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Engineering Saccharomyces cerevisiae for the high-level production of free fatty acids.

Author
Fernandez-Moya, Ruben

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Engineering *Saccharomyces cerevisiae* for the high-level production of free fatty acids.

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Chemical and Biochemical Engineering

By

Ruben Fernandez-Moya

Dissertation Committee:
Professor Nancy A. Da Silva, Chair
Professor Suzanne B. Sandmeyer
Professor Szu-Wen Wang

2017

DEDICATION

To

Marcella Pacheco Garciaguirre

One of the most caring, loving, lively, and passionate people I have ever met. You will always be missed.

'If I have seen further than others,

it is by standing upon the shoulder of giants'

Isaac Newton

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LIST OF ABBREVIATIONS

2-PS	2-pyrone synthase
6-MSA	6-methylsalicylic acid
ACP	Acyl carrier protein
ACS	Acetyl-CoA synthetase
ADIFAB	Acrylodan-labeled intestinal fatty acid binding
ALD	Acetaldehyde dehydrogenase
AT	Acetyl transferase
ATP	Adenosine triphosphate
CoA	Coenzyme A
COBRA	Constraint based reconstruction and analysis
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
eFAS	*Escherichia coli* fatty acid synthase
FA	Fatty acid
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FAS	Fatty acid synthase
FBA	Flux balance analysis
GC-MS	Gas-chromatography mass spectrometry
hFAS	*Homo sapiens* fatty acid synthase
HPLC	High performance liquid chromatography
IDT DNA	Integrated DNA Technologies
KR	Ketoacyl reductase
KS	Ketoacyl synthase

LB	Luria-Bertani
LCFA	Long-chain fatty acid
LD	Lipid droplet
MAE	Malic enzyme
MCS	Multiple cloning site
MCFA	Medium-chain fatty acid
MC-FFA	Medium-chain free fatty acid
MPT	Malonyl/palmitoyl transferase
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PKS	Polyketide synthase
PPT	Phosphopantetheinyl transferase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SE	Steryl ester
TAG	Triacylglyceride
TAL	Triacetic acid lactone
TCA	Tricarboxylic acid
TE	Thioesterase
TSS	Transcription start site
WT	Wildtype

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I want to take the opportunity to thank Dr. Andreas Schirmer for the chance to intern at REG Life Sciences for three months during my last summer as a PhD candidate. There I was able to broaden my knowledge in strain and metabolic engineering to bacteria and oleaginous yeast, as well as learn insights about the biorenewable chemical production industry. GC-MS has been a crucial instrument for my PhD dissertation results, and Dr. Felix Grün repaired the GC-MSs at the Mass Spectroscopy Facility on Campus, enabling accurate and consistent fatty acid measurements.

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CURRICULUM VITAE

EDUCATION

Nov 2017 **Ph. D. in Chemical and Biochemical Engineering**
*PhD Thesis: '*Engineering *Saccharomyces cerevisiae* for the high-level production of free fatty acids'

July 2012 **M. S. in Chemical and Biochemical Engineering**
*Master Project: '*Integration of a whole new heterologous FAS system for production of medium chain carboxylic acids in *Saccharomyces cerevisiae'*

July 2010 **B. S. in Chemical Engineering, specialization in Biochemical Engineering**

RESEARCH EXPERIENCE

Da Silva Lab. Department of Chemical Engineering. University of California, Irvine

PhD Student and Candidate July 2012 – Nov 2017

Da Silva Lab. Department of Chemical Engineering. University of California, Irvine

MS Student October 2010 – July 2012

Department of Chemical Engineering. Autonomous University of Barcelona

Research Assistant October 2009 – June 2010

international Genetically Engineered Machine (iGEM-MIT) UAB project

Main Researcher April 2009 – November 2009

PROFESSIONAL EXPERIENCE

REG Life Sciences
Intern, Strain Engineering Team June 2017 – September 2017

Hebron, S. A. (Otsuka Chemical Group Company)
Intern June 2010 – August 2010

SCIENTIFIC PAPERS

R Fernandez-Moya, NA Da Silva (2017) 'Engineering *Saccharomyces cerevisiae* high-level synthesis of fatty acids and derived products'. *FEMS Yeast Research 17 (7)*

PB Besada-Lombana, **R Fernandez-Moya**, J Fenster, NA Da Silva (2017) 'Engineering *Saccharomyces cerevisiae* fatty acid composition for increased tolerance to octanoic acid'. *Biotechnology and Bioengineering 114 (7), 1531-1538*

R Fernandez-Moya, C Leber, J Cardenas, NA Da Silva (2015) 'Functional replacement of the *Saccharomyces cerevisiae* fatty acid synthase with a bacterial type II system allows flexible product profiles'. *Biotechnology and Bioengineering 112 (12), 2618-2623*

C Leber, B Polson, **R Fernandez-Moya**, NA Da Silva (2015) 'Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in *Saccharomyces cerevisiae*'. *Metabolic Engineering 28, 54-62*

SCIENTIFIC PRESENTATIONS

R Fernandez-Moya, C Leber, B Polson, NA Da Silva. (2014) 'Engineering *Saccharomyces cerevisiae* as a biocatalyst for the synthesis of short and medium chain fatty acids'. *American Chemical Society 247, Dallas, TX*

SCIENTIFIC POSTERS

R Fernandez-Moya, J Cardenas, C Leber, NA Da Silva (2012) 'Engineering *Saccharomyces cerevisiae* for the synthesis of short-chain carboxylic acids using a type II *FAS* system'. *American Chemical Society 243, San Diego, CA*

ABSTRACT OF THE DISSERTATION

Engineering *Saccharomyces cerevisiae* for the high-level production of free fatty acids.

By

Ruben Fernandez-Moya

Doctor of Philosophy in Chemical and Biochemical Engineering

University of California, Irvine, 2017

Professor Nancy A. Da Silva, Chair

Advances in biotechnology have enabled potential solutions to the oil-dependency of the chemical industry through renewable feedstocks for the production of current compounds, the discovery and production of new compounds with novel properties, and in some cases, the synthesis of chemicals at a lower cost than with traditional processes. The yeast *Saccharomyces cerevisiae* has been widely used in industry to produce ethanol, proteins, nutritional supplements and pharmaceuticals. A recent major focus has been the use of microorganisms to produce biofuels, commodity chemicals and specialty chemicals. Long-chain fatty acids (LCFA), medium-chain fatty acids (MCFA), and polyketides are good targets for the biochemical industry as they supply platform chemicals that can be later derived to a library of final products by chemical catalysis. The focus of our work has been the metabolic engineering of *S. cerevisiae* for the synthesis of free fatty acids of defined length. We constructed a yeast strain devoid of its native fatty acid metabolism and

expressing the heterologous type II fatty acid synthase (FAS) system from *Escherichia coli* and a C14-specific thioesterase. This FAS system increased total fatty acids and shifted the fatty acid profile, increasing the percentage of C14 fatty acids from less than 1% to 33%. Yeast β-oxidation was engineered for MCFA production by a series of pathway interventions. Disruption of two genes, the essential gene *ECI1* for mono-unsaturated fatty acid (mUFA) degradation and *FAA2* for the peroxisomal MCFA activator, led to an increase in C12:1 production from 50 mg/L to 1 g/L in oleic acid medium. CRISPR/Cas9 was used to swap the native *FAS1* and *FAS2* promoters to the strong constitutive *TEF1* promoter to increase expression of the yeast FAS. This resulted in a 3.8-fold increase in the production of total MCFA from glucose media relative to the *faa2*Δ *eci1*Δ strain. LCFA production and secretion was increased by deleting three genes for enzymes involved in fatty acid activation and three genes for enzymes in the fatty acid degradation pathway. Most importantly, a novel approach overexpressing genes for a lipid particle-forming enzyme (*DGA1*) and a triacylglycerol lipase (*TGL3*) further increased the production of secreted LCFA in shake flask culture to 2.2 g/L, the highest by *S. cerevisiae* to date. Finally, we performed preliminary work to increase acetyl-CoA pools for the synthesis of the closely related polyketides by downregulating competing pathways, such as fatty acid synthesis and the glyoxylate shunt. We evaluated downregulation of *FAS1* and *FAS2* using the CRISPR interference (CRISPRi) system, and also a more traditional promoter engineering strategy.

CHAPTER 1:

Introduction

MOTIVATION

Metabolic engineering of microorganisms to convert agricultural waste biomass into fuels and biochemicals is a promising sustainable route to produce 'drop-in' molecules with current required properties, and to produce new compounds with novel characteristics (Langeveld et al., 2010). The United States Department of Agriculture (USDA) projected a 3-fold growth of the global biobased chemicals market from 2010 to 2025, reaching an approximately $500 billion industry (USDA, Market Potential and Projections Through 2025, 2008). Attention has focused on biofuels, but near-term opportunities for biobased products are particularly promising for biobased chemicals (Figure 1.1). The high volume and low-price required for biofuels makes it an unsuitable as a candidate to replace fossil-fuels in the short-term, while high-value lower-volume biorenewable platform chemicals provide a promising market to incentivize the establishment of a robust biobased industry (Bozell and Petersen, 2010).

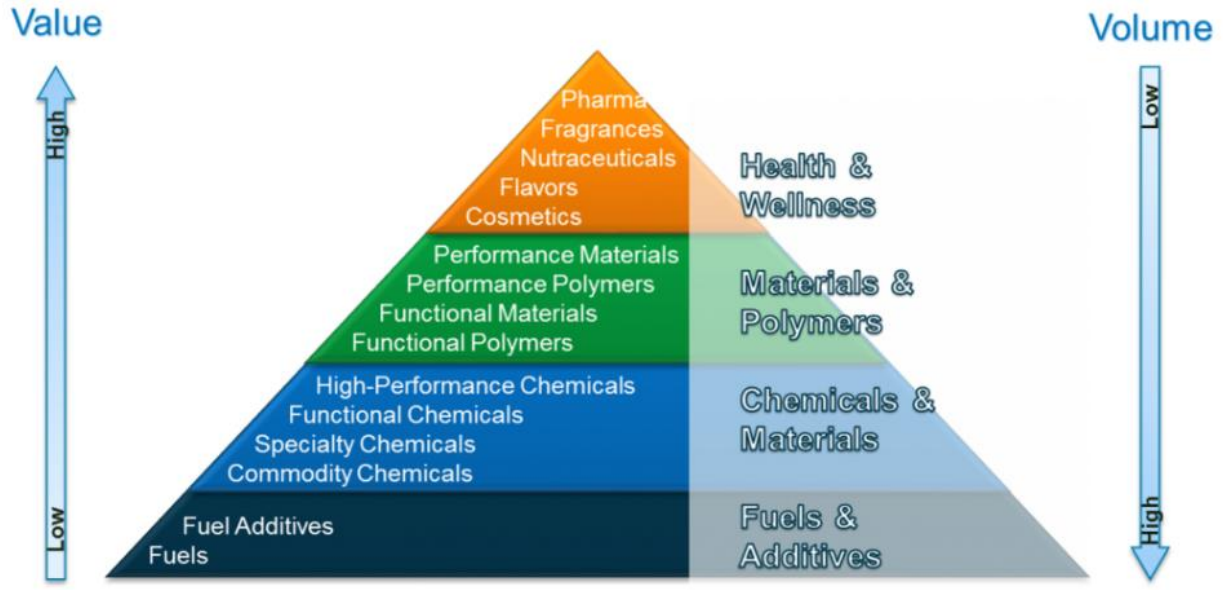

Figure 1.1. Bioproducts market pyramid. (Source: https://www.cbirc.iastate.edu/overview/market-sectors/)

It has been proposed that the most feasible and efficient transition the current chemical industry should undergo is moving towards the production of building block intermediates with multiple functional groups from biological feedstocks (Werpy and Petersen, 2004). Functional replacement of the platform chemicals produced from petroleum should include molecules with highly reduced carbon backbones in conjunction with highly reactive functional groups (Figure 1.2). Polyketides and fatty acid biosynthesis provide a promising solution route to generate a vast library of platform chemicals (Nikolau et al., 2008).

Fatty acids (FA) have been essential to society for centuries, with principal end-uses of FA and FA derivatives ranging from food products to soaps and synthetic organic detergents, rubber compounding, synthetic rubber polymerization, paints, varnishes, etc. (Ruston, 1952), making them well-known in the chemical industry. As an example of potential applications, different strategies to generate thermoplastics and thermosetting polymers from either FA or their derivatives have recently been reviewed (Ronda et al., 2011).

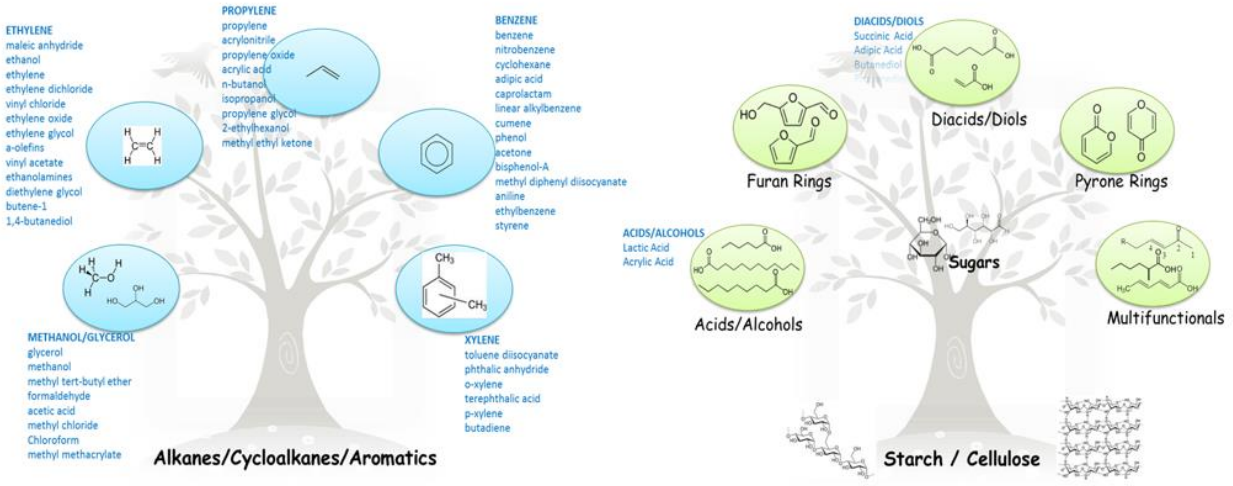

Figure 1.2. Comparison between the current petrochemical industry and the envisioned bio-based industry. (Source: https://www.cbirc.iastate.edu/overview/market-sectors/).

Fatty acid production from renewable feedstocks might be achieved through different available technologies. The most direct but inefficient route is through the extraction of oils from higher plants; however, this has proven to not be economically viable as a substitute for petroleum-based chemicals due to insufficient availability of inexpensive feedstocks, most of them competing with the food supply (Hill et al., 2006). Another route to produce industrial levels of fatty acids to meet demand is by engineering microbes to produce high levels of fatty acids from renewable feedstocks (Ruffing, 2013). Great efforts has been dedicated to the improvement of titers and productivity of lipid production using recombinant microorganisms (Lee et al., 2015; Li et al., 2008). However, microbial production of lipids requires cell lysis and separation, since lipids are intracellular products that do not leave the cell. Therefore, biosynthesis of free fatty acids (LC-FFA) is more desirable and promising. The most common application of long-chain free fatty acids (LC-FFA) is for biofuel production, since they can be easily converted to fatty acid methyl-esters (FAME) or fatty acid ethyl-ester (FAEE) (Vasudevan and Briggs, 2008). Medium-chain free fatty acids (MC-FFA) are particularly interesting due to their potential as platform chemicals, including for direct transformation to α-olefins (Kraus and Riley, 2012) and fatty alcohols (Manyar et al., 2010) through chemical catalysis.

Saccharomyces cerevisiae has been successfully used in industry over many years for applications that range from flavors, fragrances, nutritional supplements, and ethanol to pharmaceuticals (Krivoruchko and Nielsen, 2015; Nielsen et al., 2013). This yeast species has been greatly investigated and characterized (Engel et al., 2014). This has enabled the scientific community to build a large library of molecular biology tools including an array of different promoters (Blazeck et al., 2012; Fang et al., 2011; Partow et al., 2010; Shen et al., 2012), origins of replications providing different copy numbers (Clarke and Carbon, 1980; Futcher and Cox, 1983; Kikuchi, 1983), and

extensive strategies to transform exogenous DNA, manipulate the yeast genome, and express heterologous genes (Jakočiūnas et al., 2016; Da Silva and Srikrishnan, 2012). Human interest in *S. cerevisiae* started with its ability to ferment sugar to ethanol. Alcohol is produced in this yeast for carbon and energy storage, even with an excess of oxygen and glucose (Pronk et al., 1996). A major focus has been engineering *S. cerevisiae* metabolism to redirect the flux toward ethanol to other more valuable biochemical products (Borodina and Nielsen, 2014). In nature, other yeast species exist that accumulate lipids as carbon and energy source instead. They are thus of great interest to the biobased chemical industry; however their metabolism, physiology, and genomic characterization, and molecular biology tools are still limited (Ledesma-Amaro and Nicaud, 2016; Madzak et al., 2004; Papanikolaou and Aggelis, 2011). For this reason, engineering *S. cerevisiae* to produce fatty acids is still of great interest. In addition, the work in *S. cerevisiae* (as a model organism) is often translated successfully to other yeast. Recently, it has been shown that strategies to produce fatty acids, fatty alcohols, fatty acid ethyl esters, and alkanes translated successfully to *Y. lipolityca* (Ledesma-Amaro et al., 2016; Xu et al., 2016), the most studied oleaginous yeast.

Polyketides are complex organic molecules produced by many living organisms via polyketide synthases (PKS). Polyketides are synthesized in a manner analogous to fatty acids, and therefore are many similarities between the polyketide and fatty acid synthases: utilization of the same substrates, similar chemical reactions, and similar structures (Smith and Tsai, 2007). Although the function of the polyketide in the original host is not always known, many of these molecules have been shown to have beneficial pharmacological relevance, while others are known for being toxins or virulence factors (O'Hagan, 1993). The complexity of their structures and their potential as pharmaceuticals have raised interest for more than 50 years (Hertweck, 2009). Recently, polyketides have not only

been recognized as good drug candidates, but also as new unique platform chemicals that could serve the current and future chemical industry (Nikolau et al., 2008).

In this thesis, we focused on the production of fatty acids in the model organism *S. cerevisiae*. Most of the work done on engineering yeast for the synthesis of high-levels of fatty acids has happened in the last decade, and focused mainly on long-chain fatty acids (LCFA) since yeast naturally produces C16 and C18 fatty acid species (Chen et al., 2014; Li et al., 2014; Lian and Zhao, 2015; Runguphan and Keasling, 2014; Zhou et al., 2016a). Fatty acids can be converted to other products either through conventional chemical catalysis or by engineering cells to catalyze those final conversion steps. *S. cerevisiae* has been successfully engineered to produce fatty alcohols (d Espaux et al., 2017; Feng et al., 2015; Guo et al., 2016; Tang and Chen, 2015; Tang et al., 2017; Teixeira et al., 2017; Zhou et al., 2016b), fatty acid ethyl esters (de Jong et al., 2014, 2015, 2016; Shi et al., 2014; Thompson and Trinh, 2014; Valle-Rodríguez et al., 2014), and alkanes/alkenes (Buijs et al., 2015; Kang et al., 2017; Zhou et al., 2016a). However, the research on medium chain fatty acid (MCFA) production is not as extensive as for LCFA, since engineering *S. cerevisiae* to produce shorter fatty acids than it naturally produces is challenging. Production of MCFA is of great interest since they are not as abundant in nature as LCFA, and it would expand the library of potential molecules (and applications) that can be produced from the fatty acid metabolism. The native yeast fatty acid synthase (FAS) is a very large and complex enzyme that requires extensive enzyme engineering to alter its native product (Gajewski et al., 2017; Zhu et al., 2017). Also, MCFA are highly toxic to yeast (Legras et al., 2010; Stevens and Hofmeyr, 1993), adding another level of complexity. Higher yields of FA in yeast are required for economic viability, and to achieve this, upstream pathways to generate acetyl-CoA and downstream pathways involved in fatty acid metabolism need to be optimized, and competing pathways must be downregulated.

OBJECTIVES

The overall goal of this research was to engineer the yeast *S. cerevisiae* for the high-level production of fatty acids, focusing on medium chain fatty acids. The work presented in this dissertation has helped to broaden the scientific knowledge on the engineering of microbes to produce non-native products that can replace current petrochemical compounds, to produce molecules that could serve as new materials precursors or novel pharmaceuticals, and to improve product specifications. The first objective was to reduce the fatty acid average chain length produced in yeast by introducing a heterologous fatty acid synthase. In this study, the type II FAS from *Escherichia coli* (eFAS) was expressed and characterized in S*. cerevisiae*. eFAS was chosen because it is made up of discrete enzymes, which allow for individual expression optimization, and the exposure of intermediates that can be terminated at a specific step. The second objective was to evaluate a completely different approach for the production of MC-FFA. Yeast β-oxidation was engineered to stop fatty acid degradation at a longer chain length (C8, C10 and C12), and further pathway optimization was performed to increase the flux toward β-oxidation. The third objective was to engineer *S. cerevisiae* to produce high-levels of LCFA. Native fatty acid metabolism downstream of the fatty acid synthase was disrupted (β-oxidation and fatty acid activation), and the co-overexpression of neutral lipid-forming enzymes (for triacylglycerides) and lipases resulted in high levels of free fatty acids. The fourth objective was to initiate studies testing new approaches to increase acetyl-CoA pools for acetyl-CoA derived products, including polyketides.

Objective 1:

The aim was to produce MCFA by introducing a heterologous type II FAS from *Escherichia coli* (eFAS) in *S. cerevisiae*. Specific objectives included:

1.1. Testing individual protein expression of essential eFAS genes in *S. cerevisiae* and determination of *in vitro* activity.

1.2. Integration of the genes for the type II FAS into the yeast genome, determination of *in vivo* activity, and characterization of FA distribution of engineered strains.

1.3. Deletion of native yeast fatty acid synthesis machinery in strain with eFAS integrated.

1.4. Production of fatty acids and measurement of FA concentration and profile with only eFAS (native FAS deleted).

Objective 2:

The second aim was to produce MC-FFA by disrupting the pathway for monounsaturated fatty acid degradation and further optimization of MCFA metabolism. Specific objectives included:

2.1. Disruption of a MC-FA specific degradation genes, disruption of a monounsaturated fatty acid degradation gene and targeting a medium-chain thioesterase to the peroxisomes.

2.2. Elimination of cytosolic MCFA elongation activity, and optimization of peroxisomal biogenesis to increase total β-oxidation activity.

2.3. Up-regulation of malonyl-CoA pools/flux and fatty acid synthesis.

2.4. Targeting of a library of medium-chain thioesterases in the most productive strains.

Objective 3:

The aim was to engineer *S. cerevisiae* to produce and secrete high-levels LC-FFA to the medium and avoid re-importation. Specific objectives included:

3.1. Deletion of several long chain acyl-CoA synthetases genes (fatty acid activators).

3.2. Removal of a combination of genes involved in the fatty acid degradation pathway.

3.3. Combination of interventions related to fatty acid activation and degradation.

3.4. Overexpression of endogenous lipid droplet-forming enzymes and endogenous lipases.

Objective 4:

The aim was to evaluate new approaches to increase synthesis of acetyl-CoA derived products in *S. cerevisiae*. Specific objectives included:

4.1. Downregulation of native cytosolic FAS and competing pathways.

4.2. Characterization of heterologous pathways to generate acetyl-CoA from pyruvate and acetaldehyde.

REFERENCES

Blazeck, J., Garg, R., Reed, B., and Alper, H.S. (2012). Controlling promoter strength and regulation in Saccharomyces cerevisiae using synthetic hybrid promoters. Biotechnol Bioeng *109*, 2884–2895.

Borodina, I., and Nielsen, J. (2014). Advances in metabolic engineering of yeast Saccharomyces cerevisiae for production of chemicals. Biotechnol J *9*, 609–620.

Bozell, J.J., and Petersen, G.R. (2010). Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. Green Chemistry *12*, 539.

Buijs, N.A., Zhou, Y.J., Siewers, V., and Nielsen, J. (2015). Long-chain alkane production by the yeast Saccharomyces cerevisiae. Biotechnol Bioeng *112*, 1275–1279.

Chen, L., Zhang, J., Lee, J., and Chen, W.N. (2014). Enhancement of free fatty acid production in Saccharomyces cerevisiae by control of fatty acyl-CoA metabolism. Appl Microbiol Biotechnol *98*, 6739–6750.

Clarke, L., and Carbon, J. (1980). Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature *287*, 504–509.

Engel, S.R., Dietrich, F.S., Fisk, D.G., Binkley, G., Balakrishnan, R., Costanzo, M.C., Dwight, S.S., Hitz, B.C., Karra, K., Nash, R.S., et al. (2014). The reference genome sequence of Saccharomyces cerevisiae: then and now. G3 (Bethesda) *4*, 389–398.

D Espaux, L., Ghosh, A., Runguphan, W., Wehrs, M., Xu, F., Konzock, O., Dev, I., Nhan, M., Gin, J., Reider Apel, A., et al. (2017). Engineering high-level production of fatty alcohols by Saccharomyces cerevisiae from lignocellulosic feedstocks. Metab Eng *42*, 115–125.

Fang, F., Salmon, K., Shen, M.W.Y., Aeling, K.A., Ito, E., Irwin, B., Tran, U.P.C., Hatfield, G.W., Da Silva, N.A., and Sandmeyer, S. (2011). A vector set for systematic metabolic engineering in Saccharomyces cerevisiae. Yeast *28*, 123–136.

Feng, X., Lian, J., and Zhao, H. (2015). Metabolic engineering of Saccharomyces cerevisiae to improve 1-hexadecanol production. Metab Eng *27*, 10–19.

Futcher, A.B., and Cox, B.S. (1983). Maintenance of the 2 microns circle plasmid in populations of Saccharomyces cerevisiae. J Bacteriol *154*, 612–622.

Gajewski, J., Pavlovic, R., Fischer, M., Boles, E., and Grininger, M. (2017). Engineering fungal de novo fatty acid synthesis for short chain fatty acid production. Nat Commun *8*, 14650.

Guo, W., Sheng, J., Zhao, H., and Feng, X. (2016). Metabolic engineering of Saccharomyces cerevisiae to produce 1-hexadecanol from xylose. Microb Cell Fact *15*, 24.

Hertweck, C. (2009). The biosynthetic logic of polyketide diversity. Angew Chem Int Ed Engl *48*, 4688–4716.

Hill, J., Nelson, E., Tilman, D., Polasky, S., and Tiffany, D. (2006). Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci U S A *103*, 11206–11210.

Jakočiūnas, T., Jensen, M.K., and Keasling, J.D. (2016). CRISPR/Cas9 advances engineering of microbial cell factories. Metab Eng *34*, 44–59.

De Jong, B.W., Shi, S., Siewers, V., and Nielsen, J. (2014). Improved production of fatty acid ethyl esters in Saccharomyces cerevisiae through up-regulation of the ethanol degradation pathway and expression of the heterologous phosphoketolase pathway. Microb Cell Fact *13*, 39.

De Jong, B.W., Shi, S., Valle-Rodríguez, J.O., Siewers, V., and Nielsen, J. (2015). Metabolic pathway engineering for fatty acid ethyl ester production in Saccharomyces cerevisiae using stable chromosomal integration. J Ind Microbiol Biotechnol *42*, 477–486.

De Jong, B.W., Siewers, V., and Nielsen, J. (2016). Physiological and transcriptional characterization of Saccharomyces cerevisiae engineered for production of fatty acid ethyl esters. FEMS Yeast Res *16*, fov105.

Kang, M.-K., Zhou, Y.J., Buijs, N.A., and Nielsen, J. (2017). Functional screening of aldehyde decarbonylases for long-chain alkane production by Saccharomyces cerevisiae. Microb Cell Fact *16*, 74.

Kikuchi, Y. (1983). Yeast plasmid requires a cis-acting locus and two plasmid proteins for its stable maintenance. Cell *35*, 487–493.

Kraus, G., and Riley, S. (2012). A Large-Scale Synthesis of α-Olefins and α,ω-Dienes. Synthesis (Mass) *44*, 3003–3005.

Krivoruchko, A., and Nielsen, J. (2015). Production of natural products through metabolic engineering of Saccharomyces cerevisiae. Curr Opin Biotechnol *35*, 7–15.

Langeveld, J.W.A., Dixon, J., and Jaworski, J.F. (2010). Development Perspectives Of The Biobased Economy: A Review. Crop Sci *50*, S–142.

Ledesma-Amaro, R., and Nicaud, J.-M. (2016). Yarrowia lipolytica as a biotechnological chassis to produce usual and unusual fatty acids. Prog Lipid Res *61*, 40–50.

Ledesma-Amaro, R., Dulermo, R., Niehus, X., and Nicaud, J.-M. (2016). Combining metabolic engineering and process optimization to improve production and secretion of fatty acids. Metab Eng *38*, 38–46.

Lee, S.Y., Kim, H.M., and Cheon, S. (2015). Metabolic engineering for the production of hydrocarbon fuels. Curr Opin Biotechnol *33*, 15–22.

Legras, J.L., Erny, C., Le Jeune, C., Lollier, M., Adolphe, Y., Demuyter, C., Delobel, P., Blondin, B., and Karst, F. (2010). Activation of two different resistance mechanisms in Saccharomyces cerevisiae upon exposure to octanoic and decanoic acids. Appl Environ Microbiol *76*, 7526–7535.

Li, Q., Du, W., and Liu, D. (2008). Perspectives of microbial oils for biodiesel production. Appl Microbiol Biotechnol *80*, 749–756.

Li, X., Guo, D., Cheng, Y., Zhu, F., Deng, Z., and Liu, T. (2014). Overproduction of fatty acids in engineered Saccharomyces cerevisiae. Biotechnol Bioeng *111*, 1841–1852.

Lian, J., and Zhao, H. (2015). Recent advances in biosynthesis of fatty acids derived products in Saccharomyces cerevisiae via enhanced supply of precursor metabolites. J Ind Microbiol Biotechnol *42*, 437–451.

Madzak, C., Gaillardin, C., and Beckerich, J.-M. (2004). Heterologous protein expression and secretion in the non-conventional yeast Yarrowia lipolytica: a review. J Biotechnol *109*, 63–81.

Manyar, H.G., Paun, C., Pilus, R., Rooney, D.W., Thompson, J.M., and Hardacre, C. (2010). Highly selective and efficient hydrogenation of carboxylic acids to alcohols using titania supported Pt catalysts. Chem Commun (Camb) *46*, 6279–6281.

Nielsen, J., Larsson, C., van Maris, A., and Pronk, J. (2013). Metabolic engineering of yeast for production of fuels and chemicals. Curr Opin Biotechnol *24*, 398–404.

Nikolau, B.J., Perera, M.A.D.N., Brachova, L., and Shanks, B. (2008). Platform biochemicals for a biorenewable chemical industry. Plant J *54*, 536–545.

O'Hagan, D. (1993). Biosynthesis of fatty acid and polyketide metabolites. Nat Prod Rep *10*, 593–624.

Papanikolaou, S., and Aggelis, G. (2011). Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. European Journal of Lipid Science and Technology *113*, 1031–1051.

Partow, S., Siewers, V., Bjørn, S., Nielsen, J., and Maury, J. (2010). Characterization of different promoters for designing a new expression vector in Saccharomyces cerevisiae. Yeast *27*, 955–964.

Pronk, J.T., Steensma, H.Y., Van Dijken, J. P. (1996). Pyruvate Metabolism in Saccharomyces cerevisiae. Yeast *12, 1607-1633*

Ronda, J.C., Lligadas, G., Galià, M., and Cádiz, V. (2011). Vegetable oils as platform chemicals for polymer synthesis. European Journal of Lipid Science and Technology *113*, 46–58.

Ruffing, A.M. (2013). Metabolic engineering of hydrocarbon biosynthesis for biofuel production. In Liquid, Gaseous and Solid Biofuels - Conversion Techniques, Z. Fang, ed. (InTech),.

Runguphan, W., and Keasling, J.D. (2014). Metabolic engineering of Saccharomyces cerevisiae for production of fatty acid-derived biofuels and chemicals. Metab Eng *21*, 103–113.

Ruston, N.A. (1952). Commercial uses of fatty acids. J Am Oil Chem Soc *29*, 495–498.

Shen, M.W.Y., Fang, F., Sandmeyer, S., and Da Silva, N.A. (2012). Development and characterization of a vector set with regulated promoters for systematic metabolic engineering in Saccharomyces cerevisiae. Yeast *29*, 495–503.

Shi, S., Valle-Rodríguez, J.O., Siewers, V., and Nielsen, J. (2014). Engineering of chromosomal wax ester synthase integrated Saccharomyces cerevisiae mutants for improved biosynthesis of fatty acid ethyl esters. Biotechnol Bioeng *111*, 1740–1747.

Da Silva, N.A., and Srikrishnan, S. (2012). Introduction and expression of genes for metabolic engineering applications in Saccharomyces cerevisiae. FEMS Yeast Res *12*, 197–214.

Smith, S., and Tsai, S.-C. (2007). The type I fatty acid and polyketide synthases: a tale of two megasynthases. Nat Prod Rep *24*, 1041–1072.

Stevens, S., and Hofmeyr, J.-H. (1993). Effects of ethanol, octanoic and decanoic acids on fermentation and the passive influx of protons through the plasma membrane of Saccharomyces cerevisiae. Appl Microbiol Biotechnol *38*.

Tang, X., and Chen, W.N. (2015). Enhanced production of fatty alcohols by engineering the TAGs synthesis pathway in Saccharomyces cerevisiae. Biotechnol Bioeng *112*, 386–392.

Tang, X., Feng, L., Chen, L., and Chen, W.N. (2017). Engineering*Saccharomyces cerevisiae* for Efficient Biosynthesis of Fatty Alcohols Based on Enhanced Supply of Free Fatty Acids. ACS Omega *2*, 3284–3290.

Teixeira, P.G., Ferreira, R., Zhou, Y.J., Siewers, V., and Nielsen, J. (2017). Dynamic regulation of fatty acid pools for improved production of fatty alcohols in Saccharomyces cerevisiae. Microb Cell Fact *16*, 45.

Thompson, R.A., and Trinh, C.T. (2014). Enhancing fatty acid ethyl ester production in Saccharomyces cerevisiae through metabolic engineering and medium optimization. Biotechnol Bioeng *111*, 2200–2208.

Valle-Rodríguez, J.O., Shi, S., Siewers, V., and Nielsen, J. (2014). Metabolic engineering of Saccharomyces cerevisiae for production of fatty acid ethyl esters, an advanced biofuel, by eliminating non-essential fatty acid utilization pathways. Applied Energy *115*, 226–232.

Vasudevan, P.T., and Briggs, M. (2008). Biodiesel production--current state of the art and challenges. J Ind Microbiol Biotechnol *35*, 421–430.

Werpy, T., and Petersen, G. (2004). Top value added chemicals from biomass. Volume I. Results of screening for potential candidates from sugar and synthesis gas. . National Renewable Energy Laboratory. US Department of Energy.

Xu, P., Qiao, K., Ahn, W.S., and Stephanopoulos, G. (2016). Engineering Yarrowia lipolytica as a platform for synthesis of drop-in transportation fuels and oleochemicals. Proc Natl Acad Sci U S A *113*, 10848–10853.

Zhou, Y.J., Buijs, N.A., Zhu, Z., Qin, J., Siewers, V., and Nielsen, J. (2016a). Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. Nat Commun *7*, 11709.

Zhou, Y.J., Buijs, N.A., Zhu, Z., Gómez, D.O., Boonsombuti, A., Siewers, V., and Nielsen, J. (2016b). Harnessing Yeast Peroxisomes for Biosynthesis of Fatty-Acid-Derived Biofuels and Chemicals with Relieved Side-Pathway Competition. J Am Chem Soc *138*, 15368–15377.

Zhu, Z., Zhou, Y.J., Krivoruchko, A., Grininger, M., Zhao, Z.K., and Nielsen, J. (2017). Expanding the product portfolio of fungal type I fatty acid synthases. Nat Chem Biol *13*, 360–362.

CHAPTER 2:

Literature overview

Fernandez-Moya, R., and Da Silva, N.A. (2017). Engineering Saccharomyces cerevisiae for high-level synthesis of fatty acids and derived products. FEMS Yeast Res *17*.

ABSTRACT

Fatty acids and fatty acid derivatives are important biorenewable products, as well as precursors for further transformation via chemical catalysis. This minireview focuses on recent advances in increasing the production of fatty acids and derived products in the yeast *Saccharomyces cerevisiae*. The engineering of upstream pathways to increase levels of the required precursors, fatty acid synthase systems to increase expression and to modify chain length, and downstream pathways to produce free fatty acids, fatty acid ethyl esters, fatty alcohols, and alkanes are highlighted, and current challenges are discussed.

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INTRODUCTION

Accelerating concern about the pace of climate change, depletion of oil reserves, and the environmental impact of production methods, has led to increased interest in renewable routes for generation of the large fossil fuel-based library of chemicals. Microbial processes allow use of renewable resources for the synthesis of bio-based products or precursors for subsequent conversion via chemical catalysis (de Jong et al. 2012). In addition, microbial-based synthesis enables the discovery and production of new compounds with novel functionalities and properties. The US Department of Energy published a list of promising bio-based products that have potential as platform chemicals for conversion to other molecules (Werpy & Petersen 2004; Bozell & Petersen 2010). Microbial polyketide/fatty acid metabolism has also been noted as an excellent source of promising products and precursor compounds (Nikolau et al. 2008; Chia et al. 2012). Fatty acids (FA) and fatty acid derivatives, such as fatty alcohols, fatty acid methyl/ethyl esters, alkenes, and hydroxy fatty acids, are important platform chemicals in the synthesis of biofuels, lubricants, perfumes, plastics, and other products (Biermann et al. 2011).

FA are macromolecules essential for many cellular processes, including building the phospholipid bilayer in the cell membrane, storing energy, and signaling (Trotter 2001). Free fatty acids (FFA) are present at low concentrations as most fatty acids are bound to proteins, cofactors or other functional groups; this protects the cell from perturbation of the cell membrane and proteins by the fatty acids. However, FFAs are often the desired form for conversion to fatty acid derivatives, for conversion by chemical catalysis to other products, and for their ability to cross the cell membrane and simplify recovery. Producing high levels of fatty acids requires large amounts of acetyl-CoA, ATP and NADPH, making it challenging to engineer (Tehlivets et al. 2007). Depending on the acyl-chain length, fatty acids can be classified in four groups. Very long-chain fatty acids (VLCFAs) have an acyl-

chain of 22 or more carbons and are used as food supplements (Rezanka 1989). Long-chain fatty acids (LCFAs), with an acyl-chain of 14 to 20 carbon molecules, are used mainly for biofuels, especially biodiesel (Knothe 2010). Medium chain fatty acids (MCFA), 6 to 12 carbon molecules, and short chain fatty acids (SCFAs), from 2 to 4 carbons, are used as precursors for biofuels and platform chemicals (Nikolau et al. 2008; Rude & Schirmer 2009).

Saccharomyces cerevisiae is a promising microorganism for the synthesis of fatty acids and fatty acid-derived products, and high titers of free fatty acids have been obtained (Leber et al. 2015; Zhou et al 2016b). Furthermore, successful interventions to increase synthesis of FFAs and fatty acid derivatives in *S. cerevisiae* can be assessed in the oleaginous yeast that have evolved to store large amounts of lipids as carbon and energy sources. This minireview focuses on the engineering of *S. cerevisiae* for the synthesis of free fatty acids and their derivatives, with primary emphasis on the recent literature demonstrating successful metabolic engineering strategies. We will first review upstream interventions that increase the substrates and cofactors needed for high-level production, and strategies that increase fatty acid synthase expression or modify FA chain length. We will then consider downstream interventions that lead to the synthesis of free fatty acids, fatty alcohols, alkanes, and other products.

ENGINEERING UPSTREAM PATHWAYS AND FATTY ACID SYNTHASE SYSTEMS

S. cerevisiae produces most fatty acids via a cytosolic type I fatty acid synthase (FAS). The primary initiator substrate for FAS is acetyl-CoA, and malonyl-CoA (generated through the carboxylation of acetyl-CoA) is the substrate for chain elongation (Leibundgut et al. 2008). Two reactions of this elongation cycle require NADPH. High-level synthesis of fatty acids and fatty acid derivatives thus requires sufficient acetyl-CoA, malonyl-CoA, and NADPH. *S. cerevisiae* can only produce saturated and monounsaturated fatty acids, as there is one Δ9-desaturase, Ole1 (Stukey et al. 1989). The common fatty acid profile of yeast thus consists of a mix of saturated and unsaturated carboxylic acids (C16, C16:1, C18, C18:1), with only 1-2% of the total fatty acids further elongated to VLCFA via membrane bound elongases in the endoplasmic reticulum or mitochondria (Welch & Burlingame 1973).

To increase the synthesis of fatty acids and fatty acid derivatives in *S. cerevisiae*, a wide range of metabolic engineering efforts have targeted the native yeast pathways or introduced heterologous pathways to increase precursor and cofactor availability. Several efforts have also focused on the FAS system (including replacement and overexpression) to increase the amount of the acyl-CoA product, to produce free fatty acids, and to modify the dominant chain length of the FA synthesized.

Increasing precursor and cofactor availability

A variety of strategies have been implemented to increase the availability of the acetyl-CoA and malonyl-CoA substrates for FA synthesis (Figure 2.1). These include engineering of central carbon metabolism to increase flux to pyruvate by down-regulating or eliminating competing pathways, and the overexpression of endogenous or heterologous genes that lead to pyruvate, acetyl-CoA, and malonyl-CoA. Efforts have also focused on increasing cytosolic NADPH levels, and modification of

yeast regulation to increase FA synthesis.

Increasing availability of malonyl-CoA

Malonyl-CoA is the extender unit for fatty acid and polyketide elongation, and the synthesis of this substrate is considered a critical limiting step (Liu et al. 2010). Conversion of acetyl-CoA to malonyl-CoA is catalyzed by the native yeast 250 kDA acetyl-CoA carboxylase (Acc1) and requires ATP (Roggenkamp et al. 1980). Thus, strategies have focused on increasing Acc1 levels and mutagenesis of Acc1 to maintain activity. In a study by Shin et al. (2012), overexpression of the native *S. cerevisiae ACC1* gene increased fatty acids and sterol levels by a factor of 4.0 and 1.7, respectively. Similarly, Runguphan & Keasling (2014) expressed *ACC1* on a multi-copy plasmid and observed an approximately 1.5-fold increase in total fatty acid and lipid content. In an alternate approach to increase acetyl-CoA carboxylase activity, an *Arabidopsis* acetyl-CoA carboxylase gene (*AAE13*) was overexpressed in *S. cerevisiae* (Wang et al. 2014), resulting in a 1.6-fold increase in lipid content. This heterologous malonyl-CoA synthetase was able to complement a yeast strain with a temperature sensitive *acc1* mutant.

The Acc1 protein is negatively regulated by Snf1 in *S. cerevisiae.* Activation of Snf1 is triggered by glucose depletion leading to partial deactivation of Acc1 via phosphorylation at one or more serine residues (Woods et al. 1994). The decrease in active acetyl-CoA carboxylase when glucose is depleted thus leads to a drop in the pool of cytosolic malonyl-CoA. Based on prior studies with the mammalian counterpart, the site-directed mutagenesis of specific serine residues has been shown to diminish Snf1 deactivation of Acc1 in three independent studies (Choi & Da Silva 2014; Hofbauer et al. 2014; Shi et al. 2014a). Two of the studies considered the effects on fatty acid production and either fatty ethyl ester (FAEE) or polyketide production. In both cases, increased expression of wild type Acc1 did not result in significant increases in FA levels. However, overexpression of the mutant Acc1 increased

the acetyl-CoA carboxylase activity and led to 3-fold increases in both polyketide and total FA titers (using $Acc1^{S1157A}$; Choi & Da Silva, 2014) and a 0.65-fold increase in total FA titer and a 2.36-fold increase in FAEE when co-expressed with a wax ester synthase (WS) (using $Acc1^{S659A,S1157A}$; Shi et al. 2014a). Acetyl-CoA carboxylase overexpression has recently been combined with several other interventions (Feng et al. 2015; Sheng et al. 2016; Zhou et al. 2016b; Guo et al. 2016; de Jong et al. 2015; Yu et al. 2017; d'Espaux et al. 2017) generally increasing FA or fatty acid-derived products levels, although limited effects or even decreases in total FA production have also been observed (Li et al. 2014). In Choi and Da Silva (2014) and Shi et al. (2014a), the greatest increase in FA was for C18:1. Interestingly, this resulted in increased resistance to the toxicity of exogenous hexanoic acid, octanoic acid, 2-propanol, and *n*-butanol (Besada-Lombana et al. 2017). Cells expressing $Acc1^{S1157A}$ had altered membrane composition and greater membrane integrity, demonstrating that this change could increase resistance for strains producing high levels of short/medium chain fatty acids.

In a recent alternate approach (d'Espaux et al. 2017), the mitochondrial acetyl-CoA carboxylase Hfa1 was overexpressed in the cytosol; the native N-terminal mitochondrial targeting signal was replaced with the Acc1 N-terminal sequence and gene expression was under the control of the strong *GAL1* promoter. Fatty alcohols levels increased approximately 2.5-fold relative to the parent strain.

Engineering central carbon metabolism to increase pyruvate and acetyl-CoA pools

Acetyl-CoA is also critical as it is the starter unit for FA synthesis, and the precursor for synthesis of malonyl-CoA by the Acc1 enzyme. The pyruvate dehydrogenase by-pass in *S. cerevisiae* supplies the cell with the required cytosolic acetyl-CoA (Pronk et al. 1996). Pyruvate gets decarboxylated to acetaldehyde and carbon dioxide by the pyruvate decarboxylase. The acetaldehyde can be converted into ethanol by an alcohol dehydrogenase, or it can be transformed

by an acetaldehyde dehydrogenase (primarily Ald6) to produce acetate and NADPH. Acetate is then converted to acetyl-CoA by acetyl-CoA synthetase (Acs1) in an energy-consuming reaction that requires coenzyme A and two ATP equivalents. An early study increasing flux through this pathway aimed to increase production of a non-fatty acid product amorphadiene. Shiba et al. (2007) overexpressed Ald6 and a heterologous mutant acetyl-CoA synthase from *Salmonella enterica* ($SeAcs^{L641P}$), which prevents deactivation by acetylation (Starai et al. 2005); these interventions increased amorphadiene titer 1.9-fold. Feng et al. (2015) increased the production of a fatty alcohol (1-hexadecanol) in *S. cerevisiae* by overexpressing Adh2, Ald6, $SeAcs^{L641P}$, and Erg10, leading to a 69% increase in the final product titer. In a similar approach, Adh2 was overexpressed in conjunction with Ald6 and $SeAcs^{L641P}$ to increase production of fatty acid ethyl esters; the FAEE specific titer increased 77% (de Jong et al. 2015).

Another study aimed to increase FA synthesis by decreasing carbon flux to ethanol. Li et al. (2014) disrupted *ADH1*, resulting in an extremely slow-growing strain that required several rounds of adaptive mutagenesis to rescue cell growth. While the final evolved strain could not grow as rapidly as the parent strain, it produced 2.1-fold more fatty acids. In the same study, an alternate approach introduced either the *E. coli* NAD^+-dependent alcohol dehydrogenase AdhE that converts the ethanol to acetyl-CoA or the *E. coli* NAD^+-dependent acylating acetaldehyde dehydrogenase EutE that converts acetaldehyde to acetyl-CoA. The strain expressing EutE increased the production of fatty acids by 17% relative to the parent strain.

Upstream interventions to increase the level of pyruvate can also lead to increased production of FA and FA-derived products. Ghosh et al. (2016) used two-scale ^{13}C metabolic flux analysis to guide interventions to increase FA production, and found that glycerol-3-phosphate dehydrogenase (Gpd1) competes for carbon flux. The deletion of *GPD1* by the authors led to a 22%

increase in FA production compared to the parent strain. When this intervention was combined with the overexpression of the *Y. lipolytica* ACL (see below) the fatty acid titer increased 56% relative to the parent strain.

The phosphoketolase pathway was introduced into *S. cerevisiae* to increase the production of fatty acid esters, as this requires a large pool of NADPH and acetyl-CoA (de Jong et al. 2014). A xylulose-5-phosphate phosphoketolase (XpkA) from *Aspergillus nidulans* was expressed in combination with a phosphotransacetylase (Pta) from *Bacillus subtilis* or an acetate kinase (Ack) from *A. nidulans*. FAEE increased 5.7-fold when XpkA and Pta were coexpressed with wax synthase WS2 from *Marinobacter hydrocarbonoclasticus* (105 µg/gDCW) relative to the strain expressing WS2 alone. A combination of XpkA and Ack had a minimal effect on FAEE production.

Introduction of ATP-citrate lyase

ATP-citrate lyase (ACL) catalyzes the conversion of citrate to acetyl-CoA and oxaloacetate using one molecule of ATP (Takeda et al. 1969). This cytosolic enzyme is present in many fungi (Hynes & Murray 2010); however, in the sequenced members of *Saccharomycetes*, it has only been found in the yeast *Y. lipolytica*. Expression of ACL has thus been evaluated as a strategy to increase the pool of cytosolic acetyl-CoA in *S. cerevisiae*, by utilizing citrate from the peroxisome and/or the mitochondria.

Tang et al. (2013) expressed the ACL from *Mus musculus* in *S. cerevisiae* and increased total FA production by 15.4% and 21.1% in the wild type and engineered (*idh1D* and/or *idh2D*) strains, respectively. Deletion of these two mitochondrial isocitrate dehydrogenases increased the intracellular level of citrate 4- to 5-fold. 1-hexadecanol production was compared for strains overexpressing ACL enzymes from *Arabidopsis thaliana* (AtACL) and from *Y. lipolytica* (YlACL) (Feng et al. 2015). The strain expressing AtACL produced 55% more 1-hexadecanol than the parent strain,

while the strain expressing YlACL produced 136% more than the parent. In a recent paper (Zhou et al. 2016b), the authors created a cycle to transport acetyl-CoA from the mitochondria to the cytosol by combining overexpression of ACL and the endogenous mitochondrial citrate transport protein Ctp1, relocation of the peroxisomal malate dehydrogenase Mdh3 into the cytosol, and expression of a heterologous malic enzyme from *Rhodospuridium toruloides*, which provides a source of NADPH in the cytosol. ACLs from different organisms were expressed and free fatty acid production was compared in a strain expressing the *E. coli* tesA' thioesterase. The ACL from *M. musculus* (MmACL) resulted in approximately 11% more FFA than the ACL from *R. toruloides*. Implementation of this 'citrate lyase cycle' (with MmACL) in a strain previously engineered to secrete fatty acids increased FFA production by approximately 18%. More recently, Ghosh et al. (2016) measured the ^{13}C metabolic fluxes of *faa1D faa4D S. cerevisiae* strains overexpressing *ACC1*, *FAS1*, and *FAS2*. Integration of the *Y. lipolytica* ACL gene did not increase the production of FFA; however, the combination of ACL with a *GPD1* deletion increased FFA levels by 35%. The metabolic flux measurements indicated an increase in the flux towards acetyl-CoA, which was offset by an increase in malate synthase consumption of acetyl-CoA. Downregulation of the malate synthase gene *MLS1* had minimal effects on increasing FA production.

Strategies to increase NADPH availability

Sufficient availability of NADPH is also critical as two molecules are required for each elongation cycle during FA synthesis. In oleaginous fungi, the malic enzyme plays the key role in providing NADPH for fatty acid synthesis as well as desaturation (Wynn et al. 1999). However, redirection of the *S. cerevisiae* mitochondrial malic enzyme to the cytosol or expression of a heterologous malic enzyme did not prove effective at increasing cytosolic levels of NADPH (Moreira dos Santos et al. 2004) or production of fatty alcohols (Runguphan & Keasling 2014). The expression

of a heterologous $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GapN) from the bacteria *Streptococcus mutans* was evaluated as an alternate strategy to increase the pool of NADPH for the synthesis of FA-derived products (Shi et al. 2014b). When expressed in combination with the endogenous NAD^+-dependent GapDH in a strain engineered to produce FAEEs, no increase in FAEE levels was observed. Recently, the native *TDH3* gene was replaced by two heterologous enzymes, the $NADP^+$-dependent GapDH from *Kluyeromyces lactis* or the non-phosphorylating GapN from *Bacillus cereus* (d'Espaux et al. 2017); however fatty alcohol levels did not increase relative to the parent strain. The interventions to increase NADPH via heterologous enzymes have thus had limited or no effect on the synthesis of FA derivatives. A recent metabolic flux analysis (d'Espaux et al. 2017) determined that 60% of the cytosolic NADPH was consumed by the $NADP^+$-dependent glutamate dehydrogenases, Gdh1 and Gdh3. Deletion of the native *GDH1* gene led to a 2.7-fold increase in fatty alcohol levels.

Engineering yeast regulation to increase FA and FA-derived products

Yeast lipid metabolism involves a broad number of reactions that are tightly regulated at the transcriptional and post-transcriptional level (Nielsen 2009), making the system difficult to engineer. Therefore, strategies to increase FA synthesis have included the knockout of negative regulators reported to down-regulate lipid synthesis. Feng et al. (2015) looked at two families of transcriptional regulators, those involved with regulation of *INO1* and the protein kinase Snf1. The Upstream Activating Sequence (UAS) of *INO1* is also found upstream of many genes associated with the phospholipid biosynthesis pathway (Lopes et al. 1991). Individual deletions of the *INO1* regulators Pah1, Rpd3, Sin3, Opi1, Ume6, Itc1, Isw2, and Mot1 led to mixed results. Four of the knockouts (*pah1Δ*, *rpd3Δ*, *mot1Δ*, or *opi1Δ*) increased the production of 1-hexadecanol 60-170% relative to the parent strain, with the highest titer (122 mg/L) observed for *rpd3Δ*. The other four knockouts (*ume6Δ*,

itc1Δ, *sin3Δ*, or *iswΔ2*) decreased the production of this C16 fatty alcohol. Deletion of the transcriptional regulator *SNF1* in a strain overexpressing *ACC1* did not increase the production of 1-hexadecanol. Sheng et al. (2016) used the same strategy to increase the production of medium-chain fatty alcohols but found that the deletion of *RPD3* did not increase titer. In the recent work by d'Espaux et al. (2017), deletion of *RPD3* or *OPI1*, and overexpression of the positive regulator *INO2*, failed to increase fatty alcohol levels relative to the parent strain (d'Espaux et al. 2017). In another study (Teo et al. 2015), *OPI1* and *RPD3* were deleted to increase fatty acyl-CoA levels; the *opi1Δ* strain expressing a wax ester increased production of FAEE by 2.7-fold, while *rpd3Δ* slightly decreased FAEE levels.

Overexpression of native and heterologous fatty acid synthases

S. cerevisiae has a cytosolic type I fatty acid synthase (FAS) and a mitochondrial type II FAS (Dittrich et al. 1998). The major role of the mitochondrial FAS is to produce octanoic-ACP, which is a precursor of lipoic acid (Brody et al. 1997). However, the majority of fatty acids are produced by the cytosolic FAS, a large multifunction $\alpha_6\beta_6$ heterododecamer encoded by two genes: *FAS1* (for the β subunit) and *FAS2* (for the α subunit). Unlike the mammalian and bacterial FAS, the yeast FAS does not have a thioesterase subunit; instead a malonyl/palmitoyl transferase (MPT) transfers the acyl-moieties to coenzyme A. Therefore, the product is a saturated acyl-chain (primarily C16 and C18) bound to CoA (Sumper et al. 1969), not a FFA. MPT shows a broad specificity for fatty acids and is not the main factor in the final chain length of the product. The final FA profile depends on the equilibrium of substrates and cofactors, as well as difference in substrate preferences by the catalytic subunits of FAS (Tehlivets et al. 2007).

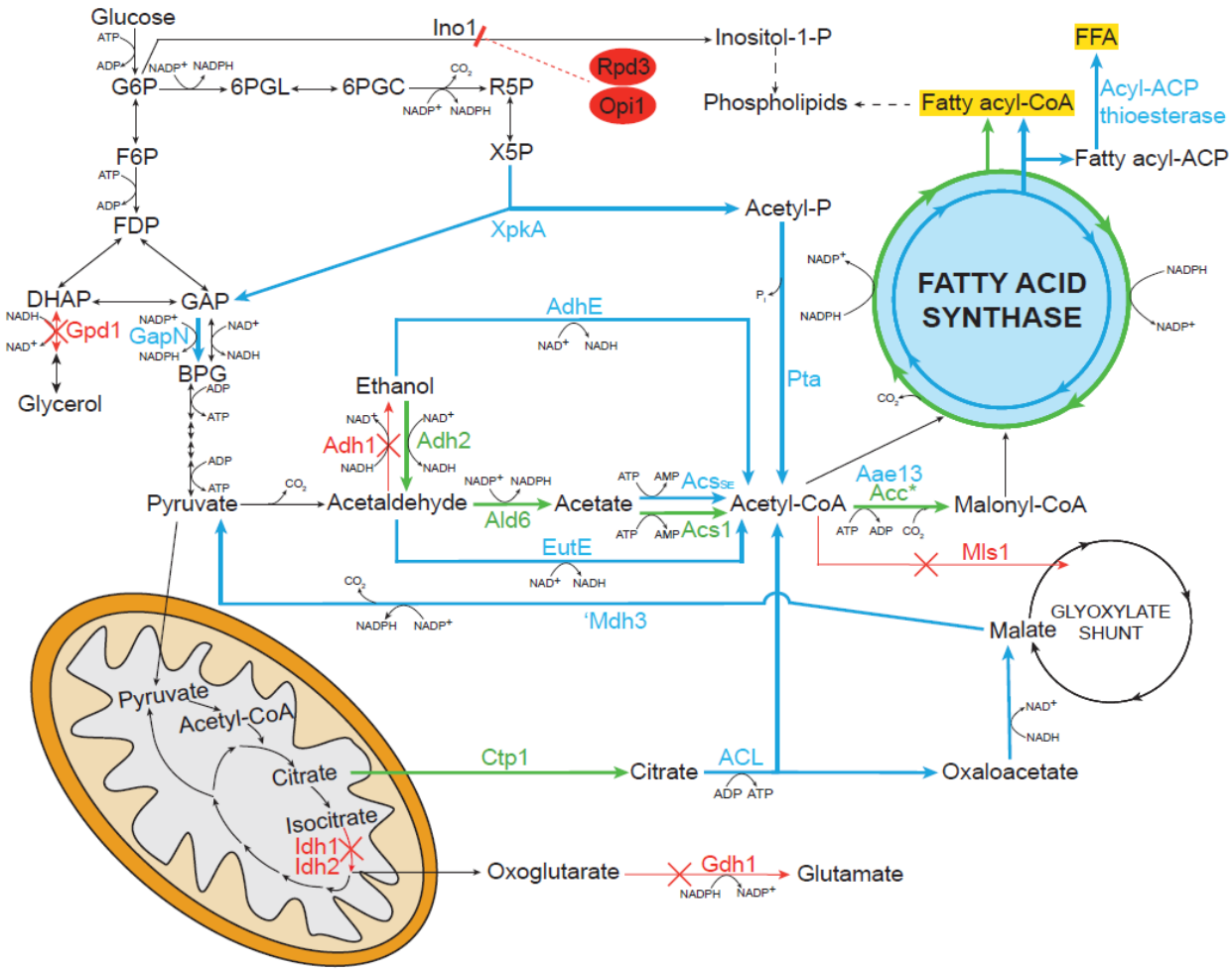


Figure 2.1. Engineering of upstream pathways and fatty acid synthase systems**.** Pathways have been engineered to increase the substrates and cofactors required for the synthesis of fatty acids, and to increase fatty acid synthase expression or modify fatty acid chain length. The native yeast pathways are shown in black, with downregulation/deletion in red and upregulation in green. Heterologous proteins and pathways are shown in blue. Acc* includes native Acc1, Acc1 variants, and the cytosolic variant of Hfa1.

To increase the synthesis of fatty acids and to modify chain length, several recent studies have focused on both the native and heterologous FAS enzymes (Figure 2.1). The level of FAS expression, the source of the FAS enzyme(s), and the mechanism for cleaving the acyl chain all affect the level, form, and chain length of the fatty acid formed.

Overexpression of FAS enzymes for increased production

Equimolar amounts of Fas1 and Fas2 subunits are found in the yeast fatty acid synthase, but regulation of the two genes is not identical (Chirala 1992; Schüller et al. 1994). While both *FAS1* and *FAS2* have an ICRE upstream activation site and respond similarly to general transcription factors, cytoplasmic FAS levels are determined primarily by the amount of Fas1 protein (Wenz et al. 2001). A down-stream regulatory site for the *FAS2* gene is positively regulated by Fas1. The overexpression of only *FAS1* led to an increase in the specific activity of FAS while overexpression of only *FAS2* did not. When both genes were overexpressed, FAS specific activity was even higher than for *FAS1* alone. In addition to transcriptional regulation, the levels of the unassembled FAS subunits in the cytosol are also controlled post-translationally by two distinct degradation pathways for the two subunits (Schüller et al. 1992; Egner et al. 1993).

Interestingly, when either *FAS1* or *FAS2* were overexpressed in a wild type strain, the amount of fatty acids produced decreased, and the FA profile showed an increase in the percent of C16 (Shin et al. 2012). In contrast, overexpression of only *FAS1* in a high sterol producer strain increased both C16 and C18 fatty acids by 2.5-fold. Runguphan & Keasling (2014) replaced the native *FAS1*, *FAS2*, and *ACC1* promoters with the strong constitutive *TEF1* promoter to increase the titers of FFAs, fatty alcohols, and fatty acid ethyl esters. In a strain expressing the *E. coli* tesA' thioesterase, strong constitutive expression of the three genes increased the level of secreted FFAs approximately 11-fold. In *faa1Δ* and *faa1Δfaa4Δ* strains (lacking the Faa1 and/or Faa4 activators and producing higher levels of FFAs), overexpression of *FAS1*, *FAS2*, and *ACC1* increased secreted FFA titers by 1.35-fold and 1.94-fold, respectively. Overexpression of the three genes in a strain expressing a fatty acyl-CoA reductase (mFAR1) improved fatty alcohol levels by 96%. In the same study, overexpression of the three genes increased fatty acid ethyl ester levels 4- to 5-fold in three different engineered yeast

strains.

Research has also focused on the introduction of heterologous fatty acid synthases to increase the synthesis of LCFA, long chain FAEE, and long chain alkenes. Eriksen et al. (2015) expressed four type I FAS enzymes (with their respective phophopantetheine transferases for FAS activation) from *Actinomyces* in *S. cerevisiae*. All heterologous FAS were able to complement growth in a *fas1Δ* strain, and produced a similar FA profile with C16:0, C16:1, C18:0 and C18:1 as the predominant FA molecules. In a *fas1Δ* yeast strain, overexpression of the Type I FAS from *Corynebacterium ammoniagenes* resulted in an approximately 1.5-fold increase in C16:1 relative to the wild type FAS. In a strain with the intact native FAS and expressing a wax synthase/acyl-coenzyme A:diacylglycerol (WS/DGAT) and the native fatty acid activator *FAA1*, expression of this *C. ammoniagenes* FAS increased FAEE titer 6-fold relative to the strain expressing WS/DGAT alone.

In more recent study, the FAS from *Rhodospuridium toruloides* was expressed in a strain engineered to produce and secrete high-levels of fatty acids (Zhou et al. 2016b). The genomic integration of the RtFAS genes under the control of the *TEF1* promoter increased FFA production from 0.80 g/L to 0.92 g/L. However, a comparison of overexpression of the native FAS and overexpression of RtFAS showed no significant difference in FFA levels.

