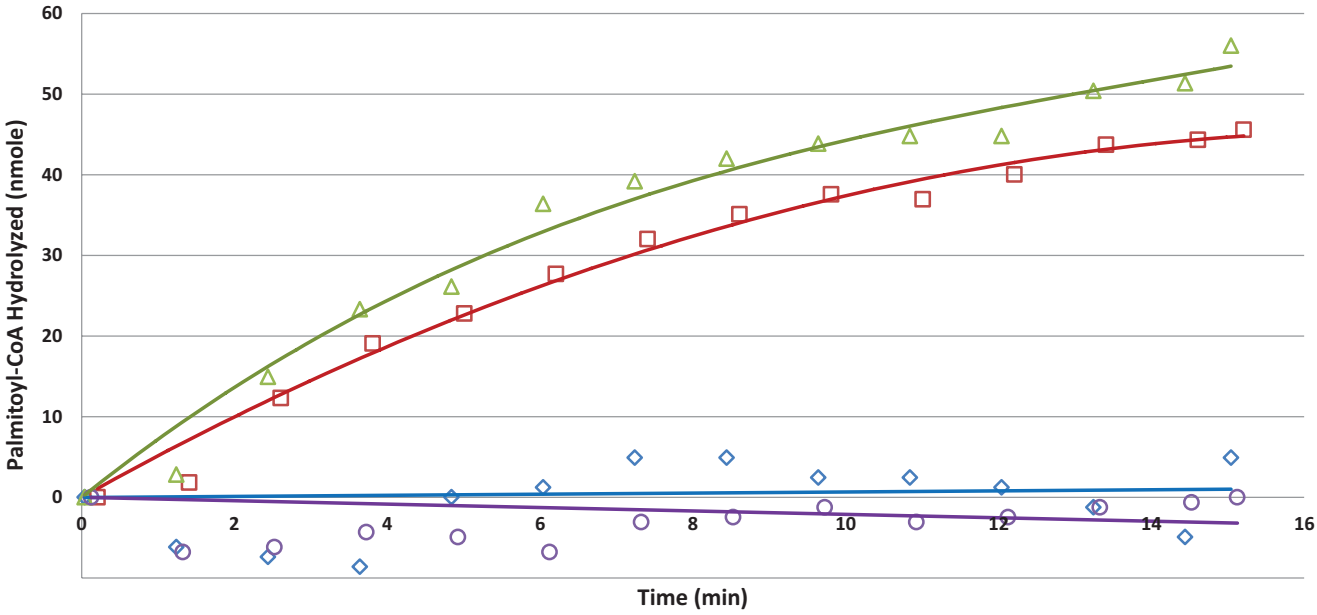

Figure 1. Confirmation of holo-hFAS formation by AcpS and Sfp. An in vitro DTNB assay demonstrated the successful transfer of the phosphopantetheine arm from coenzyme-A to the acyl carrier protein domain of hFAS. Strain BYPACPS expressing pXP218-hFAS (□) and strain BYPSFP expressing pXP218-hFAS (△). Negative controls consisted of strain BY4741 expressing pXP218-hFAS (○) and the host strain BY4741 (◊). Two independent experiments showed similar profiles.

3-ketoacyl reductase and/or enoyl reductase domains of hFAS to be active. During the FAS enzymatic reaction, a 3-fold decrease in absorbance (due to conversion to NADP+) was observed in the holo-hFAS strain when compared to negative controls lacking the PPT or the hFAS (data not shown).

In Vitro Fatty Acid Production by hFAS

An in vitro acrylodan-labeled intestinal fatty acid binding protein (ADIFAB) assay was used to confirm the production of active hFAS in the yeast strain. Strain BY4741 carried two plasmids for expression of hFAS and AcpS (pXP842-ACPS and pXP841-hFAS) or hFAS and Sfp (pXP842-SFP and pXP841-hFAS) under the control of the glucose-repressed *ADH2* promoter. The strains were incubated for 48 h in 1% glucose YPD media. The 6x-histidine tagged hFAS was then purified from both strains and combined with the ADIFAB reaction mixture. As shown in Figure 2, similar levels of palmitic acid were produced in vitro by hFAS/AcpS and hFAS/Sfp. The negative control lacking a PPT produced negligible fatty acid levels. In vitro fatty acid synthesis confirms the production and functionality of all seven domains in hFAS; any inactive domain would have prohibited the production of fatty acids.

hFAS In Vivo Activity via Growth Complementation

To demonstrate hFAS activity in vivo, we used a growth complementation assay. Cell viability and growth characteristics were determined in a *FAS2* knockout strain. The yeast FAS complex, composed of Fas1 and Fas2, is responsible for bulk fatty acid synthesis in the absence of exogenous fatty acid supplementation (Tehlivets et al., 2007). The *FAS2* knockout strain is deficient in the de novo synthesis of fatty acids; therefore, we supplemented the BYΔFAS2 strain with C_{14} fatty acids to enable cellular growth. Strain BYΔFAS2, was co-transformed with two plasmids carrying *FASN* and *acpS* under the constitutive *PGK1* promoter (pXP218-hFAS and pXP222-ACPS). The resulting colonies were streaked on synthetic minimal plates lacking fatty acids. Subsequent colonies were then individually re-suspended in water and plated (roughly 100 cells/plate) on synthetic plates lacking fatty acids. This procedure eliminated any residual fatty acid carryover and reduced the probability of cell cannibalization by lowering cell density. The co-expression of hFAS and the AcpS PPT allowed the BYΔFAS2 haploid to survive on synthetic minimal plates lacking fatty acids (Fig. 3A). Colonies were small in size compared to wild-type strain (BY4741), likely due to lower in vivo fatty acid levels. This is supported by our observation that colony size for strain BYΔFAS2 is directly correlated with supplemented fatty acid levels (data not shown). Cell growth not only confirmed that holo-hFAS is actively produced in vivo, but also demonstrated that hFAS activity and expression levels are sufficient to support yeast growth. Under a microscope, yeast cells appeared to have a standard morphology (Fig. 3B).

Removal of hFAS Thioesterase Domain

The cleavage of the growing fatty acid acyl chain from the ACP is mediated by the TE domain. The native C-terminal TE domain on hFAS cleaves primarily C_{16} fatty acyl-ACP

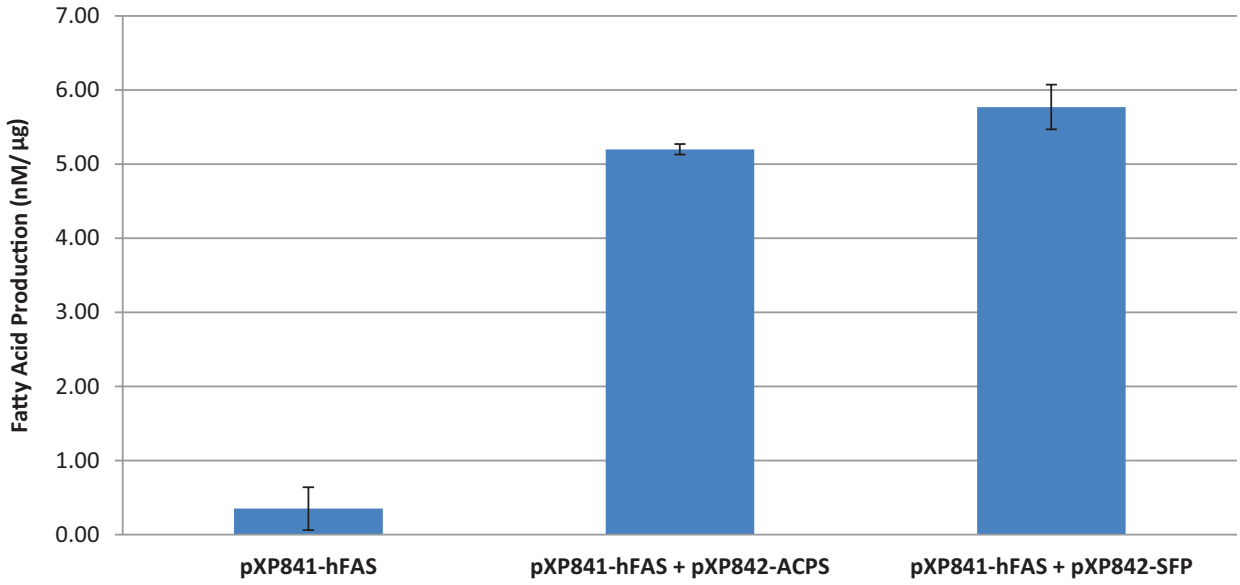

Figure 2. Confirmation of hFAS activity by fatty acid synthesis. An in vitro ADIFAB assay demonstrated fatty acid synthesis using both AcpS and Sfp PPTs. Strain BY4741 carried pXP841-hFAS and pXP842-ACPS or pXP842-SFP. The negative control was strain BY4741 carrying pXP841-hFAS. Readings were subtracted from buffer fluorescence (negligible level) and calculations assumed palmitic acid ($Q = 6.6$, $K_d = 21.4$ nM, $R_{max} = 1.16$). Results were normalized per µg hFAS and are expressed as mean ± error from two independent experiments.

esters and is tethered to the ACP domain via a linking region (Leibundgut et al., 2008; Maier et al., 2008, 2010). In order to create SCFAs, this highly specific TE domain was removed and complemented with a short chain specific TE. Due to the C-terminal location and isolation from the core scaffold, the removal of the TE domain becomes feasible, while maintaining the functionality and structure of adjacent domains. This also prevents substrate competition between the native hFAS TE and the heterologous short chain TE.