FAS replacement and modification to alter FA profile

S. cerevisiae produces primarily C16, C16:1, C18, and C18:1 fatty acids (Sumper et al. 1969). However, there is great interest in producing specific chain lengths for industrial applications, particularly short/medium chain fatty acids. The closed and complex structure of the type I yeast FAS does not allow the entry of an independent thioesterase (TE). Thus, initial efforts at engineering yeast to produce FA of defined length focused on the introduction of heterologous FAS enzymes. A type I

human FAS (hFAS) was expressed in *S. cerevisiae* in combination with several medium chain thioesterases (Leber & Da Silva 2014). The mammalian FAS has an open and flexible structure that facilitates the diffusion of heterologous TEs (Maier et al. 2010). In addition, the mammalian FAS has a dedicated TE domain on the C-terminus, that is isolated from the core scaffold. Replacement of the hFAS TE domain by independent or linked heterologous thioesterases allowed the early cleavage of elongating acyl-ACP. A strain expressing the hFASDTE, the *B. subtilis* phosphopantetheinyl transferase (PPT), and the medium chain TE from rat mammary gland (*TEII*) produced 17-fold more octanoic acid (C8) than the control strain. When the *TEII* thioesterase was linked to the hFAS, titers increased to 82 mg/L of C8 and a total of 111 mg/L C6 to C10 fatty acids, a 64-fold increase over the wild type strain. Partial codon optimization of the hFAS was shown to substantially improve C6-C10 production in a strain engineered for reduced beta-oxidation activity (as discussed further below) (Leber et al. 2016).

In an alternate approach, the dissociated type II FAS system from *E. coli* was introduced into *S. cerevisiae* (Fernandez-Moya et al. 2015). The type II systems allow more flexibility in fine-tuning the expression of limiting enzymes, and allows introduction of 'terminating' enzymes to produce a wider variety of final products. Eight enzymes (the minimum required for a functional *E. coli* FAS) were integrated into the *S. cerevisiae* genome, and several thioesterases were tested on a multicopy plasmid. Similar to the mammalian FAS, this heterologous type II FAS could complement the knockout of the yeast FAS and support growth. With the *E. coli* FAS system and the *fatB* thioesterase from *Ricinus communis* (leading to accumulation of primarily C14), the housekeeping functions of the native FAS were unnecessary and a *fas2D S. cerevisiae* strain was used. Although growth was slower relative to the parent strain, total FA production increased 1.7-fold and the FA profile shifted, increasing C14 from <1% to 33% of total fatty acids, and substantially decreasing C18 production.

In a related study, two type II FAS systems were recently expressed in *S. cerevisiae* to enable

production of lipoic acid in the cytoplasm (Lian & Zhao 2016). The aim was to increase cytosolic acetyl-CoA pools by expressing the *E. coli* PDH complex, which requires lipoic acid to function. However, in eukaryotes, the lipoic acid precursor, octanoyl-ACP, is produced by the type II FAS in the mitochondria. Addition of the native mitochondrial type II FAS to the cytosol failed to support growth of an *acs1Δacs2Δ* yeast strain expressing the *E. coli* PDH. However, introduction of the type II FAS from *E. coli* provided sufficient octanoyl-ACP for the PDH to be active (indirectly measured by cell growth).

More recently, a type I FAS from *Mycobacterium vaccae* (MvFAS) was expressed in *S. cerevisiae* to increase the production of docosanol (Yu et al. 2017). MvFAS was chosen for its ability to elongate fatty acyl-ACP to C26. Expression of a fusion MvFAS with its native PPT activator complemented growth of *fas1Δfas2Δ* and *fas1Δfas2Δelo2Δelo3Δ* strains, indicating that this heterologous FAS can produce sufficient C16/C18 fatty acids, and can also elongate acyl-ACP further and provide the VLCFA essential for yeast survival.

Recent work has included studies on the direct engineering of the yeast type I FAS to produce medium chain fatty acids. In an innovative study, Xu et al. (2016) engineered the Y. *lipolytica* FAS for MCFA production. The authors then created a truncated *FAS1* gene with the malonyl/palmitoyl transacylase (MPT) replaced by various fused thioesterases. The proximity of the medium-chain thioesterases to the FAS elongation chamber led to an almost 3-fold increase in C14 when tesA' from *E. coli* was fused, and a 6-fold increase in C12 when a thioesterase from *Umbellularia californica* was fused. This approach may prove successful in *S. cerevisiae* as well.

The *R. toruloides* FAS and *Aplanochytrium kerguelense* FAS differ from most fungal FAS, as they have two ACP domains instead of one. Mutants of the *R. toruloides* and *A. kerguelense* FAS were constructed by replacing one of the two ACP domains with a medium chain thioesterase from

Acinetobacter bayly (Zhu et al. 2017). When overexpressed in *S. cerevisiae*, 5-13 times more medium chain FFA was secreted than with the wild-type (unmutated) enzymes, and production of C18 fatty acids decreased. A similar approach was taken with the native *S. cerevisiae* FAS. The thioesterase domain was linked at different positions, either before or after the native ACP domain, or at the end of the α-chain (Fas2) subunit. This strategy increased the production of MC-FFA in the supernatant by 3-fold. Mutations in the KS domain to block elongation further increased production. The best mutant *FAS2* was integrated into a *pox1Δ S. cerevisiae* strain and cultured at pH 6.0; the total MC-FFA reached 175 mg/L, and the final OD was half that of the parent strain. A similar strategy was effective in increasing β-ketoacids, demonstrating the general effectiveness of the approach.

In another recent study (Gajewski et al. 2017b), rationally designed mutations in the condensation domain (KS) and the transferase domains (MPT and AT) of the *S. cerevisiae* FAS led to an increase in the medium chain FFA secreted to the media. The mutations on the FAS were based on their previous work with the *Corynebacterium ammoniagenes* FAS using an *in vitro* and *in silico* approach (Gajewski et al. 2017a). Several single and double point mutations in the KS domain increased C6 production 9- to 12-fold and C8 production 56-fold in a *fas1Δfas2Δ* strain, relative to the wild type with the native FAS. Mutation R1834K in the MPT domain led to a 23-fold increase in the extracellular C8 titer (100 mg/L). The authors combined the most promising mutations to create a final FAS mutant (I306A-G1250S-R1834K) that produced 464 mg/L of total MCFA (primarily C6 and C8) in complex media. The native thioesterases that might be releasing the medium-chain acyl-CoAs produced by the FAS mutants were considered. A *eht1Δeeb1Δmgl2Δ* strain carrying the mutant FAS produced minimal levels of medium chain FFAs compared to the parent strain, which may indicate the role of these previously identified thioesterases in the processing of medium chain acyl-CoAs in *S. cerevisiae.*

ENGINEERING DOWNSTREAM PATHWAYS TO DESIRED PRODUCTS

Utilizing the FAS systems described above, *S. cerevisiae* can be engineered to produce fatty acids of varying lengths, and as either acyl-CoAs or free fatty acids. A large number of studies have focused on the subsequent engineering of downstream pathways (Figure 2.2) to increase titer and yield of these fatty acids and to produce fatty acid derivatives, including fatty acid ethyl esters, fatty alcohols, and alkanes and alkenes.

Downstream pathway interventions to increase fatty acid pools

In *S. cerevisiae*, free fatty acids are quickly activated by acyl-CoA synthase to produce fatty acyl-CoAs (Scharnewski et al. 2008); these acyl-CoAs are essential to cellular metabolism and are used in phospholipid bilayer formation, energy storage and recycling, and signaling functions (Tehlivets et al. 2007). In addition, both CoA-bound and free fatty acids can be degraded via β-oxidation in the peroxisome (Hettema & Tabak 2000). Several strategies have been implemented to either increase the levels of the CoA-bound fatty acids or to increase the levels and secretion of free fatty acids; these include eliminating degradation via β-oxidation, preventing FFA activation, and increasing FFA synthesis (Figure 2.2-A,B).

Elimination of β*-oxidation*

One straightforward intervention to increase FA production is to deactivate the native β-oxidation pathway for these molecules. In *S. cerevisiae*, β-oxidation occurs only in the peroxisomes (Kunau et al. 1988). The first step is the translocation of the acyl-CoA and free fatty acids from the cytosol to the peroxisome. Acyl-CoA molecules can cross the peroxisomal membrane using the ATP binding cassette transporter Pxa1/Pxa2, which forms a complex that allows the transport of the large -CoA bound fatty acids (Hettema et al. 1996). Medium chain free fatty acids are thought to cross the

peroxisomal membrane by simple diffusion and spontaneous flipping (Hettema & Tabak 2000). Once the FFAs enter the peroxisome they are activated to the CoA form by the fatty acid activator Faa2 (localized on the matrix side of the peroxisomal membranes). The β-oxidation cycle for saturated fatty acids in *S. cerevisiae* requires three peroxisomal proteins, Pox1, Fox2, and Pot1 (Hiltunen et al. 2003).

Elimination of Pox1 was evaluated to prevent FA loss by β-oxidation and thus increase FA titers. In strains engineered for increased FA synthesis, deletion of *POX1* had a minimal effect on lipid content and total intracellular FA (Runguphan & Keasling 2014). Similarly, in work by Li et al. (2014) and Leber et al. (2015), the *POX1* deletion alone did not increase FA levels. However, in another study (Valle-Rodríguez et al. 2014), the *POX1* deletion had limited effect on total lipids but increased intracellular FFAs 4-fold. With expression of a wax synthase, 48% more FAEE was produced relative to the parent strain. Other studies have focused on blocking fatty acyl-CoA transport into the peroxisomes and/or the activation of the free fatty acids. Deletion of *PXA2*, encoding a subunit of the peroxisomal long chain acyl-CoA transporter, did not increase FA levels (Runguphan & Keasling 2014). Single knockouts of *FAA2* and *PXA2* resulted in a small increase in the production of FAEE (Thompson & Trinh 2014), while the combination had no effect. (Leber et al. 2015) showed that a combination of three β-oxidation-related gene knockouts increased intracellular FA levels. When *FAA2* and *PXA1* were disrupted, cells accumulated 1.4-fold more FA relative to the parent strain. The deletion of *POX1, FAA2,* and *PXA1* increased the total intracellular FA levels by 1.6-fold. A combination of gene knockouts for both β-oxidation and cytosolic free fatty acid activation (discussed below) have substantially increased free fatty acid levels. In addition, recent work has combined the *POX1* deletion with several other interventions to produce a variety of fatty acid-derived products (de Jong et al. 2015; Zhou et al. 2016b).

Transport to the peroxisome and activation of medium chain fatty acids differs from that for long chain fatty acids. A study by Leber et al. (2016) considered the specific interventions required to decrease degradation of medium chain FFAs in *S. cerevisiae*. A strain (expressing a partially codon-optimized human FAS with a linked medium chain thioesterase) was engineered with three gene deletions: *FAA2*, a peroxisomal medium chain fatty acyl-CoA synthetase, *ANT1*, a peroxisomal ATP transporter necessary for the activation of MCFA (van Roermund et al. 2001), and *PEX11,* a peroxisomal membrane protein implicated for the medium chain fatty acid activation (van Roermund et al. 2000). Hexanoic, octanoic and decanoic acid levels increased 68-, 181-, and 101-fold, respectively, relative to the very low amounts in the parent strain, and 10-, 4-, and 2-fold relative to a strain with the generic β-oxidation gene knockouts: *POX1, FAA2,* and *PXA1*.

Prevention of free fatty acid activation

The reduction of free fatty acid activation to the acyl-CoA form allows both an increase in the pool of FFAs and transport from the cell to the medium. The relevant activation enzymes are the acyl-CoA synthetases Faa1, Faa2, Faa3, Faa4, and Fat1 (Black & DiRusso 2007). Faa1 and Faa4 are the primary enzymes for activation of long chain FFAs (Scharnewski et al. 2008). Faa1 has high activity for carbon chains of 12 to 16, but can activate hexanoic acid and stearic acid as well (Knoll et al. 1994); Faa4 shows overlapping functionalities and activity with Faa1. Both activators account for 99% of the total activation of myristic and palmitic acid. Medium-chain FFAs in *S. cerevisiae* are activated by the peroxisomal Faa2, which shows substrate specificity for C8-C14 (Knoll et al. 1994). Faa3 has significantly lower activity than Faa2 and Faa1, and is not essential for exogenous fatty acid activation (Johnson et al. 1994). Fat1 is a multifunctional enzyme involved in both fatty acid transport and activation of long- and very long-chain FFA (Zou et al. 2002).

Disruption of the fatty acyl-CoA synthase gene *FAA1* increased the amount of fatty acid

secreted to the media by 2-fold, and also increased intracellular FA levels (Michinaka et al. 2003). When both *FAA1* and *FAA4* were disrupted, fatty acid secretion increased (Scharnewski et al. 2008); however, the additional deletion of *FAA2* or *FAA3* did not further increase secreted FFA levels. Other studies with knockouts in *FAA1* or *FAA4* have shown comparable results, leading to an increase in secreted and total FA levels (Li et al. 2014). Leber et al. (2015) included a deletion of the fatty acyl-CoA synthetase gene *FAT1*; the *faa1Δ faa4Δ fat1Δ* strain secreted 60% more long chain FFA relative to the *faa1Δ faa4Δ* parent strain.

Preventing both free fatty acid activation and β-oxidation can further increase extracellular fatty acid levels. In strains expressing a heterologous thioesterase, combined deletions of *FAA4* and *POX1* increased the FFA secreted to the medium by 54% over the *FAA4* deletion alone (Runguphan & Keasling 2014). Li et al. (2014) found that deletion of *FAA1, FAA4,* and *POX1* increased the FFA by 31% relative to deletion of *FAA1* and *FAA4*. In the study by Leber et al. (2015), a strain with six deletions (*faa1Δ faa4Δ fat1Δ* and *pox1Δ faa2Δ pxa1Δ)* produced 1.3 g/L of FFA, 165% higher than with the three activator knockouts alone.

Increasing the synthesis of free fatty acids

In addition to preventing degradation and activation, strategies have focused on increasing synthesis of free fatty acids. An acyl-CoA thioesterase can be expressed to obtain FFA from the acyl-CoA form. Runguphan & Keasling (2014) demonstrated the impact of expressing an independent heterologous thioesterase from *E. coli* ‘tesA (lacking the N-terminal membrane signal) that has both acyl-ACP and acyl-CoA activities. Expression of ‘tesA increased FFA level from 5 mg/L to 164 mg/L in a *faa1Δ* strain, and to 207 mg/L in a *faa1Δ faa4Δ* strain. This approach (‘tesA expression combined with the fatty acid activator disruptions) has recently been implemented in combination with several other interventions (Zhou et al. 2016a). Expression of ‘tesA in a *faa1Δ faa4Δ pox1Δ hfd1Δ* strain

increased the FFA in the medium by 20% compared to the parent strain. When a heterologous FAS from *R. toruloides*, Acc1, and a 'citrate lyase cycle' were overexpressed in this strain, the FFA levels reached 1 g/L in minimal medium. Further culture optimization in a fed-batch bioreactor increased the final titer to 10.4 g/L (the highest reported), although at ~33% lower yield than in batch.

S. cerevisiae has only one known acyl-CoA thioesterase, Tes1 (Jones et al. 1999). This thioesterase is targeted to the yeast peroxisomes and has broad substrate specificity (Maeda et al. 2006). The overexpression of the native Tes1 in a *faa1Δ faa4Δ* strain increased total FFA production from 77 to 115 mg/L (Li et al. 2014). Surprisingly, overexpression of a truncated version of the protein without the peroxisomal targeting signal decreased FA production relative to wild type. In the same study, overexpression of the 'tesA from *E. coli* and a thioesterase from *Cinnamomum camphorum* slightly decreased FA production. Chen et al. (2014) overexpressed the mammalian medium-chain peroxisomal thioesterase Acot5 from *M. musculus* without the native targeting signal (Chen et al. 2014). Alone there was no impact; however, in a *faa1Δ faa4Δ* strain, secreted FFA increased from 327 mg/L to 492 mg/L when Acot5' was overexpressed.

Yeast store fatty acids as neutral lipids, primarily triacylglycerols (TAG) and steryl esters (SE), forming lipid droplets (Leber et al. 1994). In *S. cerevisiae*, TAGs are synthesized by Dga1 and Lro1 from diacylglycerides (DAG) and fatty acyl-CoA (Oelkers et al. 2000; Dahlqvist et al. 2000), and steryl esters are synthesized by Are1 and Are2 from fatty acyl-CoA and sterol (Zweytick et al. 2000). Deletion of *DGA1*, *LRO1*, *ARE1*, and *ARE2* is generally innocuous to *S. cerevisiae* (Sandager et al. 2002), demonstrating that formation of lipid bodies and storage of neutral lipids is non-essential. In an alternate approach to the expression of acyl-CoA thioesterases, Leber et al. (2015) utilized the lipid recycle process to increase the synthesis of free fatty acids from the acyl-CoA form. Triacylglyceride and steryl ester synthesis enzymes were overexpressed in combination with various lipases that

hydrolyze TAGs and SEs. The highest FFA levels were achieved with overexpression of Dga1 (the major contributor to TAG synthesis in *S. cerevisiae*) and Tgl3 (the major TAG lipase). In a strain with FFA activation and β-oxidation impaired, FFA production increased from 1.3 g/L to 2.2 g/L (in shake flask) when overexpressing the proteins for TAG synthesis and recycle.

A distinctly different approach to produce MCFA utilizes a reversal of the β-oxidation pathway. Dellomonaco et al. (2011) created a reverse β-oxidation pathway in *E. coli* to produce alcohols and carboxylic acids of various chain lengths and functionalities. A reverse β-oxidation pathway was recently introduced in the *S. cerevisiae* cytosol to produce n-butanol, medium chain FFA, and medium chain FAEE (Lian & Zhao 2015). A *adh1*Δ*adh4*Δ*gpd1*Δ*gpd2*Δ strain with the reverse β-oxidation pathway and expressing the medium-chain thioesterase, CpFatB1, produced 6.1-fold more octanoic acid (~8 mg/L) relative to the strain expressing CpFatB1 alone.

Introduction of pathways to fatty acid-derived products

The production of carbon chain molecules with functional groups other than carboxylic acids widens the portfolio of biochemicals and materials that can be synthesized via subsequent chemical catalysis. Fatty acids can be converted to fatty-acid derivatives for direct use or use as platform chemicals; these include fatty acid esters, fatty alcohols, and fatty alkanes and alkenes. To obtain fatty acid-derived products in *S. cerevisiae*, heterologous enzymes have been introduced for the conversion of acyl-CoA and/or FFA to the desired compound (Figure 2.2A).

Fatty acid ethyl esters from acyl-CoAs

Fatty acid esters, primarily fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE), constitute a group of molecules with a variety of uses, e.g., as high-value lubricants, cosmetics, pharmaceuticals, and biodiesel (Jetter & Kunst 2008; Westfall & Gardner 2011). Wax esters, formed

by the esterification of a fatty acid and a fatty alcohol, are important storage lipids in some prokaryotes (Wältermann et al. 2007). The enzymes that catalyze their production can also mediate the formation of TAGs, as they are wax ester synthase/acyl-CoA:diacylglycerol acyltransferases (WS/DGATs). Identified WS/DGATs have broad substrate specificity, and several studies have considered the formation of FAEE using these enzymes.

Five heterologous wax ester synthases were expressed and characterized for their substrate specificity in *S. cerevisiae* (Shi et al. 2012). Different substrate preferences for the alcohol (acyl acceptor) were observed *in vitro*, and all increased *in vivo* FAEE production from negligible levels to 1.3-6.3 mg/L. Overexpression of the WS/DGAT from *M. hydrocarbonoclasticus* (WS2) resulted in the highest FAEE titer. In combination with overexpression of Acc1, FAEE production was further increased to 8.2 mg/L. Most subsequent studies have employed this wax synthase. (de Jong et al. 2014) expressed WS2 with enzymes of the PDH bypass (Adh2, Ald6, Acs_{se}^{L641P}) and increased FAEE specific titers 2.7-fold relative to WS2 alone. In combination with a heterologous phosphoketolase pathway (*xpkA* and *pta* from *A. nidulans*), FAEE specific titers increased 1.7-fold relative to WS2 alone. WS2 was also overexpressed in a yeast strain devoid of neutral lipid accumulation (*dga1D, lro1D, are1D, are2D*) and fatty acid degradation, resulting in a final titer of 17.2 mg/L (Valle-Rodríguez et al. 2014). The *ws2* gene was randomly integrated into the δ sequences of *S. cerevisiae* (Shi et al. 2014b). The final strain had an average *ws2* copy number of 5.4 per cell, and produced 6-fold more FAEE (34 mg/L) than the strain expressing *ws2* on a multi-copy plasmid; however, long-term stability of the integrants was an issue. Further engineering of the cells to increase acyl-CoA availability and NAPDH levels increased FAEE titers to 48 mg/L. In another study (Eriksen et al. 2015), a combination of WS2, an activated heterologous type I FAS from *C. ammoniagenes*, and Faa1 overexpression led to a 6.3-fold increase in FAEE production (to 10.5 mg/g DCW).

Alternate wax synthases have also been evaluated. Expression of the WS/DGAT from *Acinetobacter calcoaceticus* increased FAEE production in yeast from nearly negligible levels to 1.2 mg/L (Runguphan & Keasling 2014). Overexpression of *ACC1*, *FAS1*, and *FAS2*, and disruption of β-oxidation, increased FAEE titers to 5.44 mg/L in 0.2% glucose and 1.8% galactose media. Other strategies to increase FAEE have considered the *S. cerevisiae* acyl-CoA binding protein Acb1 in combination with the nonspecific WS/DGAT from *Acinetobacter baylyi* (Atfa). Acb1 binds to acyl-CoAs in the cytosol; this helps to increase solubility and provides protection from hydrolysis by thioesterases (Feddersen et al. 2007). Deletion of *ACB1* in a strain expressing *atfa* increased production of FAEE by 48% (Thompson & Trinh 2014). Interestingly, in a study by Shi et al. (2014b), the overexpression of *ACB1* with *ws2* increased the production of FAEE by 18%. When *atfa* was expressed in wild type and *faa2Δ pxa2Δ acb1Δ* strains (Thompson & Trinh 2014), the use of nitrogen-limited media increased FAEE production approximately 3.5-fold (to 2.9 mg/L and 11.7 mg/L, respectively) relative to non-nitrogen limited media. Expression of *ws2* instead of *atfA* led to a further increase in FAEE levels (13.9 mg/L). In particular, ethyl decanoate levels were substantially higher under nitrogen-limited conditions. In another study with WS2 (Shi et al. 2014a)., coexpression of $Acc1^{S659A,S1157A}$ increased FAEE titer from 4.7 mg/L to 15.8 mg/L.

In an alternate approach, the reversal of β-oxidation in *S. cerevisiae* was used to produce medium-chain FAEE (Lian & Zhao 2015). Strains expressing the full reverse β-oxidation pathway produced 2.8-fold (~0.40 mg/L) and 2.1-fold (~0.25 mg/L) more ethyl octanoate and ethyl decanoate than the parent strain, respectively. Overexpression of endonegous acyl-CoA/ethanol O-acyltransferase genes *EEB1* or *EHT1* failed to increase the medium-chain FAEE titers; this agrees with prior work (Saerens et al. 2006, 2008).

Another study (Teo et al. 2015) focused on producing short- and branched-chain alkyl esters

to improve the properties of biodiesel. *OPI1* and *RPD3* were first deleted to boost the fatty acyl-CoA levels, and isobutanol pathway mitochondrial enzymes were overexpressed to increase the pool of alcohol presursors. Wax ester synthases WS2 and Maqu_0168 from *Marinobacter sp.* were expressed and the strains produced higher levels of fatty acid isoamyl esters, fatty acid isobutyl esters, and fatty acid active amyl ester relative to the parent strain. The two wax ester synthases showed different alcohol specificity; WS2 produced primarily fatty acid isobutyl esters (127 mg/L) and Maqu_0168 produced primarily fatty acid isoamyl esters (151 mg/L). Of the fatty acid esters produced, 92% had a fatty acid component of 16 or 18 carbons.

In *Y. lipolytica*, cytosolic expression of the *A. baylyi* wax ester synthase AtfA had limited effect on FAEE production (Xu et al. 2016). However, when AtfA was targeted to the endoplasmic reticulum and to the peroxisome, the FAEE level increased 15- to 20-fold, respectively. This approach may also prove useful in *S. cerevisiae*.

Table 2.1. Summary of interventions and results for production of long chain free fatty acids, medium chain free fatty acids, and fatty acid ethyl esters

Product (LC-FFA)	Interventions	Media	Scale	Titer (g/L)	%Theor. Yield[3]	Productivity (mg/L·h)	Reference
C16-C18	TesA' Acc1↑ Fas1↑ Fas2↑ *faa1Δ faa4Δ pox1Δ*	CSM	Flask	0.4	5.7	4.2	(Runguphan & Keasling 2014)
C16-C18	EutE *faa1Δ faa4Δ pox1Δ*	MM*[1]	Flask	0.124	1.8	1.7	(Li et al. 2014)
C16-C18	Acot5' *faa1Δ faa4Δ*	CSM	Flask	0.5	7.0	20	(Chen et al. 2014)
C16-C18	Dga1↑ Tgl3↑ *faa1Δ faa4Δ fat1Δ faa2Δ pox1Δ pxa1Δ*	YPD+G418	Flask	2.2	31	45	(Leber et al. 2015)
C16-C18	TesA' RtFAS RtME MmACL 'Mdh3↑ Ctp1↑ Acc1↑ *faa1Δ faa4Δ pox1Δ hfd1Δ*	MM*	Flask	1.0	14	14	(Zhou et al. 2016b)
C16-C18	TesA' RtFAS RtME MmACL 'Mdh3↑ Ctp1↑ Acc1↑ *faa1Δ faa4Δ pox1Δ hfd1Δ*	MM*	Fed-batch	10.4	9.3	87	(Zhou et al. 2016b)

Product (MC-FFA)	Interventions	Media	Scale	Titer (mg/L)	%Theor. Yield[3]	Productivity (mg/L·h)	Reference
C6-C10	humanFAS-TEII fused	YPD	Flask	111	1.5	2.3	(Leber & Da Silva 2014)
C6-C10	humanFAS-TEII fused *faa2Δ ant1Δ pex11Δ*	YPD	Flask	119	1.6	2.5	(Leber et al. 2016)
C6-C10	Reverse β-oxidation CpFatB1 SeAcs^{L641P} *adh1Δ adh4Δ gpd1Δ gpd2Δ*	CSM	Flask	11	0.15	0.24	(Lian & Zhao 2015)
C6-C12	Mutated ScFAS (heterologous TE AcTesA' domain fused) *pox1Δ*	MM*	Flask	175	2.4	1.8	(Zhu et al. 2017)
C6-C10	Mutated ScFAS (I306A, R1834K, G1250S)	YPD	Flask	464	6.3	6.5	(Gajewski et al. 2017b)

Product (FAEE)	Interventions	Media	Scale	Titer (mg/L)	%Theor. Yield[3]	Productivity (mg/L·h)	Reference
C16-C18	WS2 Acc1↑	CSM	Flask	8.2	0.091	ND	(Shi et al. 2012)
C16-C18	AtfA Acc1↑ Fas1↑ Fas2↑ *pxa2Δ pox1Δ*	CSM/d[2]	Flask	5.4	0.077	0.032	(Runguphan & Keasling 2014)
C10-C18	WS2 *faa2Δ acb1Δ pxa2Δ*	CSM/d (high C/N)	Flask	14	0.20	0.29	(Thompson & Trinh 2014)
C10-C18	WS2 *faa2Δ acb1Δ pxa2Δ*	CSM/d (high C/N)	Fed-Batch	25	0.093	0.15	(Thompson & Trinh 2014)
C16-C18	WS2 *are1Δ are2Δ dga1Δ lro1Δ pox1Δ*	CSM	Flask	17	0.25	ND	(Valle-Rodríguez et al. 2014)
C16-C18	WS2 (integrated) SeAcs^{L641P} Adh2↑ Acc1^{S1157A,S659A}↑ Ald6↑ Acb1↑ *are1Δ are2Δ dga1Δ lro1Δ pox1Δ*	CSM	Flask	4.4	0.063	0.044	(de Jong et al. 2015)
C16-C18	WS2 (multiple integrated copies) GapN Acb1↑	CSM	Flask	48	0.69	ND	(Shi et al. 2014b)
C16-C18	WS2 (multiple integrated copies) XpkA Ack (phosphoketolase pathway)	CSM	Flask	5.1 mg/gDCW	ND	ND	(de Jong et al. 2014)
C16-C18	WS2 *opi1Δ*	CSM	Flask (Hi-Den)	33	0.48	0.70	(Teo et al. 2015)
C8-C10	Reverse β-oxidation Eeb1↑ or Eht1↑	CSM/d	Tube	0.75	0.011	0.016	(Lian & Zhao 2015)

[1] Minimal media with vitamins supplement. [2] 10% dodecane layer was added. [3] 0.35 g FFA/g glucose for LCFA and LC-FAEE. 0.37 g FFA/g glucose for MCFA and MC-FAEE.

Fatty alcohols from acyl-CoAs and FFAs

Fatty alcohols are used primarily for soap and detergents, but also for lubricants, personal care products, and emulsifiers (Rupilius & Ahmad 2006). Biosynthesis of fatty alcohols requires fatty acyl-CoA esters or free fatty acids, and an enzyme or group of enzymes to catalyze the two-step reduction of the carboxyl group. The identified enzymes that can perform this reaction in *S. cerevisiae* are shown in Figure 2.2A.

Several heterologous alcohol-forming fatty acyl-CoA reductase (FAR) enzymes have been previously expressed in *S. cerevisiae*, showing substrate specificities from C14 to C26 (Rowland et al. 2006; Domergue et al. 2010; Hellenbrand et al. 2011). To produce fatty alcohols in *S. cerevisiae,* Runguphan and Keasling (2014) overexpressed the NADPH-dependent fatty acyl-CoA reductase mFAR1 from *M. musculus*. The strain produced 47.4 mg/L versus negligible levels in the control strains. When Acc1, Fas1, and Fas2 were also expressed, fatty alcohol titer increased to 93.4 mg/L and was primarily 1-hexadecanol (91.1% of total fatty alcohols). Expression of a FAR from *Tyto alba* resulted in close to 22 mg/L intracellular 1-hexadecanol when expressed in a wild type strain, and over 35 mg/L when expressed in a *dga1Δ* strain (Tang & Chen 2015). Interestingly, extracellular levels were much lower than intracellular levels. In a study by Feng et al. (2015), expression of the *T. alba* FAR resulted in 45 mg/L of 1-hexadecanol. Deletion of *RPD3*, overexpression of Acc1, and overexpression of ACL from *Y. lipolytica* increased 1-hexadecanol titer to > 300 mg/L. Batch cultures with high cell density increased titer further to 655 mg/L, and fed-batch tube cultures reached 1.11 g/L. Combining these strategies with interventions for increased xylose utilization led to a strain able to produce 790 mg/L of 1-hexadecanol in batch cultures with 4% xylose and high cell density (Guo et al. 2016). In a recent paper (d'Espaux et al. 2017), strong expression of mFAR1, combined with overexpression of *OLE1* and *ACC1*^{S659A,S1157A} and deletion of *HFD1*, *DGA1*, *ADH6*, and *GDH1*, led to a

strain producing 1.2 g/L of fatty alcohols in shake flask and 6 g/L in a fed-batch bioreactor; these are the highest titers reported to date. Interestingly, when the native Δ9 desaturase *OLE1* was overexpressed, the fatty alcohol production increased 4-fold compared to the parent strain. A similar approach was taken in *Y. lipolytica,* and overexpression of a mammalian Δ9 desaturase increased lipid (TAG) synthesis (Qiao et al. 2015). This strategy could be a general intervention to increase free fatty acid and fatty acid-derived products.

Another route to fatty alcohols uses free fatty acids instead of fatty acyl-CoA as substrate. This conversion is accomplished by two sequential reactions catalyzed by two discrete enzymes. The first is the reduction of free fatty acids to fatty aldehydes by a NADPH- and ATP-dependent carboxylic acid reductase (CAR) (Akhtar et al. 2013). The second reaction is catalyzed by an alcohol dehydrogenase (ADH). Zhou et al. (2016b) introduced a CAR from *Mycobacterium marinum* and a PPT into a *S. cerevisiae* strain previously engineered to produce high-levels of FFA. When overexpressed with the native alcohol dehydrogenase Adh5*,* this pathway produced approximately 7-fold more fatty alcohols than an alternate two-step pathway from the fatty acyl-CoA (that utilized the aldehyde-forming fatty acyl-CoA reductase (ACR) from *A. baylyi* or the aldehyde-forming fatty acyl-ACP/-CoA reductase (AAR) from *Synechococcus elongatus*). Adh5 was found to be the most efficient endogenous ADH for conversion of the fatty aldehydes to fatty alcohols. The combination of a bi-functional fatty acyl-CoA reductase from *Marinobacter aquaeolei* with the CAR pathway, and deletion of competing pathways (*hfd1Δpox1Δ faa1/4Δ adh6Δ*), led to a fatty alcohol titer of 120 mg/L. Subsequent glucose limited fed-batch culture increased titer to 1.51 g/L. Dynamic control of *FAA1* expression has also been used (Teixeira et al. 2017), leading to a 41% increase in specific fatty alcohol level relative to the control strain. In another recent paper, expression of the *M. marinum* CAR and the *M. musculus* peroxisomal thioesterase Acot5' in a *faa1Δfaa4Δdga1Δ* yeast strain resulted in 31.2

mg/L of fatty alcohols (Tang et al. 2017).

To avoid competition for intermediates with the endogenous cytosolic ALR/ADH, the heterologous FAR was targeted to the peroxisome (Zhou et al. 2016a) (Figure 2.2B). With the bifunctional FAR, fatty alcohol titer increased 2.7-fold relative to the cytosolic pathway. Deletion of competing pathways and strategies to increase size and number of peroxisomes further improved product titer. Addition of a dodecane layer to the media to avoid product evaporation increased fatty alcohol levels to 193 mg/L. The engineered strains produced primarily C16 and C18 fatty alcohols.

To reduce the carbon chain length of the final fatty alcohol, *S. cerevisiae* β-oxidation was engineered by targeting the fatty acyl-CoA reductase from *T. alba* to the peroxisome (Sheng et al. 2016). Various targeting signals were tested, and the endogenous peroxisome biogenesis machinery was engineered by overexpressing Pex5, Pex7 and/or combinations with Pex3 and Pex19 to optimize FAR targeting. Overexpression of FAR (with the N-terminal PTS2) and Pex7 resulted in approximately 50 mg/L of 1-decanol, 200 mg/L of 1-dodecanol, and 500 mg/L of 1-hexadecanol.

A recent study focused on the production of docosanol, a C22:0 fatty alcohol (Yu et al. 2017). Elo2 and Elo1 were overexpressed in a $Acc1^{S659A,S1157A}$ parent strain to increase C22 fatty acyl-CoA, and *ELO3* was deleted to prevent elongation of C22 acyl-CoA to C26. In addition, a heterologous FAS from *M. vaccae* (capable of synthesizing very long-chain fatty acyl-CoA) and a FAR from *A. thaliana* (AtFAR) were introduced. The final engineered strain produced 83.5 mg/L, an 80-fold improvement over a strain with only *elo3Δ* and AtFAR overexpression.

Wax esters (WE) can be biosynthesized from fatty acyl-CoA and fatty alcohol, and can be used for cosmetics, lubricants, surfactants, microencapsulation, and other products (Miwa 1984). Very long-chain WEs have been synthesized in *S. cerevisiae* by expressing a heterologous fatty acyl-CoA reductase (FAR) and wax ester synthase (WS), and engineering the endogenous yeast fatty acid

elongation machinery (Wenning et al. 2017). Several FARs and WSs were tested, and then combined with the overexpression of the native elongase gene *ELO2* and deletion of *ELO3.* Overexpression of FAR from *M. aquaeolei* in this strain led to 7.84 mg/gDCW of very long-chain fatty alcohols. When the wax ester synthase from *Simmondsia chinensis* was introduced with the FAR, wax ester titer reached 14.98 mg/L, with 27% having > 38 carbons.

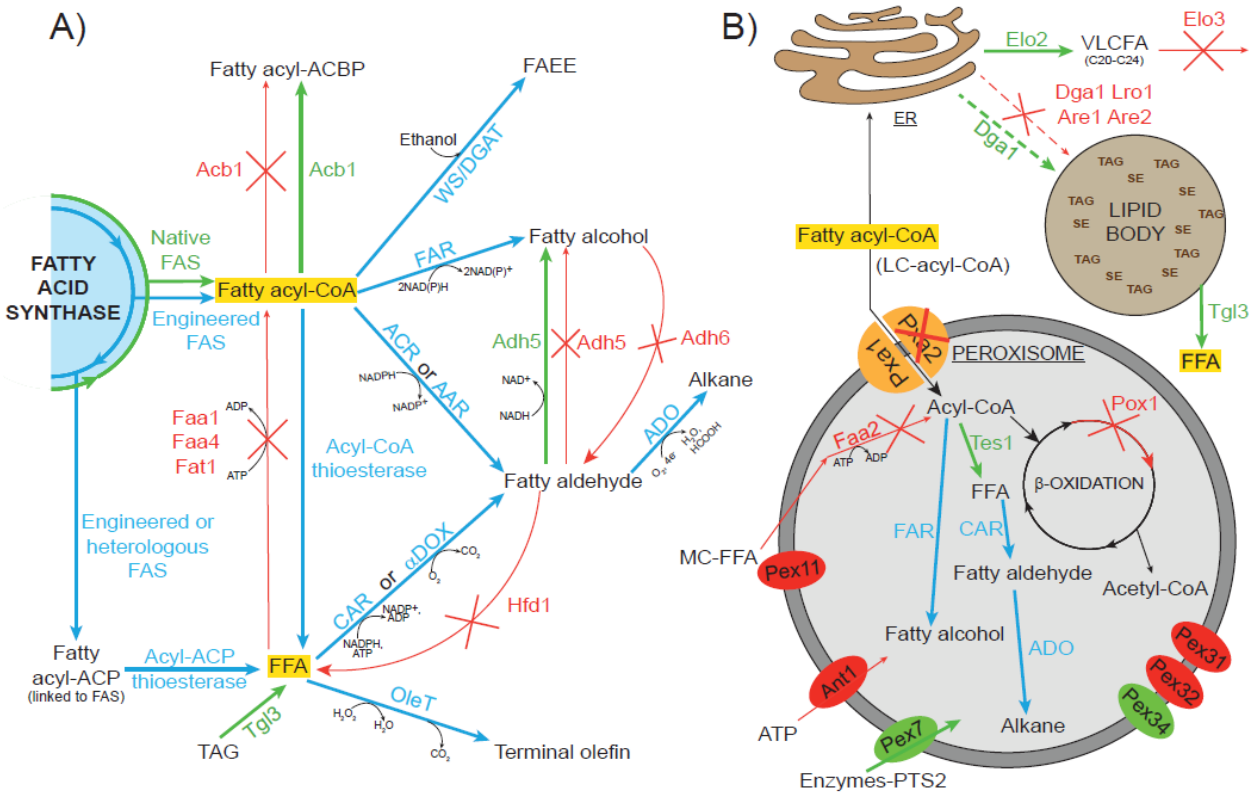


Figure 2.2. Engineering of downstream pathways. **(A)** Pathways to increase free fatty acid levels and to produce fatty acid ethyl esters, fatty alcohols, alkanes, alkenes, and terminal olefins. **(B)** Peroxisomal pathways engineered to reduce β-oxidation and localize product synthesis. Neutral lipid and VLCFA pathways engineered to increase product synthesis. The native yeast pathways are shown in black, with downregulation/deletion in red and upregulation in green. Heterologous proteins and pathways are shown in blue.

Fatty alkanes and alkenes

Long-chain alkanes are of special interest due to their potential to increase cetane number in biodiesel (Knothe 2010). Long-chain alkanes have been produced in *S. cerevisiae* by the expression of two enzymes from *S. elongatus*: the fatty acyl-ACP/-CoA reductase (AAR) that converts fatty acyl-CoA to fatty aldehydes, and the fatty aldehyde deformylating oxygenase (ADO) (Buijs et al. 2015) (**Figure 2.2A**). *S. cerevisiae HFD1* encodes a dehydrogenase that can catalyze the conversion of hexadecenal to hexadecenoic acid; its deletion was essential to increase the production of alkanes. The deletion of *HFD1* and the expression of ADO was sufficient to increase alkane production significantly. Expression of AAR increased the alkane level by an additional 18% to 22 μg/gDCW. Since the AAR activity in yeast appeared limiting, a carboxylic acid reductase (CAR) from *M. marinum* was expressed in a *S. cerevisiae* strain that had been engineered to produce high-levels of FFA (Zhou et al. 2016b). A 2.7-fold increase in alkane and alkene production was observed with the CAR pathway compared to the AAR pathway. Elimination of competing pathways and optimization of CAR and ADO expression increased the alkane/alkene titer to 0.82 mg/L. Building on this work, a comparative study of aldehyde decarbonylases from plants, insects, and cyanobacteria was performed (Kang et al. 2017). Only the ADO enzymes from cyanobacteria resulted in synthesis of alkanes and alkenes from fatty aldehydes in yeast. Expression of the ADO from *Crocosphaera watsonii* in the engineered strain led to a final alkane/alkene titer of 1.14 mg/L. Interventions proven to work in *S. cerevisiae* have also shown promising results in the oleaginous yeast *Y. lipolytica* (Xu et al. 2016).

The major by-product in the alkane-producing pathway is fatty alcohols produced from the fatty aldehyde intermediate by endogenous aldehyde reductases (ALR). Yeast peroxisomes have been used to compartmentalize the alkane biosynthetic pathway to avoid cytosolic ALR activity (Zhou et al. 2016a). Peroxisomal targeting increased titers for both strategies relative to the cytosolic

pathways. The combination of CAR and ADO again produced higher alkanes titer than AAR and ADO. Elimination of competing pathways and peroxisome biogenesis engineering further increased titer to 3.55 mg/L.

Another route to the intermediate fatty aldehyde from free fatty acids is the use of an α-fatty acid dioxygenase (α-DOX) (Koeduka et al. 2002). An advantage of this enzyme is that it does not require NADPH. α-DOX from *Oryza sativa* was introduced into *S. cerevisiae* along with a fatty aldehyde deformylating oxygenase (Foo et al. 2017). Alkane production from FFA added to the media was first evaluated, and then a *faa1Dfaa4D* yeast strain producing high-levels of FFA was utilized. However, the final titer was low, 42.4 µg/L tetradecane and 31.1 µg/L hexadecane.

Terminal alkenes represent good candidates for drop-in biofuel and platform chemicals (Rude & Schirmer 2009). The first reported work on the production of terminal olefins in *S. cerevisiae* looked at eight different P450 fatty acid decarboxylases (OleT) (Chen et al. 2015). OleT catalyzes the one-step reaction of free fatty acids to terminal olefins. A codon-optimized OleT from *Jeotgalicoccus* sp. had the broadest product profile and produced the highest alkene titers, 54.5 µg/L. Engineering of the cell to increase substrate availability (free fatty acids), produce higher levels of enzyme cofactors H_2O_2 and heme, and optimize enzyme expression led to a strain that produced over 2 mg/L, a 38.3-fold increase over the wild type strain expressing OleT. Another group focused on targeting the OleT enzyme to the yeast peroxisomes (Zhou et al. 2016a). Peroxisomal compartmentalization increased alkene levels by 40% (to ~0.18 mg/L) compared to cytosolic expression.

Table 2.2. Summary of interventions and results for production of long- and medium-chain fatty alcohols, alkanes, alkenes and terminal alkenes.

Product (Fatty Alcohol)	Interventions	Media	Scale	Titer (mg/L)	%Theor. Yield[3]	Productivity (mg/L·h)	Reference
Long-chain	mFAR1 MaME Acc1↑ Fas1↑ Fas2↑ *pxa2Δ pox1Δ*	CSM	Flask	98	1.4	0.58	(Runguphan & Keasling 2014)
Long-chain	TaFAR YlACL Acc1↑*rpd3Δ*	CSM	Tubes (Hi-Den)	330	4.9	3.44	(Feng et al. 2015)
Long-chain	TaFAR YlACL Acc1↑*rpd3Δ*	CSM/d[2]	Fed-batch	1110	3.3	23.1	(Feng et al. 2015)
Long-chain	CsXR CtXDH PpXKS TaFAR YlACL Acc1↑*rpd3Δ* evolved	CSM/d	Tubes (Hi-Den)	790	5.8	16.5	(Guo et al. 2016)
Long-chain	CsXR CtXDH PpXKS TaFAR YlACL Acc1↑*rpd3Δ* evolved	CSM/d	Fed-batch	1200	2.5	17.1	(Guo et al. 2016)
Long-chain	MaquFAR CAR Adh5↑ *faa1Δ faa4Δ pox1Δ hfd1Δ adh6Δ*	MM*[1]	Flask	115	1.1	1.60	(Zhou et al. 2016a)
Long-chain	MaquFAR CAR Adh5↑ *faa1Δ faa4Δ pox1Δ hfd1Δ adh6Δ*	MM*	Fed-batch	1510	1.4	15.7	(Zhou et al. 2016a)
Long-chain	MaquFAR (peroxisomal targeting) *are1Δ are2Δ dga1Δ lro1Δ*	MM*/d	Flask	193	1.9	2.68	(Zhou et al. 2016b)
Long-chain	TaFAR-PTS2 (peroxisomal targeting) Acc1↑ Pex7↑ (haploid)	CSM/d (high C/N)	Tubes (Hi-Den)	832	12	17.3	(Sheng et al. 2016)
Long-chain	TaFAR-PTS2 (peroxisomal targeting) Acc1↑ Pex7↑ (diploid)	CSM/d (high C/N)	Tubes (Fed-batch)	1300	9.6	36.1	(Sheng et al. 2016)
Long-chain	mFAR1 Acc1*↑ Fas1↑ Fas2↑ Ole1↑ *dga1Δ hfd1Δ adh6Δ gdh1Δ*	YPD/d	Fed-batch	6000	17	27.8	(d'Espaux et al. 2017)
Medium-chain	TaFAR-PTS2 (peroxisomal targeting) Acc1↑ Pex7↑ (haploid)	CSM/d (high C/N)	Tubes (Hi-Den)	275	3.8	5.73	(Sheng et al. 2016)
Medium-chain	TaFAR-PTS2 (peroxisomal targeting) Acc1↑ Pex7↑ (diploid)	CSM/d (high C/N)	Tubes (Fed-batch)	447	3.1	12.4	(Sheng et al. 2016)

Product (Alkane/Alkene)	Interventions	Media	Scale	Titer (mg/L)	%Theor. Yield[3]	Productivity (mg/L·h)	Reference
Alkane (C13-C17)	SeAAR SeADO EcFNR/Fd *hfd1Δ*	MM	Flask	22 µg/gDCW	ND	ND	(Buijs et al. 2015)
Alkane (C14-C16)	αDOX SeADO *faa1Δ faa4Δ*	CSM	Flask	73.5 µg/L	0.0013	0.0015	(Foo et al. 2017)
Alkane/Alkene (C13-C17)	MmCAR SeADO NpADO SeFNR/Fd EcFNR/Fd *hfd1Δ pox1Δ adh5Δ*	MM*	Flask	0.82	0.0095	0.0114	(Zhou et al. 2016a)
Alkane/Alkene (C11-C17)	MmCAR SeADO CwADO SeFNR/Fd EcFNR/Fd *hfd1Δ pox1Δ adh5Δ*	MM*	Flask	1.14	0.0021	0.0158	(Kang et al. 2017)
Alkane/Alkane (C13-C17)	MmCAR SeADO Pex34↑ *hfd1Δ pox1Δ pex30Δ pex31Δ* (peroxisomal targeting)	MM*	Flask	3.55	0.0409	0.0493	(Zhou et al. 2016b)
T-alkene (C15-C19)	OleT Hem3↑ *faa1Δ faa4Δ ctt1Δ cta1Δ ccp1Δ*	YPD+ G418	Fed-Batch	3.7	0.0244	0.0257	(Chen et al. 2015)
T-alkene (C15-C17)	OleT EcFNR/Fd (peroxisomal targeting)	MM*	Flask	0.18	0.0021	0.0025	(Zhou et al. 2016b)

[1] Minimal media with vitamins supplement. [2] 10% dodecane layer was added. [3] 0.34 g/g glucose was used for LC-fatty alcohols. 0.36 g/g glucose for MC-fatty alcohols. 0.29 g/g glucose for Alkanes/Alkenes.

SUMMARY AND OUTLOOK

Fatty acids and their derivatives are important as both biorenewable products and platform chemicals. This mini-review has focused on recent advances in the engineering of *S. cerevisiae* to produce these compounds, focusing on fatty acyl-CoAs and free fatty acids of various lengths, fatty acid ethyl esters, fatty alcohols, and fatty alkanes. As summarized in Table 2.1 and Table 2.2, titers have reached 1-10 g/L for long chain FFAs and fatty alcohols. However, titers of medium-chain FFAs, FAEEs, and particularly alkanes remain low. In addition, considerable progress must be made to increase the low (and often very low) yields from glucose for all products. Recent advances in microbial metabolic modeling, metabolic flux analysis, high-throughput screening, and genome engineering should accelerate understanding of yeast metabolism and the implementation of successful engineering strategies. Research on increasing fatty acid production in *S. cerevisiae* has occurred over a relatively short period of time, and given the rapid progress over the past 3-5 years, further pathway and enzyme engineering will substantially increase titer, yield, and productivity. Special focus should be placed on enzyme discovery and engineering to increase activity, specificity, and new functionality. This will enable manipulation of yeast metabolism to produce new multifunctionalized fatty acid derived products, that are of greater interest as platform chemicals. Furthermore, many of the interventions have already proven effective in *Y. lipolytica* and other oleaginous yeast for increasing free fatty acids, FAEE, fatty alcohols, and alkanes/alkenes. As titers of the MCFAs (particularly hexanoic, octanoic, and decanoic acid), fatty alcohols, and alkanes increase, product toxicity will become a critical issue to address. In addition, the increasing interest in the use of lignocellulosic feedstocks for the production of fatty acids and fatty acid-derived products will bring new challenges, including the toxicity of by-products of feedstock pretreatment and sugar utilization. Continuing research to increase product levels and decrease toxicity in the easily engineered *S.*

cerevisiae should lead to insights for producing these compounds in this yeast, the oleaginous yeast, and other yeast species.

REFERENCES

Akhtar MK, Turner NJ & Jones PR (2013) Carboxylic acid reductase is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities. *Proc Natl Acad Sci U S A* 110: 87–92.

Besada-Lombana PB, Fernandez-Moya R, Fenster J & Da Silva NA (2017) Engineering Saccharomyces cerevisiae fatty acid composition for increased tolerance to octanoic acid. *Biotechnol Bioeng* 114: 1531–1538.

Biermann U, Bornscheuer U, Meier MAR, Metzger JO & Schäfer HJ (2011) Oils and fats as renewable raw materials in chemistry. *Angew Chem Int Ed Engl* 50: 3854–3871.

Black PN & DiRusso CC (2007) Yeast acyl-CoA synthetases at the crossroads of fatty acid metabolism and regulation. *Biochim Biophys Acta* 1771: 286–298.

Bozell JJ & Petersen GR (2010) Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. *Green Chemistry* 12: 539.

Brody S, Oh C, Hoja U & Schweizer E (1997) Mitochondrial acyl carrier protein is involved in lipoic acid synthesis in *Saccharomyces cerevisiae*. *FEBS Lett* 408: 217–220.

Buijs NA, Zhou YJ, Siewers V & Nielsen J (2015) Long-chain alkane production by the yeast Saccharomyces cerevisiae. *Biotechnol Bioeng* 112: 1275–1279.

Chen B, Lee D-Y & Chang MW (2015) Combinatorial metabolic engineering of Saccharomyces cerevisiae for terminal alkene production. *Metab Eng* 31: 53–61.

Chen L, Zhang J, Lee J & Chen WN (2014) Enhancement of free fatty acid production in Saccharomyces cerevisiae by control of fatty acyl-CoA metabolism. *Appl Microbiol Biotechnol* 98: 6739–6750.

Chia M, Schwartz TJ, Shanks BH & Dumesic JA (2012) Triacetic acid lactone as a potential biorenewable platform chemical. *Green Chemistry* 14: 1850.

Chirala SS (1992) Coordinated regulation and inositol-mediated and fatty acid-mediated repression of fatty acid synthase genes in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* 89: 10232–10236.

Choi JW & Da Silva NA (2014) Improving polyketide and fatty acid synthesis by engineering of the yeast acetyl-CoA carboxylase. *J Biotechnol* 187: 56–59.

Dahlqvist A, Stahl U, Lenman M, Banas A, Lee M, Sandager L, Ronne H & Stymne S (2000) Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proc Natl Acad Sci U S A* 97: 6487–6492.

Dellomonaco C, Clomburg JM, Miller EN & Gonzalez R (2011) Engineered reversal of the β-oxidation cycle for the synthesis of fuels and chemicals. *Nature* 476: 355–359.

Dittrich F et al. (1998) Fatty acid elongation in yeast--biochemical characteristics of the enzyme system and isolation of elongation-defective mutants. *Eur J Biochem* 252: 477–485.

Domergue F et al. (2010) Three Arabidopsis fatty acyl-coenzyme A reductases, FAR1, FAR4, and FAR5, generate primary fatty alcohols associated with suberin deposition. *Plant Physiol* 153: 1539–1554.

Egner R, Thumm M, Straub M, Simeon A, Schuller H-J & Wolf DH (1993) Tracing Intracellular Proteolytic Pathways. *J Biol Chem* 268: 27269–27276.

Eriksen DT, HamediRad M, Yuan Y & Zhao H (2015) Orthogonal Fatty Acid Biosynthetic Pathway Improves Fatty Acid Ethyl Ester Production in Saccharomyces cerevisiae. *ACS Synth Biol* 4: 808–814.

d'Espaux L et al. (2017) Engineering high-level production of fatty alcohols by Saccharomyces cerevisiae from lignocellulosic feedstocks. *Metab Eng* 42: 115–125.

Feddersen S, Neergaard TBF, Knudsen J & Faergeman NJ (2007) Transcriptional regulation of phospholipid biosynthesis is linked to fatty acid metabolism by an acyl-CoA-binding-protein-dependent mechanism in Saccharomyces cerevisiae. *Biochem J* 407: 219–230.

Feng X, Lian J & Zhao H (2015) Metabolic engineering of Saccharomyces cerevisiae to improve 1-hexadecanol production. *Metab Eng* 27: 10–19.

Fernandez-Moya R, Leber C, Cardenas J & Da Silva NA (2015) Functional replacement of the Saccharomyces cerevisiae fatty acid synthase with a bacterial type II system allows flexible product profiles. *Biotechnol Bioeng* 112: 2618–2623.

Foo JL, Susanto AV, Keasling JD, Leong SSJ & Chang MW (2017) Whole-cell biocatalytic and de novo production of alkanes from free fatty acids in*Saccharomyces cerevisiae*. *Journal of Biochemical and Microbiological Technology and Engineering* 114: 232–237.

Gajewski J, Buelens F, Serdjukow S, Janßen M, Cortina N, Grubmüller H & Grininger M (2017a) Engineering fatty acid synthases for directed polyketide production. *Nat Chem Biol* 13: 363–365.

Gajewski J, Pavlovic R, Fischer M, Boles E & Grininger M (2017b) Engineering fungal de novo fatty acid synthesis for short chain fatty acid production. *Nat Commun* 8: 14650.

Ghosh A et al. (2016) (13)C Metabolic Flux Analysis for Systematic Metabolic Engineering of S. cerevisiae for Overproduction of Fatty Acids. *Frontiers in bioengineering and biotechnology* 4: 76.

Guo W, Sheng J, Zhao H & Feng X (2016) Metabolic engineering of Saccharomyces cerevisiae to produce 1-hexadecanol from xylose. *Microb Cell Fact* 15: 24.

Hellenbrand J, Biester E-M, Gruber J, Hamberg M & Frentzen M (2011) Fatty acyl-CoA reductases of birds. *BMC Biochem* 12: 64.

Hettema EH, van Roermund CW, Distel B, van den Berg M, Vilela C, Rodrigues-Pousada C, Wanders RJ & Tabak HF (1996) The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of Saccharomyces cerevisiae. *EMBO J* 15: 3813–3822.

Hettema EH & Tabak HF (2000) Transport of fatty acids and metabolites across the peroxisomal membrane. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1486: 18–27.

Hiltunen JK, Mursula AM, Rottensteiner H, Wierenga RK, Kastaniotis AJ & Gurvitz A (2003) The biochemistry of peroxisomal β-oxidation in the yeast*Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 27: 35–64.

Hofbauer HF et al. (2014) Regulation of gene expression through a transcriptional repressor that senses acyl-chain length in membrane phospholipids. *Dev Cell* 29: 729–739.

Hynes MJ & Murray SL (2010) ATP-citrate lyase is required for production of cytosolic acetyl coenzyme A and development in Aspergillus nidulans. *Eukaryotic Cell* 9: 1039–1048.

Jetter R & Kunst L (2008) Plant surface lipid biosynthetic pathways and their utility for metabolic engineering of waxes and hydrocarbon biofuels. *Plant J* 54: 670–683.

Johnson DR, Knoll LJ, Rowley N & Gordon JI (1994) Genetic analysis of the role of Saccharomyces cerevisiae acyl-CoA synthetase genes in regulating protein N-myristoylation. *J Biol Chem* 269: 18037–18046.

Jones JM, Nau K, Geraghty MT, Erdmann R & Gould SJ (1999) Identification of peroxisomal acyl-CoA thioesterases in yeast and humans. *J Biol Chem* 274: 9216–9223.

De Jong BW, Shi S, Siewers V & Nielsen J (2014) Improved production of fatty acid ethyl esters in Saccharomyces cerevisiae through up-regulation of the ethanol degradation pathway and expression of the heterologous phosphoketolase pathway. *Microb Cell Fact* 13: 39.

De Jong BW, Shi S, Valle-Rodríguez JO, Siewers V & Nielsen J (2015) Metabolic pathway engineering for fatty acid ethyl ester production in Saccharomyces cerevisiae using stable chromosomal integration. *J Ind Microbiol Biotechnol* 42: 477–486.

De Jong E, Higson A, Walsh P & Wellisch M (2012) Product developments in the bio-based chemicals arena. *Biofuels, Bioproducts and Biorefining* 6: 606–624.

Kang M-K, Zhou YJ, Buijs NA & Nielsen J (2017) Functional screening of aldehyde decarbonylases for long-chain alkane production by Saccharomyces cerevisiae. *Microb Cell Fact* 16: 74.

Knoll LJ, Johnson DR & Gordon JI (1994) Biochemical studies of three Saccharomyces cerevisiae acyl-CoA synthetases, Faa1p, Faa2p, and Faa3p. *J Biol Chem* 269: 16348–16356.

Knothe G (2010) Biodiesel and renewable diesel: A comparison. *Progress in energy and combustion science* 36: 364–373.

Koeduka T, Matsui K, Akakabe Y & Kajiwara T (2002) Catalytic properties of rice alpha-oxygenase. A comparison with mammalian prostaglandin H synthases. *J Biol Chem* 277: 22648–22655.

Kunau WH, Bühne S, de la Garza M, Kionka C, Mateblowski M, Schultz-Borchard U & Thieringer R (1988) Comparative enzymology of beta-oxidation. *Biochem Soc Trans* 16: 418–420.

Leber C, Choi JW, Polson B & Da Silva NA (2016) Disrupted short chain specific β-oxidation and improved synthase expression increase synthesis of short chain fatty acids in Saccharomyces cerevisiae. *Biotechnol Bioeng* 113: 895–900.

Leber C, Polson B, Fernandez-Moya R & Da Silva NA (2015) Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in Saccharomyces cerevisiae. *Metab Eng* 28: 54–62.

Leber C & Da Silva NA (2014) Engineering of Saccharomyces cerevisiae for the synthesis of short chain fatty acids. *Biotechnol Bioeng* 111: 347–358.

Leber R, Zinser E, Zellnig G, Paltauf F & Daum G (1994) Characterization of lipid particles of the yeast, Saccharomyces cerevisiae. *Yeast* 10: 1421–1428.

Leibundgut M, Maier T, Jenni S & Ban N (2008) The multienzyme architecture of eukaryotic fatty acid synthases. *Curr Opin Struct Biol* 18: 714–725.

Li X, Guo D, Cheng Y, Zhu F, Deng Z & Liu T (2014) Overproduction of fatty acids in engineered Saccharomyces cerevisiae. *Biotechnol Bioeng* 111: 1841–1852.

Lian J & Zhao H (2016) Functional Reconstitution of a Pyruvate Dehydrogenase in the Cytosol of Saccharomyces cerevisiae through Lipoylation Machinery Engineering. *ACS Synth Biol* 5: 689–697.

Lian J & Zhao H (2015) Reversal of the β-oxidation cycle in Saccharomyces cerevisiae for production of fuels and chemicals. *ACS Synth Biol* 4: 332–341.

Liu T, Vora H & Khosla C (2010) Quantitative analysis and engineering of fatty acid biosynthesis in E. coli. *Metab Eng* 12: 378–386.

Lopes JM, Hirsch JP, Chorgo PA, Schulze KL & Henry SA (1991) Analysis of sequences in the INO1 promoter that are involved in its regulation by phospholipid precursors. *Nucleic Acids Res* 19: 1687–1693.

Maeda I, Delessert S, Hasegawa S, Seto Y, Zuber S & Poirier Y (2006) The peroxisomal Acyl-CoA thioesterase Pte1p from Saccharomyces cerevisiae is required for efficient degradation of short straight chain and branched chain fatty acids. *J Biol Chem* 281: 11729–11735.

Maier T, Leibundgut M, Boehringer D & Ban N (2010) Structure and function of eukaryotic fatty acid synthases. *Q Rev Biophys* 43: 373–422.

Michinaka Y, Shimauchi T, Aki T, Nakajima T, Kawamoto S, Shigeta S, Suzuki O & Ono K (2003) Extracellular secretion of free fatty acids by disruption of a fatty acyl-CoA synthetase gene in Saccharomyces cerevisiae. *J Biosci Bioeng* 95: 435–440.

Miwa TK (1984) Structural Determination and Uses of Jojoba Oil. *J Am Oil Chem Soc* 407–410.

Moreira dos Santos M, Raghevendran V, Kötter P, Olsson L & Nielsen J (2004) Manipulation of malic enzyme in Saccharomyces cerevisiae for increasing NADPH production capacity aerobically in different cellular compartments. *Metab Eng* 6: 352–363.

Nielsen J (2009) Systems biology of lipid metabolism: from yeast to human. *FEBS Lett* 583: 3905–3913.

Nikolau BJ, Perera MADN, Brachova L & Shanks B (2008) Platform biochemicals for a biorenewable chemical industry. *Plant J* 54: 536–545.

Oelkers P, Tinkelenberg A, Erdeniz N, Cromley D, Billheimer JT & Sturley SL (2000) A lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast. *J Biol Chem* 275: 15609–15612.

Pronk JT, Steensma HY & Van Dijken, JP (1996) Pyruvate Metabolism in Saccharomyces cerevisiae. *Yeast* 12: 1607-1633.

Qiao K, Imam Abidi SH, Liu H, Zhang H, Chakraborty S, Watson N, Kumaran Ajikumar P & Stephanopoulos G (2015) Engineering lipid overproduction in the oleaginous yeast Yarrowia lipolytica. *Metab Eng* 29: 56–65.

Rezanka T (1989) Very-long-chain fatty acids from the animal and plant kingdoms. *Prog Lipid Res* 28: 147–187.

Van Roermund CW, Drissen R, van Den Berg M, Ijlst L, Hettema EH, Tabak HF, Waterham HR & Wanders RJ (2001) Identification of a peroxisomal ATP carrier required for medium-chain fatty acid beta-oxidation and normal peroxisome proliferation in Saccharomyces cerevisiae. *Mol Cell Biol* 21: 4321–4329.

Van Roermund CW, Tabak HF, van Den Berg M, Wanders RJ & Hettema EH (2000) Pex11p plays a primary role in medium-chain fatty acid oxidation, a process that affects peroxisome number and size in Saccharomyces cerevisiae. *J Cell Biol* 150: 489–498.

Roggenkamp R, Numa S & Schweizer E (1980) Fatty acid-requiring mutant of Saccharomyces cerevisiae defective in acetyl-CoA carboxylase. *Proc Natl Acad Sci U S A* 77: 1814–1817.

Rowland O, Zheng H, Hepworth SR, Lam P, Jetter R & Kunst L (2006) CER4 encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis. *Plant Physiol* 142: 866–877.

Rude MA & Schirmer A (2009) New microbial fuels: a biotech perspective. *Curr Opin Microbiol* 12: 274–281.

Runguphan W & Keasling JD (2014) Metabolic engineering of Saccharomyces cerevisiae for production of fatty acid-derived biofuels and chemicals. *Metab Eng* 21: 103–113.

Rupilius W & Ahmad S (2006) The Changing World of Oleochemicals. *Palm Oil Developments* 15–28.

Saerens SMG, Delvaux F, Verstrepen KJ, Van Dijck P, Thevelein JM & Delvaux FR (2008) Parameters affecting ethyl ester production by Saccharomyces cerevisiae during fermentation. *Appl Environ Microbiol* 74: 454–461.

Saerens SMG, Verstrepen KJ, Van Laere SDM, Voet ARD, Van Dijck P, Delvaux FR & Thevelein JM (2006) The Saccharomyces cerevisiae EHT1 and EEB1 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. *J Biol Chem* 281: 4446–4456.

Sandager L, Gustavsson MH, Ståhl U, Dahlqvist A, Wiberg E, Banas A, Lenman M, Ronne H & Stymne S (2002) Storage lipid synthesis is non-essential in yeast. *J Biol Chem* 277: 6478–6482.

Scharnewski M, Pongdontri P, Mora G, Hoppert M & Fulda M (2008) Mutants of Saccharomyces cerevisiae deficient in acyl-CoA synthetases secrete fatty acids due to interrupted fatty acid recycling. *FEBS J* 275: 2765–2778.

Schüller HJ, Hahn A, Tröster F, Schütz A & Schweizer E (1992) Coordinate genetic control of yeast fatty acid synthase genes FAS1 and FAS2 by an upstream activation site common to genes involved in membrane lipid biosynthesis. *EMBO J* 11: 107–114.

Schüller HJ, Schütz A, Knab S, Hoffmann B & Schweizer E (1994) Importance of general regulatory factors Rap1p, Abf1p and Reb1p for the activation of yeast fatty acid synthase genes FAS1 and FAS2. *Eur J Biochem* 225: 213–222.

Sheng J, Stevens J & Feng X (2016) Pathway Compartmentalization in Peroxisome of Saccharomyces cerevisiae to Produce Versatile Medium Chain Fatty Alcohols. *Sci Rep* 6: 26884.

Shi S, Chen Y, Siewers V & Nielsen J (2014a) Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. *MBio* 5: e01130–14.

Shi S, Valle-Rodríguez JO, Khoomrung S, Siewers V & Nielsen J (2012) Functional expression and characterization of five wax ester synthases in Saccharomyces cerevisiae and their utility for biodiesel production. *Biotechnol Biofuels* 5: 7.

Shi S, Valle-Rodríguez JO, Siewers V & Nielsen J (2014b) Engineering of chromosomal wax ester synthase integrated Saccharomyces cerevisiae mutants for improved biosynthesis of fatty acid ethyl esters. *Biotechnol Bioeng* 111: 1740–1747.

Shiba Y, Paradise EM, Kirby J, Ro D-K & Keasling JD (2007) Engineering of the pyruvate dehydrogenase bypass in Saccharomyces cerevisiae for high-level production of isoprenoids. *Metab Eng* 9: 160–168.

Shin G-H, Veen M, Stahl U & Lang C (2012) Overexpression of genes of the fatty acid biosynthetic pathway leads to accumulation of sterols in Saccharomyces cerevisiae. *Yeast* 29: 371–383.

Starai VJ, Gardner JG & Escalante-Semerena JC (2005) Residue Leu-641 of Acetyl-CoA synthetase is critical for the acetylation of residue Lys-609 by the Protein acetyltransferase enzyme of Salmonella enterica. *J Biol Chem* 280: 26200–26205.

Stukey JE, McDonough VM & Martin CE (1989) Isolation and characterization of OLE1, a gene affecting fatty acid desaturation from Saccharomyces cerevisiae. *J Biol Chem* 264: 16537–16544.

Sumper M, Riepertinger C, Lynen F & Oesterhelt D (1969) Die Synthese verschiedener Carbonsauren durch den Multienzymkomplex der Fettsauresynthese aus Hefe und die Erklarung ihrer Bildung. *Eur J Biochem* 10: 377–387.

Takeda Y, Suzuki F & Inoue H (1969) [27] ATP citrate lyase (citrate-cleavage enzyme). *Citric Acid Cycle*, pp. 153–160. Elsevier.

Tang X & Chen WN (2015) Enhanced production of fatty alcohols by engineering the TAGs synthesis pathway in Saccharomyces cerevisiae. *Biotechnol Bioeng* 112: 386–392.

Tang X, Feng H & Chen WN (2013) Metabolic engineering for enhanced fatty acids synthesis in Saccharomyces cerevisiae. *Metab Eng* 16: 95–102.

Tang X, Feng L, Chen L & Chen WN (2017) Engineering*Saccharomyces cerevisiae* for Efficient Biosynthesis of Fatty Alcohols Based on Enhanced Supply of Free Fatty Acids. *ACS omega* 2: 3284–3290.

Tehlivets O, Scheuringer K & Kohlwein SD (2007) Fatty acid synthesis and elongation in yeast. *Biochim Biophys Acta* 1771: 255–270.

Teixeira PG, Ferreira R, Zhou YJ, Siewers V & Nielsen J (2017) Dynamic regulation of fatty acid pools for improved production of fatty alcohols in Saccharomyces cerevisiae. *Microb Cell Fact* 16: 45.

Teo WS, Ling H, Yu A-Q & Chang MW (2015) Metabolic engineering of Saccharomyces cerevisiae for production of fatty acid short- and branched-chain alkyl esters biodiesel. *Biotechnol Biofuels* 8: 177.

Thompson RA & Trinh CT (2014) Enhancing fatty acid ethyl ester production in Saccharomyces cerevisiae through metabolic engineering and medium optimization. *Biotechnol Bioeng* 111: 2200–2208.

Trotter PJ (2001) The genetics of fatty acid metabolism in Saccharomyces cerevisiae. *Annu Rev Nutr* 21: 97–119.

Valle-Rodríguez JO, Shi S, Siewers V & Nielsen J (2014) Metabolic engineering of Saccharomyces cerevisiae for production of fatty acid ethyl esters, an advanced biofuel, by eliminating non-essential fatty acid utilization pathways. *Applied energy* 115: 226–232.

Wältermann M, Stöveken T & Steinbüchel A (2007) Key enzymes for biosynthesis of neutral lipid storage compounds in prokaryotes: properties, function and occurrence of wax ester synthases/acyl-CoA: diacylglycerol acyltransferases. *Biochimie* 89: 230–242.

Wang Y, Chen H & Yu O (2014) A plant malonyl-CoA synthetase enhances lipid content and polyketide yield in yeast cells. *Appl Microbiol Biotechnol* 98: 5435–5447.

Welch JW & Burlingame AL (1973) Very long-chain fatty acids in yeast. *J Bacteriol* 115: 464–466.

Wenning L, Yu T, David F, Nielsen J & Siewers V (2017) Establishing very long-chain fatty alcohol and wax ester biosynthesis in Saccharomyces cerevisiae. *Biotechnol Bioeng* 114: 1025–1035.

Wenz P, Schwank S, Hoja U & Schüller HJ (2001) A downstream regulatory element located within the coding sequence mediates autoregulated expression of the yeast fatty acid synthase gene FAS2 by the FAS1 gene product. *Nucleic Acids Res* 29: 4625–4632.

Werpy T & Petersen G (2004) Top value added chemicals from biomass. Volume I. Results of screening for potential candidates from sugar and synthesis gas. . *National Renewable Energy Laboratory. US Department of Energy*.

Westfall PJ & Gardner TS (2011) Industrial fermentation of renewable diesel fuels. *Curr Opin Biotechnol* 22: 344–350.

Woods A, Munday MR, Scott J, Yang X, Carlson M & Carling D (1994) Yeast SNF1 is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase in vivo. *J Biol Chem* 269: 19509–19515.

Wynn JP, bin Abdul Hamid A & Ratledge C (1999) The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. *Microbiology (Reading, Engl)* 145 (Pt 8): 1911–1917.

Xu P, Qiao K, Ahn WS & Stephanopoulos G (2016) Engineering Yarrowia lipolytica as a platform for synthesis of drop-in transportation fuels and oleochemicals. *Proc Natl Acad Sci U S A* 113: 10848–10853.

Yu T, Zhou YJ, Wenning L, Liu Q, Krivoruchko A, Siewers V, Nielsen J & David F (2017) Metabolic engineering of Saccharomyces cerevisiae for production of very long chain fatty acid-derived chemicals. *Nat Commun* 8: 15587.

Zhou YJ, Buijs NA, Zhu Z, Gómez DO, Boonsombuti A, Siewers V & Nielsen J (2016a) Harnessing Yeast Peroxisomes for Biosynthesis of Fatty-Acid-Derived Biofuels and Chemicals with Relieved Side-Pathway Competition. *J Am Chem Soc* 138: 15368–15377.

Zhou YJ, Buijs NA, Zhu Z, Qin J, Siewers V & Nielsen J (2016b) Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. *Nat Commun* 7: 11709.

Zhu Z, Zhou YJ, Krivoruchko A, Grininger M, Zhao ZK & Nielsen J (2017) Expanding the product portfolio of fungal type I fatty acid synthases. *Nat Chem Biol* 13: 360–362.

Zou Z, DiRusso CC, Ctrnacta V & Black PN (2002) Fatty acid transport in Saccharomyces cerevisiae. Directed mutagenesis of FAT1 distinguishes the biochemical activities associated with Fat1p. *J Biol Chem* 277: 31062–31071.

Zweytick D, Leitner E, Kohlwein SD, Yu C, Rothblatt J & Daum G (2000) Contribution of Are1p and Are2p to steryl ester synthesis in the yeast Saccharomyces cerevisiae. *Eur J Biochem* 267: 1075–1082.

CHAPTER 3:

Functional replacement of the *Saccharomyces cerevisiae* fatty acid synthase with a bacterial type II system allows flexible product profiles

Fernandez-Moya, R., Leber, C., Cardenas, J., and Da Silva, N.A. (2015). Functional replacement of the Saccharomyces cerevisiae fatty acid synthase with a bacterial type II system allows flexible product profiles. Biotechnol Bioeng *112*, 2618–2623.

*__RFM__, CL, JC, and NAD designed the study. NAD supervised the project. __RFM__ performed a significant amount of the experimental work, running the latest *in* vitro assays, constructing the full *E. coli* FAS and evaluating the fatty acid profile *in vivo*. CL performed molecular biology experiments and constructed the initial library of vectors. JC supported molecular biology experiments and performed initial characterization of the *E. coli* FAS system. __RFM__ analyzed the data and wrote the manuscript. All authors edited and approved the final manuscript.

ABSTRACT

The native yeast type I fatty acid synthase (FAS) is a complex, rigid enzyme, and challenging to engineer for the production of medium- or short-chain fatty acids. Introduction of a type II FAS is a promising alternative as it allows expression control for each discrete enzyme and the addition of heterologous thioesterases. In this study, the native *Saccharomyces cerevisiae* FAS was functionally replaced by the *Escherichia coli* type II FAS (eFAS) system. The *E. coli acpS* + *acpP* (together), *fabB*, *fabD*, *fabG*, *fabH*, *fabI*, *fabZ* and *tesA* were expressed in individual *S. cerevisiae* strains, and enzyme activity was confirmed by *in vitro* activity assays. Eight genes were then integrated into the yeast genome, while *tesA* or an alternate thioesterase gene, *fatB* from *Ricinus communis* or TEII from *Rattus novergicus*, was expressed from a multi-copy plasmid. Native FAS activity was eliminated by knocking out the yeast *FAS2* gene. The strains expressing only the eFAS as *de novo* fatty acid source grew without fatty acid supplementation demonstrating that this type II FAS is able to functionally replace the native yeast FAS. The engineered strain expressing the *R. communis fatB* thioesterase increased total fatty acid titer 1.7-fold and shifted the fatty acid profile towards C_{14} production, increasing it from <1% in the native strain to more than 30% of total fatty acids, and reducing C_{18} production from 39% to less than 8%.

INTRODUCTION

The United States Department of Agriculture (USDA) has projected a 3-fold growth of the global biorenewable chemicals market from 2010 to 2025, reaching approximately $500 billion (USDA, Market Potential and Projections Through 2025, 2008). Attention has focused on biofuels, but near-term opportunities for biobased products are particularly promising for chemicals (Bozell and Petersen, 2010). Functional replacement of the platform chemicals produced from petroleum should include molecules with highly reduced carbon backbones in conjunction with highly reactive functional groups, and polyketide and fatty acid (FA) biosynthesis provide a promising solution route to generate a vast library of such compounds (Nikolau et al., 2008).

S. cerevisiae produces cytosolic *de novo* fatty acids using a complex and iterative fatty acid synthase (FAS). The yeast type I FAS is composed of Fas1 and Fas2 subunits in an $\alpha_6\beta_6$ complex (Tehlivets et al., 2007), and the reactions are catalyzed by separate domains on the polypeptide chains. The primary products are C_{16} and C_{18} acyl-CoA esters, and the yeast FAS complexity and rigidity makes it a challenging enzyme to engineer to reduce this chain length (Leibundgut et al., 2008). As an alternative, our laboratory recently expressed, characterized and engineered the human type I FAS to produce short-chain fatty acids in *S. cerevisiae* (Leber and Da Silva, 2013). The higher flexibility of the hFAS structure allows replacement of the native thioesterase (TE) domain with one cleaving at the desired chain length. One disadvantage of type I FAS systems is the iterative use of domains in a single enzyme complex, and the limited ability to intervene in the series of reaction steps. In contrast, bacterial, plant, and yeast mitochondrial fatty acid synthases are type II FASs, dissociated systems consisting of individual enzymes of the catalytic pathway codified by separate gene sequences (White et al., 2005). These systems allow the flexibility to engineer the pathway to produce alternate final FA–based products and to facilitate the metabolic optimization of the biosynthesis pathway. A good

example of the flexibility to produce different end-products by the dissociated type II FAS is the production of methyl ketones in an engineered *E. coli* strain by the introduction of a 3-ketoacyl-ACP thioesterase (shmks2) from *Solanum habrochaites* (Park et al., 2012).

In this study, we replaced the native FAS system of *S. cerevisiae* with a type II FAS (from *E. coli*) and heterologous thioesterases, and then demonstrated the use of this system for the production of medium-chain fatty acids. Competition between the native and the heterologous FAS was removed by knocking-out the yeast *FAS2* gene, leaving the final strain with only the *E. coli* type II FAS as a source of *de novo* fatty acid synthesis.

MATERIALS AND METHODS

Vector and Strain Construction

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* and *in vivo* studies. 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). 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 of *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, 2000). Isolation of total genomic yeast DNA for integration and checking was performed as described in Sambrook and Russell (2001).

The *E. coli* type II FAS gene sequences were amplified from *E. coli* XL1-Blue (Stratagene, Santa Clara, CA) with a C- or N-terminal 6xHis-tag and inserted into the pXP219 vector (Table 3.2). The thioesterase gene sequence from *R. communis* (RC) was amplified from pXZ18 (Zhang et al., 2011), and the thioesterase gene sequence from *R. novergicus* (TEII) was amplified from pJLA502-TEII (Naggert et al., 1991). Both sequences were independently inserted into the pXP219 vector. The *acpS*, *fabI*, and *fabZ* gene sequences were excised from pXP219-acpS, pXP219-fabI, and pXP219-fabZ, respectively, and inserted into the pRF320 vector.

acpP, *acpS*, *fabB*, *fabD*, *fabG*, *fabH*, *fabI*, *fabZ*, and *tesA* were amplified from *E. coli* XL1-Blue using the primers shown in Table 3.3.A. The placement of the 6x-histidine tags was based on prior reports in the literature. If this information was not found (i.e., for *acpP*, *acpS*, and *tesA*), the C-terminus was chosen. Thioesterase RC was amplified from pXZ18 and TEII was amplified from pJLA502-TEII using the primers shown in Table 3.3A. PCR fragments were digested with restriction enzymes XhoI and SpeI and isolated on a 1% agar gel. DNA was extracted using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Backbone DNA (pXP219) and genes were ligated at a 1:3 molar ratio for 20 minutes at room temperature using Thermo Scientific Fermentas Rapid DNA Ligation Kit (Thermo Fisher, San Jose, CA) to generate pXP219-acpP, pXP219-acpS, pXP219-fabB, pXP219-fabD, pXP219-fabG, pXP219-fabH, pXP219-fabI, pXP219-fabZ, pXP219-tesA, pXP219-RC, and pXP219-TEII.

S. cerevisiae strain BY4741 (Open Biosystems, Huntsville, AL) was the base strain. Single and double gene integrations were performed using two-piece (gene cassette, selection marker) transformation as previously described (Fang et al., 2011). *acpS* under the control of P_{PGK1} was integrated at one copy into the genome of strain BY4741, creating strain BY-aS. PCR was used to generate fragment P_{PGK1}-*ACPS*-T_{CYC1} from vector pXP219-acpS and fragment loxP-*LEU2*-loxP from plasmid pXP222 using the primers shown in Table 3.3.B. The P_{PGK1}-*ACPS*-T_{CYC1} fragment had 50 bp homologous sequences on the 3' end to fragment loxP-*LEU2*-loxP on the 5' end. The flanking 5' and 3' ends of the adjoined segment had 50 bp overlapping sequences up- and down-stream of the target *URA3* locus. *acpS* integration into the *URA3* locus was confirmed by PCR analysis using primers that annealed upstream and downstream of the chromosomal site (Table 3.3.F).

The *CYC1* terminator on pXP320 was replaced by the *ADH2* terminator. Primers For-ADH2t and Rev-ADH2t (Table 3.3.C) were used to amplify the *ADH2* terminator from pJC702. The PCR

product and pXP320 were digested with XbaI and XhoI and isolated on a 1% agar gel. DNA was extracted using Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Backbone DNA and terminator were ligated at a 1:3 molar ratio for 20 min at room temperature using a Thermo Scientific Fermentas Rapid DNA Ligation Kit (Thermo Fisher, San Jose, CA), generating plasmid pRF320. Plasmids pRF320-acpS, pRF320-fabI, and pRF320-fabZ were constructed using the primers shown in Table 3.3.A and the same procedure as for pXP219-versions described above.

fabG under the control of P_{PGK1} was integrated at one copy into the genome of strain BY4741. PCR was used to generate fragment P_{PGK1}-*fabG*-T_{CYC1} from vector pXP219-fabG and fragment loxP-URA3-loxP from plasmid pXP219 using the primers shown in Table S-4. The P_{PGK1}-*fabG*-T_{CYC1} fragment had 50 bp homologous sequences on the 3' end to the fragment loxP-*URA3*-loxP on the 5' end. The flanking 5' and 3' ends of the adjoined segment had 50 bp overlapping sequences up- and down-stream of the target *MET15* locus. *fabG* integration into the *MET15* locus was confirmed by PCR analysis using primers that annealed upstream and downstream of the chromosomal site (Table 3.3.F).

fabD under the control of P_{PGK1} was integrated at one copy into the genome of strain BY-G (BY4741 *met15::*P_{PGK1}-*fabG*-T_{CYC1}). PCR was used to generate fragment P_{PGK1}-*fabD*-T_{CYC1} from vector pXP219-fabD and fragment loxP-*URA3*-loxP from plasmid pXP219, using the primers shown in Table 3.3.D. The flanking 5' and 3' ends of the adjoined segment had 50 bp overlapping sequences up- and down-stream of the target URA3 locus. *fabD* integrations into the *URA3* locus was confirmed by PCR analysis using primers that annealed upstream and downstream of the chromosomal site.

fabH under the control of P_{PGK1} and *acpS* under the control of P_{ADH2} were simultaneously integrated at one copy each into the genome of strain BY-DG (BY4741 met*15::*P_{PGK1}-*fabG*-T_{CYC1} *ura3::*P_{PGK1}-*fabD*-T_{CYC1}). PCR was used to generate fragment P_{PGK1}-*fabH*-T_{CYC1} from vector pXP219-

fabH, using primers Forward1-LEU2 and Reverse1-LEU2 (Table 3.3.D). Primers Forward2-LEU2 and Reverse2-LEU2 were used to generate fragment P_{TEF1}-*acpS*-T_{ADH2}-loxP-*HIS3*-loxP from plasmid pRF320-acpS. The first fragments had 50 bp homologous sequences on the 3' end to fragment P_{TEF1}-*acpS*-T_{ADH2}-loxP-*HIS3*-loxP on the 5' end. The flanking 5' and 3' ends of the adjoined segment had 50 bp overlapping sequences up- and down-stream of the target *LEU2* locus. *fabH* and *acpS* integrations into the *LEU2* locus were confirmed by PCR analysis using primers that annealed upstream and downstream of the chromosomal site.

acpP under the control of P_{PGK1}, and *fabZ* under the control of P_{ADH2}, were simultaneously integrated at one copy each into the genome of strain BY-aSDGH (BY4741 met*15::*P_{PGK1}-*fabG*-T_{CYC1} *ura3::*P_{PGK1}-*fabD*-T_{CYC1} *leu2::*P_{PGK1}-*fabH*-T_{CYC1}-P_{TEF1}-*acpS*-T_{ADH2}). PCR was used to generate fragment P_{PGK1}-*acpP*-T_{CYC1} from vector pXP219-acpP, using primers Forward1-Ty15 and Reverse1-Ty15 (Table 3.3.D). Primers Forward2-Ty15 and Reverse2-Ty15 were used to generate fragment P_{TEF1}-*fabZ*-T_{ADH2}-loxP-HIS3-loxP from plasmid pRF320-fabZ. The first fragments had 50 bp homologous sequences on the 3' end to fragment P_{TEF1}-*fabZ*-T_{ADH2}-loxP-*HIS3*-loxP on the 5' end. The flanking 5' and 3' ends of the adjoined segment had 50 bp overlapping sequences up- and down-stream of the target *LEU2* locus. *fabZ* and *acpP* integrations into the *Ty1-5* locus were confirmed by PCR analysis using primers that annealed upstream and downstream of the chromosomal site.

fabB under the control of P_{PGK1}, and *fabI* under the control of P_{ADH2}, were simultaneously integrated at one copy each into the genome of strain BY4741. PCR was used to generate fragment P_{PGK1}-*fabB*-T_{CYC1} from vector pXP219-fabB, using primers Forward1-Ty11 and Reverse1-Ty11 of Table S-4. Primers Forward2-Ty11 and Reverse2-Ty11 shown on Table 3.3.D were used to generate fragment P_{TEF1}-*fabI*-T_{ADH2}-loxP-*HIS3*-loxP from plasmid pRF320-fabI. The first fragments had 50 bp homologous sequences on the 3' end to fragment P_{TEF1}-*fabI*-T_{ADH2}-loxP-*HIS3*-loxP on the 5' end. The

flanking 5’ and 3’ ends of the adjoined segment had 50 bp overlapping sequences up- and down-stream of the target *Ty15* locus. *fabB* and *fabI* integrations into the *Ty1-5* locus were confirmed by PCR analysis using primers that annealed upstream and downstream of the chromosomal site.

To knockout the *FAS2* gene, nested polymerase chain reaction (PCR) was used to increase the homologous sequence, upstream and downstream, to approximately 100 bp. PCR was run using primers FAS2-KO-M-for and FAS2-KO-M-rev with pXP214 (*MET15* marker) to generate a piece with approximately 25 bp of homology upstream and downstream the *FAS2* gene sequence in the yeast genome. Gel electrophoresis with 1% agar gel was used to separate and to visualize DNA products. In order to increase the homologous sequence of the inserting DNA with the yeast genome, the DNA product from the previous step was used as template in a PCR reaction with primers Nes1-F-FAS2KO and Nes1-R-FAS2KO, which have 24 bp of homology with the template and add 36 bp of homology with the yeast genome on each end of the insert. This process was repeated with primers Nes2-F-FASKO and Nes2-R-FAS2KO to have a total homology of approximately 100 bp on each side.

Table 3.1: List of strains

Strains	Description	Source
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY1G	BY4741 *ura3*::P_{PGK1}-*acpS*-T_{CYC1}	This study
BY6G	BY4741 *met15::*P_{PGK1}-*fabG*-T_{CYC1} *ura3::*P_{PGK1}-*fabD*-T_{CYC1} *leu2::*P_{PGK1}-*fabH*-T_{CYC1}-P_{TEF1}-*acpS*-T_{ADH2} *ty1-5::*P_{PGK1}-*acpP*-T_{CYC1}-P_{TEF1}-*fabZ*-T_{ADH2}	This study
BY8G	BY6G *ty1-1::*P_{PGK1}-*fabB*-T_{CYC1}-P_{TEF1}-*fabI*-T_{ADH2}	This study
BY4741ΔFAS2	BY4741 fas2Δ0	This study
BY8GΔFAS2	BY8G fas2Δ0	This study

Table 3.2: List of plasmids

Plasmids	Description	Source
pXP219	2µ vector, *PGK1* promoter, *CYC1* terminator, *URA3* selectable marker	Fang et al., 2011
pXP219-acpP	pXP219 harboring *acpP* with 6xHis-tag on C-terminus	This study
pXP219-acpS	pXP219 harboring *acpS* with 6xHis-tag on C-terminus	This study
pXP219-fabB	pXP219 harboring *fabB* with 6xHis-tag on N-terminus	This study
pXP219-fabD	pXP219 harboring *fabD* with 6xHis-tag on N-terminus	This study
pXP219-fabG	pXP219 harboring *fabG* with 6xHis-tag on N-terminus	This study
pXP219-fabH	pXP219 harboring *fabH* with 6xHis-tag on N-terminus	This study
pXP219-fabI	pXP219 harboring *fabI* with 6xHis-tag on N-terminus	This study
pXP219-fabZ	pXP219 harboring *fabZ* with 6xHis-tag on N-terminus	This study
pXP219-tesA	pXP219 harboring *tesA* with 6xHis-tag on C-terminus	This study
pXP219-RC	pXP219 harboring RC with 6xHis-tag on C-terminus	This study
pXP219-TEII	pXP219 harboring TEII with 6xHis-tag on C-terminus	This study
pJF702	*CEN/ARS* vector, *ADH2* promoter, *ADH2* terminator, *LEU2* selectable marker	This study
pXP320	*CEN/ARS* vector, *TEF1* promoter, *CYC1* terminator, *HIS3* selectable marker	Fang et al., 2011
pRF320	*CEN/ARS* vector, *TEF1* promoter, *ADH2* terminator, *LEU2* selectable marker	This study
pRF320-acpS	pRF320 harboring *acpS* with 6xHis-tag on C-terminus	This study
pRF320-fabI	pRF320 harboring *fabI* with 6xHis-tag on N-terminus	This study
pRF320-fabZ	pRF320 harboring *fabZ* with 6xHis-tag on N-terminus	This study

Table 3.3: List of primers

Table 3.3.A. Primers used to amplify, 6xHis-tag, and clone FAS enzymes into pXP219

Gene	Direction	Primer name	Primer sequence, 5' -> 3'
acpP	Forward	acpP-for-speI	GACCTCCC-ACTAGT-AAAAAA-ATGAGCACTATCGAAGAACG
	Reverse	acpP-rev-histag	TGGTAATC-CTCGAG-TTA-ATGATGATGATGATGATG-CGCCTGGTGGCCGTTGA
acpS	Forward	acpS-for-speI	GACCTCCC-ACTAGT-AAAAAA-ATGGCAATATTAGGTTTAGG
	Reverse	acpS-rev-histag	TGGTAATC-CTCGAG-TTA-ATGATGATGATGATGATG-ACTTTCAATAATTACCG
fabB	Forward	fabB-for-speI-histag	GACCTCCC-ACTAGT-AAAAAA-ATG-CATCATCATCATCATCAT-AAACGTGCAGTGATTAC
	Reverse	fabB-rev	TGGTAATC-CTCGAG- TTAATCTTTCAGCTTGCGCA
fabD	Forward	fabD-for-pmeI-histag	GACCTCCC-GTTTAAAC-AAAAAA-ATG-CATCATCATCATCATCAT-ACGCAATTTGCATTTGT
	Reverse	fabD-rev	TGGTAATC-CGGTCCG-TTAAAGCTCGAGCGCCGCTG
fabG	Forward	fabG-for-speI-histag	GACCTCCC-ACTAGT-AAAAAA-ATG-CATCATCATCATCATCAT-AATTTTGAAGGAAAAAT
	Reverse	fabG-rev	TGGTAATC-CTCGAG- TCAGACCATGTACATCCCGC
fabH	Forward	fabH-for-speI-histag	GACCTCCC-ACTAGT-AAAAAA-ATG-CATCATCATCATCATCAT-TATACGAAGATTATTGG
	Reverse	fabH-rev	TGGTAATC-CTCGAG- CTAGAAACGAACCAGCGCGG
fabI	Forward	fabI-for-speI-histag	GACCTCCC-ACTAGT-AAAAAA-ATG-CATCATCATCATCATCAT-GGTTTTCTTTCCGGTAA
	Reverse	fabI-rev	TGGTAATC-CTCGAG- TTATTTCAGTTCGAGTTCGT
fabZ	Forward	fabZ-for-speI-histag	GACCTCCC-ACTAGT-AAAAAA-ATG-CATCATCATCATCATCAT-ACTACTAACACTCATAC
	Reverse	fabZ-rev	TGGTAATC-CTCGAG- TCAGGCCTCCCGGCTACGAG
tesA	Forward	tesA-for-speI	GACCTCCC-ACTAGT-AAAAAA-ATGATGAACTTCAACAATGT
	Reverse	tesA-rev-histag	TGGTAATC-CTCGAG-TTA-ATGATGATGATGATGATG-TGAGTCATGATTTACTA
RC	Forward	RC-for-speI	TCCC-ACTAGT-AAAAAA-ATGGTAGCAACAGCAGCGGCAGCA
	Reverse	RC-rev-histag	GGGACTCGAGTTAATGATGATGATGATGATGGGCGCTTTCAACCGGAATTTG
TEII	Forward	TEII-for-speI	GACCTCCC-ACTAGT-AAAAAA-ATGGAGACAGCAGTCAATGCTAAGAGTCCCAGGAATGAAAAGGTTTTGAACTGT
	Reverse	TEII-rev-histag	TGGTAATC-CTCGAG-TCA-ATGATGATGATGATGATG-AGTGAGTGACGAGAGTTCCAA

Table 3.3.B. Primers used to amplify, 6xHis-tag, and clone FAS enzymes into pXP219

Gene	Locus	Marker	Direction	Primer Sequence
acpS	***URA3***	***LEU2***	Forward1	GTTTTGACCATCAAAGAAGGTTAATGTGGCTGTGGTTTCAGGGTCCATAA GGCATTTGCAAGAATTACTCGTG
			Reverse1	CCCGGGGATCCTCTAGAGTCGACCGGCCGCAAATTAAAGCCTTCGAGCG
			Forward2	CGCTCGAAGGCTTTAATTTGCGGCCggtcgactctagaggatcCCCGGG
			Reverse2	GCGTATATAGTTTCGTCTACCCTATGAACATATTCCATTTTGTAATTTCGTG TCGAATTCGAGCTCGGTACCCGGG

Table 3.3.C. Primers used to amplify, 6xHis-tag, and clone FAS enzymes into pXP219

Terminator	Direction	Primer name	Primer sequence, 5' → 3'
ADH2	Forward	For-ADH2t	5'-TCCCctcgagAGTCGACATGCATCCTAGGTTTAAACATGC-3'
	Reverse	Rev-ADH2t	5'-TCCCTCTAGAGTCGAGGGGAGCAAACGCGCTGGGAGCAAA-3'

Table 3.3.D. Primers used to integrate the *E. coli* FAS genes into the *S. cerevisiae* genome.

Locus	Type of Integration	Gene/s	Direction	Primer Sequence 5' → 3'
MET15	Single, 2-piece, double crossover	**fabG**	forward1	GGCAAATGGCACGTGAAGCTGTCGATATTGGGGAACTGTGGT GGTTGGCAAGGCATTTGCAAGAATTACTCGTG
			reverse1	CCCGGGGATCCTCTAGAGTCGACCGGCCGCAAATTAAAGCCTT CGAGCG
			forward2	CGCTCGAAGGCTTTAATTTGCGGCCggtcgactctagaggatcCCCG GG
			reverse2	GCAAATAAAACACTATTGATTGCTTAAAAGGGCAATCCGACTA TATCTGGAATTCGAGCTCGGTACCCGGG
URA3	Single, 2-piece, double crossover	**fabD**	forward1	GTTTTGACCATCAAAGAAGGTTAATGTGGCTGTGGTTTCAGGG TCCATAAGGCATTTGCAAGAATTACTCGTG
			reverse1	CCCGGGGATCCTCTAGAGTCGACCGGCCGCAAATTAAAGCCTT CGAGCG
			forward2	CGCTCGAAGGCTTTAATTTGCGGCCggtcgactctagaggatcCCCG GG
			reverse2	CCAATTTTTTTTTTTTCGTCATTATAGAAATCATTACGACCGAG ATTCCCGGGAATTCGAGCTCGGTACCCGGG
LEU2	Tandem, 2-piece, double crossover	**fabH acpS**	forward1	CCATGTATAATCTTCATTATTACAGCCCTCTTGACCTCTAATCAT GAATGTTAGGCATTTGCAAGAATTACTCGTG
			reverse1	aaggattcgcggtaatattg**aaaaagg** GGCCGCAAATTAAAGCCTTCGAGCG
			forward2	CGCTCGAAGGCTTTAATTTGCGGCC cctttttcaatattaccgcgaatcctt
			reverse2	GCGTATATAGTTTCGTCTACCCTATGAACATATTCCATTTTGTA ATTTCGTGTCGAATTCGAGCTCGGTACCCGGG
Ty 1-5	Tandem, 2-piece, double crossover	**acpP fabZ**	forward1	CACAGAGTTGTATTTGCGCTTCTGAGCGATGCTTCCGAGATTG TTGAAGCAAGGCATTTGCAAGAATTACTCGTG
			reverse1	aaggattcgcggtaatattg**aaaaagg** GGCCGCAAATTAAAGCCTTCGAGCG
			forward2	CGCTCGAAGGCTTTAATTTGCGGCCcctttttcaatattaccgcgaatcc tt

Locus	Type of Integration	Gene/s	Direction	Primer Sequence 5' → 3'
			reverse2	GATTATTGAAGAGGGATGCGTTTGGTACAATAAAAAACATAG GTTCCCAAACCGAATTCGAGCTCGGTACCCGGG
Ty 1-1	Tandem, 2-piece, double crossover	**fabB fabI**	forward1	GACTTCTAGTATTATCTGTATATCTAATATTATAGTCTCTAACA ACAGTGGAATAGGCATTTGCAAGAATTACTCGTG
			reverse1	aaggattcgcggtaatattg**aaaaagg** GGCCGCAAATTAAAGCCTTCGAGCG
			forward2	CGCTCGAAGGCTTTAATTTGCGGCC ccttttttcaatattaccgcgaatcctt
			reverse2	CATGTATGAAACTGGGAATTCTGATAAATTTTGTCATAACTGTT GGGATTCCGAATTCGAGCTCGGTACCCGGG

Table 3.3.E. Primers used to integrate the *E. coli* FAS genes into the *S. cerevisiae* genome.

Gene	Direction	Primer name	Primer Sequence
FAS2	Forward	FAS2-KO-M-for	CAACTTTGGCTGGGATGGCTCAAACCCGGGataacttcgtataGCATACAT
	Reverse	FAS2-KO-M-rev	ATGTCTTTCAATGCAGCACCACCGCCCGGGataacttcgtataATGTATGC
	Forward	Nes1-F-FAS2KO	TTAACACTGAAAGGGTTGTTGAAATCGGTCCTTCTCCAACTTTGGCTGGG ATGGCTCAAA
	Reverse	Nes1-R-FAS2KO	ACGGCTGGAGCGTTTTTGTTAACGCGTACGATTTCGATGTCTTTCAATGC AGCACCACCG
	Forward	Nes2-F-FAS2KO	GATGGATTGAAACTCAAGATGTTTTTTTGAAGGATTTTAACACTGAAAGG GTTGTTGAAA
	Reverse	Nes2-R-FAS2KO	CTTCGGCAGCCTTTTTGGCGTTACCGTGCAGTTCAACGGCTGGAGCGTTT TTGTTAACGC

Table 3.3.F. Loci check integration/knockout primers.

Locus	Direction	Primer Sequence
FAS2	Forward	TGTTGTTGTCGTTGTTGTCCCAGC
	Reverse	CCAGTAAGTCGCTCCAGATTT
LEU2	Forward	GGAATTCTAACAATTATCAAATTGTCCG
	Reverse	CAGCCTGTACATCTGCTTCCC
MET15	Forward	CTCCTCGAGGATTTAGGAATCC
	Reverse	GCGGGATCGAACCGCTGATCC
Ty 1-1	Forward	GGCCTGTGCGTGTTCATAAGGG
	Reverse	ACATGATATCATAACTTACCAG
Ty 1-5	Forward	GCTAATCTTTGGATATAGGGC
	Reverse	CAGATCACTTATACAGCTTTACACAG
URA3	Forward	CTAGGGAAGACAAGCAACG
	Reverse	GTGAAGTCATTGACACAGTCTG3

Media and Cultivation

Luria-Bertani (LB) medium containing 100 µg/mL ampicillin was used for *E. coli* cultivation. *S. cerevisiae* strains were cultivated in complex YPD, selective SDC-A, or SD minimal medium containing 2% dextrose (see Supplementary Material). For *FAS2* knockout strains without the integrated eFAS, the medium was supplemented with 2 mM myristic acid and tween 40 (0.5%). *S. cerevisiae* strains were cultivated 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 x 125 mm culture tubes for inoculation to an OD of 0.1. Cells for fatty acid measurement studies were grown in shake flasks containing 50, 200 or 250 mL medium.

Analytical Methods

The protein purification, NADPH and ADIFAB assay protocols were modified from Leber and Da Silva (2013). Activity of AcpS, AcpS, FabD, FabH, and FabG was determined via a NADPH assay using equimolar amounts of purified *E. coli* FAS enzymes in the NADPH reaction buffer. For the ADIFAB assay, purified type II FAS enzymes (5-15 µM final conc.) were combined with acetyl-CoA (50 µM final conc.), malonyl-CoA (150 µM final conc.), and NADPH (150 µM final conc.) to a final volume of 500 µL. The reaction mixture was placed on a microfuge tube rotator at 37˚C for 1 h. 500 µL of 0.2 µM ADIFAB suspended in measuring buffer was then added and the 1 mL solution was incubated at 37˚C for 20 min. The mixture was transferred to a quartz cuvette and a Bowman Series 2 luminescence spectrometer was used to take readings at wavelengths of 432 and 505 nm. FA extraction and quantification via GC-MS followed that in Leber and Da Silva (2013).

Statistical Analysis

Three independent enzyme isolation/ADIFAB experiments were performed, with two

independent assays per experiment (n=6). For the fatty acid analysis, three independent experiments were performed (n=3). A one-way analysis of variance (ANOVA) was used, followed by Dunnett's multiple comparison test. Values were considered statistically significant when p-values were <0.05. Data are reported as mean $\pm$ standard error of the mean (SEM), unless otherwise stated.

RESULTS AND DISCUSSION

As a first step, we chose a minimum set of required enzymes, and then tested the expression and *in vitro* activity of the enzymes synthesized in yeast. Nine genes (*acpS, acpP, fabB, fabD, fabG, fabH*, *fabI, fabZ*, *tesA*) coding for nine proteins are required to produce fatty acids in yeast using this type II FAS. The genes, enzymes, and relevant reactions are shown in Table 3.4, and have been extensively reviewed in the literature, e.g., White et al. (2005).

Table 3.4. List of genes, proteins, and reactions.

Gene	Protein	Reaction	Ref.
acpP	Acyl carrier protein (ACP)		White et al., 2005
acpS	ACP synthase	apo-ACP + 4'-Phosphopantetheine Group → ACPSH	White et al., 2005
fabB*	β-ketoacyl-ACP synthase I	(n)acyl-ACP + malonyl-ACP → (n+2)β-ketoacyl-ACP acetoacetyl-ACP + malonyl-ACP → β-ketobutyryl-ACP + CO_2 + ACPSH	White et al., 2005
fabD	Malonyl-CoA:ACP transacylase	malonyl-CoA + ACPSH → malonyl-ACP	White et al., 2005
fabG	β-ketoacyl-ACP reductase	β-ketoacyl-ACP + NADPH + H^+ → β-hydroxyacyl-ACP + $NADP^+$	White et al., 2005
fabH	β-ketoacyl-ACP synthase III	malonyl-ACP + acetyl-CoA → acetoacetyl-ACP	White et al., 2005
fabI	Enoyl-ACP reductase	enoyl-ACP + NADH+H^+ → acyl-ACP + NAD^+	White et al., 2005
fabZ†	β-hydroxyacyl-ACP dehydratase	β-hydroxyacyl-ACP → enoyl-ACP + H_2O	White et al., 2005
tesA	Thioesterase I	acyl-ACP → free fatty acid + ACPSH	Cho and Cronan, 1993

* Two *E. coli* enzymes FabB (β-ketoacyl-ACP synthase I) and FabF (β-ketoacyl-ACP synthase II) catalyze the condensation of malonyl-CoA with the fatty acyl-ACP growing chain. We expressed only β-ketoacyl-ACP synthase (FabB), since expression of FabF in active form has proven challenging (Magnuson et al., 1995).

† There are two genes in *E. coli* that encode β-hydroxyacyl-ACP dehydrases, FabA and FabZ. The broad substrate specificity of FabZ and the low activity of FabA for β-hydroxybutyryl-ACP (Heath and Rock, 1996) made FabZ a better choice for our work.

Each of the genes (except *acpP*) were introduced into an independent yeast strain on a 2μ-based plasmid (Table 3.2). AcpS activates apo-ACP (encoded by *acpP*), transferring to it the 4'-phosphopantetheine from coenzyme A, resulting in holo-ACP (Flugel et al., 2000). Active ACP was obtained using *S. cerevisiae* strain BY1G (Table 3.1), with one-copy of the *acpS* gene integrated into the genome, and carrying a plasmid harboring *acpP*. Yeast strains expressing the different type II FAS proteins were cultivated in selective SDC-A medium, the cells were harvested, and the proteins were extracted and purified using Ni-NTA columns. Western blots of the purified proteins were used to verify their expression in yeast (Figure 3.1), and two *in vitro* activity assays were performed to confirm their activity (Figure 3.2).

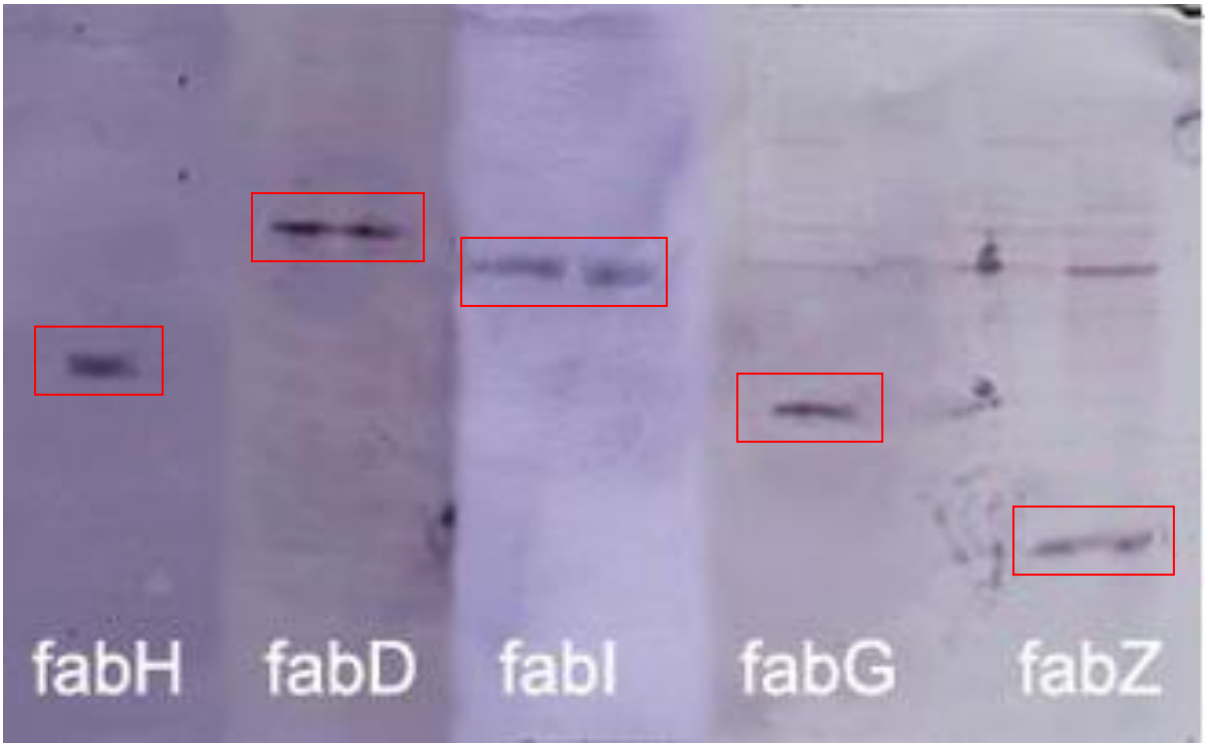


Figure 3.1. Western blot analysis of purified fabH-6xHis, fabD-6xHis, fabI-6xHis, fabG-6xHis and fabZ-6xHis. The image was created from independent Western blot images. Relative sizes among enzymes are not to scale.

A NADPH colorimetric assay (Bays et al., 2009) was used to directly verify activity of FabG, and thus the activity of the upstream enzymes AcpP, AcpS, FabD, FabH (Figure 3.2). The assay results (3.3.A) demonstrate that these five enzymes were actively expressed in yeast. To determine if all of the type II FAS proteins are active and can work together when expressed in *S. cerevisiae*, an *in vitro* acrylodan-labeled intestinal fatty acid binding protein (ADIFAB) assay was used. The purified enzymes were combined with acetyl-CoA, malonyl-CoA and NADPH, and the concentrations of free fatty acids (FFAs) were measured (Figure 3.3.B).

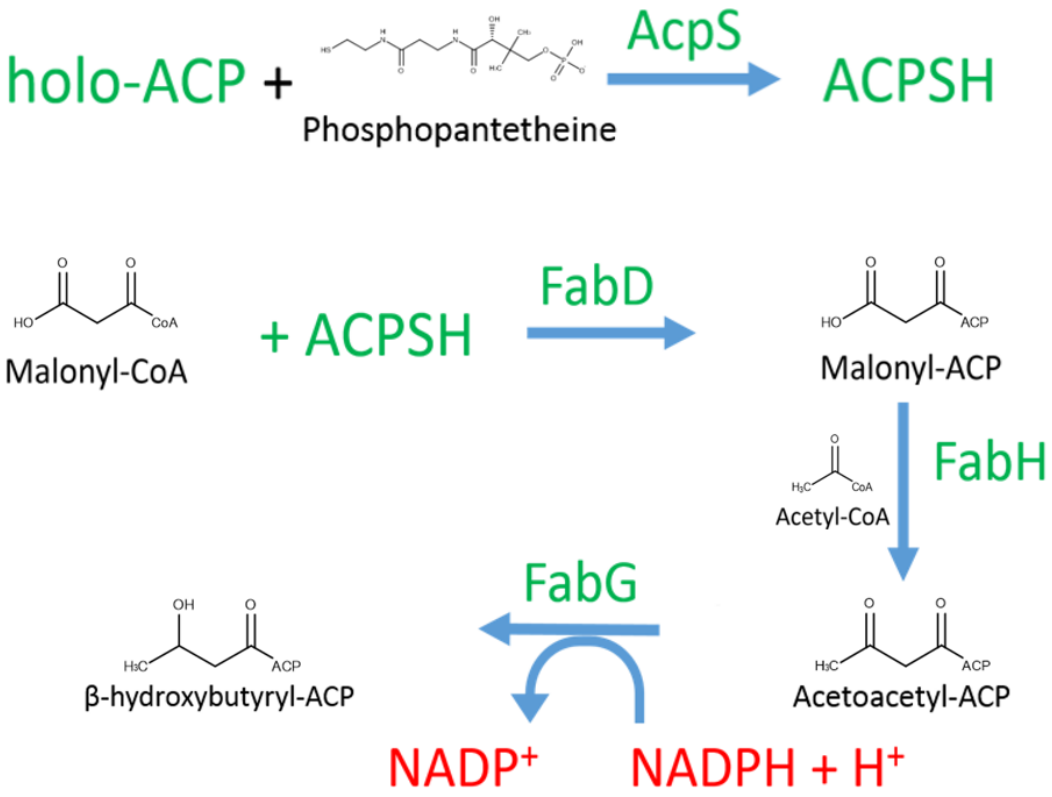


Figure 3.2. Pathway for the production of β-hydroxybutyryl-ACP. The reduction of the keto group to alcohol requires NADPH as a reducing agent, and its depletion can be monitored.

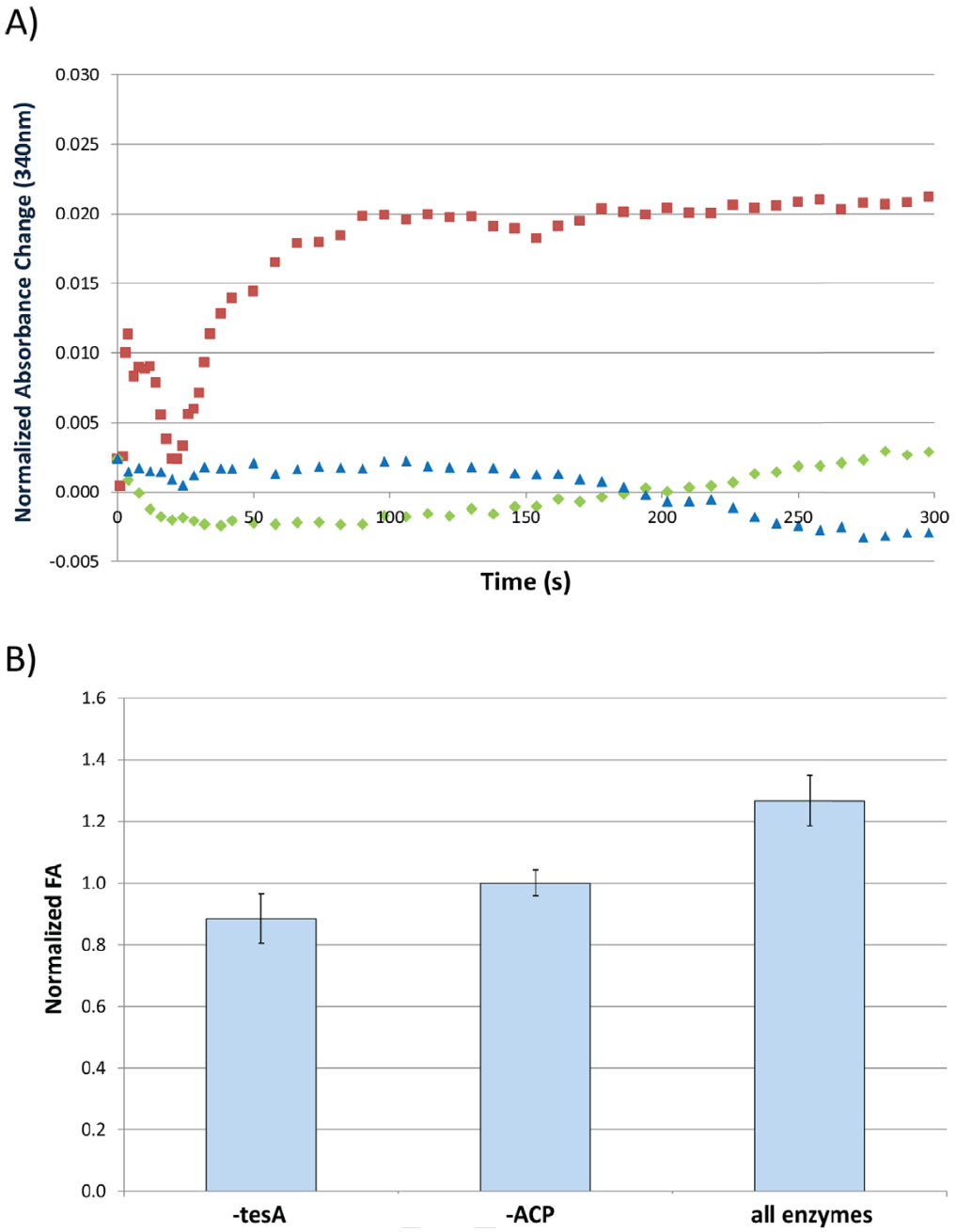


Figure 3.3. **A)** Confirmation of the activity of the AcpP, AcpS, FabD, FabH, and FabG proteins expressed in *S. cerevisiae*. An *in vitro* NADPH absorbance assay demonstrated the successful expression and activity of five of the nine genes (Figure 3.2) needed to express the type II FAS in yeast. Complete mix (squares) consisted of buffered ACP, FabD, FabH, FabG, acetyl-CoA, NADPH, and malonyl-CoA. Negative controls consisted of a complete mix minus FabG (triangles), and complete mix minus ACP (diamonds). Three independent experiments showed similar profiles. **B)** Confirmation of type II FAS activity by *in vitro* fatty acid synthesis. An *in vitro* ADIFAB assay demonstrated fatty acid synthesis when ACP, FabB, FabD, FabG, FabH, FabI, FabZ, and TesA were mixed with acetyl-CoA, malonyl-CoA, and NADPH. Negative controls were the reaction mix lacking *E. coli* thioesterase I TesA and the reaction mix lacking the acyl carrier protein ACP. Three independent experiments (with two independent replicates per experiment) were performed. Values were normalized to the -ACP control to allow comparisons across experiments. Results are expressed as mean ± SEM (n=6). The negative controls were statistically lower ($p<0.05$).

The negative controls, lacking either TesA (thioesterase activity) or holo-ACP (AcpP and AcpS) (essential prosthetic group), had a background concentration of FFA, likely due to carryover from the protein elutes and non-specific binding of the ADIFAB protein. However, the FFA level for the mixture with all enzymes was higher than the controls ($p<0.05$), demonstrating that the *E. coli* type II enzymes expressed in yeast are able to produce FFAs *in vitro* from malonyl-CoA and acetyl-CoA. The trends shown in Figure 1B were seen in each of the three independent experiments.

After confirming activity of the enzymes produced in *S. cerevisiae*, we introduced all nine genes into one strain. Eight genes (*acpS, acpP, fabB, fabD, fabG, fabH*, *fabI, fabZ*) were integrated into the BY4741 genome and the thioesterase gene was carried on a 2μ-based plasmid. The rationale for the plasmid-based TE was two-fold: (1) the ability to quickly introduce different TEs and (2) the observed overproduction of free fatty acids in *E. coli* in the presence of high levels of cytosolic thioesterase activity (Lu et al., 2008), as this reaction is the bottleneck for FA production. All genes were integrated via double crossover to ensure stability (Da Silva and Srikrishnan, 2012). Single copies of *fabG* and *fabD* were sequentially integrated; the remaining six genes (*acpP*, *acpS*, *fabB*, *fabH*, *fabI*, and *fabZ*) were integrated in pairs as a single PCR product (Figure 3.4). For the latter integrations, different promoters and terminators were used to avoid repeat sequences and thus avoid loss by homologous recombination. The nine genes were under either the *PGK1* or the *TEF1* promoter.

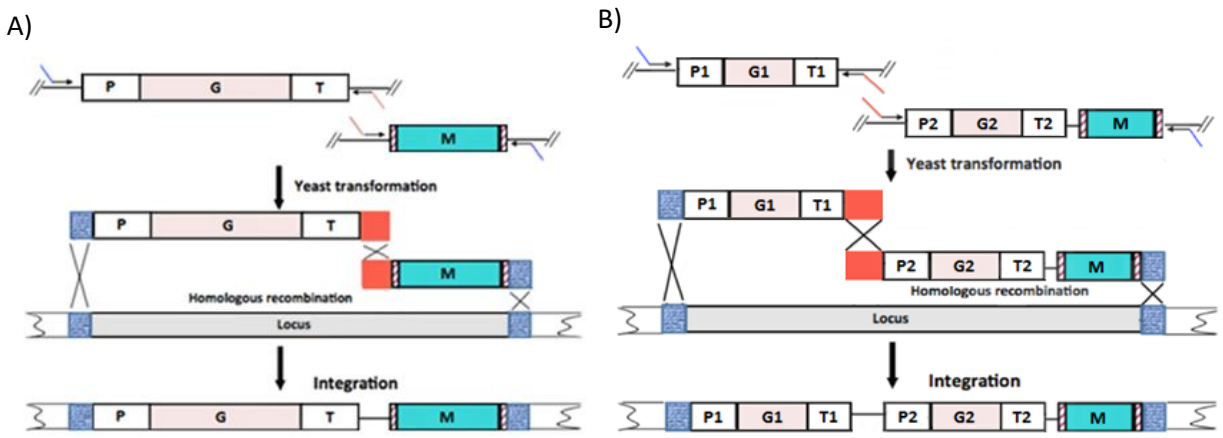


Figure 3.4. Schematic comparison of the single gene and tandem gene integration. **A)** Double crossover integration of gene G into one locus. P1 is promoter P_{PGK1} and T1 is terminator T_{CYC1}. **B)** Integration of gene G1 and gene G2. P1 is P_{PGK1}, P2 is P_{TEF1}, T1 is T_{CYC1}, and T2 is T_{ADH2}. The construct P1-G1-T1 was obtained through PCR using a pXP219-based plasmid as template. The construct P2-G2-T2-M (e.g., M=*LEU2* marker) was obtained through PCR using a pRF320-based plasmid as template.

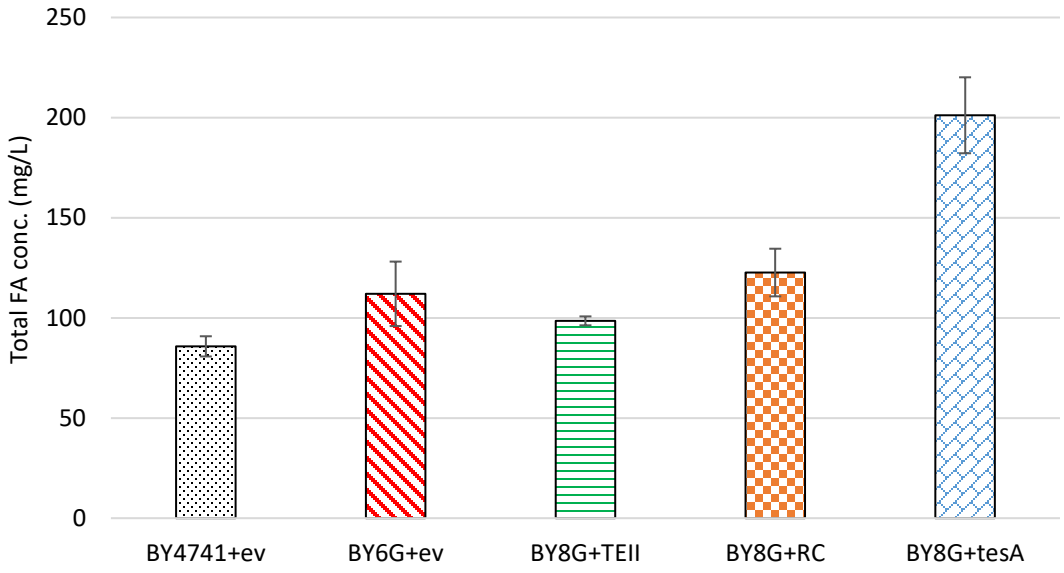
Three strains were used for our expression studies: BY4741 (parent strain), BY6G (all genes except *fabB* and *fabI* integrated), and BY8G (all eight genes integrated) (Table I). BY4741 and BY6G are control strains that do not carry the full type II FAS. Three different thioesterase genes (*tesA*, RC, and TEII) were cloned into pXP219 vectors (Table 3.1). TesA is natively located in the periplasm of *E. coli* and shows substrate preference for C_{16} and C_{18} acyl intermediates (Cho and Cronan, 1993). TEII (thioesterase II), from *Rattus novergicus* mammary gland, shifted the *in vitro* FA production profile of the rat FAS from long chain fatty acids (C_{16} and C_{18}) to short-chain fatty acids (C_8, C_{10}, C_{12}) (Libertini and Smith, 1978). In our earlier studies, this TE proved effective for producing C_8 and C_{10} when overexpressed with the human FAS in yeast (Leber and Da Silva, 2013). RC is a palmitoyl-ACP thioesterase from *Ricinus communis* (*fatB*); when overexpressed in *E. coli*, the production of myristic acid (C_{14}) increased more than 4-fold, accounting for 40% of the total fatty acids and increasing the total free fatty acid synthesized (Zhang et al., 2011).

Fatty acid production was first characterized in strain BY8G carrying pXP219-tesA, pXP219-

TEII, or pXP219-RC and the two control strains. Growth rates were similar for all strains, indicating minimal growth inhibition due to the expression of the heterologous type II FAS and the overexpression of the thioesterases. No difference was seen in the distribution of fatty acid chain lengths or total fatty acid produced by the strains expressing TEII or RC relative to the controls (Figure 3.5). However, strain BY8G+pXP219-tesA produced 201 mg/L total fatty acids, more than twice that for the control BY4741+pXP219 (86 mg/L). Overexpression of enzymes with acyl-CoA thioesterase activity has previously been shown to increase total FFA production in *S. cerevisiae* (Runguphan and Keasling, 2014). Therefore, the observed increase in fatty acids could be due to overexpression of the thioesterase rather than an increase due to the *E. coli* FAS system.

The lack of change in fatty acid distribution suggests that the native yeast FAS is dominant, limiting the impact of the heterologous eFAS system. Therefore, the native yeast FAS activity was eliminated to remove competition for substrates, cofactors, and reducing agents. We knocked out the *FAS2* gene in strains BY4741 and BY8G to create BY4741ΔFAS2 and BY8GΔFAS2. Deletion of the *FAS2* gene prevents *de novo* fatty acid production and is lethal to the yeast cells when fatty acids are not supplemented. The two *FAS2* deletion strains were plated on SDC-A supplemented with myristic acid to allow growth. Control strain BY4741ΔFAS2 was transformed with the empty vector pXP219, and strain BY8GΔFAS2 was transformed with vectors pXP219-tesA, pXP219-TEII, or pXP219-RC, creating three strains expressing the *E. coli* FAS system and different heterologous thioesterases.