Using PCR, we constructed an hFAS gene lacking the 807 base pairs coding for the TE. This gene was inserted into a 2µ vector under the *ADH2* promoter (pXP841-hFASΔTE). A

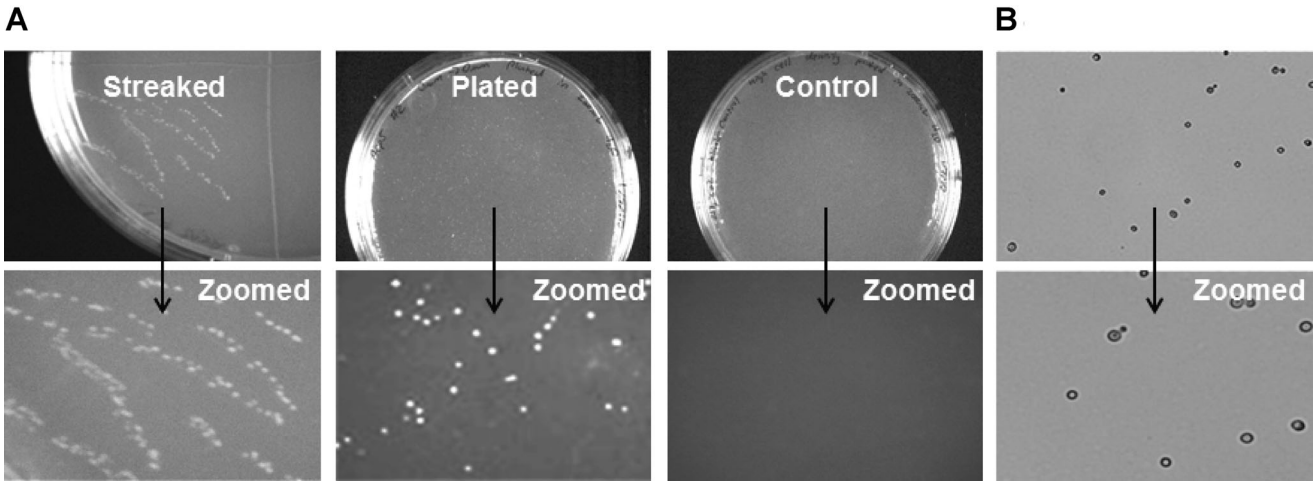

Figure 3. In vivo confirmation of holo-hFAS formation by growth complementation. **A**: Growth complementation of a *FAS2* knockout strain on synthetic plates lacking fatty acids by expression of hFAS (BYΔFAS2 carrying plasmids pXP218-hFAS and pXP222-ACPS). Transformant colonies were streaked on synthetic minimal plates lacking fatty acids. Subsequent colonies were then individually re-suspended in water and plated (roughly 100 cells/plate). The negative control was strain BYΔFAS2 carrying empty plasmids pXP218 and pXP222. **B**: Example images of yeast cells under the microscope. Cell images were taken with an Olympus BX-51 optical microscope at 40× magnification (Olympus, Center Valley, PA).

NADPH reduction assay showed equal oxidation activities of NADPH by the native hFAS and hFASΔTE. This confirms the adjacent β-ketoacyl reductase and/or enoyl reductase domains are active even though the C-terminal TE domain has been removed. The DTNB assay was used to verify the successful elimination and inactivation of the TE domain in the mutant hFAS (Fig. 4). Strain BY4741 co-expressing hFASΔTE and AcpS (pXP842-ACPS and pXP841-hFASΔTE) showed no absorption change, verifying that the hFASΔTE is unable to cleave the thioester-CoA bond of the substrate palmitic-CoA. The positive control, expressing hFAS and AcpS, exhibited a similar profile as previously observed (Fig. 1). Shorter length acyl-CoA substrates were also tested and no observable cleaving of the CoA molecule occurred with the mutant when compared to the control (data not shown). The results demonstrate the successful removal of the TE domain of the FASΔTE mutant.