A)



B)

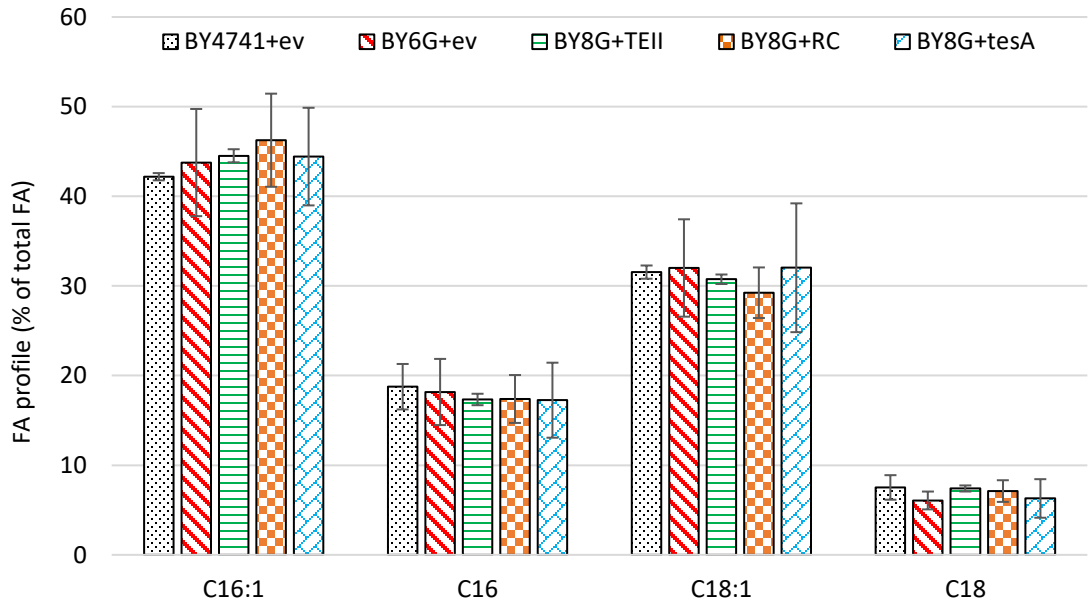


Figure 3.5. A) Total fatty acids produced by strain BY8G after 24 h using thioesterase TEII, RC, and TesA. Control strains with empty vector (ev), BY4741+pXP219 (dotted) and BY6G+pXP219 (diagonal striped) are compared to strains BY8G+pXP219-TEII (horizontal striped), BY8G+pXP219-RC (squared), and BY8G+pXP219-tesA (brick). **B)** Fatty acid profile as % of the total FA produced by each strain described above. No C14 was detected for any of the samples. Results are expressed as mean ± error from two independent experiments.

Single colonies of the four strains were plated on SDC-A without FA supplementation to test for growth, and were evaluated after 6 and 25 days (Figure 3.6). The control BY4741ΔFAS2+pXP219 did not grow, as this strain cannot produce fatty acids. Similarly, the strain with the integrated *E. coli* FAS (BY8GΔFAS2) but no thioesterase was unable to grow (data not shown). However, the three strains with the integrated *E. coli* FAS and expressing the heterologous thioesterases were viable. With the RC and TEII thioesterases, colonies grew to significant size; with TesA, the cell grew poorly generating only small colonies. It is noteworthy that the type II *E. coli* FAS system and heterologous TEs were capable of functionally replacing the native yeast FAS, producing sufficient fatty acids for growth. The RC acyl-ACP thioesterase, FatB from *R. communis,* has been previously expressed in *E. coli*, showing the highest production of FFAs in this bacteria compared to other TEs tested (Zhang et al., 2011). This result agrees with the growth of the *FAS2* knockout strains shown in Figure 3.6. While all three strains grew on plates, only BY8GΔFAS2+pXP219-RC grew well in liquid SDC-A and complex YPD media. We thus chose to focus on this strain for evaluation of the fatty acid profile and total fatty acid levels.

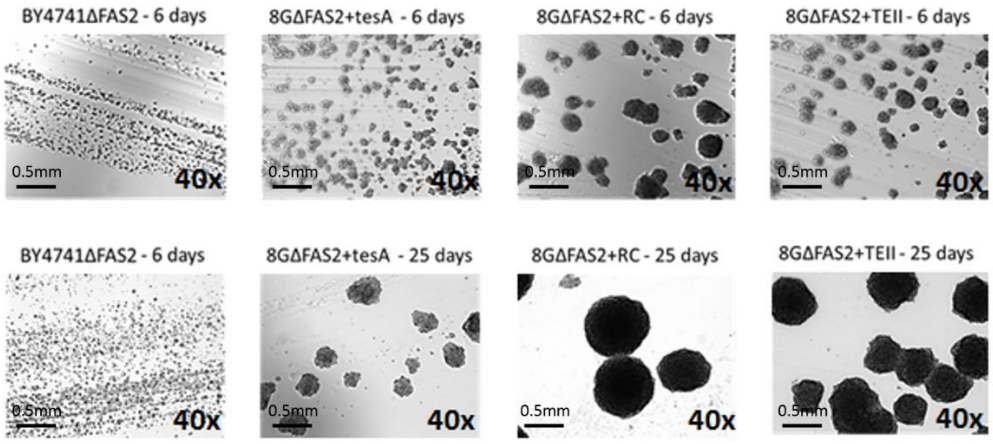


Figure 3.6. *In vivo* confirmation of heterologous *E. coli* FAS activity in strain BY8GΔFAS2 with different thioesterases: TesA, RC, and TEII. The negative control was strain BY4741ΔFAS2 carrying an empty plasmid pXP219. The ΔFAS2 strains were streaked on synthetic minimal media plates supplemented with fatty acids. Subsequent colonies were re-suspended in water, plated in the absence of FAs, and allowed to grow for 6 days (first row) and 25 days (second row). The images are 40x magnification bright-field microscopy pictures (Olympus, BX51).

Control BY4741+pXP219 and strain BY8GΔFAS2+pXP219-RC were cultivated in selective SDC-A medium, harvested at the same stage of growth (early stationary phase: 20h and 45h, respectively (Figure 3.7)), and FA levels were measured via GC-MS.

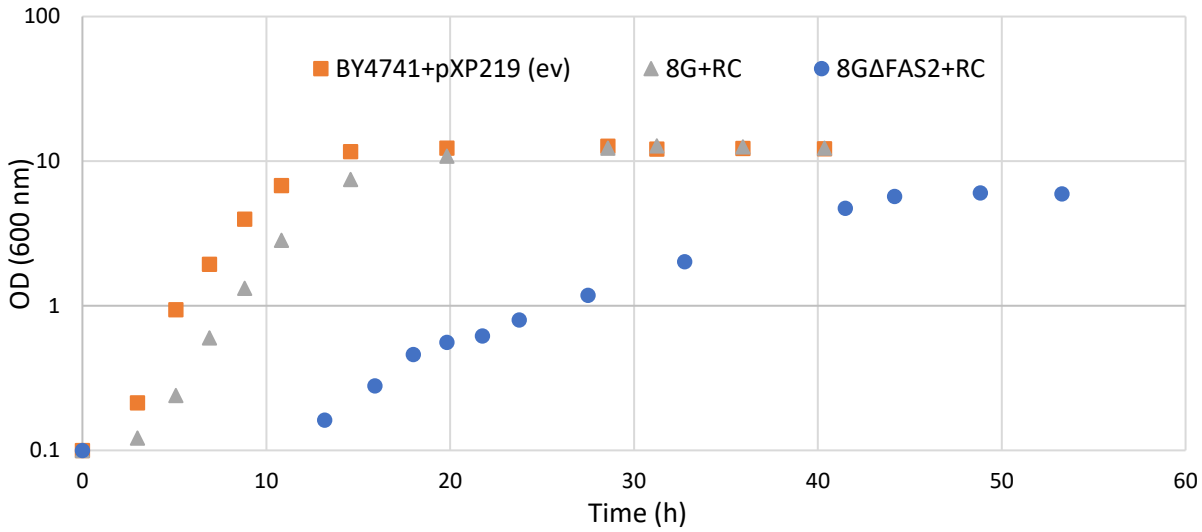


Figure 3.7. Growth curves for 8GΔFAS2+pXP219-RC (blue circles), 8G+pXP219-RC (grey triangles), and BY4741+pXP219 (orange squares) as a control. Cells were inoculated overnight in 5mL SDC(A), cells from those cultures were used to inoculate 50 mL SDC(A) at an initial OD_{600} of 0.1.

BY4741+pXP219 uses the native *S. cerevisiae* FAS to produce *de novo* fatty acids, while BY8GΔFAS2+pXP219-RC has no yeast FAS and uses only the *E. coli* FAS with the *R. communis* FatB thioesterase. Total fatty acids were 1.7-fold higher for the heterologous eFAS system (160 mg/L) relative to the control strain with the native yeast FAS (93 mg/L) (Figure 3.8.A), and 3.2-fold higher if normalized to OD. High concentrations of C_{14} fatty acids (33% of total FA) were also found in the type II eFAS strain (Figure 3.8.B), and C_{18} levels were significantly reduced (8% of total FA). Most of the C_{14} produced was monounsaturated (20% of total FA). In contrast, total C_{14} accounted for only 0.2% of total FAs (nearly undetectable) in the control strain using the native yeast FAS.

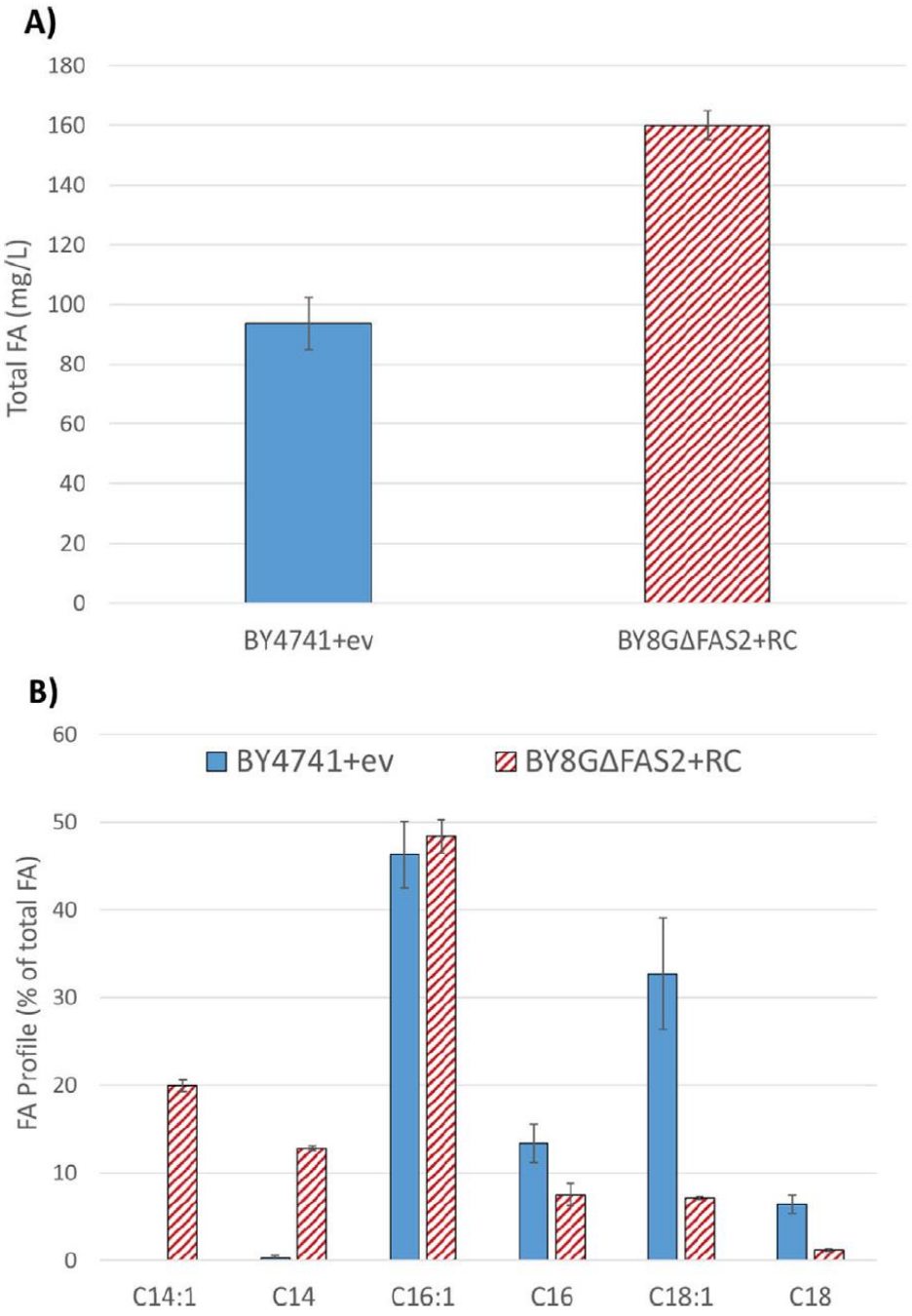


Figure 3.8. Intracellular fatty acid levels and chain length profile. **A)** Total fatty acids produced by strain BY8GΔFAS2 carrying pXP219-RC (red hatched bar), and control strain BY4741 carrying an empty vector (ev) pXP219 (blue solid bar). **B)** Profile of total fatty acids produced. Cells were harvested at early stationary phase: 20h for control and 45h for BY8GΔFAS2+pXP219-RC (Figure 3.7). No significant changes in the extracellular FA levels were measured for the control and FA production strains. Results are expressed as mean ± SEM (n=3). The differences in total fatty acid, C14:1, C14, C18:1, and C18 levels were statistically significant ($p<0.01$).

The fatty acid profile for strain BY8GΔFAS2+pXP219-RC thus showed both higher overall FA levels and significant production of C_{14} (and lower production of C_{18}) relative to the native *S. cerevisiae*. Fatty acid chain length is determined mainly by the acyl-ACP thioesterase in a type II FAS system. Interestingly, our results are similar to those observed in *E. coli*; Zhang et al., (2011) found that overexpression of *R. communis fatB* in *E. coli* increased total fatty acid levels and altered the normal FA distribution, increasing C_{14} production and decreasing C_{18}, without removing the native thioesterase. The FA profile in yeast is also dependent on elongases, especially Elo1, which can actively elongate C_{12}-C_{14} acyl-CoA primers to C_{16} and C_{18} (Dittrich et al., 1998).

The final OD of strain BY8GΔFAS2+pXP219-RC was half that of the native BY4741 (Table 3.5, Figure 3.7). In contrast, BY8G+pXP219-RC (with the intact native FAS) had a growth profile similar to the native strain. Therefore, deletion of the native *FAS2* gene and reliance on only the heterologous eFAS pathway reduced the growth of the cells. This also decreased the FA productivity (mg/L·h); although titers were substantially higher for BY8GΔFAS2+pXP219-RC, a longer growth period was needed to reach stationary phase. The heterologous pathway and the high percentage of C_{14} relative to C_{18} may be contributing factors. Better growth and productivity should be possible if the native yeast FAS is down-regulated instead of eliminated, supplementing sufficient native FAS function for healthy growth without excessive competition with the heterologous FAS system.

Table 3.5. Comparison of growth and FA production for the final engineered strain, BY8GΔFAS2+pXP219-RC, with the control, BY4741+pXP219 (ev) at stationary phase (45 h for BY8GΔFAS2+pXP219-RC and 20h for BY4741+pXP219) in SDC-A medium. Results are expressed as mean ± SEM from triplicates.

	Final OD_{600}	**C_{14} concentration (mg/L)**	**Total FA concentration (mg/L)**	**Total FA productivity mg/(L·h)**
BY4741+ev	12.2±0.2	0.20±0.02	93.5±8.7	4.7±0.4
BY8GΔFAS2+RC	6.5±0.1	52.3±1.4	160±5	3.6±0.1

CONCLUSIONS

These results demonstrate the promise of the heterologous *E. coli* FAS system for engineering yeast to produce alternate fatty acid products. The flexibility offered by this dissociated system allowed us to introduce heterologous thioesterases with the ability to cleave the ACP prosthetic group of fatty acids at different chain lengths. This system can also be used for the introduction of novel thioesterases cleaving the ACP from FA intermediates, potentially producing a large number of different final products with new chemical functionalities (Kim et al., 2015). In addition, the discrete type II FAS system allows expression to be optimized or even eliminated for each independent catalytic step.

ACKNOWLEDGEMENTS

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REFERENCES

Amberg, D., Burke, D., and Strathern, J. (2005). Methods in yeast genetics. (New York: Cold Spring Harbor Laboratory Press).

Bays, N.W., Hill, A.D., and Kariv, I. (2009). A Simplified Scintillation Proximity Assay for Fatty Acid Synthase Activity: Development and Comparison with Other FAS Activity Assays. J. Biomol. Screen. *14*, 636–642.

Bozell, J.J., and Petersen, G.R. (2010). Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. Green Chem. *12*, 539–554.

Chirala, S.S., Kuziora, M.A., Spector, D.M., and Wakil, S.J. (1987). Complementation of mutations and nucleotide sequence of FAS1 gene encoding beta subunit of yeast fatty acid synthase. J. Biol. Chem. *262*, 4231–4240.

Cho, H., and Cronan, J.E. (1993). *Escherichia coli* thioesterase I, molecular cloning and sequencing of the structural gene and identification as a periplasmic enzyme. J. Biol. Chem. *268*, 9238–9245.

Dittrich, F., Zajonc, D., Hühne, K., Hoja, U., Ekici, A., Greiner, E., Klein, H., Hofmann, J., Bessoule, J.J., Sperling, P., et al. (1998). Fatty acid elongation in yeast. Eur. J. Biochem. *252*, 477–485.

Fang, F., Salmon, K., Shen, M.W.Y., Aeling, K.A., Ito, E., Irwin, B., Tran, U.P.C., Hatfield, G.W., Da Silva, N.A., and Sandmeyer, S. (2011). A vector set for systematic metabolic engineering in *Saccharomyces cerevisiae*. Yeast *28*, 123–136.

Flugel, R.S., Hwangbo, Y., Lambalot, R.H., Cronan, J.E., and Walsh, C.T. (2000). Holo-(Acyl Carrier Protein) Synthase and Phosphopantetheinyl Transfer in *Escherichia coli*. J. Biol. Chem. *275*, 959–968.

Heath, R.J., and Rock, C.O. (1996). Roles of the FabA and FabZ beta-hydroxyacyl-acyl carrier protein dehydratases in Escherichia coli fatty acid biosynthesis. J. Biol. Chem. *271*, 27795–27801.

Hoskins, L. (2000). Yeast Transformation (high efficiency).

Kim, S., Clomburg, J.M., and Gonzalez, R. (2015). Synthesis of medium-chain length (C6–C10) fuels and chemicals via β-oxidation reversal in *Escherichia coli*. J. Ind. Microbiol. Biotechnol. *42*, 465–475.

Leber, C., and Da Silva, N. A. (2013). Engineering of *Saccharomyces cerevisiae* for the synthesis of short chain fatty acids. Biotechnol. Bioeng. *9999*, 1–12.

Leibundgut, M., Maier, T., Jenni, S., and Ban, N. (2008). The multienzyme architecture of eukaryotic fatty acid synthases. Curr. Opin. Struct. Biol. *18*, 714–725.

Libertini, L.J., and Smith, S. (1978). Purification and properties of a thioesterase from lactating rat mammary gland which modifies the product specificity of fatty acid synthetase. J. Biol. Chem. *253*, 1393–1401.

Lu, X., Vora, H., and Khosla, C. (2008). Overproduction of free fatty acids in *E. coli*: implications for biodiesel production. Metab. Eng. *10*, 333–339.

Magnuson, K., Carey, M.R., and Cronan, J.E. (1995). The putative fabJ gene of Escherichia coli fatty acid synthesis is the fabF gene. J. Bacteriol. *177*, 3593–3595.

Naggert, J., Witkowski, A., Wessa, B., and Smith, S. (1991). Expression in *Escherichia coli*, purification and characterization of two mammalian thioesterases involved in fatty acid synthesis. Biochem. J. *273*, 787–790.

Nikolau, B.J., Perera, M.A.D.N., Brachova, L., and Shanks, B. (2008). Platform biochemicals for a biorenewable chemical industry. Plant J. Cell Mol. Biol. *54*, 536–545.

Park, J., Rodríguez-Moyá, M., Li, M., Pichersky, E., San, K.Y., and Gonzalez, R. (2012). Synthesis of methyl ketones by metabolically engineered *Escherichia coli*. J. Ind. Microbiol. Biotechnol. *39*, 1703–1712.

Runguphan, W., and Keasling, J.D. (2014). Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived biofuels and chemicals. Metab. Eng. *21*, 103-113.

Sambrook, J., and Russell, D. (2001). Molecular cloning: A laboratory manual (NY: Cold Spring Harbor Laboratory Press).

Da Silva, N. A., and Srikrishnan, S. (2012). Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. FEMS Yeast Res. *12*, 197–214.

Tehlivets, O., Scheuringer, K., and Kohlwein, S.D. (2007). Fatty acid synthesis and elongation in yeast. Biochim. Biophys. Acta *1771*, 255–270.

USDA, United States Department of Agriculture (2008). U. S. Biobased Products. Market Potential and Projections Through 2025 (USDA, United States Department of Agriculture).

White, S.W., Zheng, J., Zhang, Y.M., and Rock, C.O. (2005). The structural biology of type II fatty acid biosynthesis. Annu. Rev. Biochem. *74*, 791–831.

Zhang, X., Li, M., Agrawal, A., and San, K.Y. (2011). Efficient free fatty aacid production in *Escherichia coli* using plant acyl-ACP thioesterases. Metab. Eng. *13*, 713–722.

CHAPTER 4:

Production of medium-chain free fatty acids by engineering yeast β-oxidation

ABSTRACT

Climate change, volatile oil prices and energy dependency concerns have increased the need to produce fuels and chemicals from new sustainable sources. Medium and short chain fatty acids have been identified as promising precursors for producing chemicals from biomass feedstocks. *Saccharomyces cerevisiae* can degrade long-chain fatty acids to shorter fatty acids by using the peroxisomal β-oxidation pathway. The aim of this study was to engineer the *Saccharomyces cerevisiae* β-oxidation pathway for the high-level production of medium-chain fatty acids. First, the peroxisomal medium-chain fatty acid activator *FAA2* and the enoyl-CoA isomerase *ECI1* were deleted to disrupt mono-unsaturated fatty acid degradation and avoid reactivation. The combination of these two deletions increased the production of C12:1 16-fold when cells were grown in complex media with oleic acid (~750-1 g/L of C12:1). Further pathway engineering to increase the number and size of peroxisomes was performed and medium-chain free fatty acid production was evaluated. Strategies to increase the flux through the β-oxidation pathway included the overexpression of an Acc1 variant and swapping the native *FAS1* and *FAS2* promoters to the strong constitutive *TEF1* promoter using CRISPR/Cas9. A strain with *FAA2* and *ECI1* deleted and *FAS1* and *FAS2* overexpressed increased the production of medium-chain free fatty acids in glucose medium by 14-fold and 3.8-fold, compared to the parent strain and a *faa2*Δ*eci1*Δ strain, respectively. We also evaluated if the expression and targeting of heterologous medium-chain fatty acid thioesterases to the peroxisome increased MC-FFA production by cleaving specific fatty acyl-CoA intermediates.

INTRODUCTION

Medium-chain free fatty acids (MC-FFA) have great potential as platform chemicals (Bozell and Petersen, 2010). In the last decade, there has been increased interest in controlling the chain length of the final product produced by the Fatty Acid Synthase (FAS) system. This is accomplished by stopping the acyl-ACP chain elongation using acyl-ACP thioesterases that cleave the bond between the acyl-chain and the ACP moiety. In type II FAS systems, that are composed of discrete enzymes, the product intermediates can be easily accessed by the acyl-ACP. In this case, the introduction of a thioesterase with medium-chain acyl-ACP specificity is enough to see a significant change in the total FA profile produced by the microorganism (Wu and San, 2014; Zhang et al., 2011). Most acyl-ACP thioesterases available in nature come from plants, showing a great diversity in terms of specificity and activity (Jing et al., 2011).

Reducing the length of fatty acids in *Saccharomyces cerevisiae* (with a Type I FAS) is more challenging. In Chapter 2 (Fernandez-Moya et al., 2015), we introduced the entire type II FAS from *Escherichia coli* into *S. cerevisiae*. When a C14-specific thioesterase was expressed, the percent of C14 in the yeast FA profile increased from less than 1% to 33%, and the percent of C18 decreased significantly relative to the wild type strain. Also in our lab, a human type I FAS was introduced into *S. cerevisiae*; the human FAS is a Type I FAS (a big complex with all the catalytic subunits needed to elongate the carbon chain (similar to the yeast FAS)) but with a dedicated thioesterase subunit and a more flexible and open enzyme structure (Leber and Da Silva, 2014). Co-expression of a human FAS with the native thioesterase domain replaced by the medium-chain thioesterase *TEII* and a heterologous phosphopantetheinyl transferase (PPT) resulted in 82 mg/L of C8 (29% of total fatty acids) and 111 mg/L of total medium-chain FA. Targeted β-oxidation elimination increased total

medium-chain FA to 120 mg/L (Leber et al., 2016). In a recent publication by Gajewski et al. (2017), the complex and rigid yeast FAS was modified by introducing point mutations in the ketoacyl synthase domain, malonyl-CoA/palmitoyl-ACP transferase domain, and the acyl transferase domain, and resulted in a final titer of 464 mg/L of total MC-FFA in buffered media. In another recent publication, a thioesterase domain with MC acyl-ACP specificity was tested in several positions within the peptide chain (Zhu et al., 2017). This approach led to approximately 105 mg/L of total MC-FFA after also deleting the β-oxidation process.

In yeast metabolism, there are two other processes that can be engineered to interrupt either the elongation or the degradation of the acyl-ACP or acyl-CoA chain. One is the mitochondrial type II FAS system; its main function is to provide lipoic acid that acts as a cofactor for several essential enzymes for normal TCA cycle function (Brody et al., 1997). The other process is β-oxidation, which is in charge of degrading acyl-CoA for carbon and energy.

β-oxidation is the catabolic process by which acyl-CoA molecules (activated fatty acids) are degraded to produce acetyl-CoA and redox cofactors (e.g., NADH). In yeast, β-oxidation is only found in the peroxisomes (Hiltunen et al., 2003). It is a dissociated enzyme system, which allows acyl-CoA intermediates to be potential substrates for heterologous enzymes targeted into the yeast peroxisomes. This catabolic process includes three main enzymes: Pox1, Fox2 and Fox3 (Hiltunen et al., 2003). Figure 4.1-A shows the β-oxidation reactions and the enzymes that catalyze the reaction steps. Pox1 is an acyl-CoA oxidase with broad substrate specificity, accepting from very long fatty acids to butyryl-CoA (Dmochowska et al., 1990). This differs from the narrow substrate specificity of other peroxisomal oxidases found in different yeast species and in mammals; these tend to have several acyl-CoA oxidase isoenzymes with differing length preference (Wang et al., 1999). Fox2, is a

3-hydroxyacyl-CoA dehydrogenase and an enoyl-CoA hydratase; this enzyme catalyzes two consecutives oxidation reactions and it has a broad substrate specificity (Qin et al., 1999). Fox3, is a 3-ketoacyl-Coa thiolase. The product of this enzyme is a (n-2) acyl-CoA and one molecule of acetyl-CoA (Igual et al., 1991). Figure 4.1-B shows the extra step needed for degradation of oleic acid, a cis-unsaturated fatty acid. Fox2 is not able to accept 3-enoyl-CoA as a substrate and isomerization catalyzed by the enoyl-CoA isomerase Eci1 is required (van Roermund et al., 1998). If an enzyme is introduced with the ability to cleave the –CoA group (thioesterase) from the short and medium-chain fatty acyl-CoA intermediates, short chain FFA and MC-FFA will be produced in the peroxisome.

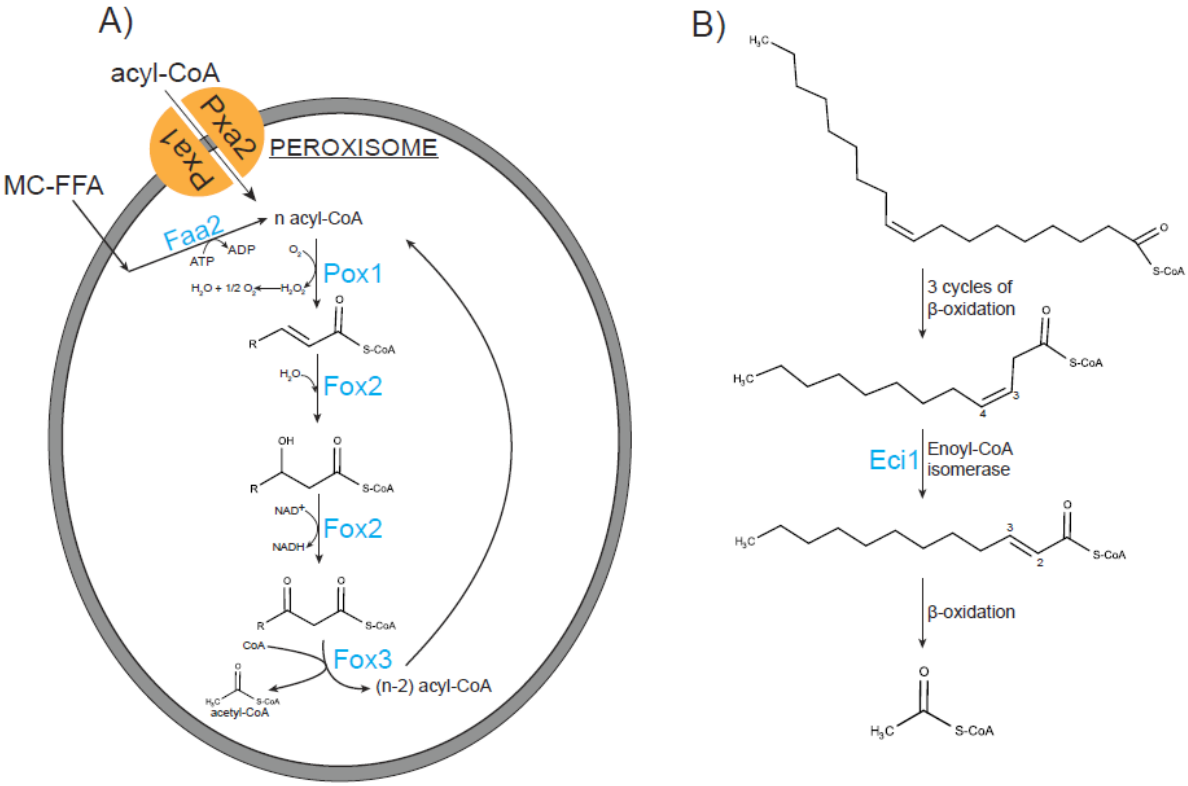


Figure 4.1. A) β-oxidation overview. Long chain FFA are activated in the cytosol and transported into the peroxisomes by Pxa1p/Pxa2p ABC transporters. Medium-chain FFA can cross the peroxisomal membrane and they are activated inside of the peroxisomal matrix **B)** Enoyl-CoA isomerase is required for oleic acid degradation. Eci1 transforms the 3-cis double bond of the oleic acid intermediate into a 2-trans double bond.

Using the peroxisome for production of biochemical compounds started with the synthesis of medium-chain polyhydroxyalkanoates (PHA) (Lee, 1996). PHA are polyesters that can be used for the production of thermoplastics and elastomers. They are made from hydroxy-fatty acids, which makes the peroxisome the perfect organelle to target the PHA synthase to in yeast (Poirier et al., 2001). The yeast peroxisome has also been used for the production of lycopene in *Pichia pastoris* (Bhataya et al., 2009). More recently, *S. cerevisiae* β-oxidation was engineered to produce MC-FFA (Chen et al., 2014). The native *POX1* (first dedicated step), was deleted and replaced by a heterologous acyl-CoA oxidase gene from *Yarrowia lipolytica*. The native *POX1* gene is the only acyl-CoA oxidase in *S. cerevisiae* and therefore has a very broad substrate specificity; on the other hand, the heterologous *POX2* from *Y. lipolytica* has a substrate specificity for long-chain acyl-CoA. This genomic intervention increased the levels of MCFA only slightly from 0.4 mg/L to 0.5 mg/L in oleic acid medium, and the strains had a growth defect.

The mechanism used by *S. cerevisiae* to transport enzymes from the cytosolic matrix to the peroxisomal matrix has been studied extensively (Kim and Hettema, 2015; Meinecke et al., 2010; Smith and Aitchison, 2013). The vast majority of peroxisomal proteins contain either a peroxisomal targeting signal 1 (PTS1) at the C-terminus, or a peroxisomal targeting signal 2 (PTS2) at the N-terminus. PTS1 and PTS2 are recognized by receptor protein Pex5 and the complex Pex7/Pex18/Pex21, respectively, in the cytosol. These receptors bring the proteins to membrane complexes that allow for translocation of the protein, and subsequent receptor release and recycling. Most peroxisomal proteins use some variant of the PTS1 tag to get directed to the peroxisomal lumen (Elgersma et al., 1996). The canonical PTS1 sequence has been found to be Ser-Lys-Leu (SKL), and it has been shown to relocate cytosolic enzymes to the peroxisomes (Hagen et al., 2015; Noguchi et al., 2013).

Acyl chains are released from the fatty acid synthase as acyl-CoA. These molecules can get transported to the peroxisome lumen by the ATP binding cassette transporter complex Pxa1/Pxa2 found in the peroxisomal membrane (Kohlwein et al., 2013) (see Figure 4.2). Free fatty acids in the media or generated intracellularly must be activated in order to be used for any cellular process (Black and DiRusso, 2007). LC-FFA (C14-C18) are activated primarily by cytosolic fatty acid activators Faa1 and Faa4 (Knoll et al., 1994; Scharnewski et al., 2008). MC-FFA are activated in the peroxisome by the peroxisomal medium-chain fatty acid activator Faa2 (van Roermund et al., 2012). ATP required for the activation of the FFA to acyl-CoA in the peroxisome is provided by the peroxisomal ATP transporter Ant1 (Palmieri et al., 2001; van Roermund et al., 2001). Recently, it has been suggested that MC-FFA cross by using the pore-forming protein Pex11, which is found in the peroxisomal membrane and allows for non-selective translocation of metabolites across the peroxisomal membrane (Mindthoff et al., 2016).

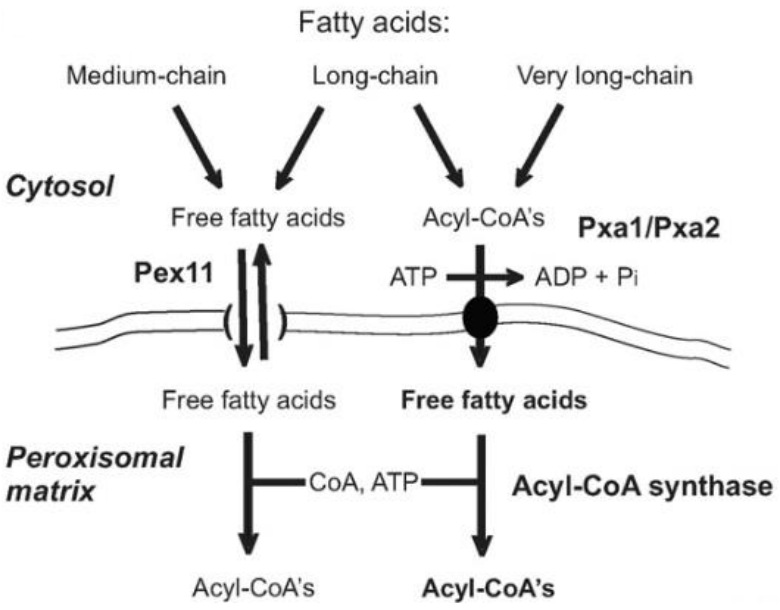


Figure 4.2. Schematic drawing of current understanding of acyl-CoA and FFA transport through the peroxisomal membrane. Adapted from Mindthoff et al., (2016)

S. cerevisiae β-oxidation has been also engineered to produce medium-chain fatty alcohols by targeting the fatty acyl-CoA reductase (FAR) from *Tyto alba* to the peroxisome (Sheng et al., 2016). To optimize FAR targeting, various targeting signals were tested, and the endogenous peroxisome biogenesis machinery was engineered by overexpressing Pex5, Pex7 and/or combinations with Pex3 and Pex19, which are involved in peroxisomal membrane protein importation and peroxisome biogenesis. Overexpression of FAR (with the N-terminal PTS2) and Pex7 resulted in approximately 50 mg/L of 1-decanol, 200 mg/L of 1-dodecanol, and 500 mg/L of 1-hexadecanol.

More recently, yeast peroxisomes have been used to compartmentalize both the fatty alcohol and alkane biosynthetic pathways from fatty acyl-CoA to avoid cytosolic aldehyde reductase/alcohol dehydrogenase activity; this strategy increased the titer of fatty alcohols by 63%, and the alkane/alkene titer by 100% relative to cytosolic expression (Zhou et al., 2016a). After eliminating competing pathways like *POX1* and *HFD1*, more than 180 mg/L of total fatty alcohols were produced.

In this study, we engineered *S. cerevisiae* to produce MC-FFA by first deleting the peroxisomal medium-chain fatty acid activator Faa2, to avoid reactivation of free fatty acids, and the peroxisomal Δ^3-cis-Δ^2-trans-enyol-CoA isomerase Eci1. The isomerase Eci1 is essential for complete degradation of unsaturated fatty acids in yeast, and deletion of the enzyme leads to a growth impairment on oleic acid: 33% growth rate compared to wild-type (Gurvitz, 1998). This is thought to be the result of accumulation of acyl-CoA intermediates. We attempted to alleviate that accumulation by overexpression of medium-chain fatty acyl-CoA thioesterases targeted to the peroxisome in a *faa2*Δ *eci1Δ* strain. We also looked at the effect of deleting the cytosolic medium-chain fatty acid elongase Elo1, and the overexpression of the acetyl-CoA carboxylase Acc1, which is a limiting step in the fatty acid production. β-oxidation and peroxisome biogenesis were further engineered to increase the size

and number of peroxisomes per cell.

To engineer yeast β-oxidation to produce MC-FFA high-level production of acyl-CoA is required to increase flux through β-oxidation. Therefore, we overexpressed Fas1, Fas2, and Acc1 (Figure 4.3). Overexpression of *FAS1* and *FAS2*, in conjunction with *ACC1*, have been shown to be beneficial for increasing fatty acids in *S. cerevisiae* (Runguphan and Keasling, 2014). In addition, we overexpressed Pox1 (acyl-CoA oxidase), which is the first step of β-oxidation and has been shown to be the rate-limiting step of this pathway (Hiltunen et al., 2003).

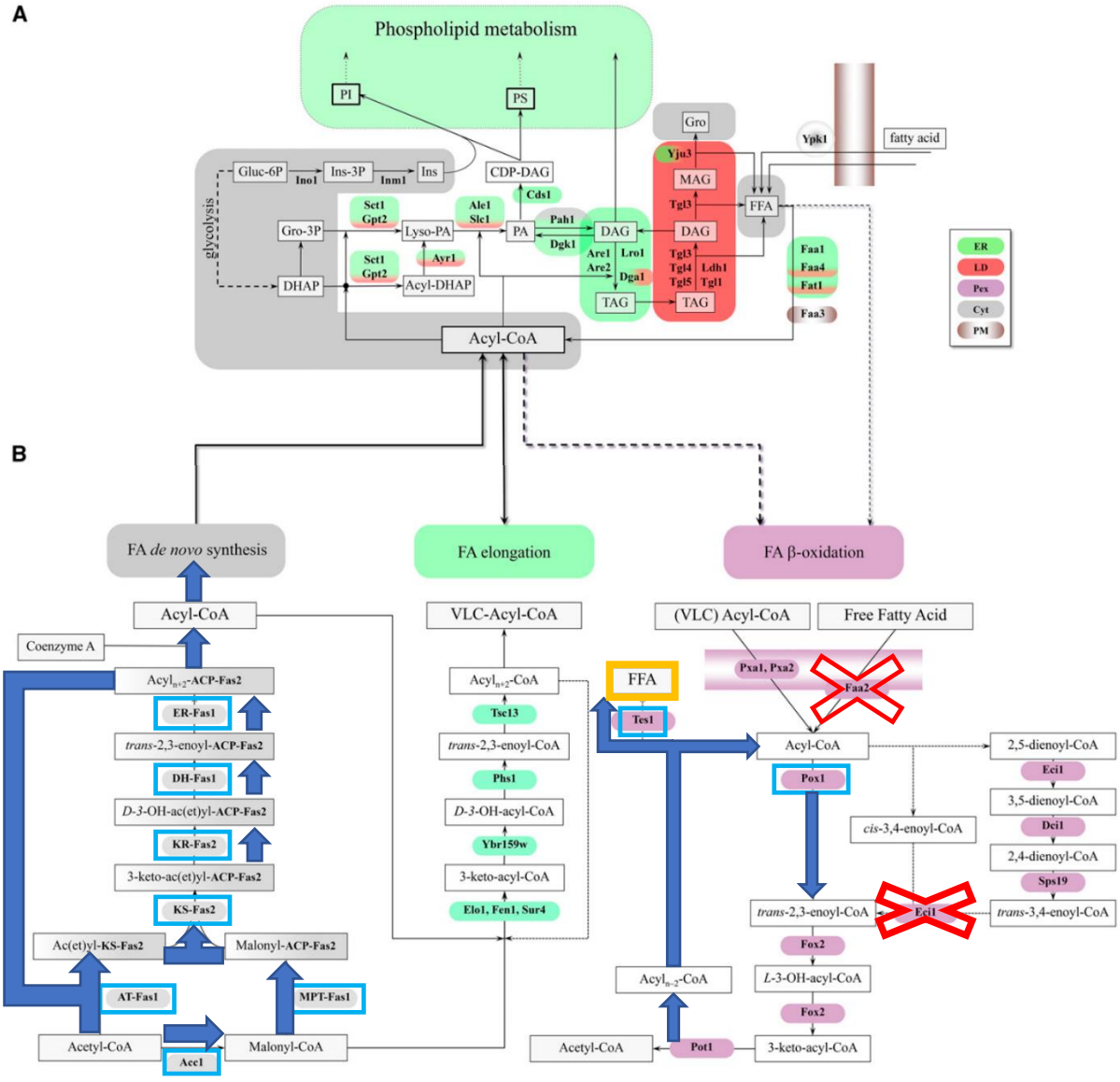


Figure 4.3. Main metabolic pathways involving acyl-CoA in *S. cerevisiae.* Enzymes that when overexpressed would benefit MC-FFA production are shown in blue boxes. Genes that when deleted would benefit MC-FFA synthesis are shown with red crosses. Fluxes that are increased are shown in blue arrows. Final short and medium chain FFA product is shown in a yellow box. Adapted from (Kohlwein et al., 2013).

Finally, we targeted medium-chain thioesterases into the peroxisomes to study their effect on the release of MC-FFA. Acyl-CoA thioesterases are enzymes that cleave the coenzyme A from the acyl group, releasing the free fatty acid molecules. To terminate the degradation cycle at longer acyl-CoA intermediates, medium and/or short-chain acyl-CoA thioesterases were expressed with a peroxisomal targeting signal. These enzymes will produce short and medium free fatty acids after –CoA cleavage, and these will not be reactivated in strains with the medium/short chain fatty acid activator gene *FAA2* deleted. The thioesterases tested were:

1. *TEII*, thioesterase from *Rattus Novergicus* (Naggert et al., 1991). A PTS1 (–SKL) C-terminus targeting signal was added to the sequence. This TE has preference for C6 and C8 acyl-CoA (Joshi et al., 2005)
2. *TES1*, native *S. cerevisiae* thioesterase with preference for C12 acyl-CoA molecules (Kal et al., 2000).
3. *BTE,* thioesterase from *Umbellularia californica.* It is very specific for C12:0 acyl-ACP species and has been shown to increase C12 production in *E. coli* (Voelker and Davies, 1994)
4. mTesA', mutant of the leaderless tesA thioesterase from *E. coli* recently constructed through a combination of *in silico* modeling and library construction/testing. It has substrate specificity for C8, C10 and C12 acyl-CoA (Grisewood et al., 2017).

MATERIALS AND METHODS

Vector and Strain Construction

Standard molecular biology procedures were carried out as described in Sambrook et al. (2001). Restriction enzymes, T5 exonuclease, Phusion polymerase, Taq ligase, 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). All sequences of gene fragments amplified by PCR were verified by DNA sequence analysis (Eton Bioscience, San Diego, CA). *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 et al., 2001). Plasmid and integrative transformations in *S. cerevisiae* were performed using a high efficiency LiAc method using DMSO (Hoskins, 2000). Isolation of total genomic yeast DNA for integration checking was performed as described by Sambrook et al. (2001).

FAA2, *ECI1* and *ELO1* knockout strains were created using double crossover recombination as previously described (Fang et al., 2011). PCR was used to generate the deletion cassettes (reusable selection marker flanked by the upstream and downstream homology sequences sequences) and knockouts deleted the full open reading frame of the gene. The deletions were confirmed by genomic PCR using primers that bind upstream and/or downstream of the chromosomal site and to the marker. PCR was used to generate fragments *loxP-Marker-loxP* from vectors pXP214 (MET17), pXP218 (URA3), pXP220 (HIS3) and pXP222 (LEU2), respectively (Fang et al., 2011). pBTEF1 was used as template to generate *loxP-KanMX-loxP* (Leber et al., 2015). faa2Δ and 3SKO strains were kindly provided by Leber et al., (2016), and fas2Δ was provided by (Leber and Da Silva, 2014) . *ECI1* knockout

PCR used backbone pXP214 and primer pair Eci1KOF1/R1 and extension primer set Eci1KOF2/R2 to create strain FE. *ELO1* knockout PCR used backbone pXP220 and primer pair Elo1KOF1/R1 and extension primer set Elo1KOF2/R2 to create strains FE-ELO1 and FETA elo1Δ. *TRP1* knockout PCR used backbone pBTEF1 and primer pair Trp1KOF1/R1 and extension primer set Trp1KOF2/R2 to create strains trp1Δ and FETAELO trp1Δ. In order to integrate the expression cassette pTEF1-ACC1m, we first swapped the p*PGK1* by the p*TEF1* in integration plasmid pIM11-ACC1m (Choi and Da Silva, 2014) using the primer set TEF1intACC1F/R. After plasmid construction, plasmid was digested with NroI and NruI to create an integration linear DNA with the *LEU2* marker and 81 and 94 bp upstream and downstream, respectively, with homology to the *MET17* empty locus.

For *PEX30*, *PEX31*, *PEX32* an *TES1* deletion, CRISPR/Cas9 system from DiCarlo et al., (2013) was adapted to create a multiplexing approach. In short, p426-SNR52p-gRNA.CAN1.Y-SUP4t, p415-GalL-Cas9-CYC1t and p414-TEF1p-Cas9-CYC1t (Figure 4.4) were acquired from Addgene. *pTEF1-MCS-CYC1t* expression cassettes from pBTEF1 were swapped by pSNR52-target sequence(ts)-gRNA-tSUP4 by amplifying it from p426-SNR52p-gRNA.CAN1.Y-SUP4t, calling this last plasmid pdgRNA (KanMX). A library of double gRNA plasmids was created with *KanMX*, *URA3*, *MET17*, *LEU2*, and *HIS3* markers. To change the target sequences to the desired ones, primers pex30gRNAfor and pex31gRNAfor were used with pdgRNA(URA3) (Figure 4.5) as template to generate the insert DNA. Primers pex30AmpRev and pex31AmpRev were used with pdgRNA(URA3) as template to generate the backbone DNA. Gibson was used to ligate both pieces and construct pdgRNA(URA3)-Pex30(ts)/Pex31(ts). A similar strategy was used to build pdgRNA(HIS3)-Pex32(ts)/Tes1(ts). Target sequences were found using the webtool Yeastriction (Mans et al., 2015). Donor DNA was created by using two 60bp primers containing with 15 3'-terminal base pairs complementary (eg. Pex30DonorF and Pex30DonorR). Final Donor DNA piece was 105 bp. p414-TEF1-Cas9 was transformed to FETAELO trp1Δ and FE-ELO1 trp1Δ

and selected on SDC(A,U) plates for the *TRP1* marker. Consequently, cells harboring p414-TEF1-Cas9 were transformed with pdgRNA(URA3)-Pex30(ts)/Pex31(ts), pdgRNA(HIS3)-Pex32(ts)/Tes1(ts), and donor DNA for Pex30, Pex31, Pex32 and Tes1. Cells were plated on SD(-ura,-his,-trp)+CSM and grown for 3-4 days.

A similar approach was followed to swap the native *FAS1* and *FAS2* promoters for the *TEF1* promoter in FE, FETA, and BY4741 strains. Atum gRNA designer (https://www.atum.bio), was used to find optimal target sequences for Cas9 within the *FAS1* and *FAS2* promoter regions. pdgRNA(URA3)-Fas1(ts)/Fas2(ts) was constructed, first by obtaining the insert with FAS1targetFor and FAS2targetFor primers, and the backbone with AmpRev, as the only primer, and pdgRNA(URA3) as template DNA for both pieces. Donor DNAs were created with TEF1_FAS1for/rev and TEF1_FAS2for/rev. Plates used to select for final intervention were SDC(A).

For plasmid construction, TEII was amplified from pXP842-TEII (Leber and Da Silva, 2014) with TEII-SpeIF/TEII-XhoIR or TEII-SpeIF/TEIISKLXhoIR, to introduce the PTS1 (-SKL at the C-terminal of the enzyme). SpeI and XhoI were used to digest pBT-URA3 (Figure 4.6) and pBT-mKanMX and PCR insert. T4 ligation was used to create the final constructs. A similar approach was used for TES1, TEIIePTS12, mTesA', and BTE cloning. For *POX1* cloning, SacII and NotI restriction enzymes were used to digest the backbone plasmid.

Table 4.1: List of strains

Strain name	Description	Source
BY4741	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
trp1Δ	BY4741 *trp1::loxP*	This study
faa2Δ	BY4741 *faa2::loxP*	Leber et al (2015)
eci1Δ	BY4741 *eci1::kanMX4*	YKO collection (Dharmacon)
FE	BY4741 *faa2::loxP eci1::loxP*	This study
3SKO	BY4741 *faa2::loxP pex11::loxP ant1::loxP*	Leber et al (2016)
fas2Δ	BY4741 *fas2::kanM4*	Leber et al (2014)
faa2Δ T-ACC1	BY4741 *faa2::loxP met15::*p*TEF1-ACC1m*-t*CYC1*	This study
FE T-ACC1	BY4741 *faa2::loxP eci1::loxP met15::*p*TEF1-ACC1m*-t*CYC1*	This study
FE-ELO1	BY4741 *faa2::loxP eci1::loxP elo1::loxP*	This study
FETA elo1Δ	BY4741 *faa2::loxP eci1::loxP elo1::loxP met15::*p*TEF1-ACC1m*-t*CYC1*	This study
FETAELO trp1Δ	BY4741 *faa2::loxP eci1::loxP elo1::loxP met15::*p*TEF1-ACC1m*-t*CYC1 trp1::loxP*	This study
SUPER	BY4741 *faa2::loxP eci1::loxP elo1::loxP trp1::loxP pex30Δ pex31Δ pex32Δ tes1Δ*	This study
SUPER T-ACC1	BY4741 *faa2::loxP eci1::loxP elo1::loxP met15::*p*TEF1-ACC1m*-t*CYC1 trp1::loxP pex30Δ pex31Δ pex32Δ tes1Δ*	This study
FE-FAS12	BY4741 *faa2::loxP eci1::loxP fas1::*p*TEF1-fas1 fas2::*p*TEF1-fas2*	This study
BY-FAS12	BY4741 fas1::pTEF1-fas1 fas2::pTEF1-fas2	This study

Table 4.2: List of plasmids

Name	Description	Orig.	Marker
pBT-URA3-empty	pTEF1--tCYC1	*2µ*	*URA3*
pBT-URA3-TSKL	pTEF1-TEIISKL-tCYC1	*2µ*	*URA3*
pBT-URA3-TES1	pTEF1-TES1-tCYC1	*2µ*	*URA3*
pBT-URA3-POX1	pTEF1-POX1-tCYC1	*2µ*	*URA3*
pBT-URA3-TSKL/POX1	pTEF1-TEIISKL-tCYC1 + pTEF1-POX1-tCYC1	*2µ*	*URA3*
pBT-URA3-TES1/POX1	pTEF1-TES1-tCYC1 + pTEF1-POX1-tCYC1	*2µ*	*URA3*
pBT-mKanMX-empty	pTEF1--tCYC1	*2µ*	*KanMX*
pBT-mKanMX-TSKL	pTEF1-TEIISKL-tCYC1	*2µ*	*KanMX*
pBT-mKanMX-TES1	pTEF1-TES1-tCYC1	*2µ*	*KanMX*
pBT-mKanMx-POX1	pTEF1-POX1-tCYC1	*2µ*	*KanMX*
pBT-mKanMX-TSKL/POX1	pTEF1-TEIISKL-tCYC1 + pTEF1-POX1-tCYC1	*2µ*	*KanMX*
pBT-mKanMX-TES1/POX1	pTEF1-TES1-tCYC1 + pTEF1-POX1-tCYC1	*2µ*	*KanMX*
pBT-mKanMX-TEII (ePTS12)	pTEF1-TEII(ePTS12)-tCYC1	*2µ*	*KanMX*
pBT-mKanMX-TEII (ePTS12)/POX1	pTEF1-TEII(ePTS12)-tCYC1 + pTEF1-POX1-tCYC1	*2µ*	*KanMX*
pBT(mKanMX)-mTesA'	pTEF1-mTesA'-tCYC1	*2µ*	*KanMX*
pBT(mKanMX)-mTesA'_ePTS1	pTEF1-mTesA'_ePTS1-tCYC1	*2µ*	*KanMX*
pBT(mKanMX)-BTE	pTEF1-BTE-tCYC1	*2µ*	*KanMX*
pBT(mKanMX)-BTE_ePTS1	pTEF1-BTE_ePTS1-tCYC1	*2µ*	*KanMX*
p414-TEF1-Cas9	pTEF1-Cas9-tCYC1	*C/A*	*TRP1*
pgRNA-Church	pSNR52-CAN1(ts)-tSUP4	*2µ*	*URA3*
pdgRNA(URA3)	pSNR52-CAN1(ts)-tSUP4 x2	*2µ*	*URA3*
pdgRNA(HIS3)	pSNR52-CAN1(ts)-tSUP4 x2	*2µ*	*URA3*
pdgRNA(URA3)-Pex30/Pex31	pSNR52-Pex30(ts)-tSUP4, pSNR52-Pex31(ts)-tSUP4	*2µ*	*URA3*
pdgRNA(HIS3)-Pex32/Tes1	pSNR52-Pex32(ts)-tSUP4, pSNR52-Tes1(ts)-tSUP4	*2µ*	*HIS3*

Table 4.3: List of primers

Reason	Name	Sequence (5' -> 3')
eci1 KO	Eci1KOF1	ACAAATTGCTCGCACAGTAAAGGAAGGAAGAACAATGGTCGACTCTAGAGGATCCCCGGG
	Eci1KOR1	ATATTGTGTGTGCGTTTTGTTTCACTGAGAAAGCGGACGAATTCGAGCTCGGTACCCGGG
	Eci1KOF2	ACTGAAAGATGAACATATGGATAGATAATTGGAGTGTATGGCACAAATTGCTCGCACAGT
	Eci1KOR2	ATGATATAGATTAATTAGCATACATATTGTCTGTACATAAGTATATTGTGTGTGCGTTTT
	Eci1CheckF	GCGGCACGAAGGAAAGAATC
	Eci1CheckR	TTCGATTATGATCGGTGTAA
elo1 KO	Elo1KOF1	TTTTAAGGAACTTGAGAGCTAAATTTGCAAGCAAGTGGTCGACTCTAGAGGATCCCCGGG
	Elo1KOR1	TAGGAAGAAAAACAGAAAAAAACAAAACAGAAGGACTGGAATTCGAGCTCGGTACCCGGG
	Elo1KOF2	AAGACTAACTGATATGAATATTAAGGTAGGATACCTTATCAA TTTTAAGGAACTTGAGAG
	Elo1KOR2	TTATTTCGAATAAAACAGTCTATATATAAGTCAAATACAAGATAGGAAGAAAAACAGAAA
	Elo1ChkFext	TCGGACCTGTGTTCAGAAAT
	Elo1ChkRint	TGAAGGTGACAATCCAGAAG
	Elo1ChkRext	TCTCGAGTAACTGAAACTGC
trp1 KO	Trp1KOF1	GTGAGTATACGTGATTAAGCACACAAAGGCAGCTTGGAGTgcaggtcgactctagaggat
	Trp1KOR1	GAGAGGGCCAAGAGGGAGGGCATTGGTGACTATTGAGCACGTGAGTATACGTGATTAAGC
	Trp1KOF2	TGCACAAACAATACTTAAATAAATACTACTCAGTAATAACcgttGAATTCgagctcggta
	Trp1KOR2	AGATCTTTTATGCTTGCTTTTCAAAAGGCCTGCAGGCAAGTGCACAAACAATACTTAAAT
	Trp1ChkF	GAGGCGGTGGAGATATTCCTTA
	Trp1ChkR	TTACGAGGATACGGAGAGAGGT
pTEF1-ACC1 int	TEF1intACC1F	GAACTGTGGTGGTTGGCAcctcaggACCGCGAATCCTTACATCACACCCA
	TEF1intACC1R	TCATttttttCGGTCCGGTTTAAACACTAGTTTTGTAATTAAAACTTAGA
	TEF1intACC1check	agattgtactgagagtgcac
pex30Δ, pex31Δ, pex32Δ, tes1Δ	pex30gRNAfor	GcagtgaaagataaatgatcCGTCAATGAGAATTAAATAAGTTTTAGAGCTAGAAATAGC
	pex31gRNAfor	GcagtgaaagataaatgatcTTACTTGTATTTTTCATAACGTTTTAGAGCTAGAAATAGC

Reason	Name	Sequence (5' -> 3')
using CRISPR/Cas9	pex32gRNAfor	GcagtgaaagataaatgatcAAGTATACGAAAATCAAAGAGTTTTAGAGCTAGAAATAGC
	tes1gRNAfor	GcagtgaaagataaatgatcCTACATTCGTATTTCATCAAGTTTTAGAGCTAGAAATAGC
	pex30AmpRev	GCTATTTCTAGCTCTAAAACTTATTTAATTCTCATTGACGgatcatttatctttcactgC
	pex31AmpRev	GCTATTTCTAGCTCTAAAACGTTATGAAAAATACAAGTAAgatcatttatctttcactgC
	pex32AmpRev	GCTATTTCTAGCTCTAAAACTCTTTGATTTTCGTATACTTgatcatttatctttcactgC
	tes1AmpRev	GCTATTTCTAGCTCTAAAACTTGATGAAATACGAATGTAGgatcatttatctttcactgC
	Pex30DonorF	TTAGGATTCGAGCTGTCTAGTTGATCCTCCGGAGTGTAAAAACTGATTTTCATGCTCGCT
	Pex30DonorR	TAATAAAGTAAATTAGAGATTATATTATGTAAAGGTAAAAACGGGAGCGAGCATGAAAAT
	Pex31DonorF	GCCCTTGCACCGACCAGTGTGAACGTTGTTGTCCATATGGGGCATGCACTCAACTATCAA
	Pex31DonorR	AATTTGGTATTCTTCCCTGGTTGTCAAGCCTTGGTTTCCCTTTATTTGATAGTTGAGTGC
	Pex32DonorF	TTGATCTTAATTTAGTTCAGTGGACATCATTTTGCTAATTTCAAGGAAAGAATAGTATTG
	Pex32DonorR	ATAATGTTAGGCAACCAACTATATATGCAGTTTAGAGGCTTAAAGCAATACTATTCTTTC
	Tes1DonorF	ATATAATATAAGAATATATATGTATGTGTTTATACGTGGGAGGGAATTGTCCTTTTATGC
	Tes1DonorR	TTCAATTCAGTATCCACCATGAGCAAGACAAGATAAGACAAGATTGCATAAAAGGACAAT
	Pex30KOcheckF	ACGGAAAGAAGCGGGAAGAG
	Pex30KOcheckR	AGAGCTGACGTTAAGACCGC
	Pex31KOcheckF	TACGAGCCTTTCAGACCTGC
	Pex31KOcheckR	AGGTTCCGCTGGAGACAATG
	Pex32KOcheckF	TTTGAGTGCCATCCTGGGTG
	Pex32KOcheckR	GCGCTGCAAGTGATGATAGC
	Tes1KOcheckF	ACAGTCTACAAACCCGCCAG
	Tes1KOcheckR	GGTCCTCGATGCTACGTCAG
	SeqgRNA1	TATCAGGGTTATTGTCTCATGAGCG
	SeqgRNA2	GGGGATGTATGGGCTAAATGTACG

Reason	Name	Sequence (5' -> 3')
dgRNA construction	BiCass1For	atttccccgaaaagtgccacctgacCATATGACTAGTtctttgaaaagataatgtatga
	BiCass1Rev	ATACGAAGTTATCCCGGGGCTAGC*tcgcga*AGACATAAAAAACAAAAAAAGCACCACCGA
	BiCass2For	GAATTCgagctcggtaCCCCCTAGGtctttgaaaagataatgtatga
	BiCass2Rev	TACGAAGTTATCCCGGGTTAATTAAAGACATAAAAAACAAAAAAAGCACCACCGA
TEII Targeting	TEII-SpeIF	GACCTCCC-ACTAGT-**AAAAAA**-atggagacagcagtcaatgctaagagtcccaggaatgaaaaggttttgaactgt
	TEII-XhoIR	TGGTAATC-CTCGAG-**tca**-agtgagtgacgagagttccaa
	TEIISKLXhoIR	TGGTAATC-CTCGAG-**tta**- *CAATTTAGA*-agtgagtgacgagagttccaa
	TEIIPTS2F	GACCTCCC-ACTAGT-AAAAAA-atg-AGTCAAAAACTTCAAAGTATTAAAGATCAACTT-gagacagcagtcaatgctaagagtc
	TEIIePTS1R	TGGTAATC-CTCGAG-**tta**- CAATTTGGATCTTCTACCTCTTCCCAATGAACC-agtgagtgacgagagttccaa
TES1 cloning	Tes1SpeIF	TCTAAGTTTTAATTACAAAACTAGTAAACAATGAGTGCTTCCAAAATGGCCATGTCCAAC
	Tes1XhoIR	GACATAACTAATTACATGACTCGAGTCAGAACTTGGCTCGAATGTCTCGTTCTGACCCGT
	SeqTes1F1	GGGAGACGATTCCAGGCCTGCA
	SeqTes1F2	CTTTGCGTATCTGTCCGATTCCT
	SeqTes1R1	TCATCGAGGTTGCGTCTTGGAA
POX1 cloning	Pox1SpeIF	TTAATTACAAAcacgtggcggccgc-AAACA-ATGACGAGACGTACTACTATTAATC
	Pox1XhoIR	TAAGCGTGACATAACTAATccgcggTCACTTGTTTATTTTCGATAAAATT
	SeqPox1F1	ACAAAGCTTCGCCTTTAACAAAC
	SeqPox1F2	GAAGATGGGTCGTGACGGTATTG
	SeqPox1F3	CAGCTTGAAAGCCACCAATACTTG
	SeqPox1F4	TGATTCACATATTTCCGATGAAAT
	SeqPox1R1	ACCATTGCCCTTGATACAATTA
BTE cloning	BTESpeIF	TTAATTACAAAACTAGTAAAAAAATGACTCTAGAGTGGAAACCGAAACC
	BTEXhoIR	CATAACTAATCTCGAGTTAAACACGAGGTTCCGCCGG
	BTEePTS1R1	ATCTTCTACCTCTTCCCAATGAACC-AACACGAGGTTCCGCCGG
	BTEePTS1R2	GTAATC-CTCGAG-tta-CAATTTGGATCTTCTACCTCTTCCCAATGAACC
TesA mutant cloning	TesAMut_P1F	CGGCGGACTAGTAAAAAAATGGCGGACACGTTATTGATTCTGGGTG
	TesAMut_P1R	TCCATTGTGGCTTTTTTTTGACCTCTTCCAAAAAAAAGGGCAGCAGCGGAACATC
	TesAMut_P2F	CTGCTGCCCTTTTTTTTGGAAGAGGTCAAAAAAAAGCCACAATGGATGCAGGATG
	TesAMut_P2R	ACTAATCTCGAGttaTGAGTCATGATTTACTAAAGGC
	TesAMutePTS1R1	ATCTTCTACCTCTTCCCAATGAACCTGAGTCATGATTTACTAAAGGC

Reason	Name	Sequence (5' -> 3')
	TesAMutePT S1R2	GTAATC-CTCGAG-tta-CAATTTGGATCTTCTACCTCTTCCCAATGAACC

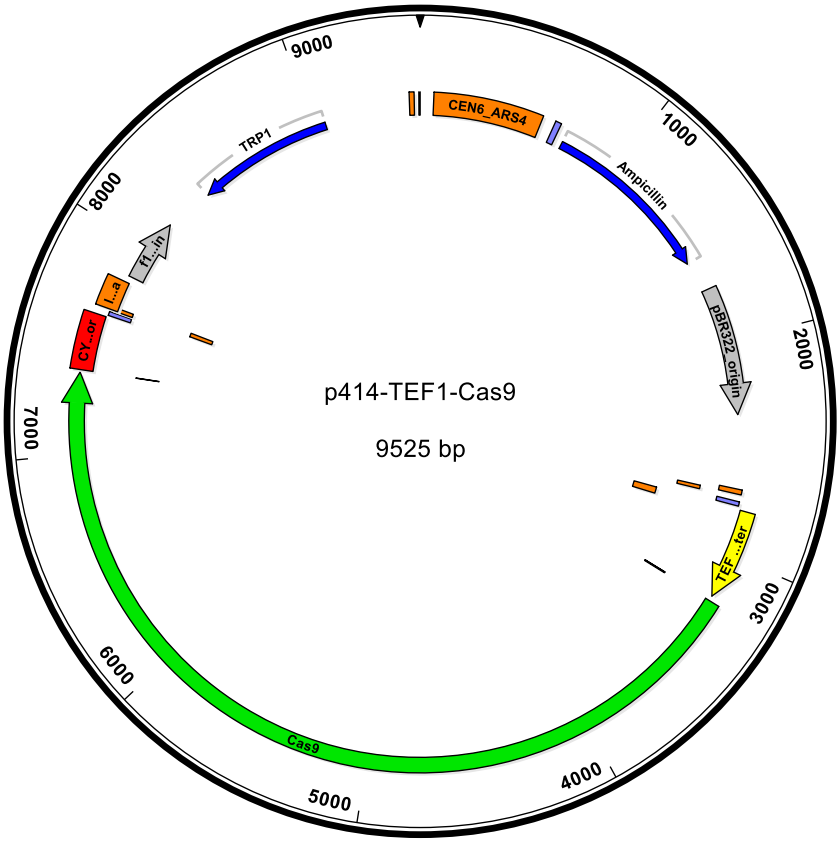


Figure 4.4. Schematic representation of p414-TEF1-Cas9. Cas9 codon-optimized for *S. cerevisiae* is shown in green. The *TEF1* promoter is shown in yellow. The *CYC1* terminator is shown in red. The *CEN/ARS* origin is shown in orange. The *TRP1* marker is shown in blue. Source: (DiCarlo et al., 2013)

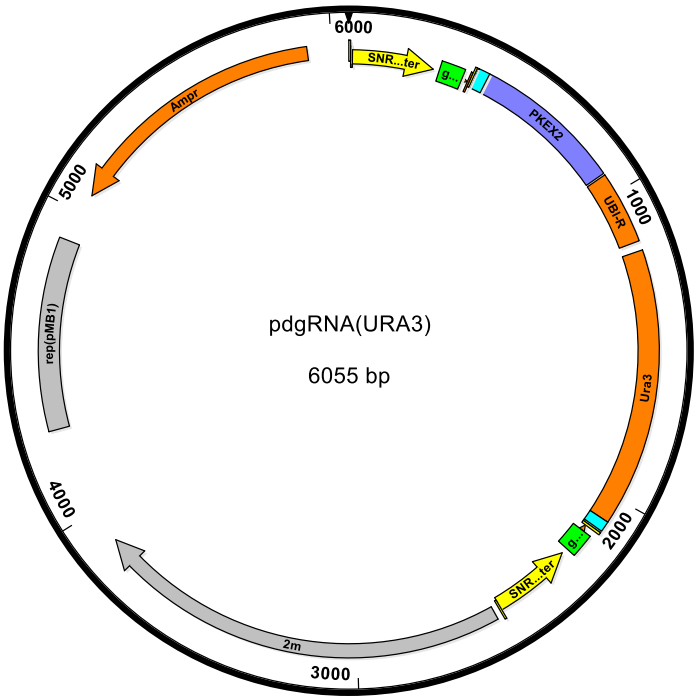


Figure 4.5. Schematic representation of pdgRNA(URA3). pdgRNA(URA3) includes two identical sgRNA expression cassettes in opposite directions. Each of these cassettes has a *SNR52* promoter shown in yellow, a structural gRNA shown in green and a very short *SUP4* terminator shown in orange (filled arrow after structural gRNA). Between the *SNR52* promoter and structural gRNA there is 20 bp target sequence.