In Vitro Fatty Acid Production Using hFASΔTE and Thioesterases CpFatB1 and TEII

SCFA production in our yeast system requires the over-expression of a short chain TE in conjunction with the heterologous hFAS. We screened 14 TEs and categorized them based on their SCFA profile and total activity. Two of the more promising short chain TEs were CpFatB1 and TEII. CpFatB1, a free-standing TE from *C. palustris*, has previously been shown to primarily produce C_8 and C_{10} length fatty acids, with minor products including C_{12}, C_{14}, C_{16}, and C_{18}, when expressed and purified from *E. coli* (Dehesh et al., 1996). TEII, a free-standing short chain enzyme from rat mammary glands, has previously been shown to work with the rat FAS (expressed in *sf9* cells), for the in vitro production of primarily C_6 and C_8 length fatty acids (Joshi et al., 2005).

Both TEs were cloned into a 2μ vector under the *ADH2* promoter (pXP842-CpFatB1 and pXP842-TEII) and transformed into strain BY4741. TE activity was detected with the in vitro DTNB assay using a variety of short chain carbon substrates. The 6x-histidine tagged TEs were purified and combined with the DTNB reaction mixture containing caprylic acid-CoA (C_8), capric acid-CoA (C_{10}), or lauric acid-CoA (C_{12}). CpFatB1 had similar activity on all tested substrates; activity was higher than that of TEII (see Supporting Information: Fig. S-1). TEII activity was highest on caprylic acid-CoA and yielded similar lower activities on capric acid-CoA and lauric acid-CoA.

Fatty acid production using the mutated hFAS and independently expressed TEs was assessed in vitro with the ADIFAB assay. hFASΔTE was co-expressed with AcpS (pXP841-hFASΔTE and pXP842-ACPS) in strain BY4741. TEs CpFatB1 and TEII were independently expressed (pXP842-CpFatB1 and pXP842-TEII) in strain BY4741. The 6x-histidine tagged hFASΔTE was purified and then combined with either independently purified 6x-histidine tagged TE. The mixture containing both hFASΔTE and TE was then added to the ADIFAB reaction mixture. Similar fatty acid production levels were observed for the CpFatB1 and TEII TEs using hFASΔTE (Fig. 5). The negative control using hFASΔTE had a noticeable fluorescence. The TE domain of the hFASΔTE mutant was completely removed, thus this signal may stem from partial acyl-CoA/ACP chains binding to the ADIFAB active site. It was also reported that after

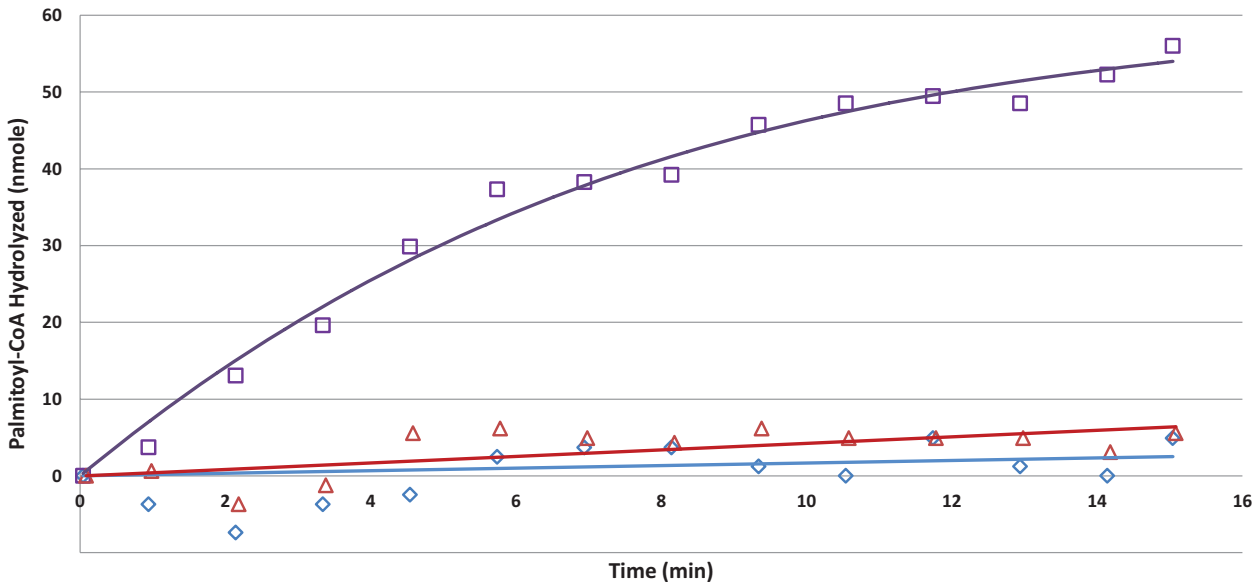

Figure 4. Confirmation of the successful removal and inactivity of the TE domain of the FASΔTE mutant. An in vitro DTNB assay demonstrated the inability of the thioesterase domain to cleave the coenzyme-A from the palmitic-CoA substrate for strain BY4741 co-expressing pXP842-ACPS and pXP841-hFASΔTE (△). The negative control was strain BY4741 (◊) and the positive control was strain BY4741 co-expressing pXP842-ACPS and pXP841-hFAS (□). Two independent experiments showed similar profiles.

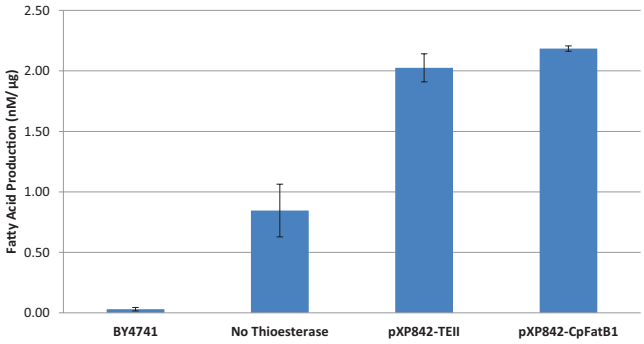

Figure 5. Confirmation of fatty acid production using the hFASΔTE mutant and short chain TEs using the in vitro ADIFAB assay. hFASΔTE was expressed in strain BY4741 carrying pXP841-hFASΔTE and pXP842-ACPS. Thioesterases were expressed in strain BY4741 carrying pXP842-CpFatB1 or pXP842-TEII. The negative controls were strain BY4741 and strain BY4741 carrying pXP841-hFASΔTE and pXP842-ACPS with no added thioesterase. Readings were subtracted from buffer fluorescence (less than 20% of measured value) and calculations assumed palmitic acid ($Q = 6.6$, $K_d = 21.4$ nM, $R_{max} = 1.16$). Results were normalized per µg hFAS and are expressed as mean ± error from two independent experiments.

removal of the TE domain from the chicken FAS, the ketoacyl synthase domain was able to elongate up to C_{22} length fatty acids, albeit at much lower rates (Singh et al., 1984). This could be occurring in the hFASΔTE mutant potentially explaining the increased fluorescence. The negative control lacking both the hFASΔTE mutant and the TE showed negligible fatty acid production.

In Vivo Short Chain Fatty Acid Production Using hFASΔTE and Thioesterases CpFatB1 and TEII

The in vitro ADIFAB assay detects total fatty acids, but cannot determine fatty acid chain lengths (under C_{16}). Therefore, to conclusively demonstrate in vivo production and to determine the length of the fatty acids being produced, detection by GC-MS and GC-GC-MS was used.

Two independent experiments were performed co-expressing hFASΔTE and TEII under the *ADH2* promoter (pXP841-hFASΔTE and pXP842-TEII) in strain BJASFP. BJASFP is a *pep4 prb1* strain, has *sfp* under the *ADH2* promoter integrated at a single copy in the genome and contains the native yeast fatty acid synthase (yFAS). While the yFAS competes with the heterologous hFAS for the same required substrate and cofactors molecules, it was retained to supply the necessary C_{16} and C_{18} length fatty acids for cellular function and growth. The cell extracts and culture broth were washed with organic solvents and methylated before chromatography runs. Nearly all of the C_6, C_8, and C_{10} fatty acids were found in the culture broth (Fig. 6). Minor C_{12} and C_{14} products were primarily intracellular, but amounted to less than 5% of the total SCFAs and MCFAs (medium chain FAs) detected. Results were similar among all strains tested.