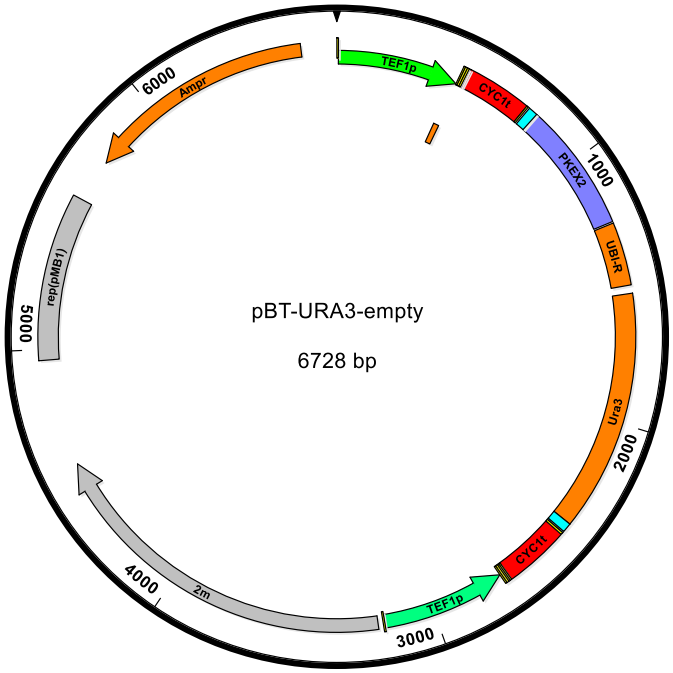


Figure 4.6. Schematic representation of pBT-URA3-empty. pBT-URA3-empty includes two identical gene expression cassettes in opposite directions. Each expression cassette has one *TEF1* (green) and one *CYC1* terminator (red). The marker is between the two expression cassettes.

Media and Cultivation

Luria-Bertani (LB) medium containing 0.1 mg/mL ampicillin for selection (Sambrook et al., 2001) was used for *E. coli* cultivation. *S. cerevisiae* strains were cultivated in complex YPD media (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.

All expression studies were conducted using YPD medium, with 200 mg/L G418 (Sigma-Aldrich, Saint Louis, MO) added for plasmid selection. Yeast strains were grown at 30°C in an air shaker (New Brunswick Scientific) at 250 rpm. Cells were inoculated from -80°C stock and grown overnight in 5 mL medium in 16 X 125 mm culture tubes. Yeast expression studies were performed in shake flasks with 50 mL medium. Cell growth was initiated at an optical density (600nm) of 0.2 (Shimadzu UV-2450 spectrophotometer; Columbia, MD) and grown for 24 h, 36 h, 48 h, or 72 h. A correlation factor was used to convert OD to dry cell weight per liter (1 OD = 0.43 g DCW/L). Media with oleic acid was prepared by adding 1% of Tween20 (Sigma Aldrich, Catalog No. P9416) to Oleic acid (Fisher Scientific, Catalog No. S25451). The mixture of oleic acid and tween20 was filter-sterilized and added to the rest of the media once it cooled down. To measure OD in media with oleic acid, 1 mL of culture of spinned down, supernatant was removed, and 1 mL of distilled water was added. This process was repeated two times, and OD was measured like explained above.

Fatty Acid Extraction and Quantification

Fatty acid extraction procedures were modified from Bligh and Dyer (1959). Undecanoic and nonadecanoic acid were used as internal standards (Sigma-Aldrich, Saint Louis, MO). Total intracellular fatty acids (free and bound) were extracted as previously described (Leber and Da Silva, 2013). Extracellular fatty acids (free fatty acids) were extracted from 5 mL culture broth by adding 500 µL 1M HCL and 5 mL of a 1:1 methanol:chloroform mixture. The solution was vortexed for 5 minutes then centrifuged for 4 minutes at 1000g. The lower chloroform layer was recovered and evaporated with nitrogen gas. Fatty acids were methylated and suspended in hexane for use in GC-MS as previously described (Leber and Da Silva, 2013).

Fatty acids were measured at the Mass Spectrometry Facility at the University of California Irvine. Fatty acids were quantified by gas-chromatography mass spectrometry (GC-MS). The oven was held at 50°C for 1 minute 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/sec) from m/z 50-650. Absolute amounts and distribution of fatty acids were determined using a total ion chromatogram and were normalized to the internal standards. Before each run, a standard curve was performed using a saturated fatty acid methyl-ester mix purchased from Sigma Aldrich (Saint Louis, MO) (catalog #49453-U).

RESULTS AND DISCUSSION

Targeting *TEII* into the peroxisome of *faa2*Δ and 3SKO strains

To avoid re-activation or degradation of MC-FFA after release from the -CoA moiety by a thioesterase, we tested the deletions of *FAA2*, *ANT1*, and *PEX11*. The medium-chain fatty acid activator encoded by *FAA2* activates MC-FFA to acyl-CoA, which are then degraded to acetyl-CoA by the β-oxidation pathway (Knoll et al., 1994). The MC-FFA reaction uses ATP and produces pyrophosphate and AMP. The enzyme Ant1, which is a peroxisomal ATP carrier, provides the required ATP for medium-chain fatty acid activation (van Roermund et al., 2001), and *PEX11* deletion strains have been associated with decreased medium-chain fatty acid oxidation (van Roermund et al., 2000). Our goal was to target the medium-chain thioesterase *TEII*, from rat mammary gland, to the peroxisome of strains with only *FAA2* deleted or with *FAA2*, *ANT1* and *PEX1* deleted, and measure if there is an increase in the MC-FFA produced when cells are cultured in oleic acid medium.

Multicopy plasmids carrying the thioesterase of *TEII* or *TEII-SKL* (TEII with peroxisomal targeting sequence) were created (Table 3.2) and transformed into three *S. cerevisiae* strains: BY4741, BY4741 with the peroxisomal medium-chain acyl-CoA synthase (activator) *FAA2* knocked out, and BY4741 with the *FAA2*, *ANT1* and *PEX11* genes deleted (3SKO strain) (Table 4.1). The strains were tested in $SD_{0.5}O_2C(A)$, which is a selective semi-defined medium with 0.5% glucose and 2% oleic acid. Oleic acid was added to the medium to provide cells with high amounts of β-oxidation substrate. Cells were grown for 90h and fatty acids from the supernatant were extracted and measured using GC-MS to determine the concentrations of C12 and C14 fatty acids secreted for each of the three strains. Large variations between colonies of the same strains were observed, which resulted in large error bars, particularly for FAA2+TSKL (Figure 4.7).

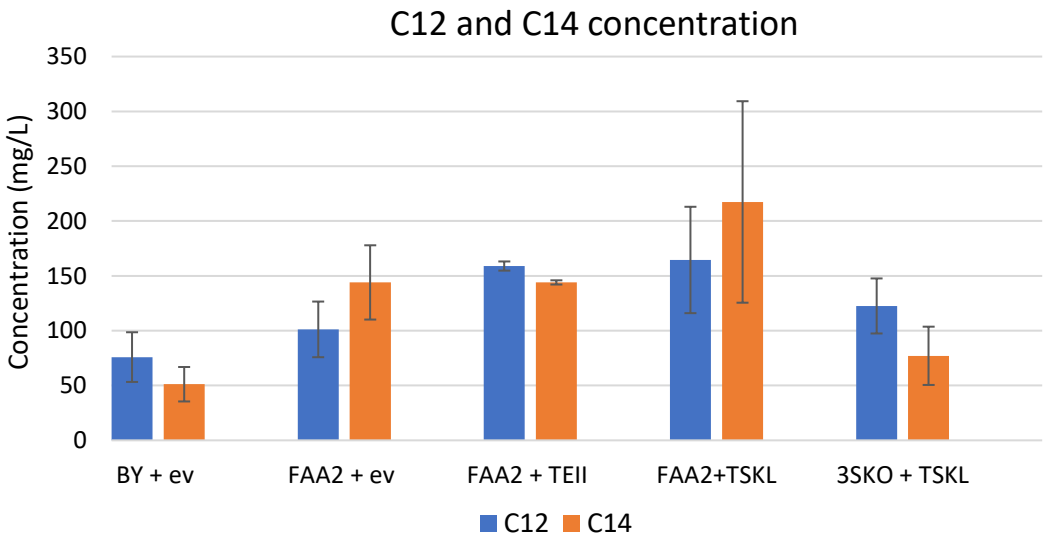


Figure 4.7. Extracelullar medium-chain free fatty acid levels of cells growing in $SD_{0.5}O_2C$(A) for 90h. BY4741 *faa2*Δ expressing thioesterase *TEII* (FAA2+TEII), BY4741 *faa2*Δ expressing thioesterase *TEII* with peroxisomal targeting signal 1 (FAA2+TSKL) and BY4741 *faa2*Δ*ant1*Δ*pex11*Δ expressing thioesterase *TEII* with peroxisomal targeting signal 1 (3SKO+TSKL) were compared to wild type BY4741 carrying an empty vector (BY+ev) and BY4741 *faa2*Δ strain carrying an empty vector (FAA2 + ev). C12 fatty acids (blue bars) and C14 fatty acids (orange bars) concentrations are shown in mg/L. Results are expressed as mean ± sd from duplicates.

Deletion of the medium-chain fatty acid activator gene *FAA2* led to a 3-fold increase in extracellular C14 but no statistically significant change in the C12 levels. When the medium-chain thioesterase *TEII* was expressed in the *faa2*Δ strain (FAA2 + TEII), the C12 levels increased approximately 50% compared to the strain harboring the empty plasmid, while C14 levels did not change. Adding the -SKL peroxisomal targeting signal to the *TEII* thioesterase (FAA2 +TSKL) did not have a significant change. Surprisingly, the strain 3SKO (*faa2*Δ *ant1*Δ *pex11*Δ) expressing *TEII*-SKL had lower titers of C14 than the *faa2*Δ strain expressing *TEII*-SKL.

To determine the distribution of intracellular MCFA versus extracellular MCFA, cells were grown in selective media with 0.5% oleic acid. Most of the C12 FFA was found in the supernatant with a ratio of approximately 3:1 (extracellular:intracellular) (Figure 4.8), while the C14 FFA was distributed evenly between the cellular mass and the supernatant.

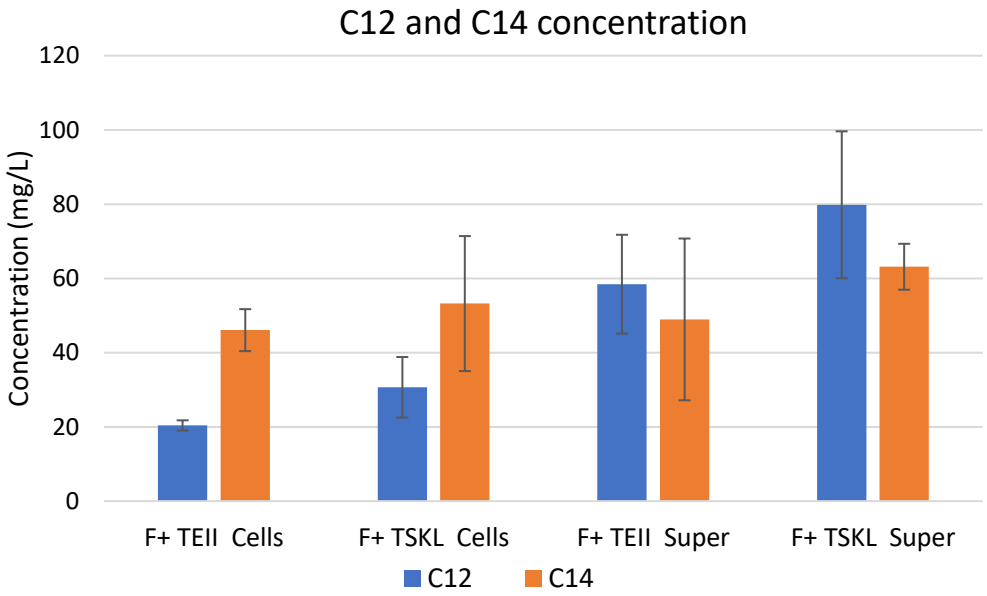


Figure 4.8. Intracellular and extracellular MC-FFA levels of cells growing in $SD_{0.5}O_{0.5}C(A)$ for 90h. BY4741 *faa2*Δ expressing thioesterase *TEII* (F+TEII), and BY4741 *faa2*Δ expressing thioesterase *TEII* with peroxisomal targeting signal 1 (F+TSKL). Intracellular levels are labeled as 'Cells' and extracellular levels are level as 'Super'. C12 fatty acids (blue bars) and C14 fatty acids (orange bars) concentrations are shown in mg/L. Results are expressed as mean ± sd from duplicates.

Disruption of monounsaturated fatty acids degradation and Acc1m overexpression

The peroxisomal Δ^3-cis-Δ^2-trans-enyol-CoA isomerase Eci1 is an auxiliary enzyme in the yeast β-oxidation of unsaturated FA. Eci1 moves the double bond from the 3-cis position to the 2-trans position (Gurvitz et al., 1998). Deletion of this gene prevents yeast cells from utilizing unsaturated fatty acids as a carbon source. Our hypothesis is that by knocking-out the *ECI1* gene we would be able to stop the β-oxidation at 3-cis-decenoyl-CoA (if the initial molecule is 9-hexadecenoyl-CoA) and 3-cis-dodecenoyl-CoA (if the initial molecule is 9-octadecenoyl-CoA). In addition, in a strain with *FAA2* deleted and expressing an active thioesterase for medium-chain fatty acyl-CoA, we could produce higher levels of C10:1 and C12:1.

Overexpression of an acetyl-CoA carboxylase variant Acc1^{S1157A} (Acc1m) that it is less sensitive to deactivation by Snf1 (activated when glucose is depleted) has been shown to increase C18:1 levels (Choi and Da Silva, 2014). Total FA produced by the strain overexpressing Acc1m (integrated at one copy under the strong constitutive *PGK1* promoter) was 3-fold higher than the total cellular FA levels of the wild type strain, and C18:1 levels were 7.3-fold higher. Similar results were reported in a later study in our lab that demonstrated that an increased percentage of C18:1 in the total cellular FA profile leads to increased resistance to several MCFA and other stressors in *S. cerevisiae* (Appendix A; Besada-Lombana et al., 2017). For the current study, *ACC1*m was expressed under the strong constitutive *TEF1* promoter instead of *PGK1*. While *PGK1* and *TEF1* promoters are generally considered strong "constitutive" promoters, expression from the *PGK1* promoter strength decreases when glucose levels become low (Partow et al., 2010), while *TEF1* promoter expression stays high.

Three strains, BY4741, BY4741 *trp1*Δ, and *faa2*Δ*eci*Δ *TEF1-ACC1m* (FE T-ACC1), were cultivated in three different media, $YPD_{0.5}O_2$, 'enriched' $SD_{0.5}O_2C(A)$, and $SD_{0.5}O_2C(A)$. There were three goals. The first was to determine if BY and the constructed BY *trp1*Δ strain have any phenotypic differences, as BY *trp1*Δ will be used in future studies. The second was to determine if the engineered strain has higher extracellular production of medium-chain FA. The third was to determine if there are any differences in MC-FFA in different media (complex, enriched semi-synthetic and semi-synthetic). As shown in Figure 4.9, that there is no major difference between BY4741 and BY4741 *trp1*Δ in terms of extracellular MC FFA after 90h. Only the engineered strain produced detectable levels of C12 (Figure 4.9-B), likely due to the fact that *FAA2* is deleted and there is higher MCFA accumulation. Interestingly, saturated C12 was not detected in the engineered strain growing in YPD; C10 and C10:1 were detected instead (Figure 4.9-A). Strain FE T-ACC1 in $YPD_{0.5}O_2$ produced 50 mg/L of C10:1 and 60 mg/L of C10, while BY and BY *trp1*Δ produced no detectable levels of these two

products. In addition, strain FE T-ACC1 produced 16-fold higher levels of C12:1 compared to the parent strain in $YPD_{0.5}O_2$. In semi-synthetic media, FE T-ACC1 produced 75% higher C12:1.

These results showed that production of MC-FFA in oleic acid medium is higher in complex media compared to semi-synthetic media, even in the optimized formulation. Also, we saw that the engineered FE T-ACC1 (faa2ΔeciΔ TEF1-ACC1m) produced higher levels of MC-FFA than the control strains in the three media tested, particularly in complex medium. Complex medium provides more nutrients; this may allow for higher flux through β-oxidation due to a faster replenishment of NAD^+ which is essential for the degradation pathway. The fact that there is a substantial amount of oleic acid in the media after the 90 h suggests that cell growth and oleic consumption are limited by other components in the medium.

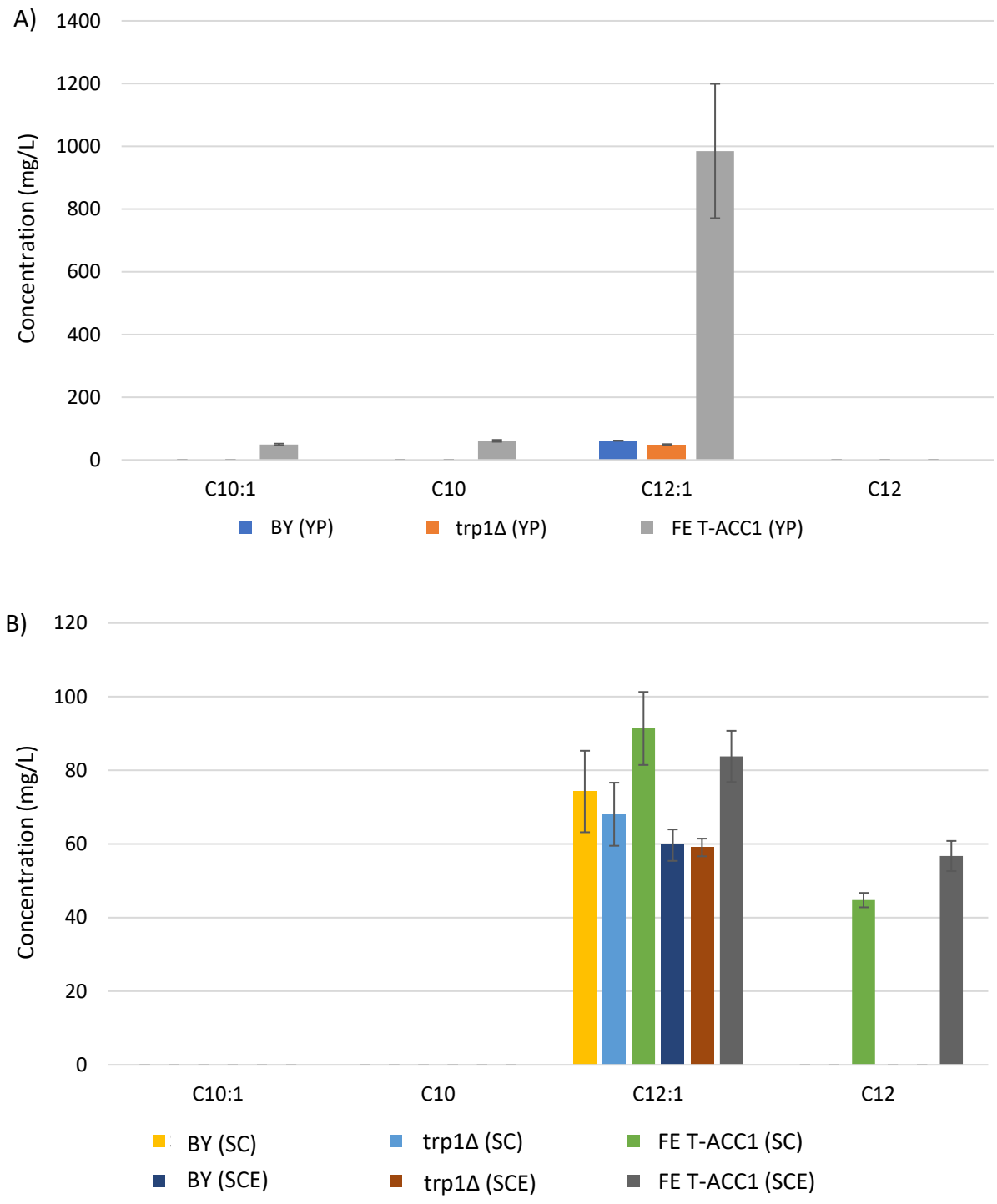


Figure 4.9. Extracellular medium-chain free fatty acid levels cells growing in (A) $YPD_{0.5}O_2$ (YP) and (B) $SD_{0.5}O_2C$(A) (SC) and enriched $SD_{0.5}O_2C$(A) (SCE) for 90h. Strains shown are BY4741 (BY), BY4741 *trp1*Δ (*trp1*Δ), and BY4741 *faa2*Δ*eci1*Δ p*TEF1-ACC1*m (FE T-ACC1). Concentrations are shown in mg/L. Results are expressed as mean ± sd from duplicates.

Effect of deletion of the cytosolic medium-chain elongase *ELO1* on MC-FFA production

S. cerevisiae has three cytosolic acyl-CoA elongases that are localized in the endoplamic reticulum (Tehlivets et al., 2007). Only one of them (Elo1) has substrate specificity for acyl-CoA shorter than 16 carbon chain length. The *ELO1* gene product has been reported to elongate C12 up to C16-C18 acyl-CoA (Toke and Martin, 1996). We showed that a *fas2*Δ strain (lacking the normal *de novo* fatty acid synthesis machinery) was able to grow on C8 and C10 as a sole source for fatty acid production (Figure 4.10). Based on these results and the known FA metabolism in *S. cerevisiae,* we speculated that the medium-chain acyl-CoA elongase is the enzyme responsible for extending the carbon chain length (with the help of auxiliary enzymes) from 8-10 carbons to the preferred C16 and C18 acyl-CoA used by yeast cells to build their phospholipid bilayer. Results in Figure 4.10 and led to the hypothesis that Elo1 could be elongating the medium-chain fatty free fatty acids if they are activated after leaving the peroxisome.

Therefore, *ELO1* was deleted in strains BY4741 *faa2*Δ *eci1*Δ p*TEF1-ACC1m* (FE T-ACC1) and BY4741 *faa2*Δ *eci1*Δ *trp1*Δ p*TEF1-ACC1m* (FETA trp1Δ) (Table 4.1). As shown in Figure 4.11, $YPD_{0.5}O_2$ was the best medium to determine the differences between strains. Therefore, this medium was used to compare FFA production in six different strains, BY4741 *trp1*Δ, BY4741 *faa2*Δ, *faa2*Δ *TEF1-ACC1m*, *faa2*Δ *eci1*Δ *TEF1-ACC1m* (FE T-ACC1), *faa2*Δ *eci1*Δ *elo1*Δ *TEF1-ACC1m* (FETA *elo1*Δ), and *faa2*Δ *eci1*Δ *elo1*Δ *trp1*Δ *TEF1-ACC1m* (FETAELO trp1Δ). As shown in Figure 4.11, only the strains with *faa2*Δ*eci1*Δ *TEF1-ACC1m* had detectable production of extracellular C10:1 FFA (~100 mg/L), and a ~9-fold increase in C12:1 production compared to the control strains. This indicates that the combination of *faa2*Δ *eci1*Δ is essential to stop the degradation of long-chain monounsaturated fatty acids and avoid reactivation, thus increasing MC-FFA synthesis.

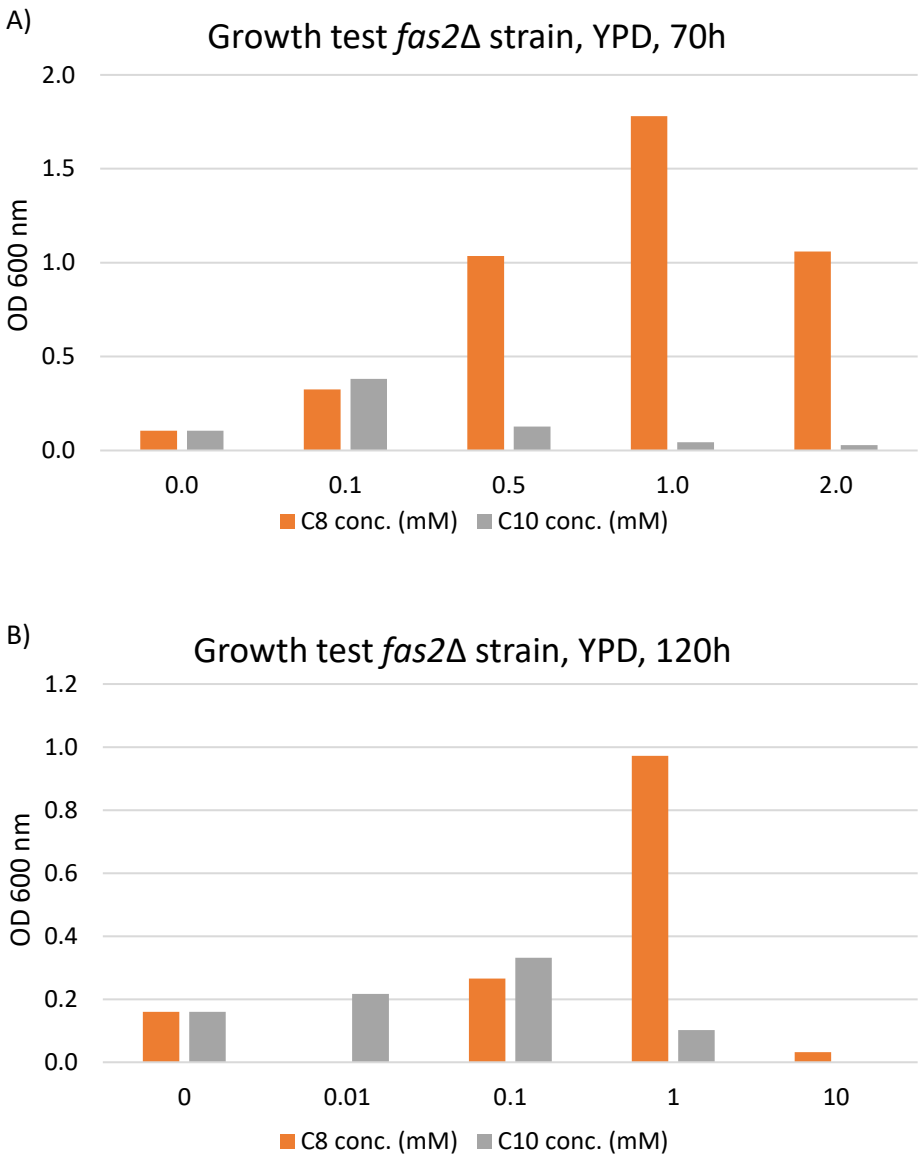


Figure 4.10. Growth measurements of *fas2*Δ *S. cerevisiae* strains in YPD with either octanoic or decanoic acid added to the media. Cells were inoculated at OD 0.1. (A) OD 600 nm measurement of cells grown for 70h in YPD and 0, 0.5, 1 and 2 mM of octanoic or decanoic acid. (B) OD 600 nm measurement of cells grown for 120h in YPD and 0, 0.1, 1 and 10 mM of octanoic or 0, 0.01, 0.1 and 1 mM of decanoic acid.

The medium-chain FFA titers in the supernatant did not increase by deleting the *ELO1* elongase (Figure 4.11). This suggests that the elongation of the MC acyl-CoA elongases is not a process that affects the final levels of extracellular MC-FFA. In addition, the deletion of *TRP1* didn't have a phenotypic effect on the FETAELO strain, similarly to what we observed previously (Figure 4.9).

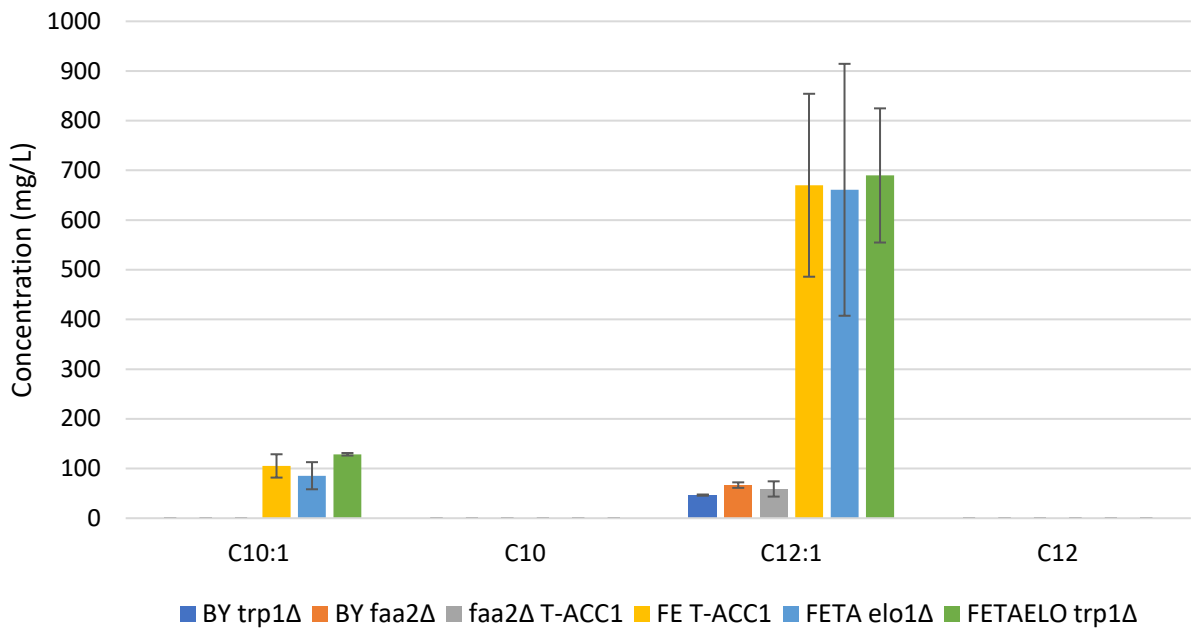


Figure 4.11. Extracellular medium-chain free fatty acid levels cells growing in $YPD_{0.5}O_2$ for 90h. Strains shown are BY4741 *trp1*Δ (*trp1*Δ) in light blue, BY4741 *faa2*Δ (BY faa2Δ) in orange, BY4741 *faa2*Δ p*TEF1-ACC1*m (faa2Δ T-ACC1) in grey, BY4741 *faa2*Δ*eci1*Δ p*TEF1-ACC1*m (FE T-ACC1) in yellow, BY4741 *faa2*Δ*eci1*Δ*elo1*Δ p*TEF1-ACC1*m (FETA elo1Δ) in dark blue, and BY4741 *faa2*Δ*eci1*Δ*elo1*Δ*trp1*Δ p*TEF1-ACC1*m (FETAELO trp1Δ) in green. Concentrations are shown in mg/L. Results are expressed as mean ± sd from duplicates.

Manipulation of size and number of peroxisomes per cell and deletion of native *TES1* thioesterase

In yeast, β-oxidation only happens in the peroxisome (Hiltunen et al., 2003). For this reason, a clear strategy to increase the flux towards the degradation of fatty acids is to increase the number of peroxisomes per cell. Previous published work demonstrated that deletion of the peroxisomal biogenesis related gene *PEX30*, and the two peroxisomal integral membrane protein encoding genes *PEX31* and *PEX32*, affects the size and number of peroxisomes per cell (Vizeacoumar et al., 2004). Deletion of these three genes increased the number of peroxisome per cell from an average of 1.32 to 6.24. In addition, *S. cerevisiae* has a native peroxisomal acyl-CoA thioesterase with a broad substrate specificity, Tes1 (Jones et al., 1999; Maeda et al., 2006). It has preference for short- and medium-chain acyl-CoA, but it can cleave long-chain acyl-CoA also. For this reason, we hypothesized

that if C14 and C16 intermediates of the oleate β-oxidation accumulate in the peroxisome, Tes1 might be cleaving them and they could leave the peroxisome. Therefore, we deleted *TES1*, *PEX30*, *PEX31*, and *PEX32*. To delete the four genes at one time, a multiplexed CRISPR/Cas9 system was adapted for our lab from previously published work in yeast (DiCarlo et al., 2013), to create SUPER strain (BY4741 *faa2*Δ*eci1*Δ*elo1*Δ*trp1*Δ*pex30*Δ*pex31*Δ*pex32*Δ*tes1*Δ) (Table 4.1). We also cloned the medium-chain thioesterase TEII (from the *Rattus novergicus* (TEII) mammary gland) with the C-terminal -SKL (Peroxisomal targeting signal 1) in pBT without *POX1* (pBT-TEII-SKL) or with the overexpression of *POX1* (pBT-TEII-SKL/POX1) to increase flux thourgh β-oxidation since it is known to be the limiting step.

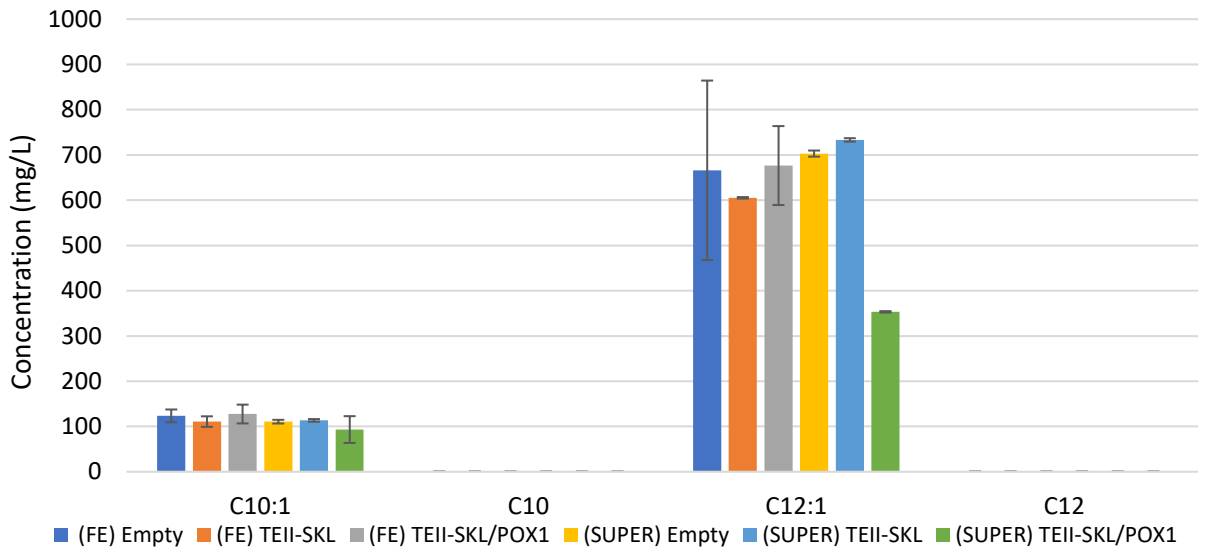


Figure 4.12. Extracellular medium-chain free fatty acid levels cells growing in $YPD_{0.5}O_2$ for 90h. Strains shown are BY4741 *faa2*Δ*eci1*Δ with empty vector [(FE) Empty] in light blue, with *TEII*-SKL [(FE) TEII-SKL] in orange, or with *TEII*-SKL and *POX1* [(FE) TEII-SKL/POX1] in grey, and BY4741 *faa2*Δ*eci1*Δ*elo1*Δ*trp1*Δ*pex30*Δ*pex31*Δ*pex32*Δ*tes1*Δ with empty vector [(SUPER) Empty] in yellow, with *TEII*-SKL [(SUPER) TEII-SKL] in dark blue, and with *TEII*-SKL and *POX1* [(SUPER) TEII-SKL/POX1]. Concentrations are shown in mg/L. Results are expressed as mean ± sd from duplicates.

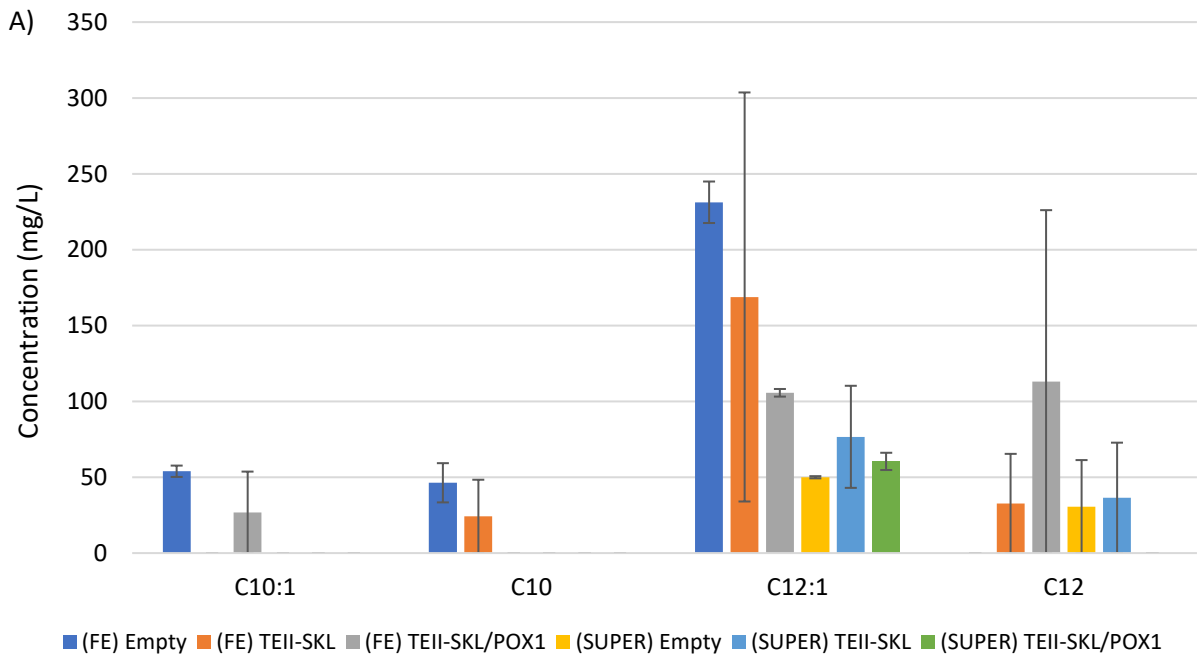
We first tested the MC-FFA productivity of the strains in non-selective medium to avoid having to use high amounts of antibiotics. There was no significant difference between the FE and

SUPER strains carrying the empty plasmid, indicating that the deletion of *PEX30*, *PEX31*, *PEX32*, and *TES1* did not affect MC-FFA production (Figure 4.12). In addition, there was no significant difference in the C10:1 levels among the various strains and plasmids. However, there were differences in the C12:1 titers. For the FE strain carrying the different plasmids, there is no statistically significant difference in C12:1 titers. Among the SUPER strains, we can conclude that overexpressing *TEII*-SKL and *POX1* in the same strain decreased the C12:1 FFA extracellular levels by approximately 2-fold. Interestingly, we didn't see this same drop in C12:1 levels in the FE strain. The other significant change is between FE TEII-SKL and SUPER TEII-SKL where C12:1 levels increased from 600 mg/L to 700 mg/L. One explanation for the similar MC-FFA levels across all the strains is that the plasmids were lost and the cells without plasmids took over the culture, especially since the results were quite similar to the strains without plasmids (Figure 4.11). To summarize, in general the SUPER strain did not increase MC-FFA compared to the FE strain, except when both strains expressed *TEII-SKL*. In addition, overexpression of *TEII-SKL* plus *POX1* decreased the levels of total MC-FFA in the SUPER strain.

MC-FFA production with selection for the TEII-SKL and TEII-SKL/POX1 plasmids

The next straight forward condition to test was to grow the engineered strains in selective complex media with oleic acid, $YPD_{0.5}O_2$ + G418, and measure growth and fatty acid titer (Figure 4.13). We observed significant differences between colonies, leading to large standard deviation of the results. Also, the addition of G418 to the media, or the sole expression of empty plasmids, reduced the C12:1 levels from more than 500 mg/L (Figure 4.9, 4.11, and 4.12) to less than 250 mg/L (Figure 4.13-A). Comparing the FE strain with the SUPER strain, FE generally produced more MC-FFA than the SUPER strain. Among FE strains, the one carrying the control plasmid (and thus not expressing the

two genes) plasmid (control) produced more MC-FFA. Among SUPER strains, there was not any statistically significant difference. Final OD of the three SUPER strains was lower than for the FE strains, especially the one expressing *TEII-SKL* and *POX1* genes (Figure 4.13-B); OD was only 2 while the FE strain expressing the same plasmid had a final OD of 24. The SUPER strain was thus very sensitive to plasmid expression, especially to the largest plasmid with two gene expression cassettes. Looking at the MC-FFA levels secreted per cell (specific concentration), we see that the strain that grew the least (SUPER+TEII-SKL/POX1) is the one that secreted more C12:1 FFA per unit cell (Figure 4.13-C).



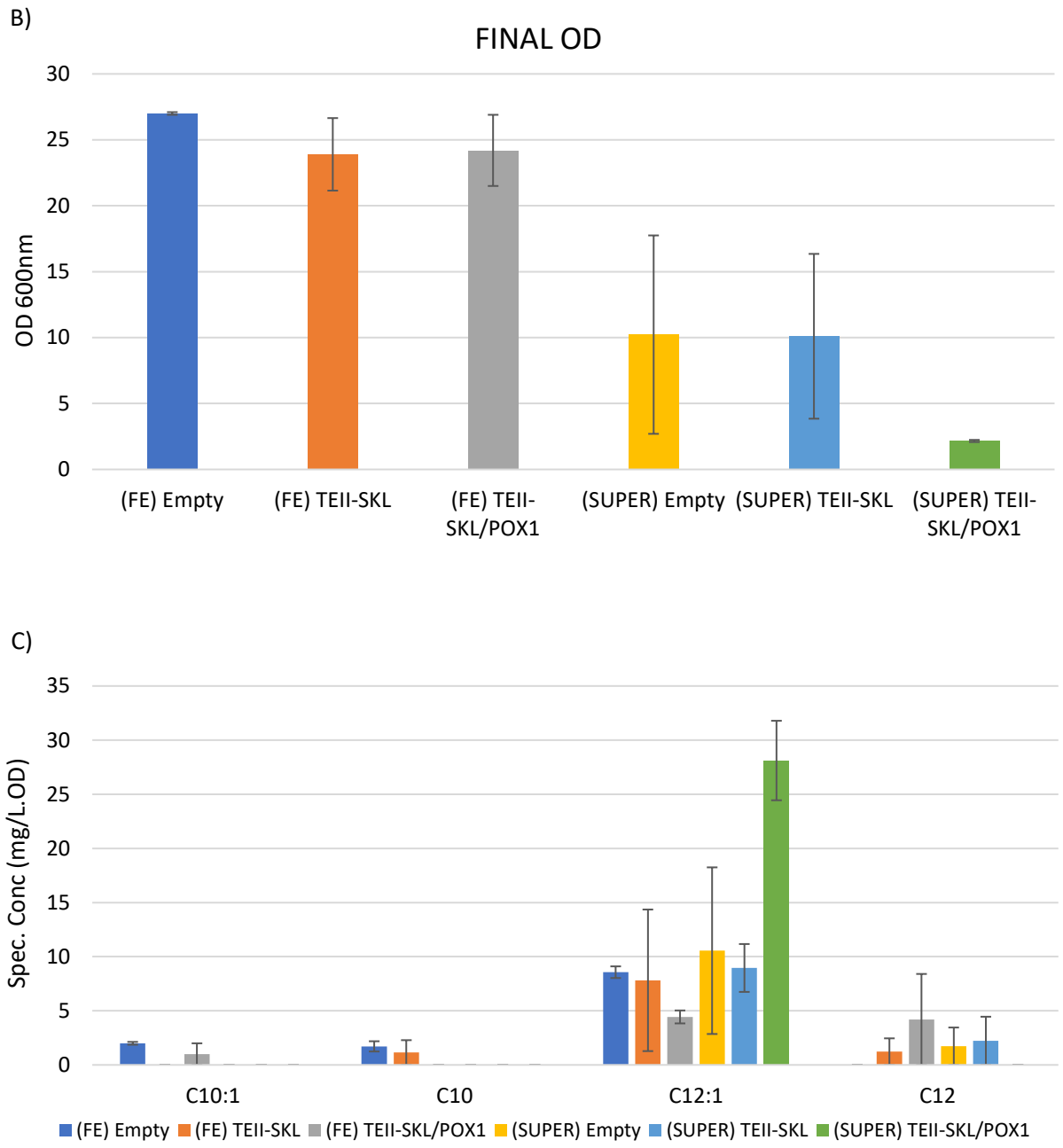


Figure 4.13. Extracellular medium-chain free fatty acid levels for cells growing in $YPD_{0.5}O_2$ + G418 for 90h. (A) Strains shown are BY4741 *faa2*Δ*eci1*Δ with empty vector [(FE) Empty] in light blue, with *TEII*-SKL [(FE) TEII-SKL] in orange, or with *TEII*-SKL and *POX1* [(FE) TEII-SKL/POX1] in grey, and BY4741 *faa2*Δ*eci1*Δ*elo1*Δ*trp1*Δ*pex30*Δ*pex31*Δ*pex32*Δ*tes1*Δ with empty vector [(SUPER) Empty] in yellow, with TEII-SKL [(SUPER) TEII-SKL] in dark blue, and with *TEII*-SKL and *POX1* [(SUPER) TEII-SKL/POX1] in green. Concentrations are shown in mg/L. (B) OD 600nm at harvesting time, 90h (C) Specific concentration of the different strains, expressed in mg/(L.OD). Results are expressed as mean ± sd from duplicates.

Characterization of MC-FFA production in medium with glucose as sole carbon source

For all experiments above, oleic acid feeding was used to increase carbon flux towards β-oxidation; however, oleic acid was not totally consumed after 90h which makes the remaining oleic acid in the media another variable to consider. In addition, the use of oleic acid makes the fatty acid extraction and detection more problematic. Earlier attempts to detect levels of medium chain free fatty acids in the supernatant of yeast cells grown in YPD lacking oleic acid were unsuccessful, primarily because of the low sensitivity and noisy results from the GC-MS at the UC Irvine Mass Spectrometry Facility. A newer GC-MS was repaired and provided much better sensitivity and less noise when measuring fatty acid methyl esters. We therefore switched to medium lacking oleic acid.

Recent papers on the production of LC-FFA and fatty acid-derivatives have used 3% glucose (Zhou et al., 2016a, 2016b; Zhu et al., 2017). We tested the previously built strains on YPD 3% and measured the MC-FFA levels in the supernatant after 48h (Figure 4.14-A). All engineered strains produced similar amounts of hexanoic acid and more C8 than BY4741. The *eci1*Δ strain produced nearly all C8 (~18 mg/L), with barely detectable levels of other MC-FFA. A possible explanation is that it is a BY4741 strain but from the Yeast Knockout Collection, not created directly from our lab BY4741 stock. Only strains with *FAA2* deleted produced C10 species. There were no significant differences between *faa2*Δ, *faa2*Δ*eci1*Δ, and *faa2*Δ*elo1*Δ strains in term of MC-FFA levels. There were not cumulative increases when *eci1*Δ was deleted in the *faa2*Δ strain, and the *elo1*Δ deletion did not increase MC-FFA. However, overexpression of the Acc1m enzyme in the *faa2*Δ*eci1*Δ strain, increased the production of C10:1, C10 and C12 2.2-fold. The large standard deviation of the MCFA measurement for the SUPER strain makes it difficult to draw any conclusions. In Figure 4.14-B, we can observe that all engineered strains had a total MC-FFA (C6-C12) higher than the control, BY4741.

Among the engineered strains, *eci1*Δ and FE-ACC1 were the ones that produced the most extracellular MC-FFA (~ 20 mg/L).

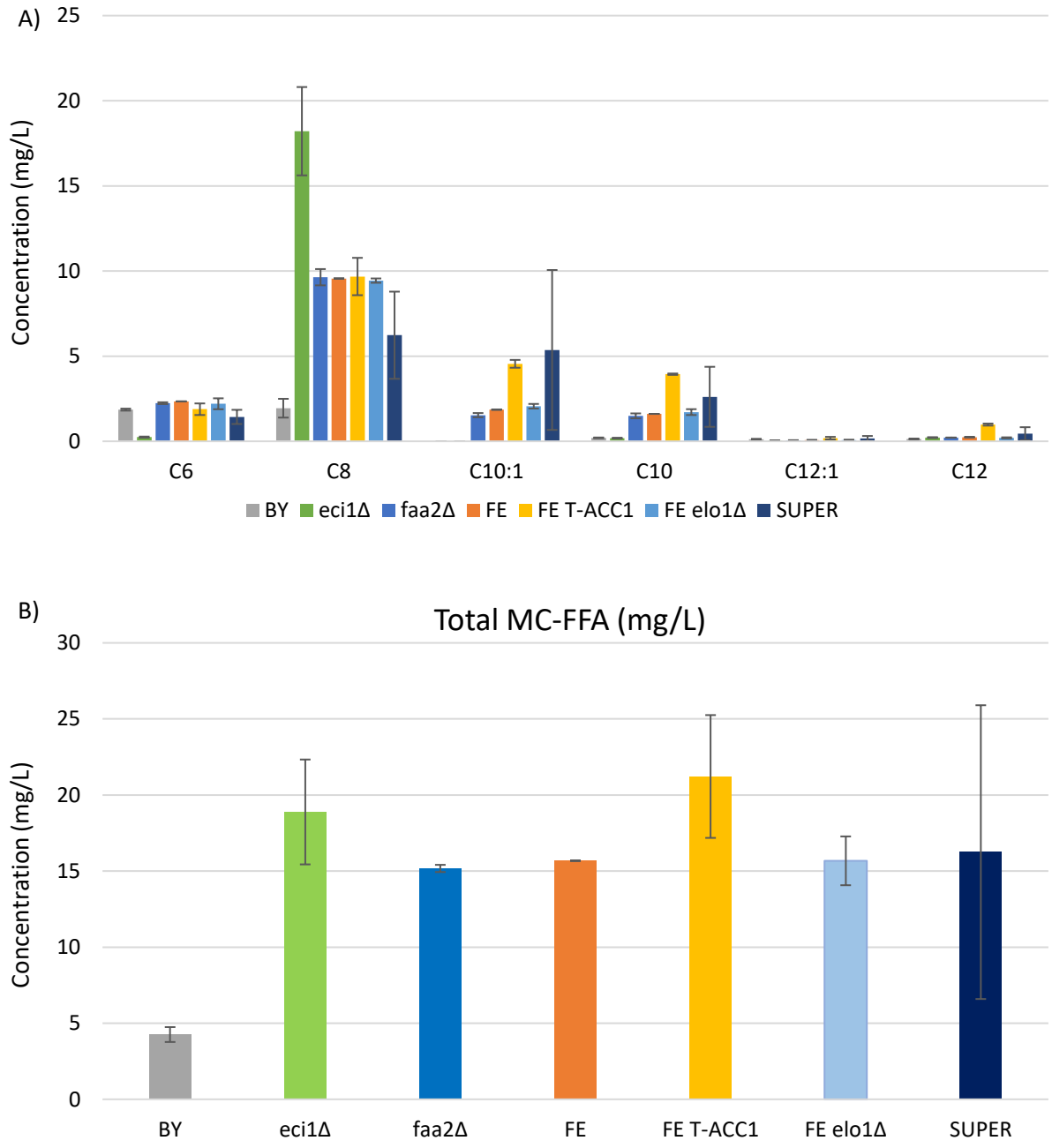


Figure 4.14. Extracellular medium-chain free fatty acid levels cells growing in YPD 3% for 48h. (A) Profile of MC-FFA. (B) Total MC-FFA produced by each of the strains. Strains shown are BY4741 (BY) in grey, BY4741 *eci1*Δ (eci1Δ) in green, BY4741 *faa2*Δ (faa2Δ) in blue, BY4741 *faa2*Δ*eci1*Δ (FE) in orange, BY4741 *faa2*Δ*eci1*Δ p*TEF1-ACC1m* (FE T-ACC1) in yellow, BY4741 *faa2*Δ*eci1*Δ*elo1Δ* (FE elo1Δ) in light blue, and BY4741 *faa2*Δ*eci1*Δ*elo1*Δ*trp1*Δ*pex30*Δ*pex31*Δ*pex32*Δ*tes1*Δ (SUPER) in dark blue. Concentrations are shown in mg/L. Results are expressed as mean ± sd from duplicates.

Overexpression of *FAS1* and *FAS2* genes in the FE (*faa2*Δ *eci1*Δ) strain

One of the limitations to increasing flux through β-oxidation in *S. cerevisiae* is the limited native production of high-levels of acyl-CoA. Overexpression of the native FAS increased the production of free fatty acids (Runguphan and Keasling, 2014). By using CRISPR/Cas9, we swapped the native *FAS1* and *FAS2* promoters in the genome for the strong constitutive *TEF1* promoter. The FE strain overexpressing the native FAS resulted in increased titers of all MC-FFA species (except C12:1) relative to BY4741 and FE (Figure 4.15-A). Interestingly, it also increased the C6 levels ~5-fold; C6 was increased only slightly in FE without FAS overexpression. The most abundant MC-FFA in the supernatant produced by FE-FAS12 was octanoic acid (28 mg/L), 3-fold higher relative to the FE strain. The second most abundant was decanoic acid (13 mg/L), which increased 8-fold compared to FE. Total MC-FFA in the supernatant increased 14-fold and 3.8-fold, with respect to BY4741 and FE, respectively (Figure 4.15-B). A control experiment with BY, BY-FAS12 (overexpression of *FAS1* and *FAS2*) and FE-FAS12 showed that BY and BY-FAS12 did not have significantly different MC-FFA profiles (data not shown). Thus, FAS overexpression only increased MC-FAA in the engineered strain.

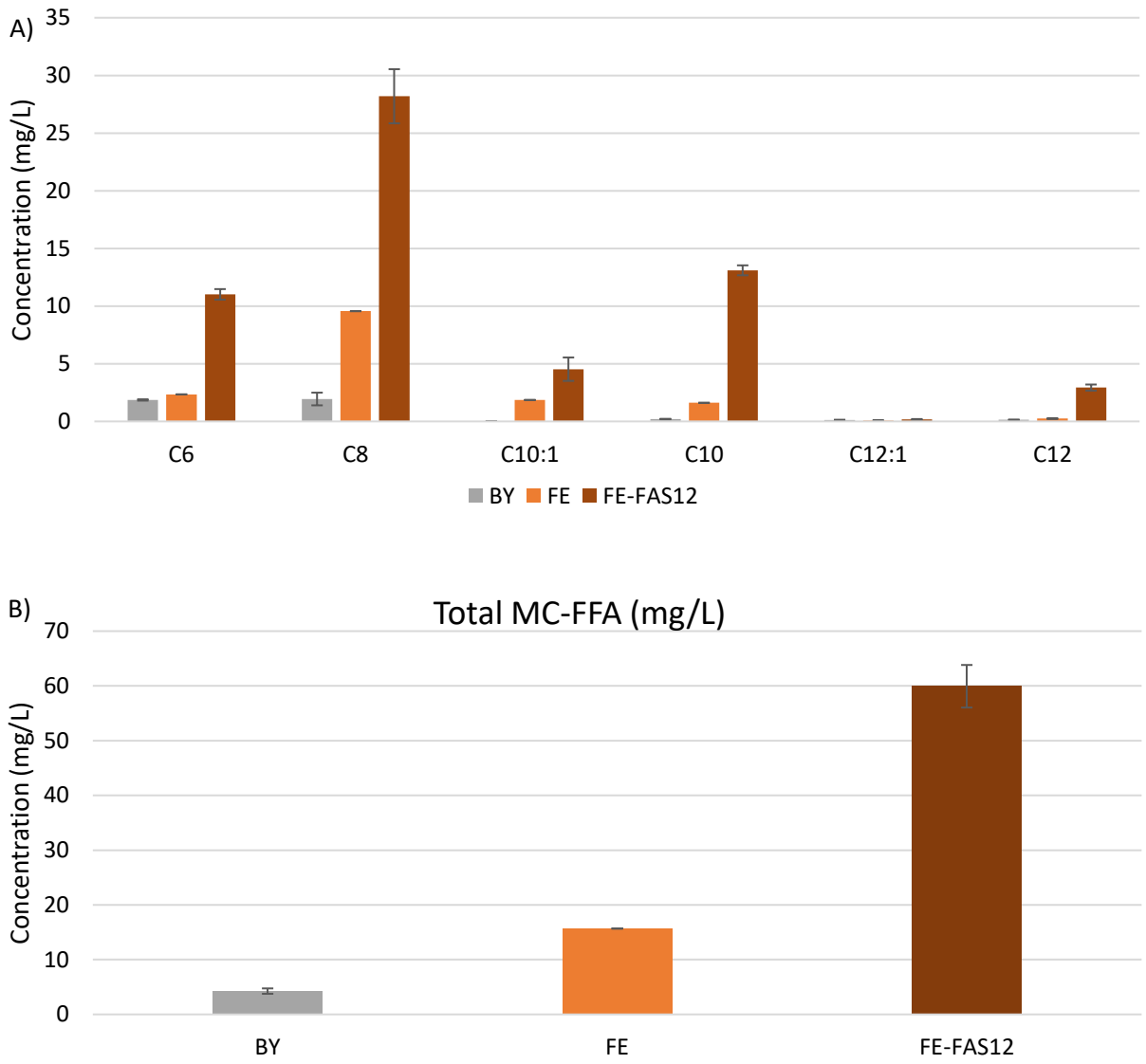


Figure 4.15. Extracellular medium-chain free fatty acid levels for cells growing in YPD 3% for 48h. (A) Profile of MC-FFA. (B) Total MC-FFA produced by each of the strains. Strains shown are BY4741 (BY) in grey, BY4741 *faa2*Δ*eci1*Δ (FE) in orange, BY4741 *faa2*Δ*eci1*Δ *TEF1-FAS1 TEF1-FAS2* (FE-FAS12) in dark red. Concentrations are shown in mg/L. Results are expressed as mean ± sd from duplicates.

Evaluation of the Acc1m overexpression effect on MC-FFA production

We integrated Acc1m to determine if the overexpression of the Acc1 variant that is less prone to deactivation (Choi and Da Silva, 2014) has the same effect on our engineered strains. We found that FE-ACC1 produced more C8, C10:1 and C10 than FE (Figure 4.16). Surprisingly, overexpression of Acc1m in the FE *elo1*Δ and SUPER strains had the opposite effect, decreasing MC-FFA production relative to the parent strains (Figure 4.16-B). In this experiment, the deletion of the medium-chain elongase *ELO1* increased the levels of total MC-FFA 2-fold compared to the parent strain FE. The increase was significant for C10:1, C10, C12:1 and C12. In addition, the SUPER strain produced 1.63-fold more MC-FFA than the parent strain FE *elo1*Δ, with C10:1 the most abundant FA species. As seen in Figure 4.15, *FAS1* and *FAS2* overexpression produced the highest MC-FFA levels, especially for C8 and C10.