The levels of extracellular fatty acids were measured and compared for the control strain (BJ5464) and four strains with the *sfp* or *acps* PPT gene integrated (BJASFP and BJAACPS), the TEII or CpFatB1 on a plasmid (pXP842-TEII or pXP842-CpFatB1) and the hFASΔTE on a plasmid (pXP841-hFASΔTE). Expression of all genes was under the control of the *S. cerevisiae ADH2* promoter. Extracellular fatty acid levels increased for all measurable SCFAs (C_6, C_8, and C_{10}) relative to the BJ5464 control (Fig. 7). The Sfp PPT performed consistently better than AcpS for hFAS activation in vivo (2.5-fold higher fatty acid levels), and TEII outperformed CpFatB1 in the production of caprylic acid (2.0-fold increase). Strain BJASFP co-expressing hFASΔTE and TEII had the highest titers of SCFAs reaching 17 mg/L C_8 and 20 mg/L total SCFA. Caprylic acid was the major SCFA product and increased 17-fold over the control due to the

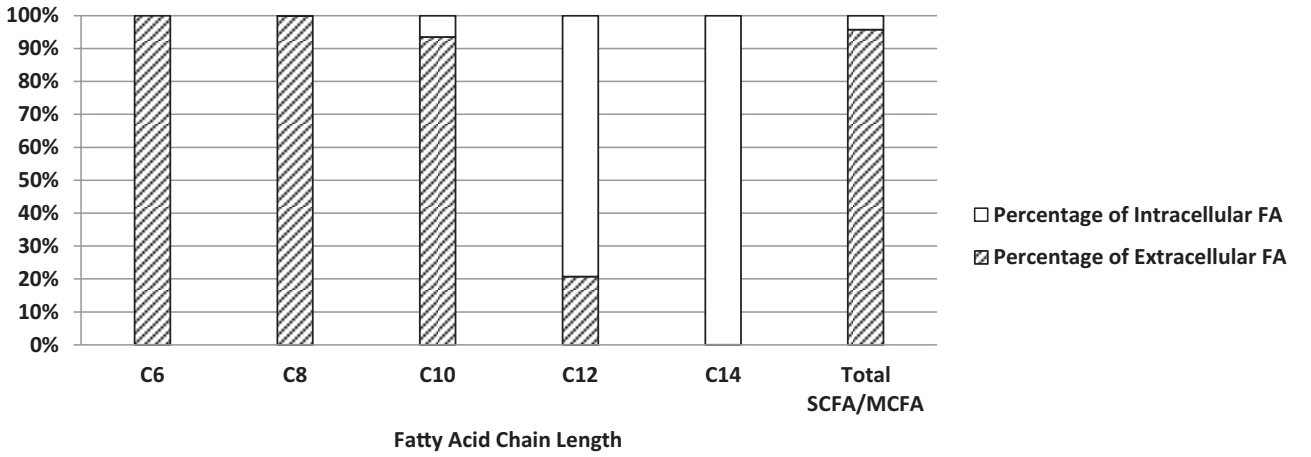

Figure 6. Intracellular and extracellular location of short chain fatty acids in *S. cerevisiae*. In vivo short chain fatty acid (SCFA, C_6–C_{10}) and medium chain fatty acid (MCFA, C_{12}–C_{14}) levels were determined by GC-MS and GC-GC-MS, and are shown for strain BJAACPS expressing pXP841-hFASΔTE and pXP842-TEII. The fraction of short and medium chain fatty acids that are intracellular (white bars) is compared to the fraction of short and medium chain fatty acids that are extracellular (slashed bars). The total MCFA/SCFA column is the sum of C_6–C_{14} length fatty acids. Error was approximately 1–2% of measured values and results were similar among all strains.

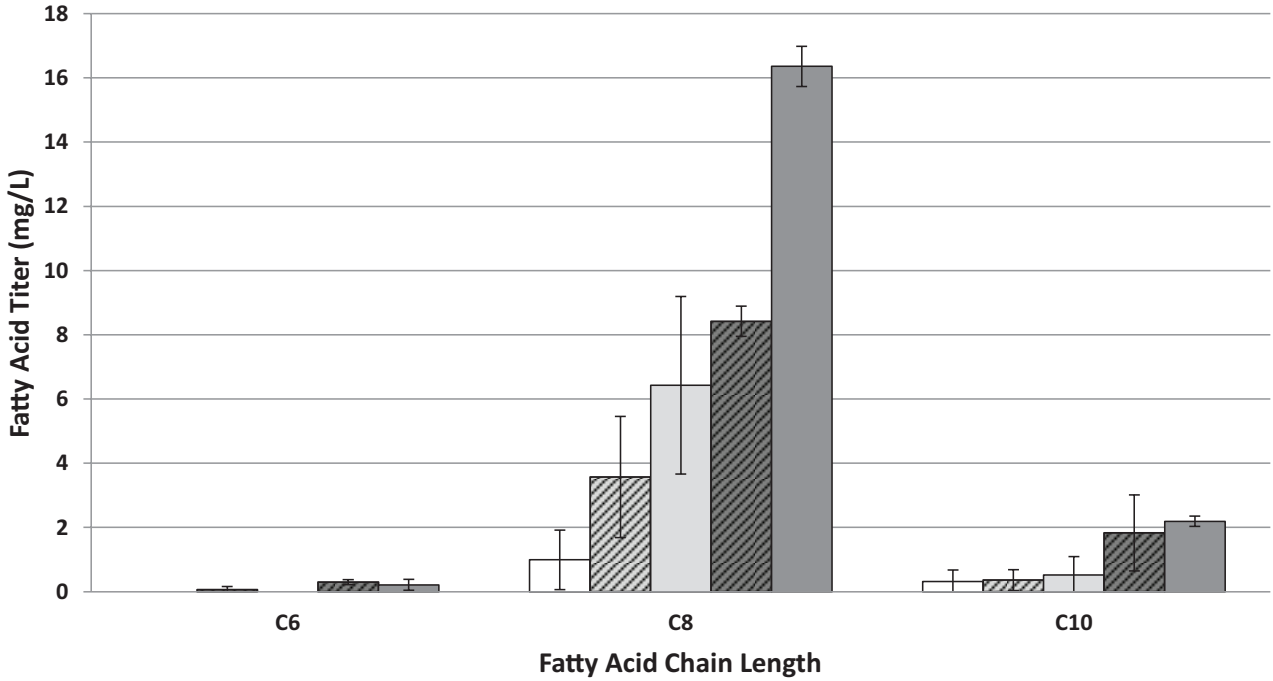

Figure 7. Extracellular short chain fatty acids produced using thioesterases TEII and CpFatB1 with hFASΔTE. Control strain BJ5464 (white bars) is compared to strain BJAACPS carrying pXP841-hFASΔTE and pXP842-CpFatB1 (light gray slashed bars), BJAACPS carrying pXP841-hFASΔTE and pXP842-TEII (light gray bars), BJASFP carrying pXP841-hFASΔTE and pXP842-CpFatB1 (dark gray slashed bars) and BJASFP carrying pXP841-hFASΔTE and pXP842-TEII (dark gray bars). Results are expressed as mean ± error from two independent experiments.