Acc1m was integrated in the highest producing strain, FE-FAS12 (Table 4.1). Only two colonies appeared on the transformation plate, and they both were tested (duplicates of each). FE-FAS12 produced ~25% more MC-FFA than the FE-FAS12 T-ACC1 strain (Figure 4-17). This result is similar to the effect of the *ACC1m* integration observed for the FE *elo1*Δ and the SUPER strains. FE-FAS12 produced more of every MC-FFA species except for C10:1. Thus, the overexpression of the Acc1m enzyme increased MC-FFA only in the FE strain (Figure 4.16), and decreased MC-FFA in all other tested strains.

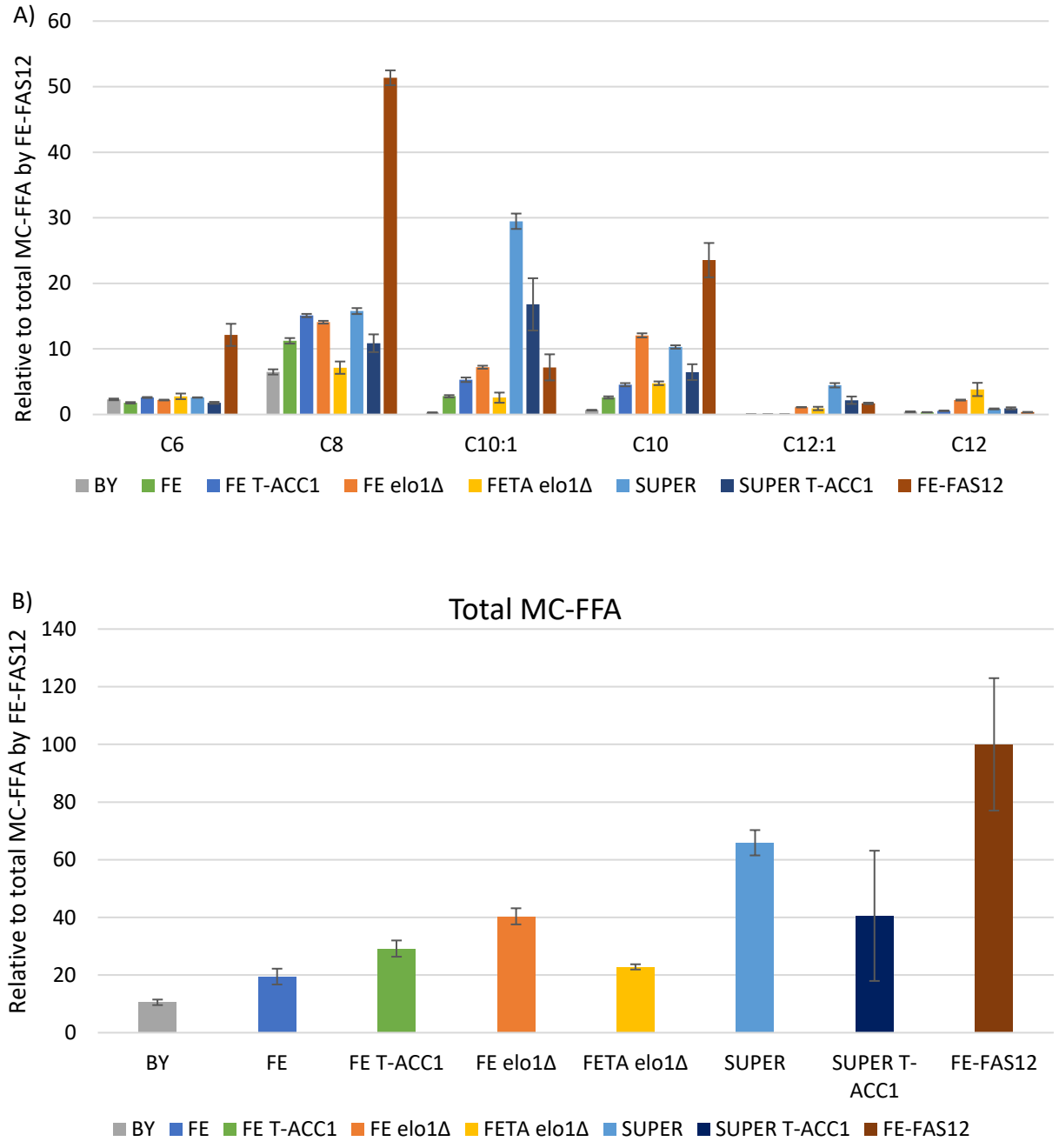


Figure 4.16. Extracellular medium-chain free fatty acid levels for cells growing in YPD 3% for 48h. (A) Profile of MC-FFA. (B) Total MC-FFA produced by each of the strains. Strains shown are BY4741 (BY) in grey, BY4741 *faa2Δeci1Δ* (FE) in blue, BY4741 *faa2Δeci1Δ* p*TEF1-ACC1m* (FE T-ACC1) in green, BY4741 *faa2Δeci1Δelo1Δ* (FE elo1Δ) in orange, BY4741 *faa2Δeci1Δelo1Δ* p*TEF1-ACC1m* (FETA elo1Δ) in yellow, BY4741 *faa2Δeci1Δelo1Δtrp1Δpex30Δpex31Δpex32Δtes1Δ* (SUPER) in light blue, BY4741 *faa2Δeci1Δelo1Δtrp1Δpex30Δpex31Δpex32Δtes1Δ* p*TEF1-ACC1m* (SUPER T-ACC1) in dark blue, and BY4741 *faa2Δeci1Δ* p*TEF1-FAS1* p*TEF1-FAS2* (FE-FAS12) in dark red. Concentrations are shown in mg/L. Results are expressed as mean ± sd from duplicates.

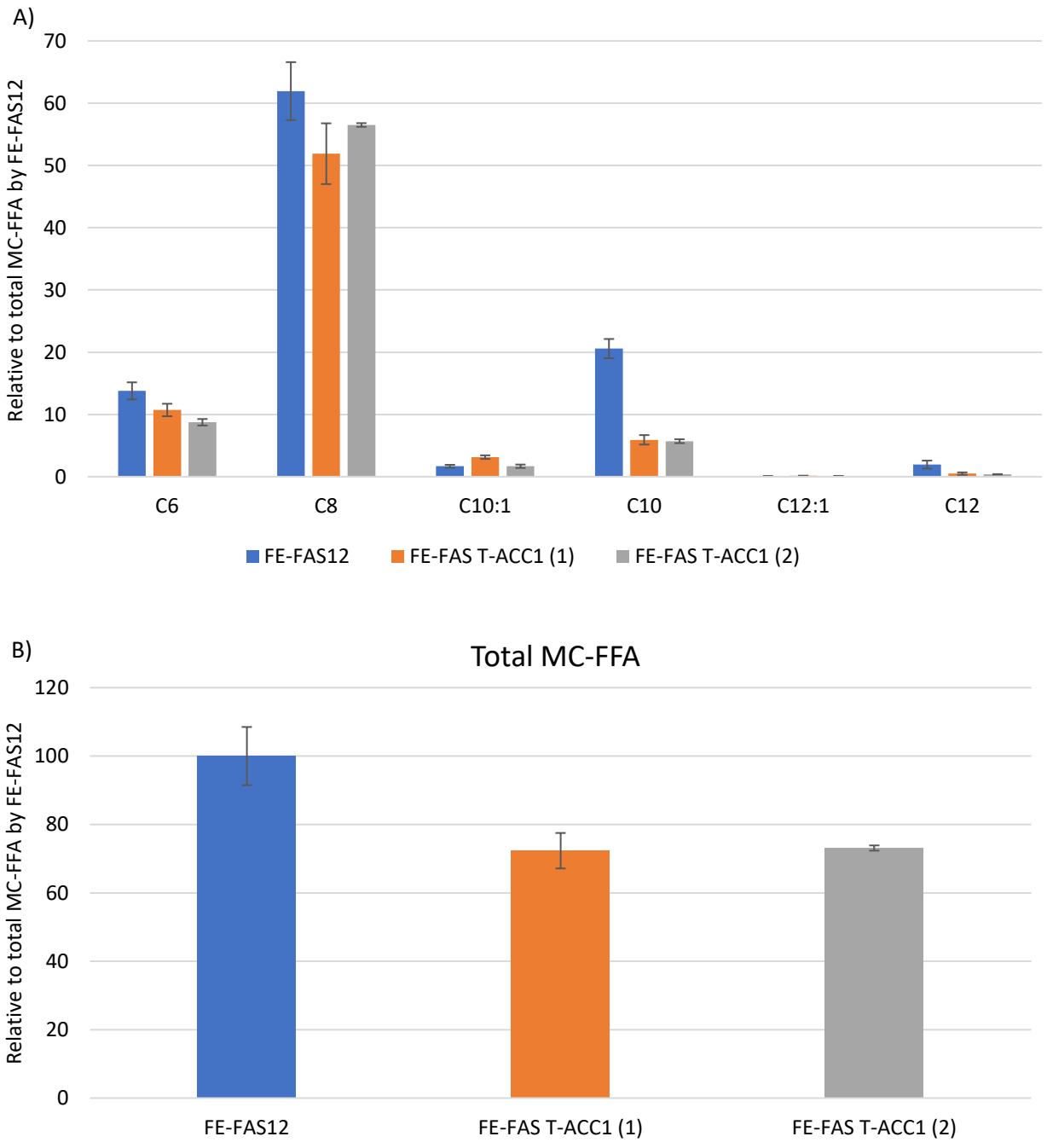


Figure 4.17. Extracellular medium-chain free fatty acid levels for cells growing in YPD 3% for 48h. (A) Profile of MC-FFA. (B) Total MC-FFA produced by each of the strains. Strains shown are BY4741 *faa2*Δ*eci1*Δ p*TEF1-FAS1* p*TEF1-FAS2* (FE-FAS12) in blue, and BY4741 *faa2*Δ*eci1*Δ p*TEF1-FAS1* p*TEF1-FAS2* p*TEF1-ACC1m* (FE-FAS12 T-ACC1), colony 1 in orange and colony 2 in grey. Concentrations are shown as relative to total MC-FFA by FE-FAS12. Results are expressed as mean ± sd from duplicates.

Targeting MCFA thioesterase into the peroxisomes of strain FE-FAS12

We have shown that FE-FAS12 is the strain that produces the most extracellular MC-FFA among all the strains created, and octanoic acid accounts for approximately 50% of the total MC-FFA. We then tested if the expression of a medium-chain acyl-CoA thioesterase, targeted or not targeted to the peroxisome, can increase the extracellular levels further. Our previous attempts at this strategy in other strains during oleic acid feeding showed mixed results. For the *faa2*Δ strain, expression of the *TEII* thioesterase increased the extracellular levels of lauric acid by 50%; however, introduction of the peroxisomal targeting signal to the enzyme did not further increase the C12 levels (Figure 4.7). Expression of the *TEII*-SKL and the native *TES1* thioesterases in the SUPER strain had no effect on MC-FFA production relative to SUPER expressing an empty plasmid (data not shown). The most important characteristic to look for in a medium-chain thioesterase to make this strategy work is a strong preference for acyl-CoA/-ACP molecules with 12 carbons or less (i.e., very low substrate specificity for long chain fatty acyl-CoA). The enzyme must be active on acyl-CoA intermediates, not only on acyl-ACP. This last requirement is important since most thioesterases from plants are only active on ACP-bound fatty acid intermediates (Jing et al., 2011).

A good candidate is the *BTE* medium-chain thioesterase from California bay (*Umbellularia californica)*, which produces oilseeds with high C12:0 content (Davies et al., 1991). This enzyme has been shown to increase lauric acid levels when expressed in *E. coli* (Lennen et al., 2010; Sherkhanov et al., 2014; Voelker and Davies, 1994). It is highly specific for C12:0, which is very important for our application; however, the main question is whether it will be active on acyl-CoA substrates, instead of only acyl-ACP. No previous work has determined its activity on acyl-CoA molecules.

Another good candidate is a new mutant of the tesA thioesterase from *E. coli*. tesA is found in the periplasm of *E. coli*, but removal of the leader sequence (‘tesA) localizes the enzyme to the cytoplasm, and it significantly increases the total FFA production (Cho and Cronan, 1995). ‘tesA has been used as the preferred thioesterase in many studies focused on the overproduction of free fatty studies due to its high activity (Davis et al., 2000; Liu et al., 2010; Steen et al., 2010). In addition, it has been shown to increase FFA production when expressed in *S. cerevisiae* (Li et al., 2014; Runguphan and Keasling, 2014; Zhou et al., 2016b). Increased interest in production of medium-chain fatty acids in recent years has resulted in the engineering of ‘tesA to change its substrate specificity (Grisewood et al., 2017). Through several cycles of *in silico* modeling refining and library construction and testing the authors were able to find a ‘tesA mutant with higher substrate specificity for medium-chain acyl-CoA than long-chain acyl-CoA. The mutant had three point mutations (M141L, Y145K, L146K) and 50% of the total FFA secreted to the media by the *E. coli* was C8, compared to 6% by the strain overexpressing the wild type variant. Although there are other published thioesterases with better substrate specificity, tesA’ has been shown to be active on acyl-CoA, while most of the other specific thioesterases come from plants and don’t show activity towards acyl-ACP (Jing et al., 2011).

In our previous experiments, a peroxisomal targeting signal 1 (PTS1) was added at the C-terminal of *TEII*. The C-terminus tag -SKL has been shown to be conserved in many peroxisomal yeast proteins (Gould et al., 1989). However, the addition of -SKL (canonical PTS1) to a heterologous protein does not ensure assure efficient targeting in all cases, and it was founed that upstream amino acids may have an important role (Distel et al., 1992). Recent work has determined a more efficient PTS1 by using random library construction and high-throughput screening (DeLoache et al., 2016). The C-terminal (LGRGRRSKL) increased the rate at which proteins were targeted to the yeast peroxisomes and the efficiency (% of total protein) of targeting. This new PTS1 (ePTS1) was added to the *BTE* and

the mutant TesA genes (mTesA) (Table 4.2), and their expression was tested for extracellular MC-FFA production in FE-FAS12, the highest producing strain. No significant change was found between the strains producing the tagged genes (BTEePTS1 and mTesAePTS1) and the non-tagged variants (BTE and mTesA) (Figure 4.18). More importantly, no difference in MC-FFA production was observed between the FE-FAS12 control expressing an empty plasmid and the rest of the plasmids tested. This could indicate two things; the first is that the production of MC-FFA in FE-FAS12 is limited by acyl-CoA availability, and it is not affected by the overexpression of medium-chain acyl-ACP/-CoA thioesterases. The second is that BTE and mTesA are not efficient enough to overcome the activity of the native peroxisomal thioesterase, Tes1.

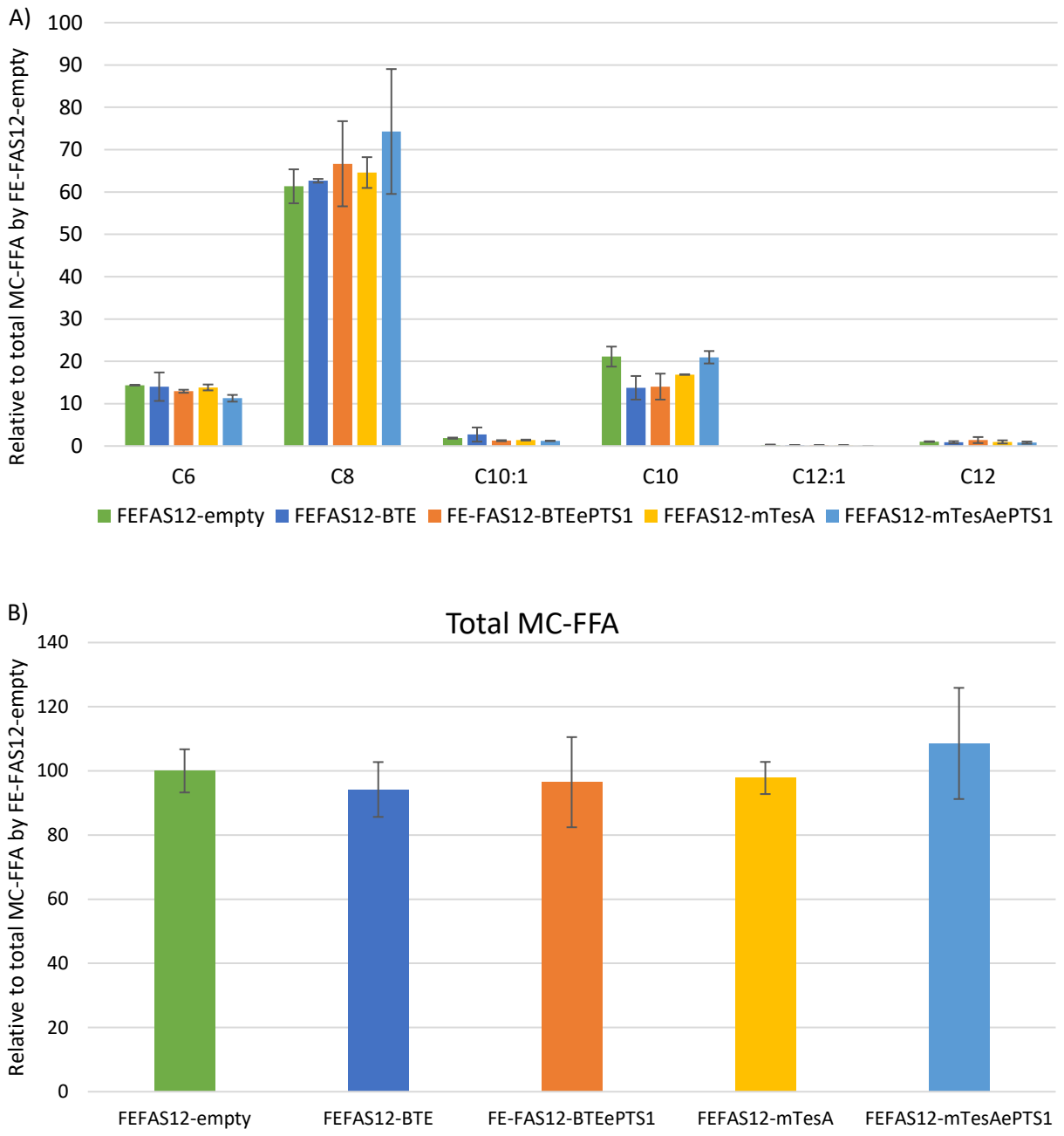


Figure 4.18. Extracellular medium-chain free fatty acid levels for cells growing in YPD+G418 3% for 48h. (A) Profile of MC-FFA. (B) Total MC-FFA produced by each of the strains. Strains shown are BY4741 *faa2*Δ*eci1*Δ p*TEF1-FAS1* p*TEF1-FAS2* (FE-FAS12) with pBT-KanMX-empty in green, with pBT-KanMX-BTE in dark blue, with pBT-KanMX-BTEePTS1 in orange, with pBT-KanMX-mTesA in yellow, and with pBT-KanMX-mTesAePTS1 in light blue. Concentrations are shown as relative to total MC-FFA by FEFAS12-empty. Results are expressed as mean ± sd from duplicates.

Targeting MCFA thioesterase into the peroxisomes of strain SUPER

To test the second hypothesis, BTE and mTesA, tagged and untagged, were overexpressed in a strain with the native peroxisomal thioesterase gene *TES1* deleted: SUPER (Table 4.1), and the fatty acid profile and total MC-FFA in the medium were determined (Figure 4.19). The cells expressing BTE without the ePTS1 produced 9% more MC-FFA than the cells expressing BTE with the ePTS1 (Figure 4.19-B). In addition, all of the strains expressing either BTE or mTesA, regardless of which variant, increased the MC-FFA levels compared to the cells harboring the empty plasmid. Most of the MC-FFA was C8 (56-69%), and the second most abundant FFA species was C10 (22-33%) (Figure 4.19-A). C8, C10:1, C10 and C12 were increased for the strains expressing both variants of BTE or mTesA, and C6 was reduced.

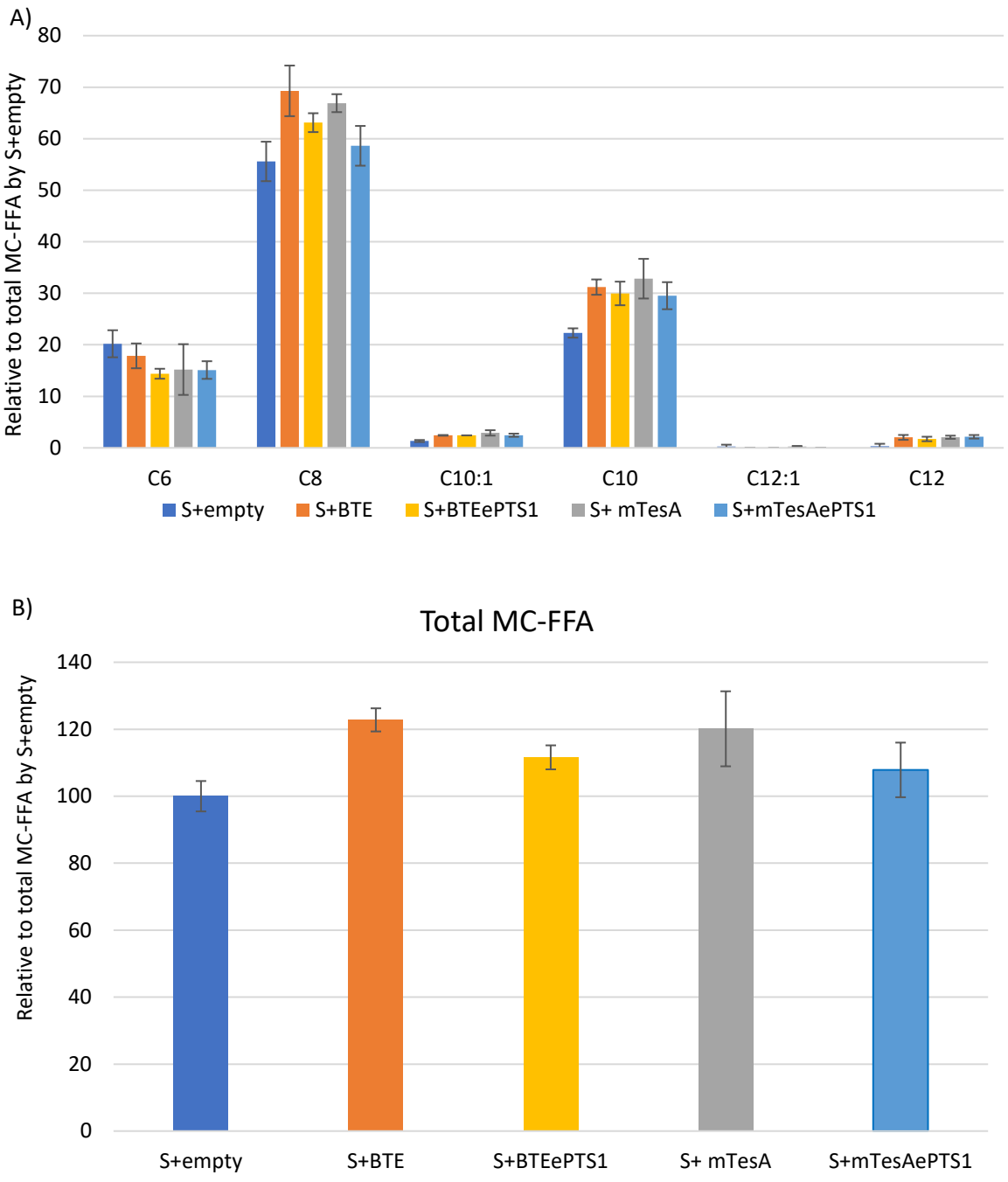


Figure 4.19. Extracellular medium-chain free fatty acid levels for cells growing in YPD+G418 3% for 48h. (A) Profile of MC-FFA. (B) Total MC-FFA produced by each of the strains. Strains shown are BY4741 *faa2Δeci1Δelo1Δtrp1Δpex30Δpex31Δpex32Δtes1Δ* (SUPER) with pBT-KanMX-empty in dark blue, with pBT-KanMX-BTE in orange, with pBT-KanMX-BTEePTS1 in yellow, with pBT-KanMX-mTesA in gray, and with pBT-KanMX-mTesAePTS1 in light blue. Concentrations are shown as relative to total MC-FFA by S+empty. Results are expressed as mean ± sd from duplicates.

CONCLUSIONS

S. cerevisiae is a model yeast species and metabolic engineering strategies that worked to improve fatty acids and fatty acid-derived products have translated successfully to the promising oleaginous yeast. In this study, we showed a novel approach to produce medium-chain free fatty acids in *S. cerevisiae*. Yeast produce primarily long chain fatty acids for several cellular processes, and can degrade them when needed for carbon and energy. We engineered the FA degradation pathway (β-oxidation) to shorten LCFA to C10 and C12 species. We found that the expression of the MCFA specific *TEII* from rat mammary gland in a *FAA2* strain increased the production of C12 species by 50% compared to a strain not expressing any thioesterase. Combination of the *FAA2* and *ECI1* deletions was sufficient to increase the production of C12:1 14-fold in cells grown in complex medium, and to increase C12 species (C12:0+C12:1) 1.83-fold in synthetic medium, both supplemented with oleic acid. In medium with glucose as the only carbon source, a *faa2ΔeciΔ* strain with *FAS1* and *FAS2* overexpressed produced 14-fold and 3.8-fold more MC-FFA relative to the original parent and *faa2ΔeciΔ* strains, respectively. This strain secreted 60 mg/L of total MC-FFA. Further interventions including overexrpression of an Acc1 variant or several heterologous medium-chain specific thioesterases did not further increase the productivity of the strain.

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REFERENCES

Besada-Lombana, P.B., Fernandez-Moya, R., Fenster, J., and Da Silva, N.A. (2017). Engineering Saccharomyces cerevisiae fatty acid composition for increased tolerance to octanoic acid. Biotechnol Bioeng *114*, 1531–1538.

Bhataya, A., Schmidt-Dannert, C., and Lee, P.C. (2009). Metabolic engineering of Pichia pastoris X-33 for lycopene production. Process Biochemistry *44*, 1095–1102.

Black, P.N., and DiRusso, C.C. (2007). Yeast acyl-CoA synthetases at the crossroads of fatty acid metabolism and regulation. Biochim Biophys Acta *1771*, 286–298.

Bozell, J.J., and Petersen, G.R. (2010). Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. Green Chemistry *12*, 539.

Brody, S., Oh, C., Hoja, U., and Schweizer, E. (1997). Mitochondrial acyl carrier protein is involved in lipoic acid synthesis in*Saccharomyces cerevisiae*. FEBS Lett *408*, 217–220.

Chen, L., Zhang, J., and Chen, W.N. (2014). Engineering the Saccharomyces cerevisiae β-oxidation pathway to increase medium chain fatty acid production as potential biofuel. PLoS ONE *9*, e84853.

Cho, H., and Cronan, J.E. (1995). Defective export of a periplasmic enzyme disrupts regulation of fatty acid synthesis. J Biol Chem *270*, 4216–4219.

Choi, J.W., and Da Silva, N.A. (2014). Improving polyketide and fatty acid synthesis by engineering of the yeast acetyl-CoA carboxylase. J Biotechnol *187*, 56–59.

Davies, H.M., Anderson, L., Fan, C., and Hawkins, D.J. (1991). Developmental induction, purification, and further characterization of 12:0-ACP thioesterase from immature cotyledons of Umbellularia californica. Arch Biochem Biophys *290*, 37–45.

Davis, M.S., Solbiati, J., and Cronan, J.E. (2000). Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in Escherichia coli. J Biol Chem *275*, 28593–28598.

DeLoache, W.C., Russ, Z.N., and Dueber, J.E. (2016). Towards repurposing the yeast peroxisome for compartmentalizing heterologous metabolic pathways. Nat Commun *7*, 11152.

DiCarlo, J.E., Norville, J.E., Mali, P., Rios, X., Aach, J., and Church, G.M. (2013). Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res *41*, 4336–4343.

Distel, B., Gould, S.J., Voorn-Brouwer, T., van der Berg, M., Tabak, H.F., and Subramani, S. (1992). The carboxyl-terminal tripeptide serine-lysine-leucine of firefly luciferase is necessary but not sufficient for peroxisomal import in yeast. New Biol *4*, 157–165.

Elgersma, Y., Vos, A., van den Berg, M., van Roermund, C.W., van der Sluijs, P., Distel, B., and Tabak, H.F. (1996). Analysis of the carboxyl-terminal peroxisomal targeting signal 1 in a homologous context in Saccharomyces cerevisiae. J Biol Chem *271*, 26375–26382.

Fang, F., Salmon, K., Shen, M.W.Y., Aeling, K.A., Ito, E., Irwin, B., Tran, U.P.C., Hatfield, G.W., Da Silva, N.A., and Sandmeyer, S. (2011). A vector set for systematic metabolic engineering in Saccharomyces cerevisiae. Yeast *28*, 123–136.

Fernandez-Moya, R., Leber, C., Cardenas, J., and Da Silva, N.A. (2015). Functional replacement of the Saccharomyces cerevisiae fatty acid synthase with a bacterial type II system allows flexible product profiles. Biotechnol Bioeng *112*, 2618–2623.

Gajewski, J., Pavlovic, R., Fischer, M., Boles, E., and Grininger, M. (2017). Engineering fungal de novo fatty acid synthesis for short chain fatty acid production. Nat Commun *8*, 14650.

Gould, S.J., Keller, G.A., Hosken, N., Wilkinson, J., and Subramani, S. (1989). A conserved tripeptide sorts proteins to peroxisomes. J Cell Biol *108*, 1657–1664.

Grisewood, M.J., Hernández-Lozada, N.J., Thoden, J.B., Gifford, N.P., Mendez-Perez, D., Schoenberger, H.A., Allan, M.F., Floy, M.E., Lai, R.-Y., Holden, H.M., et al. (2017). Computational Redesign of Acyl-ACP Thioesterase with Improved Selectivity toward Medium-Chain-Length Fatty Acids. ACS Catal *7*, 3837–3849.

Gurvitz, A., Mursula, A.M., Firzinger, A., Hamilton, B., Kilpeläinen, S.H., Hartig, A., Ruis, H., Hiltunen, J.K., and Rottensteiner, H. (1998). Peroxisomal Delta3-cis-Delta2-trans-enoyl-CoA isomerase encoded by ECI1 is required for growth of the yeast Saccharomyces cerevisiae on unsaturated fatty acids. J Biol Chem *273*, 31366–31374.

Hagen, S., Drepper, F., Fischer, S., Fodor, K., Passon, D., Platta, H.W., Zenn, M., Schliebs, W., Girzalsky, W., Wilmanns, M., et al. (2015). Structural insights into cargo recognition by the yeast PTS1 receptor. J Biol Chem *290*, 26610–26626.

Hiltunen, J.K., Mursula, A.M., Rottensteiner, H., Wierenga, R.K., Kastaniotis, A.J., and Gurvitz, A. (2003). The biochemistry of peroxisomal β-oxidation in the yeast*Saccharomyces cerevisiae*. FEMS Microbiol Rev *27*, 35–64.

Hiltunen, J.K., Schonauer, M.S., Autio, K.J., Mittelmeier, T.M., Kastaniotis, A.J., and Dieckmann, C.L. (2009). Mitochondrial fatty acid synthesis type II: more than just fatty acids. J Biol Chem *284*, 9011–9015.

Jing, F., Cantu, D.C., Tvaruzkova, J., Chipman, J.P., Nikolau, B.J., Yandeau-Nelson, M.D., and Reilly, P.J. (2011). Phylogenetic and experimental characterization of an acyl-ACP thioesterase family reveals significant diversity in enzymatic specificity and activity. BMC Biochem *12*, 44.

Jones, J.M., Nau, K., Geraghty, M.T., Erdmann, R., and Gould, S.J. (1999). Identification of peroxisomal acyl-CoA thioesterases in yeast and humans. J Biol Chem *274*, 9216–9223.

Kim, P.K., and Hettema, E.H. (2015). Multiple pathways for protein transport to peroxisomes. J Mol Biol *427*, 1176–1190.

Knoll, L.J., Johnson, D.R., and Gordon, J.I. (1994). Biochemical studies of three Saccharomyces cerevisiae acyl-CoA synthetases, Faa1p, Faa2p, and Faa3p. J Biol Chem *269*, 16348–16356.

Kohlwein, S.D., Veenhuis, M., and van der Klei, I.J. (2013). Lipid droplets and peroxisomes: key players in cellular lipid homeostasis or a matter of fat--store' ' 'em up or burn 'em down. Genetics *193*, 1–50.

Leber, C., and Da Silva, N.A. (2014). Engineering of Saccharomyces cerevisiae for the synthesis of short chain fatty acids. Biotechnol Bioeng *111*, 347–358.

Leber, C., Choi, J.W., Polson, B., and Da Silva, N.A. (2016). Disrupted short chain specific β-oxidation and improved synthase expression increase synthesis of short chain fatty acids in Saccharomyces cerevisiae. Biotechnol Bioeng *113*, 895–900.

Lee, S.Y. (1996). Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol *14*, 431–438.

Lennen, R.M., Braden, D.J., West, R.A., Dumesic, J.A., and Pfleger, B.F. (2010). A process for microbial hydrocarbon synthesis: Overproduction of fatty acids in Escherichia coli and catalytic conversion to alkanes. Biotechnol Bioeng *106*, 193–202.

Li, X., Guo, D., Cheng, Y., Zhu, F., Deng, Z., and Liu, T. (2014). Overproduction of fatty acids in engineered Saccharomyces cerevisiae. Biotechnol Bioeng *111*, 1841–1852.

Lian, J., and Zhao, H. (2016). Functional Reconstitution of a Pyruvate Dehydrogenase in the Cytosol of Saccharomyces cerevisiae through Lipoylation Machinery Engineering. ACS Synth Biol *5*, 689–697.

Liu, T., Vora, H., and Khosla, C. (2010). Quantitative analysis and engineering of fatty acid biosynthesis in E. coli. Metab Eng *12*, 378–386.

Maeda, I., Delessert, S., Hasegawa, S., Seto, Y., Zuber, S., and Poirier, Y. (2006). The peroxisomal Acyl-CoA thioesterase Pte1p from Saccharomyces cerevisiae is required for efficient degradation of short straight chain and branched chain fatty acids. J Biol Chem *281*, 11729–11735.

Meinecke, M., Cizmowski, C., Schliebs, W., Krüger, V., Beck, S., Wagner, R., and Erdmann, R. (2010). The peroxisomal importomer constitutes a large and highly dynamic pore. Nat Cell Biol *12*, 273–277.

Noguchi, M., Okumoto, K., and Fujiki, Y. (2013). System to quantify the import of peroxisomal matrix proteins by fluorescence intensity. Genes Cells *18*, 476–492.

Palmieri, L., Rottensteiner, H., Girzalsky, W., Scarcia, P., Palmieri, F., and Erdmann, R. (2001). Identification and functional reconstitution of the yeast peroxisomal adenine nucleotide transporter. EMBO J *20*, 5049–5059.

Partow, S., Siewers, V., Bjørn, S., Nielsen, J., and Maury, J. (2010). Characterization of different promoters for designing a new expression vector in Saccharomyces cerevisiae. Yeast *27*, 955–964.

Poirier, Y., Erard, N., and Petétot, J.M. (2001). Synthesis of polyhydroxyalkanoate in the peroxisome of Saccharomyces cerevisiae by using intermediates of fatty acid beta-oxidation. Appl Environ Microbiol *67*, 5254–5260.

Van Roermund, C.W., Hettema, E.H., Kal, A.J., van den Berg, M., Tabak, H.F., and Wanders, R.J. (1998). Peroxisomal beta-oxidation of polyunsaturated fatty acids in Saccharomyces cerevisiae: isocitrate dehydrogenase provides NADPH for reduction of double bonds at even positions. EMBO J *17*, 677–687.

Van Roermund, C.W., Drissen, R., van Den Berg, M., Ijlst, L., Hettema, E.H., Tabak, H.F., Waterham, H.R., and Wanders, R.J. (2001). Identification of a peroxisomal ATP carrier required for medium-chain fatty acid beta-oxidation and normal peroxisome proliferation in Saccharomyces cerevisiae. Mol Cell Biol *21*, 4321–4329.

Van Roermund, C.W.T., Ijlst, L., Majczak, W., Waterham, H.R., Folkerts, H., Wanders, R.J.A., and Hellingwerf, K.J. (2012). Peroxisomal fatty acid uptake mechanism in Saccharomyces cerevisiae. J Biol Chem *287*, 20144–20153.

Runguphan, W., and Keasling, J.D. (2014). Metabolic engineering of Saccharomyces cerevisiae for production of fatty acid-derived biofuels and chemicals. Metab Eng *21*, 103–113.

Scharnewski, M., Pongdontri, P., Mora, G., Hoppert, M., and Fulda, M. (2008). Mutants of Saccharomyces cerevisiae deficient in acyl-CoA synthetases secrete fatty acids due to interrupted fatty acid recycling. FEBS J *275*, 2765–2778.

Sheng, J., Stevens, J., and Feng, X. (2016). Pathway Compartmentalization in Peroxisome of Saccharomyces cerevisiae to Produce Versatile Medium Chain Fatty Alcohols. Sci Rep *6*, 26884.

Sherkhanov, S., Korman, T.P., and Bowie, J.U. (2014). Improving the tolerance of Escherichia coli to medium-chain fatty acid production. Metab Eng *25*, 1–7.

Smith, J.J., and Aitchison, J.D. (2013). Peroxisomes take shape. Nat Rev Mol Cell Biol *14*, 803–817.

Steen, E.J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S.B., and Keasling, J.D. (2010). Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature *463*, 559–562.

Tehlivets, O., Scheuringer, K., and Kohlwein, S.D. (2007). Fatty acid synthesis and elongation in yeast. Biochim Biophys Acta *1771*, 255–270.

Toke, D.A., and Martin, C.E. (1996). Isolation and characterization of a gene affecting fatty acid elongation in Saccharomyces cerevisiae. J Biol Chem *271*, 18413–18422.

Vizeacoumar, F.J., Torres-Guzman, J.C., Bouard, D., Aitchison, J.D., and Rachubinski, R.A. (2004). Pex30p, Pex31p, and Pex32p form a family of peroxisomal integral membrane proteins regulating peroxisome size and number in Saccharomyces cerevisiae. Mol Biol Cell *15*, 665–677.

Voelker, T.A., and Davies, H.M. (1994). Alteration of the specificity and regulation of fatty acid synthesis of Escherichia coli by expression of a plant medium-chain acyl-acyl carrier protein thioesterase. J Bacteriol *176*, 7320–7327.

Wu, H., and San, K.-Y. (2014). Efficient odd straight medium chain free fatty acid production by metabolically engineered Escherichia coli. Biotechnol Bioeng *111*, 2209–2219.

Zhang, X., Li, M., Agrawal, A., and San, K.-Y. (2011). Efficient free fatty acid production in Escherichia coli using plant acyl-ACP thioesterases. Metab Eng *13*, 713–722.

Zhou, Y.J., Buijs, N.A., Zhu, Z., Gómez, D.O., Boonsombuti, A., Siewers, V., and Nielsen, J. (2016a). Harnessing Yeast Peroxisomes for Biosynthesis of Fatty-Acid-Derived Biofuels and Chemicals with Relieved Side-Pathway Competition. J Am Chem Soc *138*, 15368–15377.

Zhou, Y.J., Buijs, N.A., Zhu, Z., Qin, J., Siewers, V., and Nielsen, J. (2016b). Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. Nat Commun *7*, 11709.

Zhu, Z., Zhou, Y.J., Krivoruchko, A., Grininger, M., Zhao, Z.K., and Nielsen, J. (2017). Expanding the product portfolio of fungal type I fatty acid synthases. Nat Chem Biol *13*, 360–362.

CHAPTER 5:

Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in *Saccharomyces cerevisiae*

Leber C, Polson B, Fernandez-Moya R & Da Silva NA (2015) Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in Saccharomyces cerevisiae. *Metab Eng* 28: 54–62.

*CL, **RFM**, and NAD designed the study. NAD supervised the project. CL performed a significant amount of the experimental work, constructing and testing fatty acid activators and β-oxidation deletion strains. BP performed a substantial amount of the free fatty acid measurement experiments. **RFM** conceived the idea for the disrupted neutral recycle and constructed the plasmids for this section. CL analyzed the data and wrote the manuscript. All authors edited and approved the final manuscript.

ABSTRACT

The production of fuels and chemicals from biorenewable resources is important to alleviate the environmental concerns, costs, and foreign dependency associated with the use of petroleum feedstock. Fatty acids are attractive biomolecules due to the flexibility of their iterative biosynthetic pathway, high energy content, and suitability for conversion into other secondary chemicals. Free fatty acids (FFAs) that can be secreted from the cell are particularly appealing due to their lower harvest costs and straightforward conversion into a broad range of biofuel and biochemical products. *Saccharomyces cerevisiae* was engineered to overproduce extracellular FFAs by targeting three native intracellular processes. β-oxidation was disrupted by gene knockouts in *FAA2*, *PXA1* and *POX1*, increasing intracellular fatty acids levels up to 55%. Disruptions in the acyl-CoA synthetase genes *FAA1*, *FAA4* and *FAT1* allowed the extracellular detection of free fatty acids up to 490 mg/L. Combining these two disrupted pathways, a sextuple mutant (Δ*faa1* Δ*faa4* Δ*fat1* Δ*faa2* Δ*pxa1* Δ*pox1*) was able to produce 1.3 g/L extracellular free fatty acids. Further diversion of carbon flux into neutral lipid droplet formation was investigated by the overexpression of *DGA1* or *ARE1* and by the co-overexpression of a compatible lipase, *TGL1*, *TGL3* or *TGL5*. The sextuple mutant overexpressing the diacylglycerol acyltransferase, *DGA1*, and the triacylglycerol lipase, *TGL3*, yielded 2.2 g/L extracellular free fatty acids. This novel combination of pathway interventions led to 4.2-fold higher extracellular free fatty acid levels than previously reported for *S. cerevisiae*. Preliminary work showed promising results in upstream pathway optimization to further increase LCFA by overexpressing an Acc1 mutant and overexpressing the native fatty acid synthase.

INTRODUCTION

The escalation and instability in crude oil costs, growing environmental concerns, competing policy agendas, and foreign dependency, have all contributed to an increased interest in using microorganisms for the production of biofuels and biochemicals. The production of fatty acids has gained significant interest due in part to their well-studied biosynthetic pathway and their suitability for conversion into other secondary chemicals (Peralta-Yahya et al., 2012). Numerous hosts have been engineered to overproduce fatty acids and lipid moieties, but fewer studies have investigated the specific production and secretion of free fatty acids (FFAs). FFAs, fatty acids that are not bound to another molecule, are desired due to their lower production and harvest costs and straightforward chemical conversion into a broad range of biofuel and biochemical products, including alkanes, alkenes, fatty acid methyl-esters, fatty acid ethyl-esters and fatty alcohols (Lennen et al., 2010; Lian and Zhao, 2014; Runguphan and Keasling, 2014; Schirmer et al., 2010; Steen et al., 2010). In *S. cerevisiae*, FFAs are not directly utilized, but are converted and 'activated' to an acyl-CoA ester by acyl-CoA synthetases. Fatty acids that are acquired from the growth medium or other exogenous sources first undergo an energy-dependent process requiring ATP and CoA forming Acyl-CoA and AMP products (Black and DiRusso, 2007). Fatty acids produced in *S. cerevisiae* are primarily C_{16} and C_{18} in length and are produced as acyl-CoA esters by the cytosolic fatty acid synthase (FAS), encoded by *FAS1* and *FAS2* (Tehlivets et al., 2007). While this cytosolic FAS is very efficient in the production of fatty acids, down-stream modifications are needed for the production and secretion of FFAs. In this study, we performed a systematic investigation on the overproduction and secretion of FFAs by removing long- and medium-chain acyl-CoA synthetases, disrupting β-oxidation processes, and

increasing neutral lipid recycle through the co-overexpression of lipid forming acyltransferases and native cellular lipases (Figure 5.1).

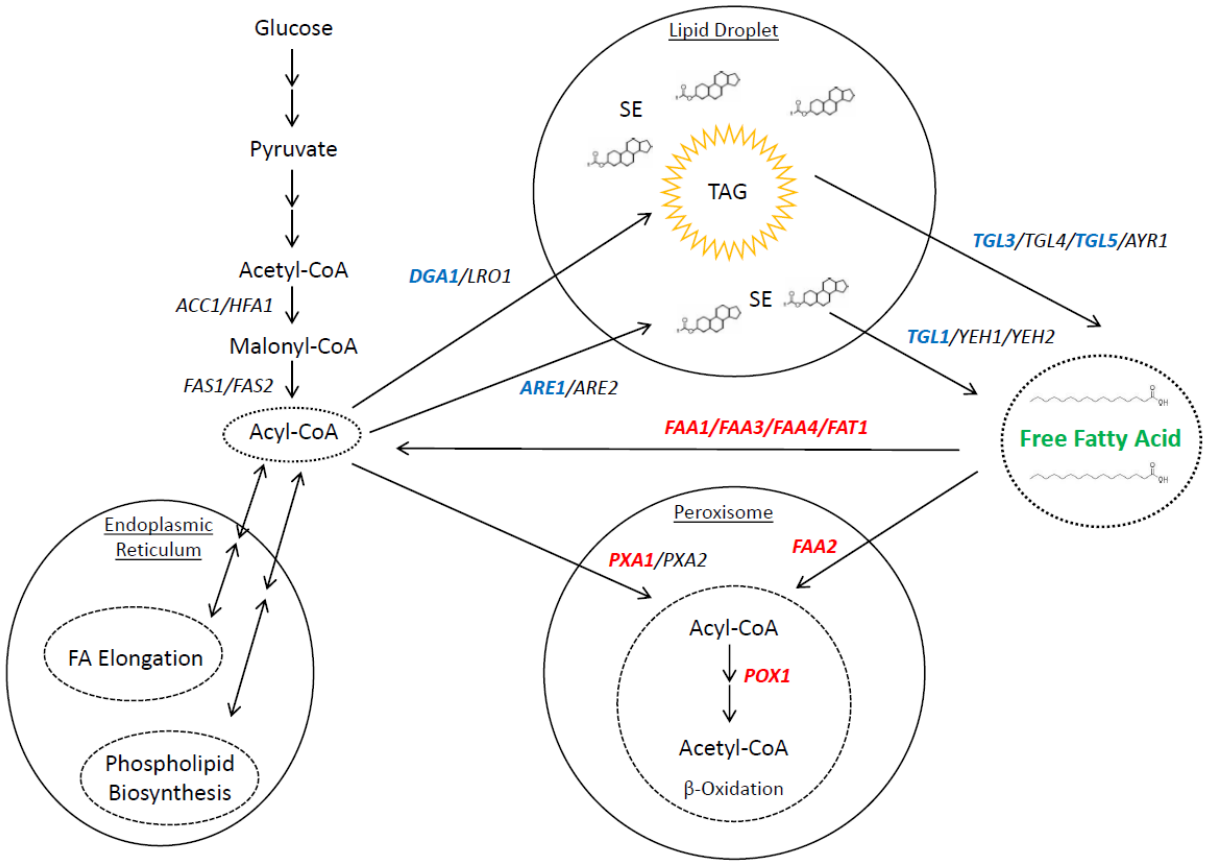


Figure 5.1. Engineered pathways for the overproduction of free fatty acids. Overexpressed genes are highlighted in blue; knocked out genes are highlighted in red. Acyl-CoA flux was increased toward lipid droplet formation by the overexpression of acyltransferases, *DGA1*, and sterol acyltransferases, *ARE1*. Lipid droplet recycle was improved by overexpression of triacylglycerol lipases *TGL3* and *TGL5* and sterol ester hydrolase *TGL1*. Free fatty acid activation and recycle was reduced by removal of acyl-CoA synthetases *FAA1*, *FAA3*, *FAA4* and *FAT1*. β-oxidation activity was disrupted by removal of *FAA2*, *PXA1* and *POX1*.

S. cerevisiae has six known acyl-CoA synthetases encoded by *FAA1*, *FAA2*, *FAA3*, *FAA4*, *FAT1* and *FAT2* (Black and DiRusso, 2007). The exact function of the peroxisomal Fat2 is still unclear, but has been shown to be non-essential for growth on oleic acid (Kohlwein et al., 2013). The majority of synthetase activity has been attributed to the long-chain acyl-CoA synthetase *FAA1* (Black and DiRusso, 2007). Previously, it was shown that by removing acyl-CoA synthetases, *FAA1* and *FAA4,*

yeast begin to secrete free fatty acids into the growth medium (Schwarnewski et al., 2008). A Δ*faa1*Δ*faa2*Δ*faa3*Δ*faa4* knockout strain did not improve fatty acid secretion levels over the double knockout Δ*faa1*Δ*faa4* strain (Schwarnewski et al., 2008). Recent studies, specifically focusing on the overproduction of FFAs in *S. cerevisiae*, have only focused on acyl-CoA synthetases *FAA1* and *FAA4*. Li et al. (2014) were able to produce approximately 80 mg/L fatty acids by disrupting *FAA1* and *FAA4*. Runguphan and Keasling (2014) produced 207 mg/L free fatty acids by deleting *FAA1* and *FAA4* and by overexpressing TesA, a native *Escherichia coli* thioesterase, and Chen et al. (2014) produced roughly 520 mg/L free fatty acids by removing *FAA1* and *FAA4* and by overexpression of *ACOT5*, a truncated *S. cerevisiae* peroxisomal acyl-CoA thioesterase 5.

β-oxidation is the process by which fatty acid molecules are broken down in the peroxisome to generate acetyl-CoA, which is then used as a building block in various metabolic functions or by the TCA cycle (Tehlivets et al., 2007). Throughout exponential and stationary growth phases, β-oxidation is a major contributor to the loss of fatty acids. Valle-Rodriguez et al. (2014) removed *POX1*, the enzyme responsible for the initial step of fatty acid degradation (oxidation of acyl-CoA to trans-2-enoyl-CoA), and observed a 4-fold increase in free fatty acid content when compared to wild-type. Li et al. (2014) removed *POX1* in their Δ*faa1*Δ*faa4* strain and increased fatty acid levels to roughly 110 mg/L. Surprisingly, Runguphan and Keasling (2014) found that removing either *POX1* or *PXA2* (coding for a subunit of the ABC transporter complex Pxa1-Pxa2 that is responsible for importing long chain fatty acids into the peroxisome) reduced fatty acid titers and biomass. While prior studies have focused on removing Pox1 for eliminating β-oxidation, this may be insufficient due to a unique set of auxiliary enzymes. In *S. cerevisiae*, unsaturated fatty acids, such as oleic acid, with the double bond between C_9 and C_{10}, can be fully oxidized only in the presence of these auxiliary enzymes (Kohlwein et al., 2013). Typically, 80% of yeast fatty acids are monounsaturated with oleic acid being a major

fatty acid product (Klug and Daum, 2014; Tehlivets et al., 2007). Additionally, to degrade *cis* or *trans* double bonds at odd-numbered positions, or *cis* double bonds at even-numbered positions, auxiliary enzymes are mandatory and some fatty acids, such as petroselinic acid, bypass Pox1 completely and instead get shuttled directly to Fox2, the second-step enzyme involved in FA degradation (Hiltunen et al., 2003).

Lipid droplet (LD) formation and utilization is a non-essential process in *S. cerevisiae* (Sandager et al., 2002). LDs serve as a storage compartment for nonpolar lipids and are primarily composed of triacylglycerols (TAG) and steryl esters (SE). These LDs are formed in the endoplasmic reticulum and are surrounded by a phospholipid monolayer with a highly hydrophobic core composed of roughly 50% TAG and 50% SE (Koch et al., 2014; Schmidt et al., 2013). Dga1 has been identified as the primary protein involved in TAG formation and has been overexpressed in yeast hosts contributing to an increase in intracellular and extracellular fatty acid levels (Blazeck et al., 2014; Kamisaka et al, 2013; Runguphan and Keasling, 2014). Kamisaka et al. (2013) found that lipid content increased by about 45% when overexpressing Dga1ΔN (a mutant lacking 29 amino acids on the N-terminus) in a Δ*snf2* host (lacking the transcriptional regulator Snf2). Runguphan and Keasling (2014) found *DGA1* overexpression led to a 150% increase in lipid content when compared to wild-type. *LRO1* has been recognized as a lesser contributor to TAG formation than *DGA1* (Sandager et al., 2002). Similarly, Are1 and Are2 have been identified as the primary proteins involved in sterol ester formation (Sandager et al., 2002; Zweytick et al., 2000). In a Δ*are1*Δ*are2* double mutant, SE synthesis was completely inhibited and in a quadruple mutant, Δ*are1*Δ*are2*Δ*dga1*Δ*lro1,* LD formation was removed (Sandager et al., 2002; Zweytick et al., 2000). Lipases, which are required for TAG and SE conversion to FFAs, are abundant in yeast, with over fourteen enzymes currently identified (Athenstaedt and Daum, 2003; Athenstaedt and Daum, 2005; Henry et al., 2012; Jandrositz et al.,

2005; Koffel et al., 2005; Ploier et al., 2013). Among these, Tgl1, Yeh1, and Yeh2 have been identified as the only SE hydrolases, and Tgl3 as the primary TAG lipase.

In the study, we engineered *S. cerevisiae*, a robust industrial organism with well-characterized metabolic pathways, to overproduce free fatty acids by increasing carbon flux from glucose into the fatty acid and neutral lipid forming pathways and by preventing the degradation and re-activation of these fatty acids. We considered the three pathways independently and in combination to increase titers and yields of extracellular free fatty acids (Figure 5.1). FFA accumulation and intracellular fatty acid levels were measured at different points during batch culture. A systematic study considered all six acyl-CoA synthetases and evaluated their removal on extracellular and intracellular fatty acid levels. Recent studies have only focused on Δ*faa1*Δ*faa4* mutants. In addition, β-oxidation was disrupted by deleting a combination of three genes coding for critical enzymes (Faa2, Pxa1, Pox1) involved in the activation, transport and degradation of fatty acids and their impact on intracellular fatty acid levels was determined. Carbon flux was then redirected into LD formation by overexpressing combinations of LD forming enzymes with the co-expression of degrading lipases to produce FFAs. To our knowledge, this is the first report to utilize native LD forming enzymes with native lipases to overproduce FFAs in yeast. A unique combination of these pathway interventions led to substantially higher FFA titers (4.2-fold increase) and yields than previously reported for *S. cerevisiae*, demonstrating the advantages of this multiple gene/pathway approach. We also performed upstream pathway engineering to increase the malonyl-CoA pool/flux by introducing an acetyl-CoA carboxylase variant that is less prone to deactivation when glucose runs out, and we overexpressed the native yeast fatty acid synthase by swapping the native promoter to a stronger promoter. Such strains can be used to generate high titers and yields of free fatty acids for bio-chemical and bio-fuel production.

MATERIALS AND METHODS

Vector and strain construction

Standard molecular biology procedures were carried out as described in Sambrook et al. (2001). Restriction enzymes, T5 exonuclease, Phusion polymerase, Taq ligase, 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 Integrated DNA technologies (IDT) (San Diego, CA). All sequences of gene fragments amplified by PCR were verified by DNA sequence analysis (Eton Bioscience, San Diego, CA). *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 et al., 2001). Plasmid and integrative transformations in *S. cerevisiae* were performed using a high efficiency LiAc method using DMSO (Hoskins, 2000). Isolation of total genomic yeast DNA for integration checking was performed as described by Sambrook et al. (2001).

Vector pBTEF1 pBTEF1 was assembled from three gBlocks (IDT, San Diego, CA) and cloned into backbone pXP842 (Shen et al., 2012) using the Gibson Method (Gibson et al., 2009). pXP842 was linearized with restriction enzymes *Sma*I and *Zra*I. gBlock #1 was amplified with primers gBlock1-F and gBlock1-R (Table 5.4). The KanMX region was further amplified to insert unique restriction sites, *BamH*I and *Hpa*I, with primers KanMX-F and KanMX-R. pBTEF1 utilizes two pTEF1 – CYC1t cassettes, set in opposite directions (Figure 5.2). pBTEF1 uses a KanMX marker for G418 selection. Genes *DGA1* or *ARE1* were inserted into the *Spe*I/*Xho*I multiple cloning site and genes *TGL1*, *TGL3* or *TGL5* were inserted into the *Pml*I/*Sac*II multiple cloning site. *DGA1*, *ARE1*, *TGL1*, *TGL3*, and *TGL5* were amplified from BY4741 genomic DNA using primers ARE1-F/R, DGA1-F/R, TGL1-F/R, TGL3-F/R and TGL5-F/R and

inserted into pBTEF1 using the Gibson Method to give pBTEF1-ARE1, pBTEF1-DGA1, pBTEF1-TGL1, pBTEF1-TGL3, and pBTEF1-TGL5, respectively. Primer sequences are given in Table 5.3. Dual gene plasmids were created by digesting pBTEF1-ARE1 or pBTEF1-DGA1 with *Bgl*II and *Not*I to linearize the backbone followed by the Gibson Method to insert TGL1, TGL3 or TGL5 PCR fragments to give pBTEF1-ARE1-TGL1, pBTEF1-DGA1-TGL3 and pBTEF1-DGA1-TGL5, respectively. A total of nine expression vectors were assembled (Table 5.2). PCR fragments were digested with restriction enzyme *Dpn*I and isolated on a 0.6% agar gel. DNA was extracted using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Backbone DNA and genes were ligated at a 1:3 molar ratio for 60 minutes at 50 C as previously described (Gibson et al., 2009).

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 as the base yeast strain (Table 5.1). Knockout strains were created using double crossover recombination as previously described (Fang et al., 2011). PCR was used to assemble the deletion cassettes (reusable selection marker flanked by the up-stream and down-stream sequences) and knockouts covered the full open reading frame of the gene. The deletions were confirmed by PCR analysis using primers that annealed up- and down-stream of the chromosomal site. PCR was used to generate fragments *loxP-URA3-loxP*, *loxP-HIS3-loxP* and *loxP-LEU2-loxP* from vectors pXP218, pXP220 and pXP222 (Fang et al., 2011). *FAA1* knockout PCR used backbone pXP218 and primer sets KO-FAA1-F/R and nested primer set KO-FAA1-FN/RN to create strain BY4741MΔFAA1. *FAA4* knockout PCR used backbone pXP222 and primer sets KO-FAA4-F/R and nested primer set KO-FAA4-FN/RN to create strain BY4741MΔFAA1ΔFAA4. *FAT1* knockout PCR used backbone pXP220 and primer sets KO-FAT1-F/R and nested primer set KO-FAT1-FN/RN to create strain BY4741MΔFAA1ΔFAA4ΔFAT1. *FAA2* knockout PCR used backbone pXP220 and primer sets KO-FAA2-F/R and nested primer set KO-FAA2-FN/RN to create strain BY4741MΔFAA2.

PXA1 knockout PCR used backbone pXP218 and primer sets KO-PXA1-F/R and nested primer set KO-PXA1-FN/RN to create strain BY4741MΔFAA2ΔPXA1. *POX1* knockout PCR used backbone pXP222 and primer sets KO-POX1-F/R and nested primer set KO-POX1-FN/RN to create strain BY4741MΔFAA2ΔPXA1ΔPOX1. The reusable selection markers were removed from strains as previously described (Fang et al., 2011). The sextuple knockout strain (BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1) was constructed using strain BY4741ΔFAA1ΔFAA4ΔFAT1 and PCR knockout fragments as described above.

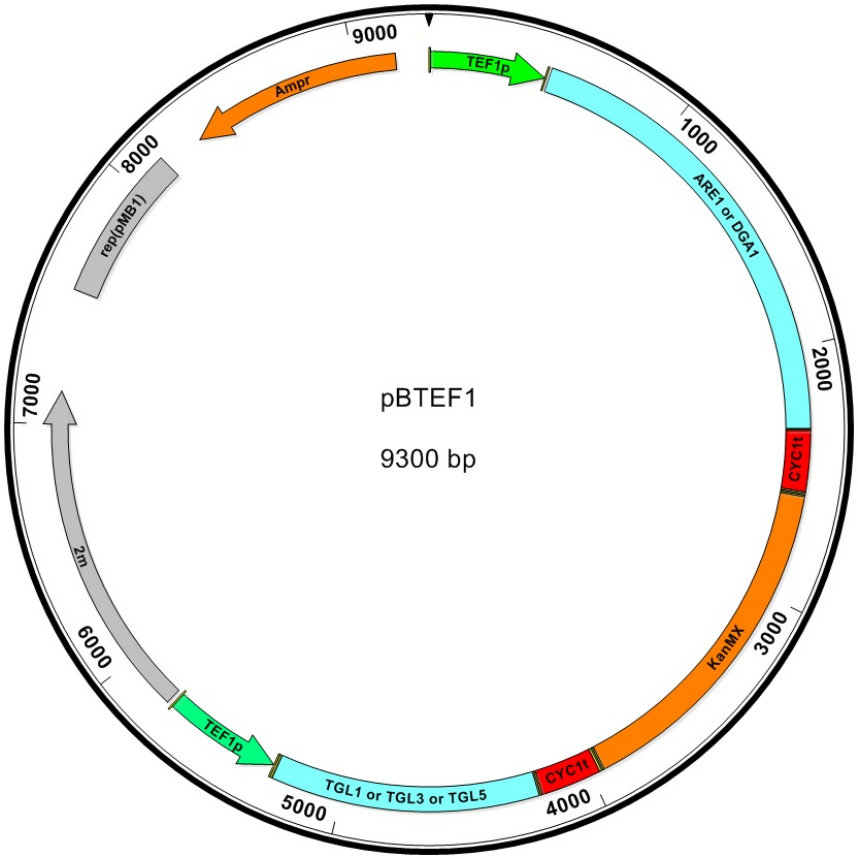


Figure 5.2. Schematic representation of vector pBTEF1. pBTEF1 includes two pTEF1 promoters (green) and two CYC1 terminators (red), each set in opposite directions. pBTEF1 uses a KanMX marker (orange) and a 2μ origin for yeast replication (grey). *DGA1* or *ARE1* were inserted into one multiple cloning site (blue) and *TGL1*, *TGL3* or *TGL5* were inserted into the other multiple cloning site.

In order to integrate the expression cassette p*PGK1-ACC1*m, we used the integration plasmid pIM11-ACC1m (Choi and Da Silva, 2014). The plasmid was digested with NroI and NruI to create an integration linear DNA with the *LEU2* marker and 81 and 94 bp upstream and downstream, respectively, with homology to the *MET17* empty locus.

To swap the native *FAS1* and *FAS2* promoters for the *TEF1* promoter in 6KO tef1 FAS1, 6KO tef1 FAS2, and 6KO tef1 FAS1/2, the CRISPR/Cas9 system (DiCarlo et al., 2013) was adapted to create a multiplexing approach. Briefly, p426-SNR52p-gRNA.CAN1.Y-SUP4t, p415-GalL-Cas9-CYC1t and p414-TEF1p-Cas9-CYC1t (Figure 4.4) were acquired from Addgene. *pTEF1-MCS-CYC1t* expression cassettes from pBTEF1 were swapped by pSNR52-target sequence(ts)-gRNA-tSUP4 by amplifying it from p426-SNR52p-gRNA.CAN1.Y-SUP4t, resulting in plasmid pdgRNA (KanMX). A library of double gRNA plasmids was created with *KanMX*, *URA3*, *MET17*, *LEU2*, and *HIS3* markers. To change the target sequences to the desired ones, primers FAS1targetfor and FAS2targetfor were used with pdgRNA(URA3) (Figure 4.5) as template to generate the insert DNA. Primer AmpRev was used with pdgRNA(URA3) as template to generate the backbone DNA. Gibson was used to ligate both pieces and construct pdgRNA(URA3)-Fas1(ts)/Fas2(ts). Target sequences were found using the webtool Atum gRNA designer (https://www.atum.bio). Donor DNAs were created with TEF1_FAS1for/rev and TEF1_FAS2for/rev. p414-TEF1-Cas9 was transformed to 6KO trp1Δ (see Chapter 4 for protocol for *trp1*Δ) and selected on SDC(A,U) plates for the *TRP1* marker. Subsequently, cells harboring p414-TEF1-Cas9 were transformed with pdgRNA(URA3)-Fas1(ts)/Fas2(ts), and donor DNA for p*TEF1* insertion. Cells were plated on SDC(A) and grown for 3-4 days.

Media and cultivation

Luria-Bertani (LB) medium containing 0.1 mg/mL ampicillin for selection (Sambrook et al., 2001) was used for *E. coli* cultivation. *S. cerevisiae* strains were cultivated in complex YPD media (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.

All expression studies were conducted using YPD medium, with 200 mg/L G418 (Sigma-Aldrich, Saint Louis, MO) added for plasmid selection. Yeast strains were grown at 30°C in an air shaker (New Brunswick Scientific) at 250 rpm. Cells were inoculated from -80°C stock and grown overnight in 5 mL medium in 16 X 125 mm^2 culture tubes. Yeast expression studies were performed in shake flasks with 20 mL or 60 mL medium. Cell growth was initiated at an optical density (600 nm) of 0.2 (Shimadzu UV-2450 spectrophotometer; Columbia, MD) and grown for 24 h, 36 h, 48 h, or 72 h. A correlation factor was used to convert OD to dry cell weight per liter (1 OD = 0.43 g DCW/L).

Fatty acid extraction and quantification

Fatty acid extraction procedures were modified from Bligh and Dyer (1959). Undecanoic and nonadecanoic acid were used as internal standards (Sigma-Aldrich, Saint Louis, MO). Total intracellular fatty acids (free and bound) were extracted as previously described (Leber and Da Silva, 2013). Extracellular fatty acids (free fatty acids) were extracted from 5 mL culture broth by adding 500 uL 1M HCL and 5 mL of a 1:1 methanol:chloroform mixture. The solution was vortexed for 5 minutes then centrifuged for 4 minutes at 1000g. The lower chloroform layer was recovered and evaporated with nitrogen gas. Fatty acids were methylated and suspended in hexane for use in GC-MS as previously described (Leber and Da Silva, 2013).

Fatty acids were measured at the Mass Spectrometry Facility at the University of California Irvine. Fatty acids were detected by gas-chromatography mass spectrometry (GC-MS) and liquid-chromatography mass spectrometry (LC-MS). GC-MS was used for all fatty acid quantification. Unlike GC-MS measurements, fatty acid detection on the LC-MS does not require derivitization; however, overall resolution is diminished. LC-MS was used only to confirm the presence of free fatty acids in the growth medium; any TAGs or SEs would be detected. The GC-MS was a Trace MS+ from Thermo Fisher (San Jose, CA) using a 30m long x 0.25 mm i.d. DB-5 column from Agilent JW Scientific (Santa Clara, CA). The oven was held at 50°C for 1 minute 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/sec) from m/z 50-650. Absolute amounts and distribution of fatty acids were determined using a total ion chromatogram and were normalized to the internal standards. Before each run, a standard curve was performed using a saturated fatty acid methyl-ester mix purchased from Sigma Aldrich (Saint Louis, MO) (catalog #49453-U). A standard curve using a mixture of saturated and unsaturated

fatty acids from Sigma Aldrich (catalog #CRM18918) demonstrated less than 13% area difference between saturated and monounsaturated fatty acids for C_{16}, C_{18} and C_{22} length fatty acids over a wide range of concentrations. LC-MS was performed using an LCT Classic (Waters, Manchester, UK) with Kinetex core shell 2.6 um C_{18} resin with 100 A pore size and 100 x 2.1 mm column (Phenomenex, Torrance, CA). Two mobile phases were used, mobile phase A consisting of 2% acetonitrile and 0.2% acetic acid in water and mobile phase B consisting of 0.2% acetic acid in acetonitrile. The LC gradient was started at 2% mobile phase B and held for one minute, then ramped up to 95% in 19 minutes and held for an additional 7 minutes. Mobile phase B was then ramped down to 2% in 3 minutes and held for an additional 5 minutes. The ion source uses an electrospray ionization method that was set to generate negative ions from fatty acids. A time of flight mass analyzer was used that was set to measure masses from 100-300 m/z or 200-1000 m/z.

Statistical significance and yield calculations

Statistical significance was determined by calculating the two-tailed probability value (p-value) using an unpaired t-test. A p-value of less than 0.05 was considered significant.

The yield of extracellular fatty acids was calculated by dividing the mass of the product formed by the total mass of carbon (glucose) supplied at inoculation. Maximum theoretical yield values assumed simplified biochemical reactions as outlined in Varman et al. (2014). The maximum theoretical yield was determined to be 0.356, 0.353, 0.351 and 0.348 g (FA)/g (glucose) for C16:0, C16:1, C18:0 and C18:1 length fatty acids, respectively. The theoretical yield assumes no glucose goes to intracellular fatty acids or to cellular growth requirements.

Table 5.1. List of strains

Strains	Description	Source
BY4741 (*S. cerevisiae*)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY4741MΔFAA1	BY4741 *faa1::loxP-URA3-loxP*	This Study
BY4741MΔFAA2	BY4741 *faa2::loxP-HIS3-loxP*	This Study
BY4741ΔFAA2	BY4741 *faa2Δ*	This Study
BY4741MΔFAA3	BY4741 *faa3::loxP-MET17-loxP*	This Study
BY4741MΔFAA4	BY4741 *faa4::loxP-LEU2-loxP*	This Study
BY4741MΔFAT1	BY4741 *fat1::loxP-HIS3-loxP*	This Study
BY4741MΔPOX1	BY4741 *pox1::loxP-LEU2-loxP*	This Study
BY4741MΔPXA1	BY4741 *pxa1::loxP-URA3-loxP*	This Study
BY4741MΔFAA1ΔFAA3	BY4741 *faa1::loxP-URA3-loxP; faa3::loxP-MET17-loxP*	This Study
BY4741MΔFAA1ΔFAA4	BY4741 *faa1::loxP-URA3-loxP; faa4::loxP-LEU2-loxP*	This Study
BY4741MΔFAA1ΔFAT1	BY4741 *faa1::loxP-URA3-loxP; fat1::loxP-HIS3-loxP*	This Study
BY4741MΔFAA2ΔPXA1	BY4741 *faa2Δ*; *pxa1::loxP-URA3-loxP*	This Study
BY4741MΔFAA3ΔFAA4	BY4741 *faa3::loxP-MET17-loxP; faa4::loxP-LEU2-loxP*	This Study
BY4741MΔFAA4ΔFAT1	BY4741 *faa4::loxP-LEU2-loxP; fat1::loxP-HIS3-loxP*	This Study
BY4741MΔFAA1ΔFAA4ΔFAT1 (3KO)	BY4741 *faa1::loxP-URA3-loxP; faa4::loxP-LEU2-loxP; fat1::loxP-HIS3-loxP*	This Study
BY4741ΔFAA1ΔFAA4ΔFAT1	BY4741 *faa1Δ; faa4Δ; fat1Δ*	This Study
BY4741MΔFAA2ΔPXA1ΔPOX1	BY4741 *faa2Δ*; *pxa1::loxP-URA3-loxP; pox1::loxP-LEU2-loxP*	This Study
BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2Δ PXA1ΔPOX1 (6KO)	BY4741 *faa1Δ; faa4Δ; fat1Δ; faa2Δ; pxa1Δ; pox1Δ*	This Study
6KO pgk1 ACC1	6KO *met17*::p*PGK1-ACC1-CYCt*	This Study
6KO pgk1 ACC1M	6KO *met17*::p*PGK1-ACC1m-CYCt*	This Study
6KO tef1 FAS1	6KO *trp1*::*loxP* p*FAS1::pTEF1*	This Study
6KO tef1 FAS2	6KO *trp1*::*loxP* p*FAS2::pTEF1*	This Study
6KO tef1 FAS1/2	6KO *trp1*::*loxP* p*FAS1::pTEF1* p*FAS2::pTEF1*	This Study

*** The inclusion of an M in the strain name indicates that selection markers have not yet been excised.**

Table 5.2. List of plasmids

Plasmids	Description	Source
pBTEF1	2μ vector, two *TEF1* promoters, two *CYC1* terminators, *KanMX* selectable marker	This Study
pBTEF1-ARE1	pBTEF1 harboring gene *ARE1*	This Study
pBTEF1-DGA1	pBTEF1 harboring gene *DGA1*	This Study
pBTEF1-TGL1	pBTEF1 harboring gene *TGL1*	This Study
pBTEF1-TGL3	pBTEF1 harboring gene *TGL3*	This Study
pBTEF1-TGL5	pBTEF1 harboring gene *TGL5*	This Study
pBTEF1-ARE1-TGL1	pBTEF1 harboring genes *ARE1* and *TGL1*	This Study
pBTEF1-DGA1-TGL3	pBTEF1 harboring genes *DGA1* and *TGL3*	This Study
pBTEF1-DGA1-TGL5	pBTEF1 harboring genes *DGA1* and *TGL5*	This Study

Table 5.3. List of primers

Cloning Primers	Sequence
gBlock1-F	5' gttccgcgcacatttccccgaaaagtgccacctgacCATATGACCGCGAATCCTTACATC 3'
gBlock1-R	5' CCTTTTATATTTCTCTACAGGGGCGCGGCGTGGGGACAATTCAACGCGTCTGTGAGGGGA 3'
KanMX-F	5' TTAATTTGGCTAGCCCCGGGGGATCCGACATGGAGGCCCAGAATACCCTC 3'
KanMX-R	5' AATTTGTTAATTAACCCGGGGTTAACCAGTATAGCGACCAGCATTCACAT 3'
ARE1-F	5' AGTTTTAATTACAAAACTAGTGTTTAAAACAATGACGGAGACTAAGGATTTGTTG 3'
ARE1-R	5' GAATGTAAGCGTGACATAACTAATCTCGAGCGTCATAAGGTCAGGTACAACGTCATA 3'
DGA1-F	5' AGTTTTAATTACAAAACTAGTGTTTAAAACAATGTCAGGAACATTCAATGATATAA 3'
DGA1-R	5' GAATGTAAGCGTGACATAACTAATCTCGAGCGTTACCCAACTATCTTCAATTCTGCA 3'
TGL1-F	5' TAAGTTTTAATTACAAAcacgtggcAAAACAATGTACTTCCCCTTTTTAGGCAG 3'
TGL1-R	5' GAATGTAAGCGTGACATAACTAATccgcggaTCATTCTTTATTTAGAGCATCC 3'
TGL3-F	5' TAAGTTTTAATTACAAAcacgtggcAAAACAATGAAGGAAACGGCGCAGGAAT 3'
TGL3-R	5' GAATGTAAGCGTGACATAACTAATccgcggaCTACCTACTCCGTCTTGCTCTT 3'
TGL5-F	5' TAAGTTTTAATTACAAAcacgtggcAAAACAATGTCTAATACCTTGCCAGTAAC 3'
TGL5-R	5' GAATGTAAGCGTGACATAACTAATccgcggaTCAATTTTGAAAAATGTCTGAA 3'
Knockout Primers	**Sequence**
KO-FAA1-F	5' CTAGAACAAACACAAAAGACAAAAAAAGACAACAATggtcgactctagaggatcCCCGGG 3'
KO-FAA1-R	5' ATGATGAGGCTTTCCTATCATGGAAATGTTGATCCAGAATTCgagctcggtaCCCGGGat 3'
KO-FAA1-FN	5' CTTGTTGTTAAAAAACTCGTTAGGATACAATAAAAACTAGAACAAACACAAAAGACAAAA 3'
KO-FAA1-RN	5' CAGTTCTAAAGCAAAAAACTGAAAAAGTGCTTTAGTATGATGAGGCTTTCCTATCATGGA 3'
KO-FAA4-F	5' CACTATTTCTTGAAAAACTAAGAAGTACGCATCAAAggtcgactctagaggatcCCCGGG 3'
KO-FAA4-R	5' AAGGGCAGGGGGGAAAGTAAAAAACTATGTCTTCCTGAATTCgagctcggtaCCCGGGat 3'
KO-FAA4-FN	5' CAGCATTAGTAACACATCATTTTTTTCTCTGTTCTTCACTATTTCTTGAAAAACTAAGAA 3'
KO-FAA4-RN	5' ATTGATGCTCTTAGAAAATGAAACGTAGTGTTTATGAAGGGCAGGGGGGAAAGTAAAAAA 3'
KO-FAT1-F	5' TATCGTTGAACTTTTAATAGGCTGCGAATACCGACTggtcgactctagaggatcCCCGGG 3'
KO-FAT1-R	5' AGGAAAAATACTTTATCCTAATTCAGGAACATCAAAGAATTCgagctcggtaCCCGGGat 3'
KO-FAT1-FN	5' AAACTAAATTGAAAAGCCCAAATCATTCAAATTCTATATCGTTGAACTTTTAATAGGCTG 3'
KO-FAT1-RN	5' TTATCAATATTACGTTTACATACGTACTCTCGTGCAAGGAAAAATACTTTATCCTAATTC 3'
KO-FAA2-F	5' TACAAAAAACGGATAAGAACAAACTTGTTTCGAAATggtcgactctagaggatcCCCGGG 3'
KO-FAA2-R	5' TTCTAGTTTGAATGTGTTCCAAATCGTCATAAGTACGAATTCgagctcggtaCCCGGGat 3'
KO-FAA2-FN	5' GAAACGCATGGCTAAGGGAAGTGGAAGAATGCAGGTTACAAAAAACGGATAAGAACAAAC 3'
KO-FAA2-RN	5' ATGGATGTGCATAGGGATATCCTACATCAAAGTTTTTTCTAGTTTGAATGTGTTCCAAAT 3'
KO-PXA1-F	5' AGACAATCTGGAAGTCTTAGAACGCATAACACAGAAggtcgactctagaggatcCCCGGG 3'
KO-PXA1-R	5' ATTCGCTAAATAAAATCTCTCCCTTTCTAGGGTGTTGAATTCgagctcggtaCCCGGGat 3'
KO-PXA1-FN	5' TCTCTAAGAAGTGAGACTAACAATGAAACCCGTTTCAGACAATCTGGAAGTCTTAGAACG 3'
KO-PXA1-RN	5' GTACAAATTCAGTTACGGAAATAATATATAAATTATATTCGCTAAATAAAATCTCTCCCT 3'
KO-POX1-F	5' AGAAAAAAAGAAAATATAATAAATTAGTATTGCGATggtcgactctagaggatcCCCGGG 3'
KO-POX1-R	5' AAACAGAGGGTTCGAAGGAAAACAGGAAACCTCTACGAATTCgagctcggtaCCCGGGat 3'
KO-POX1-FN	5' GTTTTTGAATACACACTTGACACTAATAAGTATCACAGAAAAAAAGAAAATATAATAAAT 3'
KO-POX1-RN	5' CTTGGCGACACTAGTTGAATTGAAACAAAAGTCGCAAAACAGAGGGTTCGAAGGAAAACA 3'

Name	Sequence (5' -> 3')
FAS1targetFor	gcagtgaaagataaatgatcGCTCTATTATTCGCTCATTAGTTTTAGAGCTAGAAATAGC
FAS2targetFor	gcagtgaaagataaatgatcAACTTCCGGCTTCATAATGAGTTTTAGAGCTAGAAATAGC
AmpRev	GCTATTTCTAGCTCTAAAAC
TEF1_FAS1for	TGTTGAACGATTCACTGCGACAATAATCAGAGATTACAGTACCGCGAATCCTTACATCAC
TEF1_FAS1rev	CGTGAGATAGGGTTAATGGTCTTGTGGAGTAAGCGTCCATCATTTTTTTACTAGTTTTGT
TEF1_FAS2for	TCCTAAATTTTCTCTGGTCTGCAGGCCAAAAACAACAACTACCGCGAATCCTTACATCAC
TEF1_FAS2rev	GCAAAATATGAGCTAATTCTTGCTCAACTTCCGGCTTCATCATTTTTTTACTAGTTTTGT
IntFAS2GoodRev	GCAGCTGGAGTTGGAGCAGG
FAS1IntRev	GCCGACGGCCCTAAAAAGAGC
TEF1pseqFor	GGGTGTCGTTAATTACCCGT
FAS2promGoodFor	TGTCGTTGTTGTCCCAGCCG
FAS1promForCheck	GTGTGCCTTTCCCAATCACCG

Table 5.4. List of gBlocks

gBLOCK #1 (TEF1-CYC1)
CATATGACCGCGAATCCTTACATCACACCCAATCCCCCACAAGTGATCCCCCACACACCATA GCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGT ACCACTTCAAAACACCCAAGCACAGCATACTAAATTTCCCCTCTTTCTTCCTCTAGGGTGTCG TTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCG TCGAAAAAGGCAATAAAAATTTTTATCACGTTTCTTTTTCTTGAAAATTTTTTTTTTTGATTTT TTTCTCTTTCGATGACCTCCCATTGATATTTAAGTTAATAAACGGTCTTCAATTTCTCAAGTTT CAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATA GCAATCTAATCTAAGTTTTAATTACAAAACTAGTGTTTAAACCGGACCGCTCGAGATTAGTT ATGTCACGCTTACATTCACGCCCTCCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAG ACAACCTGAAGTCTAGGTCCCTATTTATTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTT ATATTTCAAATTTTTCTTTTTTTTCTGTACAGACGCGTGTACGCATGTAACATTATACTGAAA ACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGGCTAGCCCCGGGataactt cgtataGCATACATtatacgaagttatGACATGGAGGCCCAGAATACCCTCCTTGACAGTCTTGAC GTGCGCAGCTCAGGGGCATGATGTGACTGTCGCCCGTACATTTAGCCCATACATCCCCATGT ATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGCAAAAATTACGGCTCCT CGCTGCAGACCTGCGAGCAGGGAAACGCTCCCCTCACAGACGCGTTGAATTGT

gBLOCK #2 (KanMX)
TCCCCTCACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCTGTAGAGAAATATAAAAGGTT AGGATTTGCCACTGAGGTTCTTCTTTCATATACTTCCTTTTAAAATCTTGCTAGGATACAGTT CTCACATCACATCCGAACATAAACAACCATGGGTAAGGAAAAGACTCACGTTTCGAGGCCG CGATTAAATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCG GGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCT GAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTG GCTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATG GTTACTCACCACTGCGATCCCCGGCAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATT CAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTT TGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAA TAACGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAA GTCTGGAAAGAAATGCATAAGCTTTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGA TTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGAC GAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGTGAGTT TTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAA ATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGTACTGACAATAAAAAGATTCTT GTTTTCAAGAA

gBLOCK #3 (CYC1-TEF1)
ATAAAAAGATTCTTGTTTTCAAGAACTTGTCATTTGTATAGTTTTTTTATATTGTAGTTGTTCT ATTTTAATCAAATGTTAGCGTGATTTATATTTTTTTTCGCCTCGACATCATCTGCCCAGATGC GAAGTTAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGAATGCTGGTCGCTATA CTGataacttcgtataGCATACATtatacgaagttatCCCGGGTTAATTAACAAATTAAAGCCTTCGA GCGTCCCAAAACCTTCTCAAGCAAGGTTTTCAGTATAATGTTACATGCGTACACGCGTCTGT ACAGAAAAAAAAGAAAAATTTGAAATATAAATAACGTTCTTAATACTAACATAACTATAAAA AAATAAATAGGGACCTAGACTTCAGGTTGTCTAACTCCTTCCTTTTCGGTTAGAGCGGATGT GGGGGGAGGGCGTGAATGTAAGCGTGACATAACTAATccgcggagatctgcggccgccacgtgTTT GTAATTAAAACTTAGATTAGATTGCTATGCTTTCTTTCTAATGAGCAAGAAGTAAAAAAAGT TGTAATAGAACAAGAAAAATGAAACTGAAACTTGAGAAATTGAAGACCGTTTATTAACTTA AATATCAATGGGAGGTCATCGAAAGAGAAAAAAATCAAAAAAAAAAATTTTCAAGAAAAA GAAACGTGATAAAAATTTTTATTGCCTTTTTCGACGAAGAAAAAGAAACGAGGCGGTCTCTT TTTTCTTTTCCAAACCTTTAGTACGGGTAATTAACGACACCCTAGAGGAAGAAAGAGGGGA AATTTAGTATGCTGTGCTTGGGTGTTTTGAAGTGGTACGGCGATGCGCGGAGTCCGAGAAA ATCTGGAAGAGTAAAAAAGGAGTAGAAACATTTTGAAGCTATGGTGTGTGGGGGATCACT TGTGGGGGATTGGGTGTGATGTAAGGATTCGCGGTCCTAGGGGGtaccgagctcGAATTCaac ga

RESULTS AND DISCUSSION

Characterization of acyl-CoA synthetase knockouts

All metabolic processes in *S. cerevisiae* involving fatty acids require the activated form bound to coenzyme-A as an acyl-CoA ester (Schweizer and Hofmann, 2004). Removal of acyl-CoA synthetase activity prevents free fatty acid activation, and with deletion of *FAA1* and *FAA4,* the yeast began to secrete free fatty acids into the growth medium (Schwarnewski et al., 2008). Our goal was to maximize extracellular free fatty acid levels by considering all known acyl-CoA synthetase genes. Synthetases were first deleted individually and then in combination. The knockout strains were created using double crossover recombination in parent strain BY4741. Initially, we screened five independent knockouts (*faa1*Δ*, faa2*Δ*, faa3*Δ*, faa4*Δ*, fat1*Δ) and found minimal fatty acid secretion (data not shown). The sixth acyl-CoA synthetase (Fat2) was not included as it has been shown to be a minor contributor to total intracellular acyl-CoA synthetase activity (Black and DiRusso 2007; Kohlwein et al., 2013). Double knockout combinations of three acyl-CoA synthetases (*FAA1*, *FAA3* and *FAA4*) were then constructed and screened. We identified Δ*faa1*Δ*faa4* as the only double knockout to increase extracellular fatty acid levels, confirming previous reports. Since *FAA2* and *FAA3* knockouts do not further increase fatty acid secretion (Schwarnewski et al., 2008), we then focused on the fatty acid transporter Fat1. Fat1 is a multi-functional enzyme that is responsible for long chain fatty acyl-CoA synthetase activity and fatty acid transport, and is found in the endoplasmic reticulum and lipid droplets (Black and DiRusso, 2007; Kohlwein et al., 2013). We constructed a triple knockout strain, BY4741MΔFAA1ΔFAA4ΔFAT1.

The parent (BY4741), double knockout (*faa1*Δ*faa4*Δ), and triple knockout (*faa1*Δ*faa4*Δ*fat1*Δ) strains were cultivated in complex medium and the intracellular, extracellular and total fatty acid levels were compared at 24, 48, and 72 h (Figure 5.3).

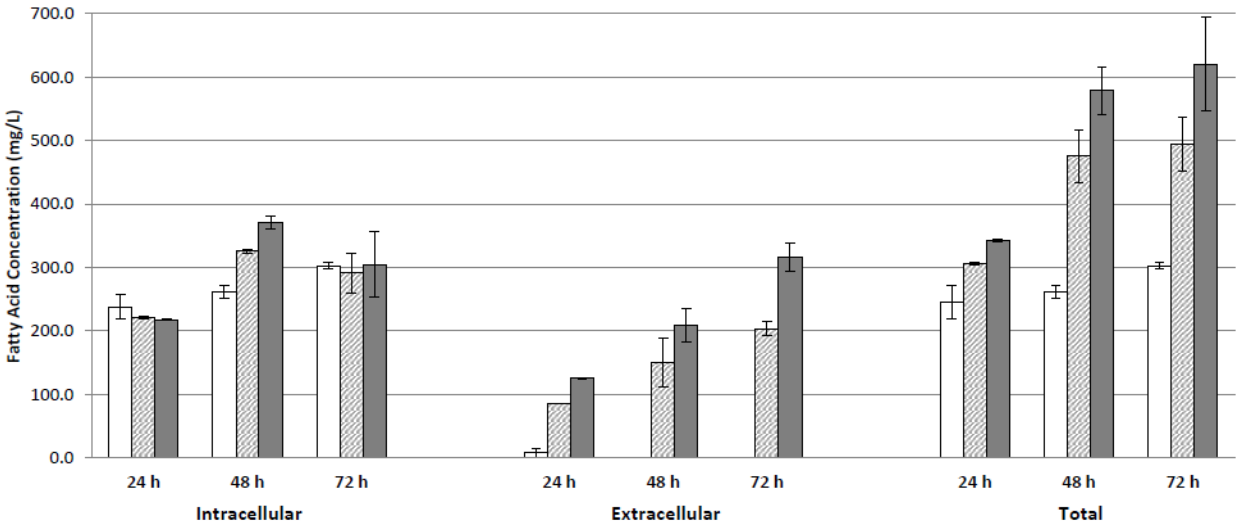


Figure 5.3. Effect of acyl-CoA synthetase disruption on fatty acid titers. Intracellular fatty acid, extracellular free fatty acid, and total fatty acid (intracellular plus extracellular) titers produced using wild type BY4741 (white bars), BY4741MΔFAA1ΔFAA4 (gray slashed bars), and BY4741MΔFAA1ΔFAA4ΔFAT1 (dark gray bars) at 24 h, 48 h, and 72 h harvest points. Error bars represent ± standard deviation between three independent experiments.

All three strains had similar growth rates and final cell densities, and similar intracellular levels of fatty acids. However, the double knockout strain (BY4741MΔFAA1ΔFAA4) produced 200 mg/L extracellular free fatty acids (FFAs) after 72 h of growth. In contrast, the parent strain had nearly undetectable levels of extracellular FFAs at 72 h. This increased total fatty acids (intracellular plus extracellular) by 60% in the *faa1*Δ*faa4*Δ strain relative to the parent. The additional knockout of *FAT1* resulted in a significant further increase in extracellular FFAs levels. Strain BY4741MΔFAA1ΔFAA4ΔFAT1 produced 320 mg/L extracellular FFAs at 72 h, with total fatty acids 110% higher than the parent strain. The triple knockout mutant (*faa1*Δ*faa4*Δ*fat1*Δ) increased

extracellular FFA levels 60% over the double knockout (*faa1*Δ*faa4*Δ) after 72 h, with an average increase of 50% for all time points. The triple knockout strain also increased total fatty acids 30% over the double knockout strain at 72 h. Markedly, strain BY4741MΔFAA1ΔFAA4ΔFAT1 secreted roughly 50% of all detected fatty acids (310 mg/L intracellular FAs vs 320 mg/L extracellular FAs). No noticeable re-importation of fatty acids was observed in either knockout strain. Recent studies have only focused on acyl-CoA synthetase genes *FAA1* and *FAA4* to overproduce FFAs in *S. cerevisiae*. However, the additional removal of *FAT1* led to substantially higher extracellular FFAs and total fatty acid levels. Consequently, our further studies utilized the triple knockout (*faa1*Δ*faa4*Δ*fat1*Δ) strain.

Disruption of β-oxidation activity

β-oxidation is the process by which fatty acid molecules are broken down in the peroxisome to generate acetyl-CoA. In yeast, full β-oxidation activity is not necessary to maintain cell viability when fatty acids are not used as the carbon source. While prior studies have disrupted β-oxidation in *S. cerevisiae* by deleting the gene for Pox1, the initial enzyme involved in the degradation of fatty acids, this may be insufficient for the complete removal of β-oxidation activity due to other auxiliary enzymes (Hiltunen et al., 2003; Kohlwein et al., 2013). Therefore, to further disrupt β-oxidation processes, we focused on three key independent enzymes of the β-oxidation pathway: Faa2, Pxa1 and Pox1. This disrupts fatty acid activation in the peroxisome (Faa2), fatty acid transport into the peroxisome (Pxa1), and fatty acid degradation inside the peroxisome (Pox1).

Initially, independent single knockouts in *FAA2*, *PXA1* and *POX1* were created and screened for intracellular fatty acid levels. After 36 h, no significant differences in intracellular fatty acid levels were observed among the three independent mutant strains (Figure 5.4). Consequently, two

additional combinatorial mutant strains were created. The double knockout (*faa2*Δ*pxa1*Δ) disrupts import and activation of fatty acids; this combination was chosen as Faa2 and Pxa1 act prior to Pox1 (Figure 5.1).

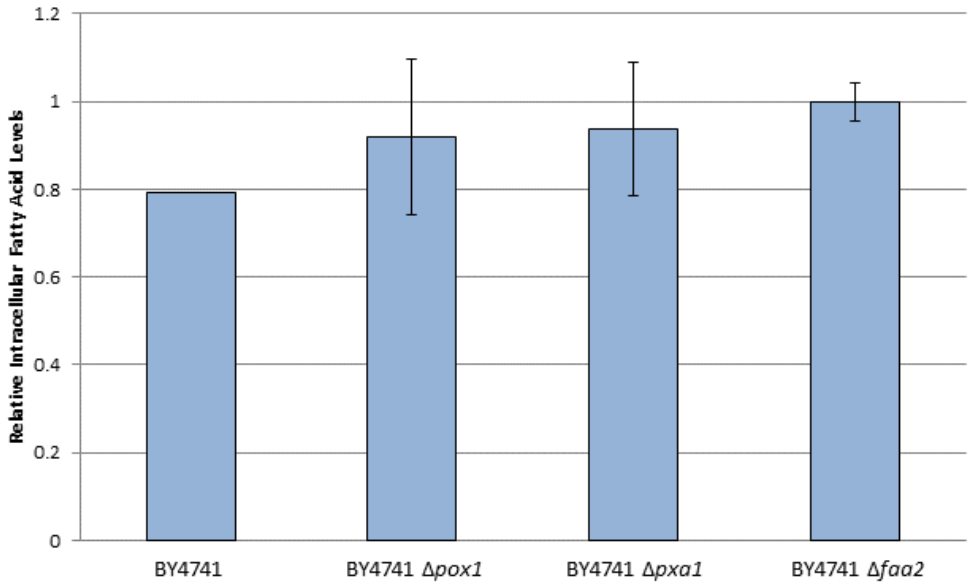


Figure 5.4. Effect of β-oxidation single knockout disruptions on intracellular fatty acid levels. Relative amounts of fatty acids are normalized to the highest fatty acid producer for each data set. Intracellular fatty acid levels produced using parent strain BY4741, BY4741MΔPOX1, BY4741MΔPXA1 and BY4741ΔFAA2 were measured after 36 h of growth. Error bars represent ± standard deviation between two independent experiments.

The triple knockout (*faa2*Δ*pxa1*Δ*pox1*Δ) was then created to disrupt the initial degradation step as well. The strains were screened for intracellular fatty acid levels at 10, 24 and 48 h of growth (Figure 5.5). At the 10 and 24 h time points, no significant differences in intracellular fatty acid levels were observed among all tested strains. This was likely due to the reduced activity of β-oxidation processes in earlier stages of cell growth. However, after 48 h, the double knockout (strain BY4741MΔFAA2ΔPXA1) and triple knockout (strain BY4741MΔFAA2ΔPXA1ΔPOX1) increased intracellular fatty acid levels by 40 and 50% over the BY4741 control, respectively (Figure 5.5). These same trends were observed when normalized to cell density. Strain BY4741MΔFAA2ΔPXA1 and strain

BY4741MΔFAA2ΔPXA1ΔPOX1 produced 410 and 470 mg/L intracellular fatty acids at 48 h, compared to 300 mg/L from parent strain BY4741. Furthermore, although the error bars are large, the general trend of increasing fatty acid concentration with sequential gene knockouts (*faa2*Δ to *faa2*Δ*pxa1*Δ *to* Δ*faa2*Δ*pxa1*Δ*pox1*) was observed in each of the independent experiments. The cumulative fatty acid increase may be due to the sequential removal of various degradation pathways available to yeast for both saturated and unsaturated fatty acids.

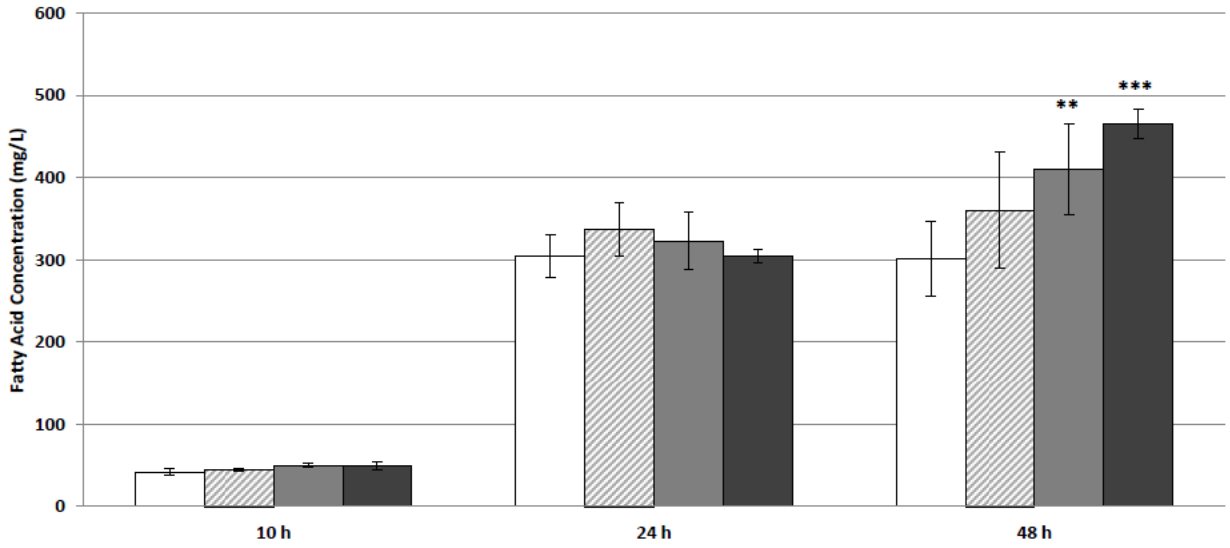
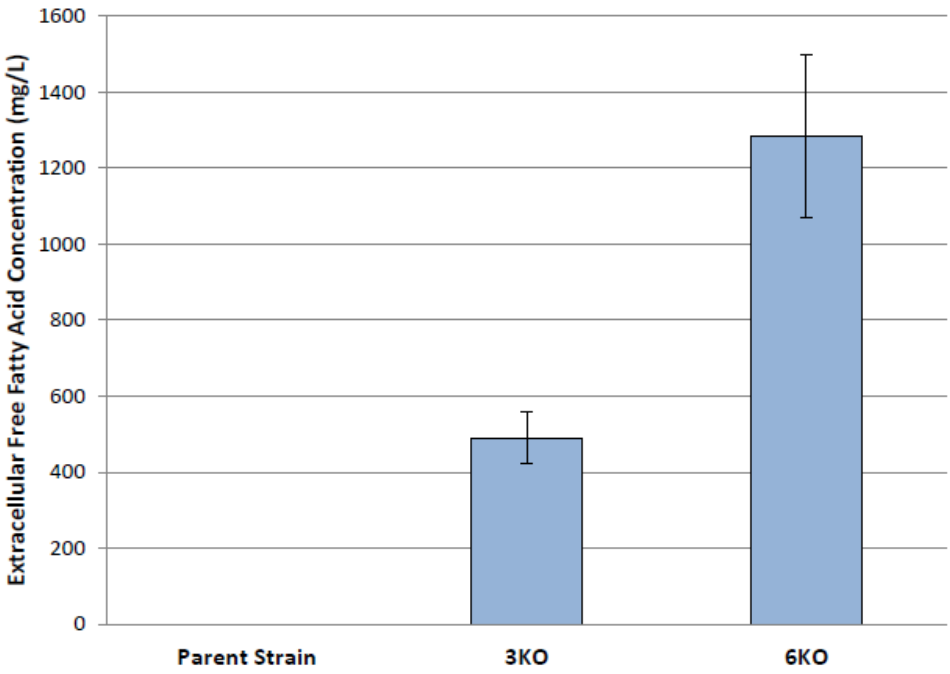


Figure 5.5. Effect of β-oxidation disruption on intracellular fatty acid titers. Intracellular fatty acid levels produced using parent strain BY4741 (White bars), BY4741ΔFAA2 (grey slashed bars), BY4741MΔFAA2ΔPXA1 (light grey bars), and BY4741MΔFAA2ΔPXA1ΔPOX1 (dark grey bars) at 10 h, 24 h, and 48 h harvest points. ** $p < 0.05$ compared to control (BY4741). *** $p < 0.01$ compared to control (BY4741). Error bars represent ± standard deviation between three independent experiments.

Independent disruption of acyl-CoA synthetase and β-oxidation activity both led to an increase in fatty acid production. Therefore, a strain disrupted in both processes was constructed. A sextuple knockout strain, BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1 (denoted 6KO), was cultured for 48 h and extracellular free fatty acid levels and growth were compared to the acyl-CoA synthetase triple knockout strain BY4741ΔFAA1ΔFAA4ΔFAT1 (denoted 3KO) (Figure 5.6). Both strains

had all markers excised. The addition of the β-oxidation knockouts resulted in 2.7-fold higher levels of extracellular free fatty acids: 1300 mg/L for the 6KO strain relative to 490 mg/L for the 3KO strain. Yield also increased from 7% to 18% of the maximum theoretical value (that assumes no glucose is used for growth or intracellular fatty acids), and productivities of FFAs increased from 10 to 27 mg/L·h. Parent strain, BY4741, was unable to produce any detectable levels of extracellular FFAs.



FA Titer (mg/L)	N.D.	490 ± 70	1300 ± 200
FA Productivity (mg/L·h)	-	10 ± 1	27 ± 5
Theoretical Yield (%)	-	7 ± 1	18 ± 3

Figure 5.6. Effect of interrupting acyl-CoA synthetase and β-oxidation activities. Extracellular free fatty acid titer (mg/L), productivity (mg/L·h) and theoretical yield (%) on glucose for parent strain (BY4741), 3KO strain (BY4741ΔFAA1ΔFAA4ΔFAT1) and 6KO strain (BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1) at 48 h. N.D. is not detected. Values are reported as average ± standard deviation from at least three independent experiments.

By combining unique gene disruptions in acyl-CoA synthetase and β-oxidation activity in our sextuple knockout strain, we were able to produce 1300 mg/L extracellular FFAs, nearly 2.5-fold higher than what has previously been reported for *S. cerevisiae*. These results demonstrate the benefit of combining disrupted acyl-CoA synthetase activity with an inefficient β-oxidation pathway for maximizing free fatty acid pools.

Overexpression of the native neutral lipid pathway in a disrupted fatty acid recycle host

Lipid droplets (LD) serve as a storage compartment for nonpolar lipids and are primarily composed of triacylglycerols (TAG) and steryl esters (SE) (Sandager et al., 2002). LDs utilize lipases to break down TAGs and SEs into free fatty acids. In this study, we overexpressed native lipid forming enzymes to increase carbon flux into the neutral lipid pathway, and overexpressed native lipases to increase the formation of FFAs. The triple knockout strain (BY4741ΔFAA1ΔFAA4ΔFAT1) and sextuple knockout strain (BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1) were used as hosts to prevent the reactivation and the reuse of free fatty acids.

For expression, we utilized the pBTEF1 vector that is capable of expressing two genes simultaneously, each under the strong constitutive p*TEF1* promoter (in opposite orientations) (Figure 5.2). pBTEF1 has a 2μ origin and contains the KanMX selection cassette. The empty pBTEF1 vector was transformed into the 3KO strain (BY4741ΔFAA1ΔFAA4ΔFAT1) and the 6KO strain (BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1), and extracellular free fatty acid titer and cell density decreased in both strains (Figure 5.7). The reduction in FFA titer may be due in part to the decreased final cell density and higher metabolic burden. To determine if there was a deleterious effect from G418 (200 mg/L), we grew the plasmid-containing strain in medium lacking G418. G418

was determined to have a negligible effect on the extracellular fatty acid levels (data not shown). Therefore, the 6KO strain carrying pBTEF1 will be considered the control strain, and the 6KO strain will be designated the parent strain.

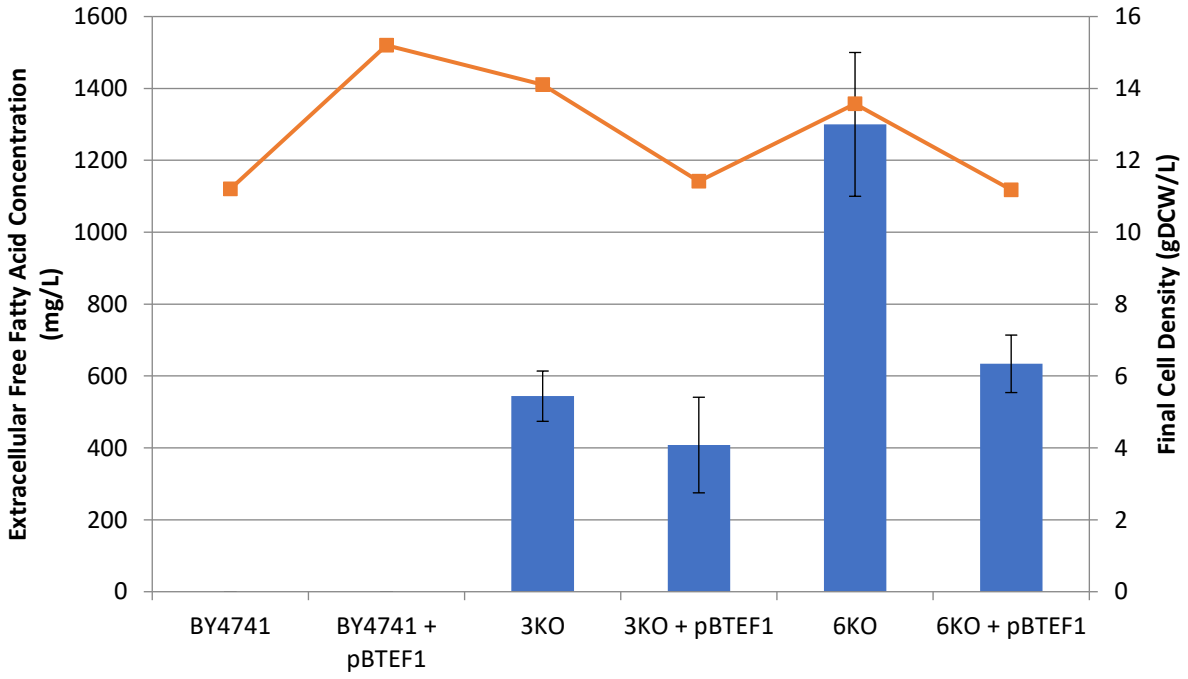


Figure 5.7. Extracellular free fatty acid levels (mg/L) (blue bars) and final cell densities (gDCW/L) (orange line) of parent strain (BY4741), 3KO (BY4741ΔFAA1ΔFAA4ΔFAT1) and 6KO (BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1) with and without the expression of empty vector pBTEF1. Optical densities were taken on a Shimadzu UV-2450 spectrophotometer (Columbia, MD) and free fatty acids were harvested after 48 h. A correlation factor was used to convert OD to dry cell weight per liter (1 OD = 0.43 gDCW/L). Extracellular free fatty acid error bars represent ± standard deviation between three independent experiments.

To improve free fatty acid production, we redirected carbon flux into LD formation and degradation. Acyl-transferases *DGA1* and *ARE1* were utilized for TAG and SE formation, respectively. Among the numerous lipases available we chose to focus on *TGL1*, *TGL3* and *TGL5*. *TGL1* is a steryl ester hydrolase, converting SEs to FFAs and sterols. *TGL3* has been identified as a major TAG lipase and *TGL4* and *TGL5* are paralogs (Athenstaedt and Daum, 2005). All enzymes except AreI are found in the lipid droplets, allowing compartmentalization of enzyme activity. Genes *DGA1*, *ARE1*, *TGL1*,

TGL3, and *TGL5* were cloned into the expression vector pBTEF1. A total of nine expression vectors (carrying single genes or a combination of two genes) were assembled (Table 5.2). The highest extracellular FFA producer (strain BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1), with disruptions in acyl-CoA synthetase and β-oxidation activity, was used in the expression studies. When overexpressing just a lipase gene (*TGL1*, *TGL3* or *TGL5*), extracellular FFAs fell after 48 h when compared to the control strain (6KO carrying the pBTEF1 empty vector) (Figure 5.8), reaching a maximum of only 340 mg/L after 48 h of growth. These findings indicate that merely overexpressing a lipase is not sufficient to successfully divert carbon flux into the neutral lipid-forming pathway in our engineered host. Furthermore, overexpression of just an acyl-transferase, using genes *ARE1* (pBTEF1-ARE1) or *DGA1* (pBTEF1-DGA1), almost completely arrested extracellular FFA production with only 2 mg/L and 50 mg/L produced, respectively. This may be due to an increase in intracellular lipid accumulation. The overexpression of *DGA1*, lacking a complimentary lipase, increased intracellular fatty acid levels by nearly 60% when normalized to cell dry weight relative to the control. These results are not surprising as the combination of both an acyl-transferase and a lipase is likely required for the enhanced production of extracellular FFAs.

We created three dual expression vectors (pBTEF1-ARE1-TGL1, pBTEF1-DGA1-TGL3 and pBTEF1-DGA1-TGL5) to co-express both an acyl-transferase and a lipase and transformed them into the 6KO strain. Surprisingly, the ARE1-TGL1 combination produced nearly undetectable levels of extracellular FFAs (Figure 5.8). Further studies could investigate extracellular free fatty acid production under anaerobic conditions where *ARE1* was found to contribute the most to SE synthesis (Kohlwein et al., 2013). However, the overexpression of the TAG acyl-transferase, *DGA1*, with the co-expression of a lipase, *TLG3* or *TGL5*, both increased extracellular FFAs (Figure 5.8). With vector

pBTEF1-DGA1-TGL5, extracellular FFA titers increased to over 600 mg/L after 48 h. Markedly, when *TGL3* was substituted for *TGL5*, extracellular FFAs increased substantially.

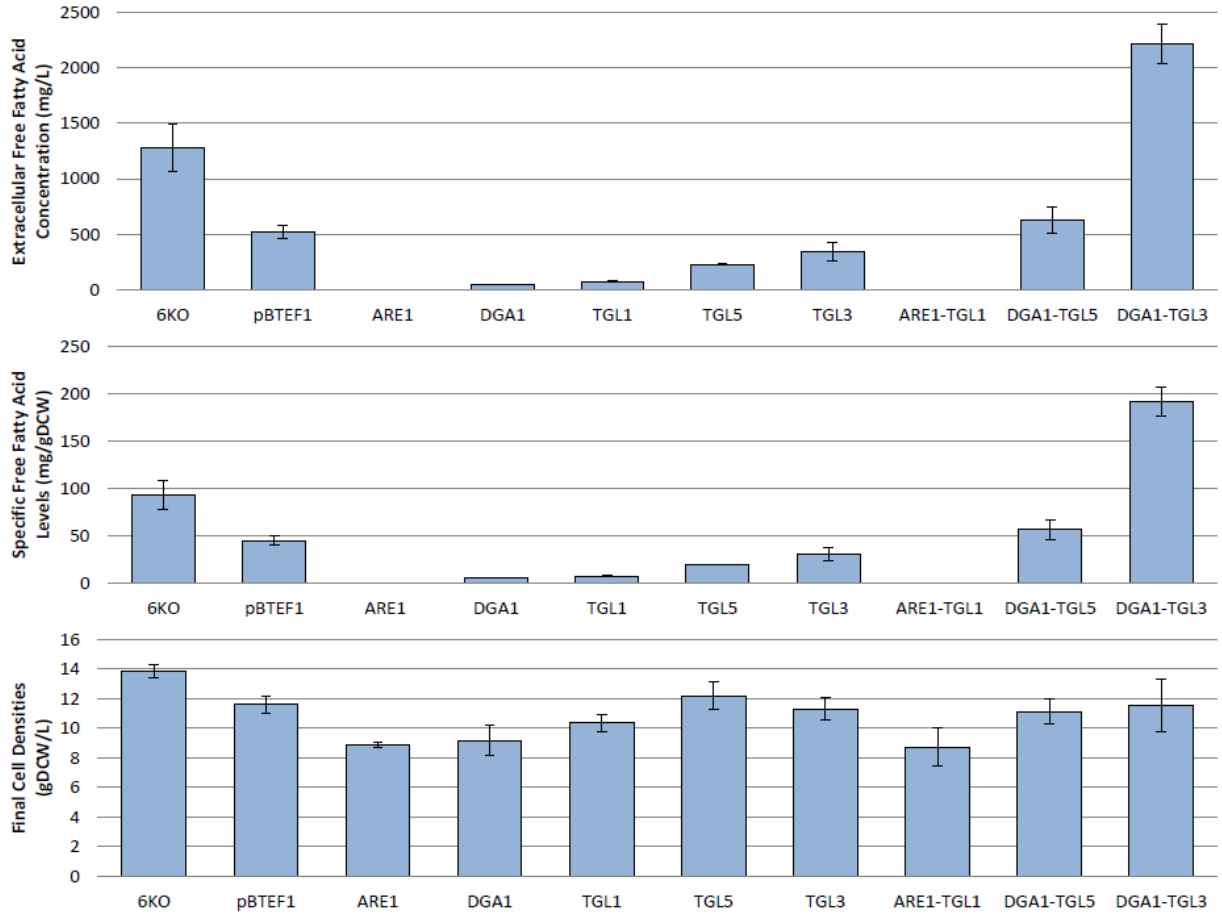


Figure 5.8. Effect of overexpressing and disrupting the neutral lipid recycle pathway in a host with limited acyl-CoA synthetase and β-oxidation activity. Extracellular free fatty acid levels were measured in strain BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1 (6KO) overexpressing acyltransferase genes *DGA1* and *ARE1* and lipase genes *TGL1*, *TGL3*, and *TGL5* alone or in combination. The control strain is 6KO strain carrying empty vector pBTEF1 (labeled pBTEF1). Top panel displays titer (mg/L), middle panel displays specific FA levels (mg/g DCW) and bottom panel displays final cell densities (g DCW/L) after 48 h of growth. Error bars represent ± standard deviation between at least two independent experiments.

Overexpression of *DGA1* and *TGL3* (pBTEF1-DGA1-TGL3) increased extracellular free fatty acid titers to over 2200 mg/L after 48 h. With this strain, 80-90% of total detected fatty acids (intracellular plus extracellular) were secreted from the cell as FFAs. This corresponds to a 4.3-fold increase in extracellular FFAs over the control strain, 6KO carrying empty vector pBTEF1. When normalized to optical density, overexpression of pBTEF1-DGA1-TGL3 increased extracellular FFAs 4.3-fold over the control and 2.1-fold over the parent 6KO strain. This combination of pathway interventions yielded nearly 32% of the maximum theoretical yield on glucose with a free fatty acid productivity of 46 mg/L·h after 48 h of growth. To our knowledge, 2200 mg/L FFA is the highest level reported and is a 4.2-fold increase over recently reported results for *S. cerevisiae*.

The chain length distribution of the extracellular free fatty acids in the 6KO control strain and in 6KO overexpressing *DGA1* and *TGL3* are shown in Figure 5.9. Only C16 and C18 FFAs were detected, and the distribution of FA chain lengths was similar in both cases, likely due to the broad substrate activities of Dga1 and Tgl3 (Athenstaedt and Daum, 2003; Athenstaedt and Daum, 2005; Sandager et al., 2002). Previously, Dga1, Are1 and Are2 were shown to have different substrate specificities *in vitro* (Oelkers et al., 2002; Yang et al., 1997). Alternating either the acyl-transferase or lipase with one that has a different substrate preference could potentially affect the FA chain length profile.

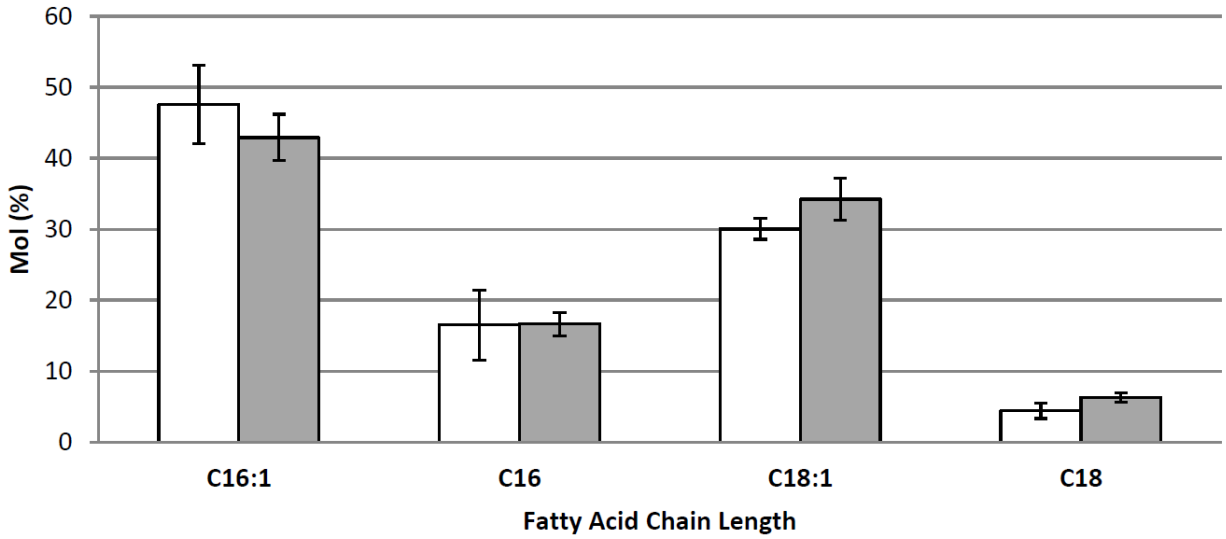


Figure 5.9. Extracellular free fatty acid chain length distribution profile for strain BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1 carrying pBTEF1 DGA1-TGL3 (white bars) and empty vector pBTEF1 (gray bars). Error bars represent ± standard deviation between three independent experiments.

The high levels of extracellular free fatty acids in our engineered strain were clearly visible (Figure 5.10). After spinning out the cells, the spent medium is clear for strain BY4741, translucent for the 3KO strain (BY4741ΔFAA1ΔFAA4ΔFAT1), and opaque for the 6KO strain (BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1) overexpressing *DGA1* and *TGL3* from plasmid pBTEF1-DGA1-TGL3. Markedly, no visible fatty acid precipitation was observed, although this was reported in a previous yeast study at lower titers (Runguphan and Keasling, 2014). Former studies using *E. coli* to produce high levels of free fatty acids did not report free fatty acid precipitation (Liu et al., 2012; Zhang et al., 2011). Media components and strain attributes could have a large effect on whether precipitation is observed. Further confirmation of free fatty acid production was verified using negative ion LC-MS. Only free fatty acids were observed.

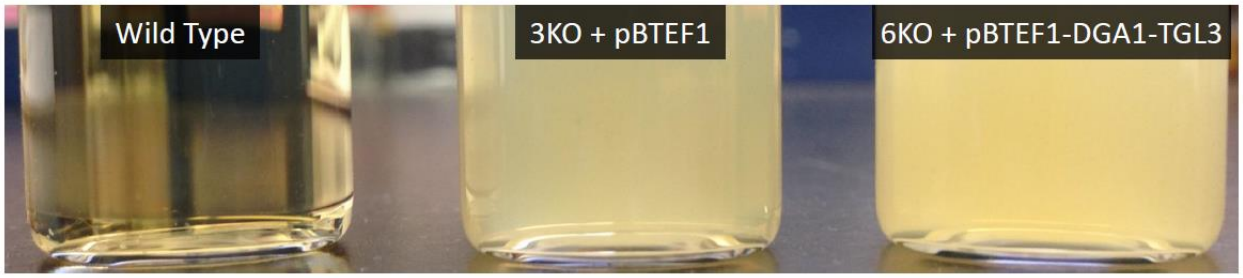


Figure 5.10. Visualization of extracellular free fatty acids. After centrifugation and removal of cells, the spent media after 48 h of growth from wild type (strain BY4741) is clear, 3KO (strain BY4741ΔFAA1ΔFAA4ΔFAT1) is translucent and 6KO (strain BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1 ΔPOX1) overexpressing pBTEF1-DGA1-TGL3 is opaque.

Overexpression of acetyl-CoA carboxylase (Acc1) to increase malonyl-CoA availability

Malonyl-CoA is the extender unit for fatty acid and polyketide elongation, and the synthesis of this substrate is considered a critical limiting step (Liu et al., 2010). Conversion of acetyl-CoA to malonyl-CoA is catalyzed by the native yeast 250 kDA acetyl-CoA carboxylase (Acc1) and requires ATP (Roggenkamp et al., 1980). Thus, strategies have focused on increasing Acc1 levels to produce fatty acids and fatty acid derived-products in multiple published studies (d Espaux et al., 2017; Guo et al., 2016; de Jong et al., 2015; Runguphan and Keasling, 2014; Shin et al., 2012). The Acc1 protein is negatively regulated by Snf1 in *S. cerevisiae.* Activation of Snf1 is triggered by glucose depletion leading to partial deactivation of Acc1 via phosphorylation at one or more serine residues (Woods et al., 1994). The decrease in active acetyl-CoA carboxylase when glucose is depleted thus leads to a drop in the pool of cytosolic malonyl-CoA. Site-directed mutagenesis of specific serine residues has been shown to diminish Snf1 deactivation of Acc1 in three independent studies (Choi and Da Silva, 2014; Hofbauer et al., 2014; Shi et al., 2014).

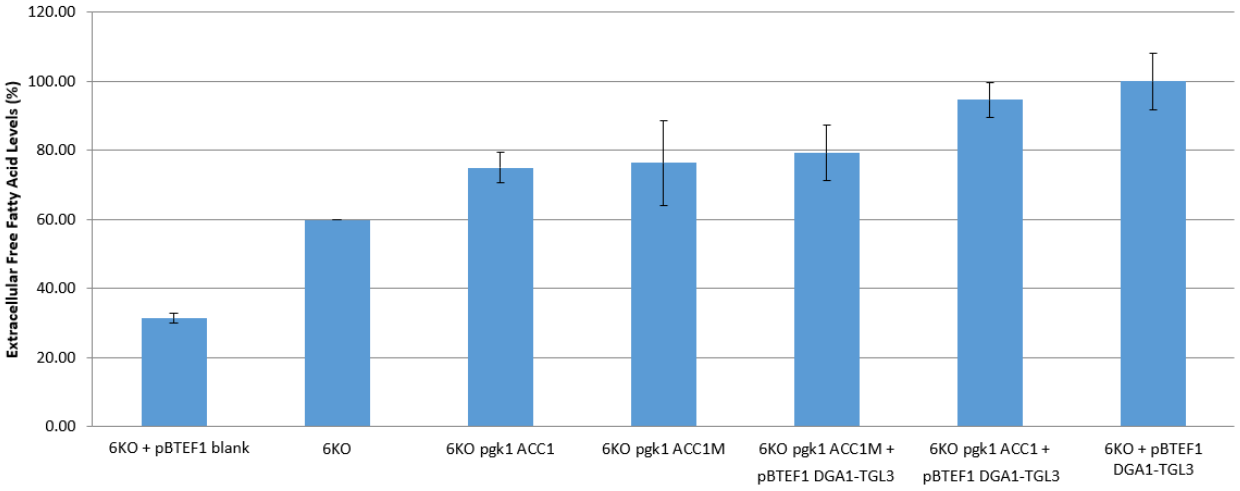
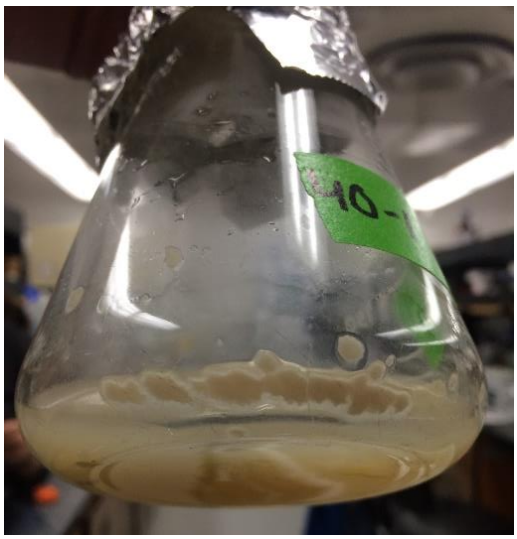


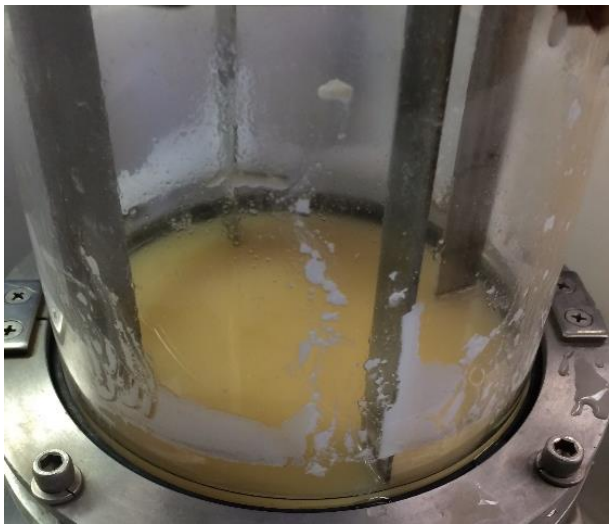
Figure 5.11. Effect of overexpressing *ACC1 or ACC1m* in BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1 (6KO) with or without the pBTEF1 DGA1-TGL3 plasmid in extracellular free fatty acid levels. Cells were grown for 48h in 2% YPD (cells expressing no plasmid) or 2% YPD+G418 for cell expressing plasmids. Control is 6KO strain expressing empty vector pBTEF1 (labeled pBTEF1). Results are presented as relative (%) to the 6KO + pBTEF1 DGA1-TGL3 extracellular LCFA levels. Error bars represent ± standard deviation between at least two independent experiments.

In this study, we tested if either the integration of an extra copy of the native *ACC1* gene or $ACC1^{S1157A}$ gene under the PGK1 promoter increased the production and secretion of LCFA to the media (Figure 5.11). Expression of *ACC1* or *ACC1m* in the 6KO strain increased LCFA production by 19-20%; thus, deactivation of *ACC1* after glucose depletion was not a critical factor in this strain. The same interventions to the 6KO strain expressing the pBTEF1 DGA1-TGL3 plasmid led to a different outcome. Overexpression of *ACC1* or *ACC1m* decreased the measured LCFA by 5% and 20%, respectively. Surprisingly, the flasks with strain 6KO pgk1 ACC1m accumulated chunks of white precipitate at the bottom (Figure 5.12). We saw that same phenomenon when the cells were cultured in a fed-batch bioreactor.

A)



B)



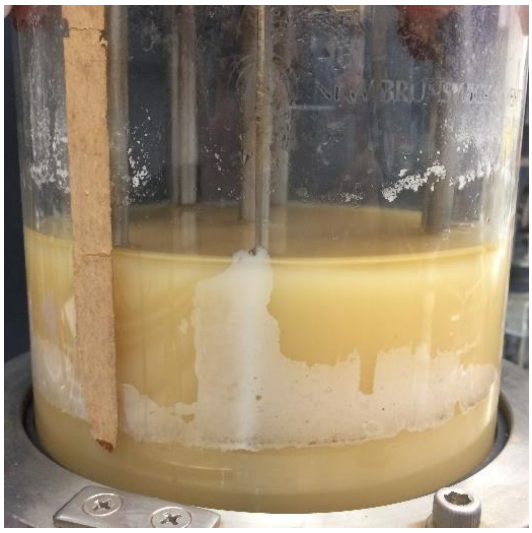
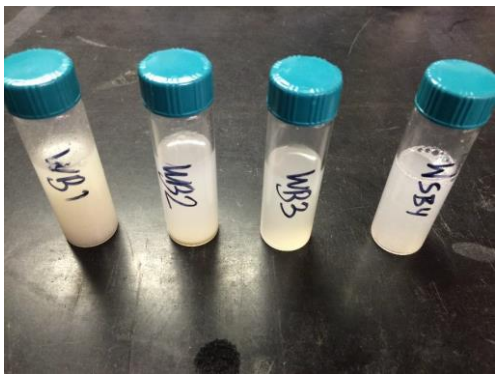


Figure 5.12. Visualization of white precipitates at the bottom of flasks and bioreactor, and the wall of bioreactors. Strain grown was 6KO pgk1 Acc1m (strain BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1 with one copy of the *ACC1m* gene integrated under the *PGK1* promoter). (A) White precipitate at the bottom of the flask after 48h (B) White precipitate on the fermenter walls after 96h of growing in a fed-batch bioreactor.