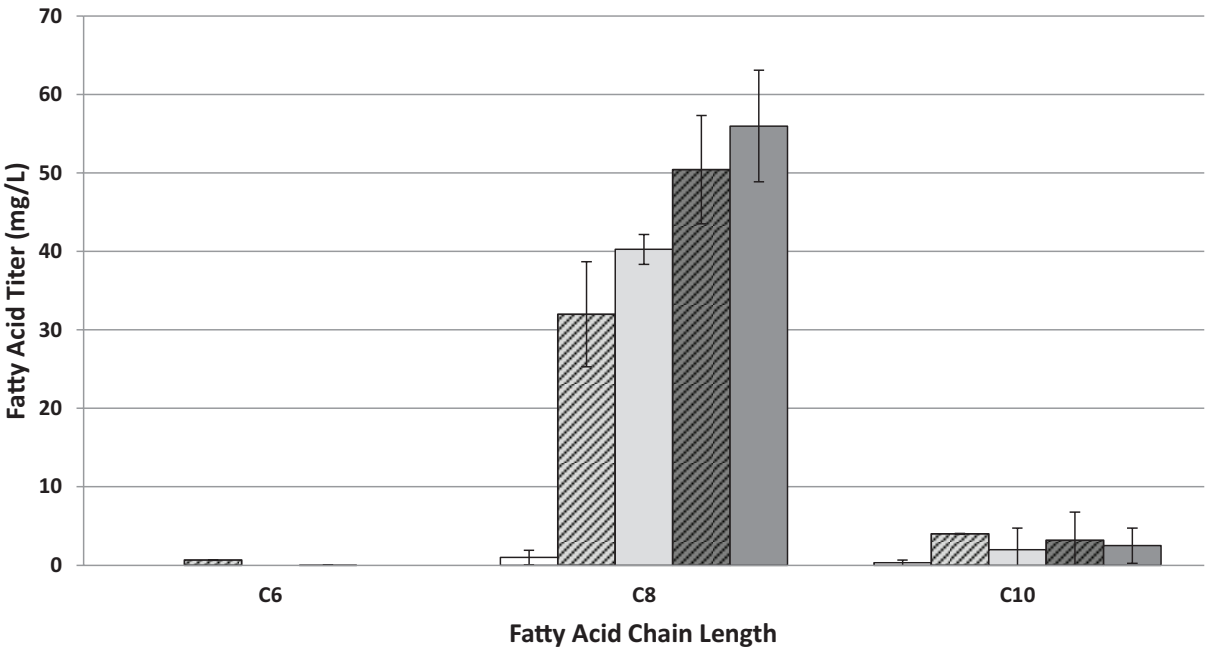

Figure 8. Extracellular short chain fatty acids produced using linked hFAS-TEII and linked hFAS-CpFatB1. Control strain BJ5464 (white bars) is compared to strain BJAACPS carrying pXP842-hFAS-CpFatB1 (light gray slashed bars), BJAACPS carrying pXP842-hFAS-TEII (light gray bars), BJASFP carrying pXP842-hFAS-CpFatB1 (dark gray slashed bars) and BJASFP carrying pXP842-hFAS-TEII (dark gray bars). Results are expressed as mean ± error from two independent experiments.

shorter chain specificity of TEII (Joshi et al., 2005; Libertini and Smith, 1978). TEII and CpFatB1 were expressed in the absence of hFASΔTE and no change in fatty acid profile from wild type was observed. All strains (expressing CpFatB1 or TEII) had similar final optical densities and growth rates to those of the control.

In Vivo Short Chain Fatty Acid Production Using Linked hFAS-TEII and hFAS-CpFatB1

In an in vitro study by Joshi et al. (2005), linking a short chain TE directly to the rat FAS maintained activity of both enzymes and shifted the fatty acid profile. To increase the efficiency of release by the TE and thus the SCFA levels produced by our strains, we created two new hFAS enzymes by replacing the native TE with short chain specific TEs TEII and CpFatB1. The 26 base amino acid hFAS ACP linker was altered to create an *Nhe*1 restriction digestion site (while maintaining the native amino acid residues) and the TE genes were ligated (Supplemental Material). The resulting *FASN-TEII* and *FASN-CpFatB1* genes were inserted into a 2μ vector under the *ADH2* promoter creating pXP842-hFAS-TEII and pXP842-hFAS-CpFatB1.

Four strains, BJASFP and BJAACPS expressing either the linked hFAS-TEII (pXP842-hFAS-TEII) or the linked hFAS-CpFatB1 (pXP842-hFAS-CpFatB1), were compared to the control strain BJ5464 (Fig. 8). Extracellular fatty acid levels increased for all measurable SCFAs relative to the BJ5464 control (Fig. 8). Linking the TE improved caprylic acid production 4- to 9-fold over the unlinked counterparts (Fig. 7), indicating the importance of the TE proximity to the FAS. Strain BJASFP expressing linked hFAS-TEII produced the highest SCFA titers, reaching 63 mg/L C_8 and 68 mg/L total SCFA. Increasing SFP synthesis using a 2 μ vector increased C_8 and SCFA levels by roughly an additional 30% to 82 mg/L and 111 mg/L, respectively. All strains had similar final optical densities and growth rates to those of the control.

Caprylic acid levels varied from 8–15 mass% or 15–28 mol % of all fatty acids produced in strain BJASFP carrying pXP842-TEII. The results were obtained in *S. cerevisiae* strains expressing the native yFAS. Higher levels are expected if native yFAS expression is downregulated during SCFA synthesis. The results also correlate with the expected activities of CpFatB1 and TEII on short chain carbon substrates, and verify the ability to utilize hFAS and exogenous TEs for in vivo SCFA production in *S. cerevisiae*.

Conclusions

Many petroleum-derived chemicals can be directly or functionally substituted with chemicals produced from renewable feedstocks. Among these compounds, SCFAs may fulfill a role as platform molecules using their functional group as a target for chemical catalysis. In this study, *S. cerevisiae* was engineered to produce SCFAs from glucose using the hFAS type I FAS. This synthase was shown to be activated by PPTs AcpS and Sfp from *E. coli* and *B. subtilis*, respectively. Overexpression of hFAS in a yeast *FAS2* knockout strain, deficient in de novo fatty acid synthesis, demonstrated the full functional replacement of the native fungal FAS by hFAS. Yeast strains expressing the native yeast FAS (for C_{16} and C_{18} production), an hFAS mutant deficient in the TE domain and short chain TEs were constructed. This resulted in a 17-fold increase in C_8 levels compared to the wild-type strain in vivo. Linking the short chain TEs to the hFAS resulted in a 4- to 9-fold increase in C_8 levels over the unlinked counterparts and a 64-fold increase over the wild-type strain. Combined over-expression of the PPT with the hFAS mutant resulted in C_8 titers up to 82 mg/L and total SCFA titers up to 111 mg/L. Additional strain and enzyme engineering can now be implemented to further improve product yield, product selectivity, and product sequestration of the SCFAs.

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