To test if the white precipitate was indeed fatty acids that had precipitated due to hitting the saturation limit, we first tested its solubility in different solvents (Figure 5.13-A). Solubility in water (WB1) was extremely low. A thin layer of white sediment was observed at the bottom of the glass

vial when hexane (WB2) was used. We found that the mix of organic solvents chloroform and methanol (1:1) (WB3), used in the first step of the FA extraction, was the solvent with the highest solubility for the white solid; however, the solution never became totally transparent. Water + detergent tween 20 (WB4) solubilized the white solid to levels similar to hexane. To determine if there were any fatty acids, we performed a fatty acid extraction with a chloroform/methanol solution of the white precipitate, and measured the fatty acid composition via GC-MS (Figure 5.13-B). 90% of the fatty acids in the white precipitate were stearic acid (saturated C18), which has the lowest solubility in water for the C16, C16:1, C18, C18:1 subgroup; and 10% of the fatty acids were palmitic acid (saturated C16). This result was expected since we have shown in previous studies that overexpression of Acc1m pushes the native yeast FA profile to longer average chain length, specifically an increase in C18 species (Choi and Da Silva, 2014). Also, saturated FA species have a lower solubility limit than the unsaturated species with same number of carbon molecules.

A)



B)

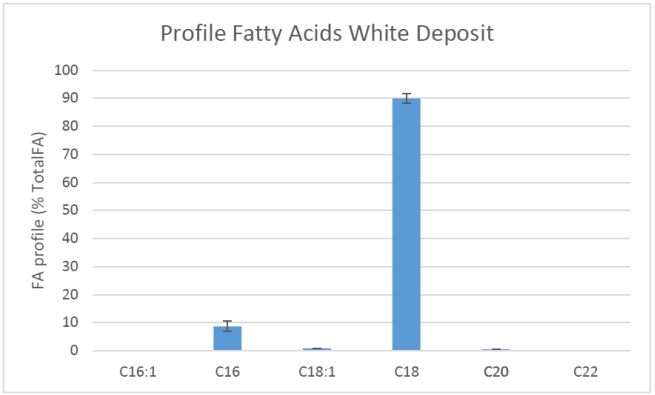


Figure 5.13. Analysis of white precipitates at the bottom of flasks and bioreactor, and the wall of bioreactors. Strain grown was 6KO (strain BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1) with one copy of the *ACC1m* gene integrated under the *PGK1* promoter. (A) WB1=water, WB2=hexane, WB3=chloroform/methanol (1:1), WB4= water+tween20 (detergent) (B) White precipitate analysis in the GC-MS after performing a fatty acid extraction.

These results suggest that the lower concentration of fatty acids in the media in the 6KO pgk1 ACC1M strain expressing pBTEF1 DGA1-TGL3 is likely a consequence of the limitation of our extraction procedure and analysis, which did not include the white precipitate formed at the bottom and walls of the flask. A potential approach to fix this issue would be to add a 10% dodecane overlay (d Espaux et al., 2017) to extract the secreted fatty acids *in situ* by the organic phase.

Increasing fatty acid synthase expression levels

S. cerevisiae has a cytosolic type I FAS that produces the majority of FAs**.** The yeast FAS is a large multifunction $\alpha_6\beta_6$ heterododecamer encoded by two genes: *FAS1* (for the β subunit) and *FAS2* (for the α subunit). Equimolar amounts of Fas1 and Fas2 subunits are found in the yeast FAS, but regulation of the two genes is not identical (Chirala 1992; Schüller *et al.* 1994). Cytoplasmic FAS levels are determined primarily by the amount of Fas1 protein (Wenz *et al.* 2001). A downstream regulatory site for the *FAS2* gene is positively regulated by Fas1. Overexpression of only *FAS1* led to an increase in the specific activity of FAS, while overexpression of only *FAS2* did not. When both genes were overexpressed, FAS-specific activity was even higher than for *FAS1* alone. Overexpression of *FAS1* and *FAS2* has been shown to increase levels of fatty acids and sterols in yeast (Runguphan and Keasling, 2014; Shin et al., 2012; Zhou et al., 2016).

To test if the enzyme cytosolic levels of the fatty acid synthase are limiting in our 6KO engineered strain, we swapped the native *FAS1* and *FAS2* promoters to the strong constitutive *TEF1* promoter using CRISPR/Cas9. Strains overexpressing only *FAS1*, only *FAS2*, or both were constructed and tested for LCFA production and secretion (Figure 5.14). As expected, total free fatty acid concentration in the medium increased approximately 25% for the 6KO strain overexpressing *FAS1*

or both genes, compared to the 6KO strain. Overexpression of only *FAS2* had no effect on the total FFA produced and or on the profile.

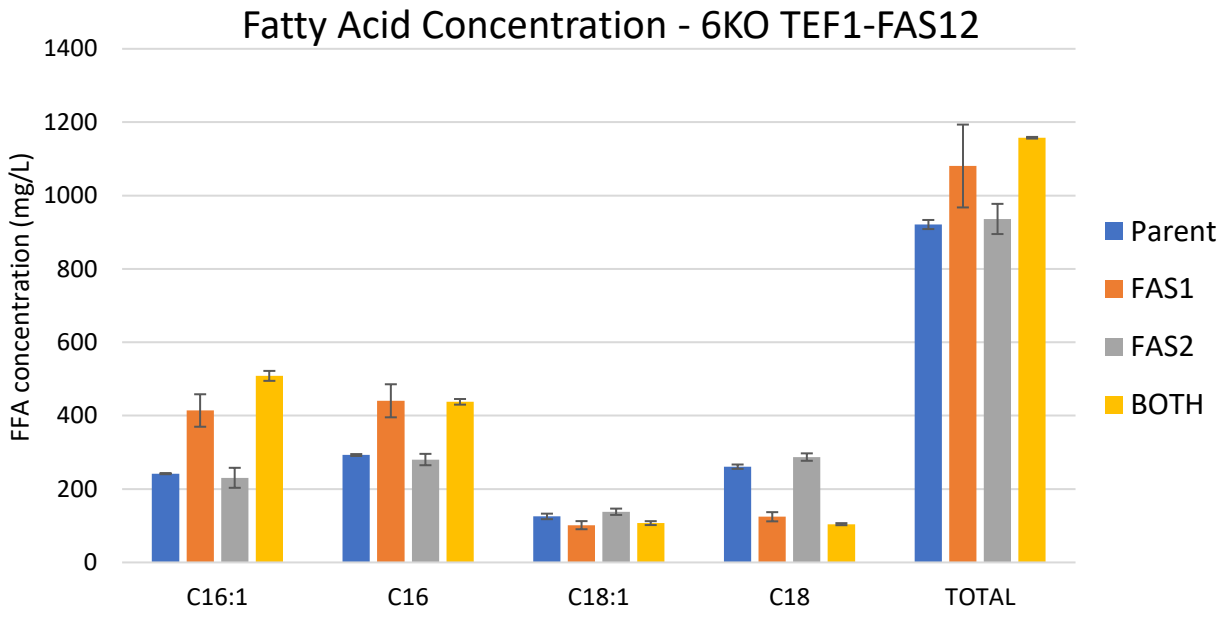


Figure 5.14. Effect of overexpressing *FAS1*, *FAS2* or both in BY4741ΔFAA1ΔFAA4ΔFAT1ΔFAA2ΔPXA1ΔPOX1 (6KO) in extracellular free fatty acid levels. Cells were grown in 2% YPD for 48h. Results are presented as mg/L of extracellular free fatty acids. Error bars represent ± standard deviation between at least two independent experiments.

These results align with previous published insights that the levels of Fas1 protein control expression of the *FAS2* gene, but not vice versa. An interesting observation is that the profile for 6KO pTEF1-FAS1 and 6KO pTEF1-FAS1/2 is clearly different than the 6KO control and 6KO pTEF1-FAS2. Levels of C16 species increased (particularly C16:1) and levels of C18 species decreased (particularly C18). One possible explanation is that when levels of FAS complex increase, the ratio of enzyme to malonyl-CoA increases and therefore there are fewer building blocks per enzyme and the average chain length gets reduced. Combination of the overexpression of *FAS1*, *FAS2*, and *ACC1m* with a 10% dodecane overlay layer would thus be interesting to try to increase the LCFA productivity in *S. cerevisiae* further.

CONCLUSIONS

S. cerevisiae was engineered to overproduce extracellular free fatty acids (FFAs) by targeting three non-essential intracellular processes. A disrupted β-oxidation host (*faa2*Δ*pxa1*Δ*pox1*Δ) increased intracellular fatty acids levels up to 55% higher than that of the control strain BY4741. Disruptions in the acyl-CoA synthetase genes *FAA1*, *FAA4* and *FAT1* resulted in extracellular FFA concentration of 490 mg/L. A sextuple mutant (*faa1*Δ*faa4*Δ*fat*1Δ*faa2*Δ*pxa1*Δ*pox1*Δ), defective in both pathways, was able to produce approximately 1.3 g/L of extracellular FFAs. By increasing carbon flux into neutral lipid droplet formation and degradation by overexpressing *DGA1* and a compatible lipase (*TGL3* or *TGL5*), extracellular FFAs were further increased. Overexpression of *DGA1* with *TGL3* (pBTEF1-DGA1-TGL3) in the sextuple mutant produced extracellular FFAs at a titer of 2.2 g/L after 48 h, and 32% of maximum theoretical yield. This titer is 4.2-fold greater than the highest level of extracellular FFAs reported for *S. cerevisiae*. These metabolic engineering strategies should also prove useful for the increased production of other fatty acid derived biomolecules in yeast. This approach provides a large pool of extracellular FFAs, which can be used directly or as precursors for further conversion into alkanes, alkenes, fatty alcohols, and fatty acid esters.

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REFERENCES

Amberg D, Burke D, Strathern J. 2005. Methods in Yeast Genetics. New York: Cold Spring Harbor Laboratory Press.

Athenstaedt, K., Daum, G., 2003. YMR313c/TGL3 encodes a novel triacylglycerol lipase located in lipid particles of *Saccharomyces cerevisiae*. J. Biol. Chem. 278, 23317–23. doi:10.1074/jbc.M302577200

Athenstaedt, K., Daum, G., 2005. Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast *Saccharomyces cerevisiae* are localized to lipid particles. J. Biol. Chem. 280, 37301–9. doi:10.1074/jbc.M507261200

Black, P.N., DiRusso, C.C., 2007. Yeast acyl-CoA synthetases at the crossroads of fatty acid metabolism and regulation. Biochim. Biophys. Acta 1771, 286–98. doi:10.1016/j.bbalip.2006.05.003

Blazeck, J., Hill, A., Liu, L., Knight, R., Miller, J., Pan, A., Otoupal, P., Alper, H.S., 2014. Harnessing *Yarrowia lipolytica* lipogenesis to create a platform for lipid and biofuel production. Nat. Commun. 5, 3131. doi:10.1038/ncomms4131

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917. doi:10.1139/o59-099

Chen, L., Zhang, J., Lee, J., Chen, W., 2014. Enhancement of free fatty acid production in *Saccharomyces cerevisiae* by control of fatty acyl-CoA metabolism. Appl. Microbiol. Biotechnol. 98, 6739–6750. doi:10.1007/s00253-014-5758-8

Chirala, S.S. (1992). Coordinated regulation and inositol-mediated and fatty acid-mediated repression of fatty acid synthase genes in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *89*, 10232–10236.

Choi, J.W., and Da Silva, N.A. (2014). Improving polyketide and fatty acid synthesis by engineering of the yeast acetyl-CoA carboxylase. J Biotechnol *187*, 56–59.

d'Espaux, L., Ghosh, A., Runguphan, W., Wehrs, M., Xu, F., Konzock, O., Dev, I., Nhan, M., Gin, J., Reider Apel, A., et al. (2017). Engineering high-level production of fatty alcohols by Saccharomyces cerevisiae from lignocellulosic feedstocks. Metab Eng *42*, 115–125.

Damodar N. Gujarati, *Basic Econometrics*, Third Edition. New York: McGraw-Hill, 1995, p.809.

De Jong, B.W., Shi, S., Valle-Rodríguez, J.O., Siewers, V., and Nielsen, J. (2015). Metabolic pathway engineering for fatty acid ethyl ester production in Saccharomyces cerevisiae using stable chromosomal integration. J Ind Microbiol Biotechnol *42*, 477–486.

DiCarlo, J.E., Norville, J.E., Mali, P., Rios, X., Aach, J., and Church, G.M. (2013). Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res *41*, 4336–4343.

Fang, F., Salmon, K., Shen, M. W. Y., Aeling, K. A., Ito, E., Irwin, B., Tran, U. P. C., Hatfield, G. W., Da Silva, N. A. and Sandmeyer, S. (2011), A vector set for systematic metabolic engineering in *Saccharomyces cerevisiae*. Yeast, 28: 123–136. doi: 10.1002/yea.1824

Gibson, D.G., Young, L., Chuang, R., Venter, J.C., Hutchison, C.A., Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343–5. doi:10.1038/nmeth.1318

Guo, W., Sheng, J., Zhao, H., and Feng, X. (2016). Metabolic engineering of Saccharomyces cerevisiae to produce 1-hexadecanol from xylose. Microb Cell Fact *15*, 24.

Henry, S. a, Kohlwein, S.D., Carman, G.M., 2012. Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. Genetics 190, 317–49. doi:10.1534/genetics.111.130286

Hiltunen, J.Kalervo., Mursula, A. M., Rottensteiner, H., Wierenga, R. K., Kastaniotis, A. J. and Gurvitz, A. (2003), The biochemistry of peroxisomal β-oxidation in the yeast *Saccharomyces cerevisiae*. FEMS Microbiology Reviews, 27: 35–64. doi: 10.1016/S0168-6445(03)00017-2

Hoskins, L. (July 5th, 2000). Yeast Transformation (high efficiency). *Fred Hutchinson Cancer Research Center*. Retrieved September 6th, 2012, from http://labs.fhcrc.org/hahn/Methods/genetic_meth/dmso_yeast_transform.html

Hofbauer, H.F., Schopf, F.H., Schleifer, H., Knittelfelder, O.L., Pieber, B., Rechberger, G.N., Wolinski, H., Gaspar, M.L., Kappe, C.O., Stadlmann, J., et al. (2014). Regulation of gene expression through a transcriptional repressor that senses acyl-chain length in membrane phospholipids. Dev Cell *29*, 729–739.

Jandrositz, A., Petschnigg, J., Zimmermann, R., Natter, K., Scholze, H., Hermetter, A., Kohlwein, S.D., Leber, R., 2005. The lipid droplet enzyme Tgl1p hydrolyzes both steryl esters and triglycerides in the yeast, *Saccharomyces cerevisiae*. Biochim. Biophys. Acta 1735, 50–8. doi:10.1016/j.bbalip.2005.04.005

Kamisaka, Y., Kimura, K., Uemura, H., Yamaoka, M., 2013. Overexpression of the active diacylglycerol acyltransferase variant transforms *Saccharomyces cerevisiae* into an oleaginous yeast. Appl. Microbiol. Biotechnol. 97, 7345–7355. doi:10.1007/s00253-013-4915-9

Klug, L., Daum, G., 2014. Yeast lipid metabolism at a glance. FEMS Yeast Res. 14, 369–88. doi:10.1111/1567-1364.12141

Koch, B., Schmidt, C., Daum, G., 2014. Storage lipids of yeasts: a survey of nonpolar lipid metabolism in *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica*. FEMS Microbiol. Rev. 1–24. doi:10.1111/1574-6976.12069

Köffel, R., Tiwari, R., Falquet, L., Schneiter, R., 2005. The *Saccharomyces cerevisiae* YLL012/YEH1, YLR020/YEH2, and TGL1 genes encode a novel family of membrane-anchored lipases that are

required for steryl ester hydrolysis. Mol. Cell. Biol. 25, 1655–68. doi:10.1128/MCB.25.5.1655-1668.2005

Kohlwein, S.D., Veenhuis, M., van der Klei, I.J., 2013. Lipid droplets and peroxisomes: key players in cellular lipid homeostasis or a matter of fat--store 'em up or burn 'em down. Genetics 193, 1–50. doi:10.1534/genetics.112.143362

Leber, C. and Da Silva, N. A. (2014), Engineering of *Saccharomyces cerevisiae* for the synthesis of short chain fatty acids. Biotechnol. Bioeng., 111: 347–358. doi: 10.1002/bit.25021

Lennen, R.M., Braden, D.J., West, R. a, Dumesic, J. a, Pfleger, B.F., 2010. A process for microbial hydrocarbon synthesis: Overproduction of fatty acids in *Escherichia coli* and catalytic conversion to alkanes. Biotechnol. Bioeng. 106, 193–202. doi:10.1002/bit.22660

Li, X., Guo, D., Cheng, Y., Zhu, F., Deng, Z. and Liu, T. (2014), Overproduction of fatty acids in engineered *Saccharomyces cerevisiae*. Biotechnol. Bioeng., 111: 1841–1852. doi: 10.1002/bit.25239

Lian, J., Zhao, H., 2014. Reversal of the β-Oxidation Cycle in *Saccharomyces cerevisiae* for Production of Fuels and Chemicals. ACS Synth. Biol. doi:10.1021/sb500243c

Liu, T., Vora, H., and Khosla, C. (2010). Quantitative analysis and engineering of fatty acid biosynthesis in E. coli. Metab Eng *12*, 378–386.

Liu, H., Yu, C., Feng, D., Cheng, T., Meng, X., Liu, W., Zou, H., Xian, M., 2012. Production of extracellular fatty acid using engineered *Escherichia coli*. Microb. Cell Fact. 11, 41. doi:10.1186/1475-2859-11-41

Oelkers, P., Cromley, D., Padamsee, M., Billheimer, J.T., Sturley, S.L., 2002. The *DGA1* gene determines a second triglyceride synthetic pathway in yeast. J. Biol. Chem. 277, 8877–81. doi:10.1074/jbc.M111646200

Peralta-Yahya, P.P., Zhang, F., del Cardayre, S.B., Keasling, J.D., 2012. Microbial engineering for the production of advanced biofuels. Nature 488, 320–8. doi:10.1038/nature11478

Ploier, B., Scharwey, M., Koch, B., Schmidt, C., Schatte, J., Rechberger, G., Kollroser, M., Hermetter, A., Daum, G., 2013. Screening for hydrolytic enzymes reveals Ayr1p as a novel triacylglycerol lipase in *Saccharomyces cerevisiae*. J. Biol. Chem. 288, 36061–72. doi:10.1074/jbc.M113.509927

Roggenkamp, R., Numa, S., and Schweizer, E. (1980). Fatty acid-requiring mutant of Saccharomyces cerevisiae defective in acetyl-CoA carboxylase. Proc Natl Acad Sci U S A *77*, 1814–1817.

Runguphan, W., Keasling, J.D., 2014. Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived biofuels and chemicals. Metab. Eng. 21, 103–13. doi:10.1016/j.ymben.2013.07.003

Sambrook J, Russell D. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Sandager, L., Gustavsson, M.H., Ståhl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H., Stymne, S., 2002. Storage lipid synthesis is non-essential in yeast. J. Biol. Chem. 277, 6478–82. doi:10.1074/jbc.M109109200

Scharnewski, M., Pongdontri, P., Mora, G., Hoppert, M., Fulda, M., 2008. Mutants of *Saccharomyces cerevisiae* deficient in acyl-CoA synthetases secrete fatty acids due to interrupted fatty acid recycling. FEBS J. 275, 2765–78. doi:10.1111/j.1742-4658.2008.06417.x

Shi, S., Chen, Y., Siewers, V., and Nielsen, J. (2014). Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. MBio *5*, e01130–14.

Schirmer, A., Rude, M. a, Li, X., Popova, E., del Cardayre, S.B., 2010. Microbial biosynthesis of alkanes. Science 329, 559–62. doi:10.1126/science.1187936

Schmidt, C., Athenstaedt, K., Koch, B., Ploier, B., Daum, G., 2013. Regulation of the Yeast Triacylglycerol Lipase Tgl3p by Formation of Nonpolar Lipids. J. Biol. Chem. 288, 19939–19948. doi:10.1074/jbc.M113.459610

Schüller, H.J., Schütz, A., Knab, S., Hoffmann, B., and Schweizer, E. (1994). Importance of general regulatory factors Rap1p, Abf1p and Reb1p for the activation of yeast fatty acid synthase genes FAS1 and FAS2. Eur J Biochem *225*, 213–222.

Schweizer, E., Hofmann, J., 2004. Microbial Type I Fatty Acid Synthases (FAS): Major Players in a Network of Cellular FAS Systems. Microbiol. Mol. Biol. Rev. 68, 501–517. doi:10.1128/MMBR.68.3.501-517.2004

Shen, M.W.Y., Fang, F., Sandmeyer, S., Da Silva, N.A., 2012. Development and characterization of a vector set with regulated promoters for systematic metabolic engineering in *Saccharomyces cerevisiae*. Yeast 29, 495–503. doi:10.1002/yea.2930

Shin, G.-H., Veen, M., Stahl, U., and Lang, C. (2012). Overexpression of genes of the fatty acid biosynthetic pathway leads to accumulation of sterols in Saccharomyces cerevisiae. Yeast *29*, 371–383.

Steen, E.J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S.B., Keasling, J.D., 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature 463, 559–62. doi:10.1038/nature08721

Tehlivets, O., Scheuringer, K., Kohlwein, S.D., 2007. Fatty acid synthesis and elongation in yeast. Biochim. Biophys. Acta 1771, 255–70. doi:10.1016/j.bbalip.2006.07.004

Valle-Rodríguez, J.O., Shi, S., Siewers, V., Nielsen, J., 2014. Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid ethyl esters, an advanced biofuel, by eliminating non-essential fatty acid utilization pathways. Appl. Energy 115, 226–232. doi:10.1016/j.apenergy.2013.10.003

Varman, A.M., He, L., You, L., Hollinshead, W., Tang, Y.J., 2014. Elucidation of intrinsic biosynthesis yields using 13C-based metabolism analysis. Microb. Cell Fact. 13, 42. doi:10.1186/1475-2859-13-42

Wenz, P., Schwank, S., Hoja, U., and Schüller, H.J. (2001). A downstream regulatory element located within the coding sequence mediates autoregulated expression of the yeast fatty acid synthase gene FAS2 by the FAS1 gene product. Nucleic Acids Res *29*, 4625–4632

Choi, J.W., and Da Silva, N.A. (2014). Improving polyketide and fatty acid synthesis by engineering of the yeast acetyl-CoA carboxylase. J Biotechnol *187*, 56–59.

DiCarlo, J.E., Norville, J.E., Mali, P., Rios, X., Aach, J., and Church, G.M. (2013). Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res *41*, 4336–4343.

D Espaux, L., Ghosh, A., Runguphan, W., Wehrs, M., Xu, F., Konzock, O., Dev, I., Nhan, M., Gin, J., Reider Apel, A., et al. (2017). Engineering high-level production of fatty alcohols by Saccharomyces cerevisiae from lignocellulosic feedstocks. Metab Eng *42*, 115–125.

Guo, W., Sheng, J., Zhao, H., and Feng, X. (2016). Metabolic engineering of Saccharomyces cerevisiae to produce 1-hexadecanol from xylose. Microb Cell Fact *15*, 24.

Hofbauer, H.F., Schopf, F.H., Schleifer, H., Knittelfelder, O.L., Pieber, B., Rechberger, G.N., Wolinski, H., Gaspar, M.L., Kappe, C.O., Stadlmann, J., et al. (2014). Regulation of gene expression through a transcriptional repressor that senses acyl-chain length in membrane phospholipids. Dev Cell *29*, 729–739.

De Jong, B.W., Shi, S., Valle-Rodríguez, J.O., Siewers, V., and Nielsen, J. (2015). Metabolic pathway engineering for fatty acid ethyl ester production in Saccharomyces cerevisiae using stable chromosomal integration. J Ind Microbiol Biotechnol *42*, 477–486.

Liu, T., Vora, H., and Khosla, C. (2010). Quantitative analysis and engineering of fatty acid biosynthesis in E. coli. Metab Eng *12*, 378–386.

Roggenkamp, R., Numa, S., and Schweizer, E. (1980). Fatty acid-requiring mutant of Saccharomyces cerevisiae defective in acetyl-CoA carboxylase. Proc Natl Acad Sci U S A *77*, 1814–1817.

Runguphan, W., and Keasling, J.D. (2014). Metabolic engineering of Saccharomyces cerevisiae for production of fatty acid-derived biofuels and chemicals. Metab Eng *21*, 103–113.

Shi, S., Chen, Y., Siewers, V., and Nielsen, J. (2014). Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. MBio *5*, e01130–14.

Shin, G.-H., Veen, M., Stahl, U., and Lang, C. (2012). Overexpression of genes of the fatty acid biosynthetic pathway leads to accumulation of sterols in Saccharomyces cerevisiae. Yeast *29*, 371–383.

Woods, A., Munday, M.R., Scott, J., Yang, X., Carlson, M., and Carling, D. (1994). Yeast SNF1 is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase in vivo. J Biol Chem *269*, 19509–19515.

Zhou, Y.J., Buijs, N.A., Zhu, Z., Qin, J., Siewers, V., and Nielsen, J. (2016). Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. Nat Commun *7*, 11709.

Yang, H., Cromley, D., Wang, H., Billheimer, J.T., Sturley, S.L., 1997. Functional Expression of a cDNA to Human Acyl-coenzyme A:Cholesterol Acyltransferase in Yeast. J. Biol. Chem. 272, 3980–3985. doi:10.1074/jbc.272.7.3980

Zhang, X., Li, M., Agrawal, A., San, K.-Y., 2011. Efficient free fatty acid production in *Escherichia coli* using plant acyl-ACP thioesterases. Metab. Eng. 13, 713–22. doi:10.1016/j.ymben.2011.09.007

Zhou, Y.J., Buijs, N.A., Zhu, Z., Qin, J., Siewers, V., and Nielsen, J. (2016). Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. Nat Commun *7*, 11709.

Zweytick, D., Leitner, E., Kohlwein, S.D., Yu, C., Rothblatt, J., Daum, G., 2000. Contribution of Are1p and Are2p to steryl ester synthesis in the yeast *Saccharomyces cerevisiae*. Eur. J. Biochem. 267, 1075–1082. doi:10.1046/j.1432-1327.2000.01103.x

CHAPTER 6:

Outlook and prospective work

INTRODUCTION

The overall goal of this thesis was to engineer yeast to produce high-levels of acetyl-CoA derived products, mainly free fatty acids. Most chapters in this dissertation focused on exploring strategies to optimize downstream pathways (after acetyl-CoA or malonyl-CoA biosynthesis) to produce medium-chain and long-chain free fatty acids. An additional approach to increase acetyl-CoA derived products is to increase the pool of acetyl-CoA or malonyl-CoA. To accomplish this, it is necessary to rewire *Saccharomyces cerevisiae* central carbon metabolism and remove or downregulate, when possible, acetyl-CoA consuming pathways to increase flux to fatty acids and polyketides. Native cytosolic fluxes to acetyl-CoA in Crabtree-positive yeast species cannot support high yields of acetyl-CoA derived products. Also, the fact that acetyl-CoA is found not only in the cytosol but also in the nucleus, the mitochondria, and the peroxisomes, makes it challenging to optimize. In this chapter, we show preliminary work on strategies to increase the production of 6-MSA and TAL polyketides, which require acetyl-CoA and malonyl-CoA.

Native cytosolic fluxes to acetyl-CoA in Crabtree-positive yeast species (like *S. cerevisiae)* are lower than in other strictly aerobic yeast species (Christen and Sauer, 2011) (Figure 6.1). In yeast, cytosolic acetyl-CoA is produced from pyruvate by three reactions in the pyruvate dehydrogenase bypass (PDH-bypass). The limiting step in engineering the pyruvate dehydrogenase (PDH)-bypass was determined to be the formation of acetyl-CoA from acetate by ACS, due to its high energy requirements and product inhibition (Shiba et al., 2007). The very energy-consuming activation of acetate in the PDH-bypass triggered the search for alternatives that would help to reduce the ATP requirements and therefore increase the yield of acetyl-CoA derived products from glucose, since less glucose needs to be oxidized by the TCA cycle to provide ATP. The most straight forward approach

was to find an enzyme that could catalyze the synthesis of acetyl-CoA from acetaldehyde. Acetylating aldehyde dehydrogenases are found in many prokaryotes and catalyze the formation of acetyl-CoA from acetaldehyde without the need of ATP molecules (Kozak et al., 2014). This type of enzyme can complement growth of yeast strains devoid of all aldehyde dehydrogenases or all the acetyl-CoA synthetases. It has been determined by metabolic flux analysis that the combination of EutE and phosphoketolase + phosphotransacetylase pathways was the most favorable when considering the energetics and CO_2 molecules lost (Meadows et al., 2016).

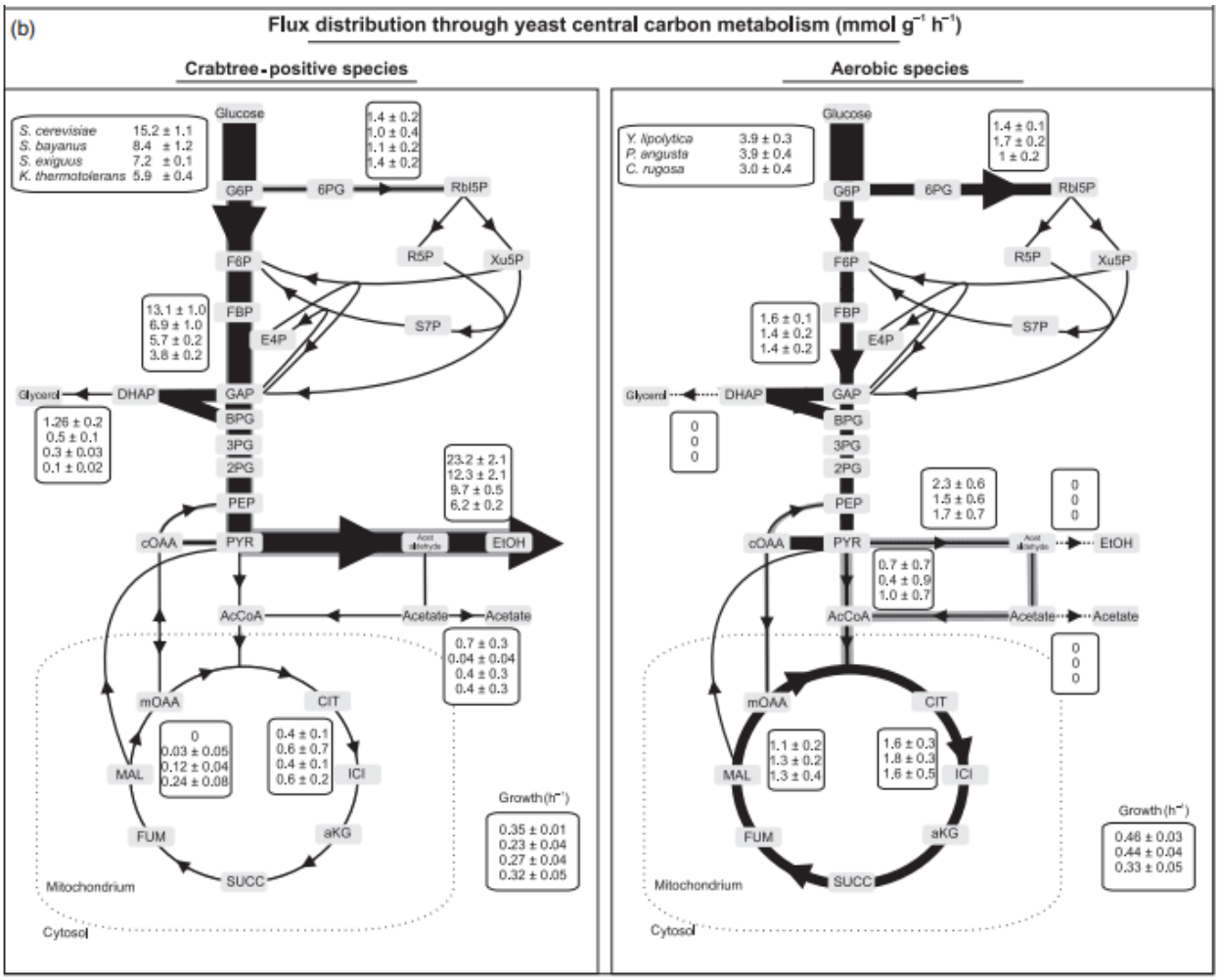

Figure 6.1. ^{13}C flux distribution of central carbon metabolism (A) four Crabtree-positive yeast species and (B) three strictly aerobic species. Cells were grown in glucose minimal media. From Christen and Sauer, (2011).

A complementary approach to increase acetyl-CoA pools is to delete or downregulate competing pathways. The main challenge of this strategy is that most acetyl-CoA consuming pathways in yeast are essential for cell survival. The main pathway that uses cytosolic acetyl-CoA is the glyoxylate shunt (Chen et al., 2012; Ghosh et al., 2016). This cycle converts acetyl-CoA to succinate and isocitrate which enter the tricarboxylic acid cycle (TCA), allowing cells to generate energy. In addition, it produces malate and oxaloacetate that can be converted into phosphoenolpyruvate, which leads to gluconeogenesis (Duntze et al., 1969). This pathway is essential for yeast growth on ethanol, acetate and fatty acids. There are two reactions within the glyoxylate shunt that consume acetyl-CoA; citrate synthase (*CIT2*) catalyzes the condensation of acetyl-CoA and oxaloacetate to produce citrate, and malate synthase (*MLS1*) forms malate from acetyl-CoA and glyoxylate. The effect of deleting one of the genes (either *MLS1* or *CIT2*) has had mixed results within the literature. Examples where it increased product formation are the production of α-santalene (Chen et al., 2013), 3-hydroxypropionic acid (Chen et al., 2014), and n-butanol (Krivoruchko et al., 2013); all improvements were between 1.2- and 2.3-fold. Another study found that deleting either of these genes reduced the production of polyhydroxybutyrate (Kocharin et al., 2012). In addition, it was shown in our lab that deletion of *MLS1* decreased the production of the TAL polyketide (Cardenas and Da Silva, 2014).

OPTIMIZATION OF UPSTREAM PATHWAYS

Rewiring PDH-bypass by combining PDHm and EutE

In collaboration with the Maranas lab (Chowdhury et al., 2014), our lab has shown that by changing the redox cofactor requirement of the *E. coli* PDH from NADH to NADPH, and expressing this enzyme in a strain with the main NADPH producing pathway eliminated (pentose phosphate pathway), the carbon flux towards acetyl-CoA could be successfully increased (Cardenas and Da Silva, 2016). This resulted in a 4.4-fold increase in polyketide production.

In a preliminary study, we wanted to test if the co-expression of the $NADP^+$-dependent PDH mutant and acetylating aldehyde dehydrogenase EutE from *E. coli* could increase the production of TAL in yeast cells growing in synthetic media. We created a trigenic (three gene expression cassettes) plasmid carrying the three PDH subunits. Through computational analysis of the different pathway options using the COBRA toolbox (Schellenberger et al., 2011), we determined that the combination of PDHm and EutE could lead to a 3-5% higher yield than EutE alone. To force the flux through the new acetyl-CoA pathways, deletion of the native acetaldehyde dehydrogenase (ALD) activity in yeast is necessary. We deleted the most active ALDs in the mitochondria and cytosol, *ALD4* and *ALD6*, respectively (Boubekeur et al., 2001). This not only prevents yeast from transforming acetaldehyde to acetate, but also decreases the production of cytosolic NADPH, since *ALD6* generates NADPH. A push and pull strategy was thus implemented, since the flux through the PDHm enzyme can replenish that drop in cytosolic NADPH.

Growth of the *ald4*Δ*ald6*Δ strain in liquid medium without acetate was very poor (< OD 2 after 48h of growth), indicating that the other aldehyde dehydrogenases left intact do not allow healthy growth of cells. After transformation of EutE, PDHm or both, we determined that cells expressing

EutE were able to growth well in minimal liquid medium lacking acetate. PDHm alone was unable to complement the Ald gene deletions, even though we were feeding lipoic acid required for PDH activity (Stephens et al., 1983).

These preliminary results show a promising approach to rewiring the central carbon metabolism of *S. cerevisiae.* Cells with the native PDH-bypass eliminated recovered growth when EutE from *E. coli* was expressed. Metabolic flux analysis of the *ald4*Δ*ald6*Δ strain, and measurement of mRNA levels for the genes coding for the PDHm could help to determine why PDHm expression was ineffective. Future work should also focus on TAL production of engineered strains.

DOWNREGULATION OF COMPETING PATHWAYS

Downregulation of *FAS2* by promoter swapping

The biosynthetic pathways with greatest demand for cytosolic acetyl-CoA in yeast are fatty acid synthesis and the glyoxylate shunt. To drive the acetyl-CoA flux toward the desired product (e.g., polyketides, MCFA from heterologous eFAS system), downregulation of the native fatty acid synthesis machinery is needed. When glucose is depleted, *S. cerevisiae* cells utilize the ethanol they have accumulated and produce acetyl-CoA that can then be directed to the synthesis of the desired product. We thus constructed a library of integration plasmids carrying the *TDH3* (Mumberg et al., 1995), full *ADH1* (Ammerer, 1983), *TEF1-mut4*, and *TEF1-mut7* (Nevoigt et al., 2006) promoters. These plasmids were used to insert the different promoters in front of the *FAS2* gene in *S. cerevisiae* strains expressing the human FAS for MCFA production, the 2-PS for TAL synthesis, or the 6-methyl salicylic acid synthase (6-MSAS) for 6-MSA production.

Preliminary studies included a TAL production screening of the different promoters driving the expression of *FAS2*. All promoters resulted in higher levels of TAL than the native *FAS2* promoter (Figure 6.2). The *ADH1* promoter was the most effective (Figure 6.2); final TAL titer after 72h was >3-fold higher than for BY4741 (WT). With these results, we showed that downregulation of the fatty acid biosynthesis pathway is a promising approach to increase pools of acetyl-CoA, and therefore an approach to test and optimize to produce other acetyl-CoA-derived products. Design of synthetic promoters that completely turn off when cell mass reaches a certain concentration might be needed to stop competing pathways that are essential for growth.

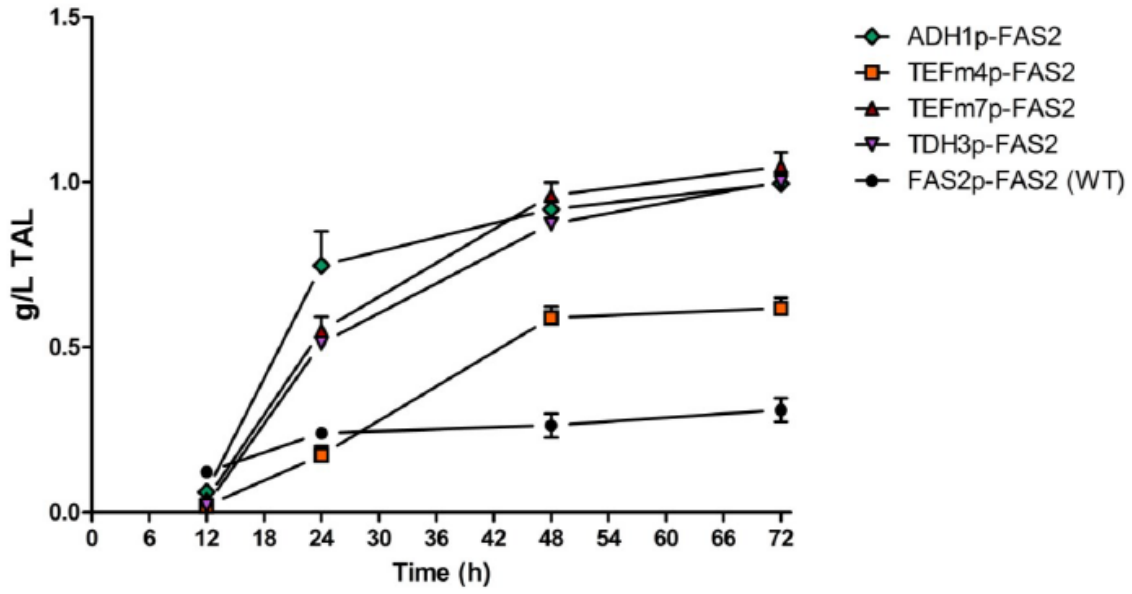

Figure 6.2. TAL profile after 72h of growing in 1% glucose YPD. Error bars represent ± standard deviation for at least three independent experiments.

Downregulation of *FAS1* and *FAS2* using CRISPRi (dCas9)

Recent advances in genetic tools have enabled modulation of gene expression in a novel way for metabolic engineering. Cas9 is a RNA-guided nuclease originally from bacteria (Doudna and Charpentier, 2014). It attaches to the specific complementary genomic DNA of the guiding RNA and creates a double stranded break. Many mutants have been published in the literature, from nickases to regulators (Dominguez et al., 2016). When the two nucleases subunits of the Cas9 protein are deactivated by two-point mutations, the protein retains the binding activity but loses the cleaving activity. New subunits with known repression or activation activities can be linked to this deactivated Cas9 protein (dCas9) to provide new dCas9 mutants with new functionalities. Gilbert et al., (2013) showed that a dCas9 targeting the native yeast *TEF1* promoter was able to repress gene expression by-18-fold in *S. cerevisiae*. In the same study, the authors were able repress the tetON promoter by

115-fold. The authors also fused dCas9 to Mxi1, a mammalian transcriptional repressor domain that is reported to interact with the histone deacetylase Sin3 homolog in yeast. The fused dCas9-Mxi1 repressed gene expression under the *TEF1* promoter by 53-fold.

To redirect malonyl-CoA from fatty acid biosynthesis to our products of interest, we need to downregulate fatty acid production in a timely manner. We want the cells to produce fatty acids until they reach a certain OD to assure fitness, and then stop fatty acid production and start expression of the heterologous synthase. In this preliminary study, we used CRISPR-dCas9 as a gene expression interference system (CRISPRi). We used a dCas9 with and without the repressor domain Mxi1. We targeted regions close to the start codon of the yeast genes *FAS1* and *FAS2* using a plasmid with two guide RNA expression cassettes. The *ADH2* promoter controlled expression of the nucleases and the polyketide synthases, forcing the downregulation of the fatty acid biosynthesis and the upregulation of polyketide synthase expression at the same time. This occurs in early stationary phase when glucose in the culture is depleted and ethanol utilization begins (Lee and DaSilva, 2005).

A strain with the polyketide synthase 6-MSAS and the NpgA gene from *Aspergillus* nidulans (a PPT to activate 6-MSAS) integrated into the genome was transformed with the plasmids shown in Table 6.1. The three strains were grown in 1% glucose YPD and 1% glucose SD(-ura,-leu)+dropout for 72h in 5mL tubes (Figure 6.3). Final ODs were similar among the three strains in the same medium, although the Mxi1-expressing strain seemed to flocculate in the minimal medium.

Table 6.1. Description of the plasmids expressed by each of the strains in Figure 6.3.

Strain	Plasmid 1	Plasmid 2
Control	pADH2-dCas9	pXP318
dCas9	pADH2-dCas9	dgRNA-FAS1/FAS2
Mxi1	pADH2-dCas9	dgRNA-FAS1/FAS2

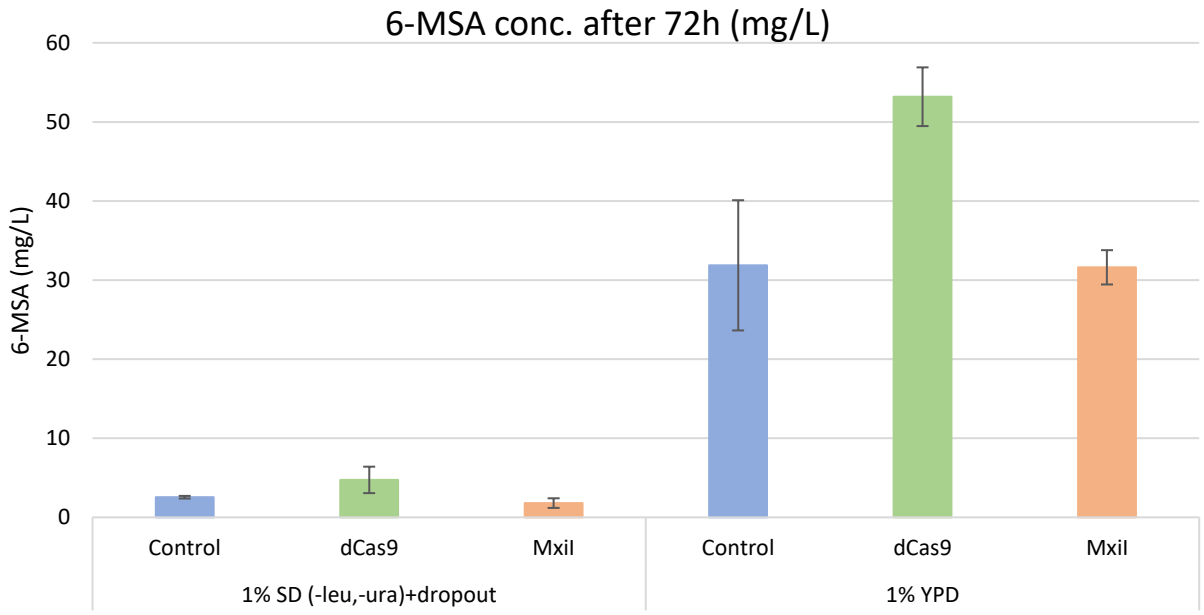

Figure 6.3. BY4741 *trp1*Δ with 6-MSAS and PPT integrated was transformed with the plasmids in Table 6.2. Cells were grown either in 1% glucose SD(-leu,-ura)+dropout or 1% glucose YPD for 72h. The culture was spun down and 6-MSA in the supernatant was measured by HPLC. Results are shown in mg/L. Error bars represent ± standard deviation for at least two independent experiments.

6-MSA levels in the supernatant increased in both minimal and complex media when dCas9 was expressed (Figure 6.3). The titers were significantly higher in complex media than in minimal media as expected for *ADH2* promoter control. When dCas9 and dgRNA with target sequences for *FAS1* and *FAS2* were expressed, the 6-MSA titer in minimal media increase 86% (from 2.6 to 4.7 mg/L) compared to the strain not expressing the dgRNA-FAS1/FAS2 plasmid. In complex media, the titer increased 67%, from 31.9 to 53.2 mg/L. Surprisingly, the 6-MSA final titer was lower when using dCas9-Mxi1 than dCas9, and similar to the control strain. This could indicate that the dCas9-Mxi1 was unable to bind efficiently to the target genomic DNA, or that a strong downregulation of *FAS1* and *FAS2* is detrimental for 6-MSA production (cells flocculated in the minimal medium). To determine the correct hypothesis, we would need to measure *FAS1* and *FAS2* mRNA levels.

These results show that using CRISPRi is very powerful tool in metabolic engineering to downregulate pathways in a timely manner. New target sequences with better efficiencies for downregulation might optimize the system presented in this preliminary study. Recently, an article was published on the efficacy of the target sequence position with respect to the transcription start site (TSS) of the gene to downregulate gene expression using CRISPRi in *S. cerevisiae* (Smith et al., 2016). The authors found that gRNA targeted to a region between the TSS and -200 bp after the TSS was more likely to be effective. We based our the target sequences used in our preliminary work on previous reports on CRISPRi in *Escherichia coli*, where the most effective targeting spot was reported to be within the gene start codon (Larson et al., 2013). It is important to note that these rules might change from species to species, since for example, the most effective region to target dCas9-KRAB in human myeloid leukemia cells for gene downregulation purposes was between -50 bp and +300 bp relative to the TSS (Gilbert et al., 2014).

SUMMARY

Higher acetyl-CoA pools or fluxes are essential to increase yields of fatty acids, polyketides, and other products synthesized from acetyl-CoA in yeast, and, thus help meet the requirements to turn the biochemical industry into an economically competitive market. We completed preliminary work aimed at increasing the efficiency of pathways leading to acetyl-CoA production. Expression of the *E. coli* acetylating aldehyde dehydrogenase EutE complemented growth of a *S. cerevisiae* strain with the two most active native aldehyde dehydrogenases *ALD4* and *ALD6* deleted. However, preliminary results on expression of a $NADP^+$-dependent PDH enzyme in this strain did not complement growth of the strain with the Ald genes deleted. This indicates that the PDHm expression in yeast needs to be optimized.

In addition, we showed that the downregulation of the fatty acid synthesis gene *FAS2* by swapping the native promoter with the *ADH1* promoter increased TAL titers >3-fold. This suggests that downregulation of the fatty acid synthase is a promising approach to increase acetyl-CoA products; however, fine-tuning is necessary to decouple growth from production. We also showed how CRISPRi can be a successful alternative method to downregulate competing pathways. The RNA-guided deactivated Cas9 targeted to the *FAS1* and *FAS2* promoters increased the 6-MSA levels 86% and 67% in minimal medium and complex medium, respectively.

REFERENCES

Boubekeur, S., Camougrand, N., Bunoust, O., Rigoulet, M., and Guérin, B. (2001). Participation of acetaldehyde dehydrogenases in ethanol and pyruvate metabolism of the yeast*Saccharomyces cerevisiae*. European Journal of Biochemistry *268*, 5057–5065.

Cardenas, J., and Da Silva, N.A. (2014). Metabolic engineering of Saccharomyces cerevisiae for the production of triacetic acid lactone. Metab Eng *25*, 194–203.

Cardenas, J., and Da Silva, N.A. (2016). Engineering cofactor and transport mechanisms in Saccharomyces cerevisiae for enhanced acetyl-CoA and polyketide biosynthesis. Metab Eng *36*, 80–89.

Chen, Y., Siewers, V., and Nielsen, J. (2012). Profiling of cytosolic and peroxisomal acetyl-CoA metabolism in Saccharomyces cerevisiae. PLoS ONE *7*, e42475.

Chen, Y., Daviet, L., Schalk, M., Siewers, V., and Nielsen, J. (2013). Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. Metab Eng *15*, 48–54.

Chen, Y., Bao, J., Kim, I.-K., Siewers, V., and Nielsen, J. (2014). Coupled incremental precursor and co-factor supply improves 3-hydroxypropionic acid production in Saccharomyces cerevisiae. Metab Eng *22*, 104–109.

Choi, J.W., and Da Silva, N.A. (2014). Improving polyketide and fatty acid synthesis by engineering of the yeast acetyl-CoA carboxylase. J Biotechnol *187*, 56–59.

Chowdhury, A., Zomorrodi, A.R., and Maranas, C.D. (2014). k-OptForce: integrating kinetics with flux balance analysis for strain design. PLoS Comput Biol *10*, e1003487.

Christen, S., and Sauer, U. (2011). Intracellular characterization of aerobic glucose metabolism in seven yeast species by 13C flux analysis and metabolomics. FEMS Yeast Res *11*, 263–272.

Dominguez, A.A., Lim, W.A., and Qi, L.S. (2016). Beyond editing: repurposing CRISPR-Cas9 for precision genome regulation and interrogation. Nat Rev Mol Cell Biol *17*, 5–15.

Doudna, J.A., and Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science *346*, 1258096.

Duntze, W., Neumann, D., Atzpodien, W., Holzer, H., and Gancedo, J.M. (1969). Studies on the Regulation and Localization of the Glyoxylate Cycle Enzymes in Saccharomyces cerevisiae. European Journal of Biochemistry *10*, 83–89.

Ghosh, A., Ando, D., Gin, J., Runguphan, W., Denby, C., Wang, G., Baidoo, E.E.K., Shymansky, C., Keasling, J.D., and García Martín, H. (2016). (13)C Metabolic Flux Analysis for Systematic Metabolic Engineering of S. cerevisiae for Overproduction of Fatty Acids. Frontiers in Bioengineering and Biotechnology *4*, 76.

Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., Stern-Ginossar, N., Brandman, O., Whitehead, E.H., Doudna, J.A., et al. (2013). CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell *154*, 442–451.

Gilbert, L.A., Horlbeck, M.A., Adamson, B., Villalta, J.E., Chen, Y., Whitehead, E.H., Guimaraes, C., Panning, B., Ploegh, H.L., Bassik, M.C., et al. (2014). Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell *159*, 647–661.

Kocharin, K., Chen, Y., Siewers, V., and Nielsen, J. (2012). Engineering of acetyl-CoA metabolism for the improved production of polyhydroxybutyrate in Saccharomyces cerevisiae. AMB Express *2*, 52.

Kozak, B.U., van Rossum, H.M., Benjamin, K.R., Wu, L., Daran, J.-M.G., Pronk, J.T., and van Maris, A.J.A. (2014). Replacement of the Saccharomyces cerevisiae acetyl-CoA synthetases by alternative pathways for cytosolic acetyl-CoA synthesis. Metab Eng *21*, 46–59.

Krivoruchko, A., Serrano-Amatriain, C., Chen, Y., Siewers, V., and Nielsen, J. (2013). Improving biobutanol production in engineered Saccharomyces cerevisiae by manipulation of acetyl-CoA metabolism. J Ind Microbiol Biotechnol *40*, 1051–1056.

Larson, M.H., Gilbert, L.A., Wang, X., Lim, W.A., Weissman, J.S., and Qi, L.S. (2013). CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat Protoc *8*, 2180–2196.

Lee, K.M., and DaSilva, N.A. (2005). Evaluation of the Saccharomyces cerevisiae ADH2 promoter for protein synthesis. Yeast *22*, 431–440.

Meadows, A.L., Hawkins, K.M., Tsegaye, Y., Antipov, E., Kim, Y., Raetz, L., Dahl, R.H., Tai, A., Mahatdejkul-Meadows, T., Xu, L., et al. (2016). Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature *537*, 694–697.

Schellenberger, J., Que, R., Fleming, R.M.T., Thiele, I., Orth, J.D., Feist, A.M., Zielinski, D.C., Bordbar, A., Lewis, N.E., Rahmanian, S., et al. (2011). Quantitative prediction of cellular metabolism with constraint-based models: the COBRA Toolbox v2.0. Nat Protoc *6*, 1290–1307.

Shiba, Y., Paradise, E.M., Kirby, J., Ro, D.-K., and Keasling, J.D. (2007). Engineering of the pyruvate dehydrogenase bypass in Saccharomyces cerevisiae for high-level production of isoprenoids. Metab Eng *9*, 160–168.

Smith, J.D., Suresh, S., Schlecht, U., Wu, M., Wagih, O., Peltz, G., Davis, R.W., Steinmetz, L.M., Parts, L., and St Onge, R.P. (2016). Quantitative CRISPR interference screens in yeast identify chemical-genetic interactions and new rules for guide RNA design. Genome Biol *17*, 45.

Stephens, P.E., Darlison, M.G., Lewis, H.M., and Guest, J.R. (1983). The pyruvate dehydrogenase complex of Escherichia coli K12. Nucleotide sequence encoding the dihydrolipoamide acetyltransferase component. Eur J Biochem *133*, 481–489.

APPENDIX A:

Engineering *Saccharomyces cerevisiae* fatty acid composition for increased tolerance to octanoic acid

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*PBL, JF, **RFM**, and NAD designed the study. NAD supervised the project. PBL performed a significant amount of the experimental work, testing octanoic acid toxicity on yeast growth. JF performed a substantial amount of the growth studies. **RFM** contributed the idea that led to the main goal of the paper. **RFM** measured the fatty acid profile of cells. PBL analyzed the data and wrote the manuscript. All authors edited and approved the final manuscript.

ABSTRACT

Biorenewable chemicals such as short and medium chain fatty acids enable functional or direct substitution of petroleum-derived building blocks, allowing reduction of anthropogenic greenhouse gases while meeting market needs of high-demand products like aliphatic alcohols and alpha olefins. However, producing these fatty acids in microorganisms can be challenging due to toxicity issues. Octanoic acid (C8) can disrupt the integrity of the cell membrane in yeast, and exogenous supplementation of oleic acid has been shown to help alleviate this. We recently engineered the *Saccharomyces cerevisiae* enzyme acetyl-CoA carboxylase by replacing serine residue 1157 with alanine to prevent deactivation by phosphorylation. Expression of $Acc1^{S1157A}$ in *S. cerevisiae* resulted in an increase in total fatty acid production, with the largest increase for oleic acid. In this study, we evaluated the effect of this modified lipid profile on C8 toxicity to the yeast. Expression of $Acc1^{S1157A}$ in *S. cerevisiae* BY4741 increased the percentage of oleic acid 3.1-fold and 1.6-fold in the absence and presence of octanoic acid challenge, respectively. Following exposure to 0.9 mM of C8 for 24 h, the engineered yeast had a 10-fold higher cell density relative to the baseline strain. Moreover, overexpressing $Acc1^{S1157A}$ allowed survival at C8 concentrations that were lethal for the baseline strain. This marked reduction of toxicity was shown to be due to higher membrane integrity as an 11-fold decrease in leakage of intracellular magnesium was observed. Due to the increase in oleic acid, this approach has the potential to reduce toxicity of other valuable bioproducts such as shorter chain aliphatic acids and alcohols and other membrane stressors. In an initial screen, increased resistance to *n*-butanol, 2-propanol and hexanoic acid was demonstrated with cell densities 3.2-fold, 1.8-fold, and 29-fold higher than the baseline strain, respectively.

INTRODUCTION

The use of renewable raw materials in lieu of petroleum-based feedstocks has become increasingly important not only for political, energetic, and economic reasons but also due to environmental concerns (Carlson, 2011; Demski et al., 2014; Lopes, 2015; Perlack et al., 2005). Higher levels of greenhouse gases derived from fossil fuel consumption have been related to climate change, affecting sea levels, thermohaline circulation and coral reefs (Liao et al., 2016). Moreover, the extraction process and utilization of fossil fuels can contaminate water and air (Chen et al., 2015). In response to these threats, there is an increasing incentive for the production of biofuels and biobased chemicals (Schwartz et al., 2016). Microbial production of short and medium chain fatty acids, such as octanoic acid, has the potential to functionally replace molecules derived from petroleum for synthesis of aliphatic alcohols (Gunukula et al., 2016) and high-demand monomers for synthesis of polyolefins (Polyolefin Comonomers-World Markets, 2005-2015). However biological production of fatty acids such as octanoic acid (C8) is hindered by the toxic effects on the microbial cell factories (Jarboe et al., 2013; Sandoval and Papoutsakis, 2016).

Inhibition of cell growth by fatty acids is the consequence of multiple factors including triggering of oxidative stress, dysfunctional regulation of internal turgor pressure, lipid peroxidation, membrane trafficking, and disruption of mitochondrial, vacuolar, and plasma membrane organization (Casal et al., 2008; Mira et al., 2010; Piper et al., 2001; Wojtczak and Więckowski, 1999). For octanoic acid, the effects on the plasma membrane are considered the main mechanism of microbial growth inhibition. When the yeast *Saccharomyces cerevisiae* was challenged with octanoic acid, transcriptome analysis showed activation of genes related to regulation of iron and phosphate starvation (Liu et al., 2013). Supplementation of the medium with Fe^{2+}, Fe^{3+} or KH_2PO_4 did not mitigate

the toxicity of C8 and had no impact on cell growth, suggesting that membrane integrity had been compromised, causing leakage of these essential nutrients and triggering the observed starvation transcriptional response (Liu et al., 2013). Cabral et al. (2001) reported that disruption of the *S. cerevisiae* cell membrane organization by octanoic acid dissipated the trans-membrane proton motive force, triggering activation of H^+-ATPase to restore pH homeostasis. The negative effects on the cell membrane are likely due to octanoic acid retention; nearly 80% of the carboxylic acid remains in the cell wall fraction after 3h of exposure, dropping to values between 30% and 50% after 12 h (Borrull et al., 2015).

To cope with membrane-related stresses such as changes in temperature, osmotic stress and exposure to membrane-damaging substances, microorganisms can modulate their membrane composition to maintain function, integrity, stability and fluidity (Arneborg et al., 1995; Bui et al., 2015; Hazel and Eugene Williams, 1990; Prashar et al., 2003; Rodríguez-Vargas et al., 2007; You et al., 2003). In general, an increase in saturated fatty acids promotes membrane stability to solvents and high temperatures due to the establishment of more van der Waals interactions, while introduction of a *cis*-unsaturation impacts membrane fluidity. Longer chain length can provide higher stability and rigidity (Sandoval and Papoutsakis, 2016). Adding exogenous oleic acid (C18:1) to the media was shown to mitigate the toxic effects of octanoic acid (Liu et al., 2013) and acetaldehyde (Matsufuji et al., 2008) on the yeast cells. Expressing the enzymes insect acyl coenzyme A Δ9 Z-desaturase (TniNPVE) and rat elongase 2 (rELO2) increased the fraction of oleic acid from 14.9% to 32% and from 29% to 44%, respectively (Yazawa et al., 2011; You et al., 2003). This resulted in improved tolerance when cells were challenged with ethanol, *n*-butanol, *n*-propanol, and 2-propanol. However, in the study by Liu et al. (2013), expression of TniNPVE failed to increase oleic acid levels enough to provide tolerance against octanoic acid. In two recently published studies, overexpression of the native Δ9-

fatty acid desaturase Ole1 increased the fraction of oleic acid from 23% to approximately 30%, and increased membrane stability (Fang et al., 2017; Nasutution et al., 2017). Based on the changes in fatty acid composition, it was hypothesized that modification of membrane rigidity activates the cell's stress response mechanism leading to increased proton efflux, diminished membrane damage and lower levels of ROS (Nasutution et al., 2017). Higher oleic acid levels were also found to ameliorate cadmium-related stresses by promoting cytoplasmatic membrane stability via inhibition of lipid peroxidation (Fang et al., 2017). These studies demonstrate that modulation of fatty acid composition results in increased membrane stability.

Our laboratory recently engineered the *S. cerevisiae* acetyl-CoA carboxylase enzyme to prevent Snf1-mediated deactivation, and thus increase the synthesis of polyketides following glucose depletion (Choi and Da Silva, 2014). This entailed identifying a critical serine residue (1157) and replacing it with alanine ($Acc1^{S1157A}$). In addition to improving polyketide titers, this strain showed a 3-fold increase in production of total fatty acids. Interestingly oleic acid was the predominant acyl chain with levels 7.3-fold higher than the baseline strain (representing a 2.3-fold increase in the percentage of oleic acid). In the current study, we evaluated how the increased proportion of oleic acid due to expression of $Acc1^{S1157A}$ mitigates the toxic effects of octanoic acid. The fatty acid profiles of the baseline and engineered strain in the presence and absence of octanoic acid were measured. We then determined the viability of the cells at increasing concentrations of C8, and studied the effects of the increased synthesis of oleic acid on membrane integrity upon exposure to lethal C8 concentrations. This is the first report where endogenous synthesis of oleic acid was able to provide tolerance against octanoic acid. The results will be important for increasing titers of octanoic acid produced in *S. cerevisiae* (Leber and Da Silva, 2014). The increased tolerance to *n*-butanol, 2-

propanol, and hexanoic acid observed demonstrates potential application when producing other toxic biobased chemicals that disrupt the yeast membrane.

MATERIALS AND METHODS

Yeast and bacterial strains

Saccharomyces cerevisiae BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)* (Brachmann et al., 1998) was used as the base yeast strain. A copy of the gene for $Acc1^{S1157A}$ (acetyl-CoA carboxylase with the mutation S1157A) under transcriptional control of the *S. cerevisiae PGK1* promoter and *CYC1* terminator, was integrated into the genome as described by Choi and Da Silva (2014). This new strain is designated BY4741-ACC1m.

Media and cultivation

Cells were inoculated from -80°C stock and grown in 5 ml of YPD non-selective complex medium (20 g/L dextrose, 20 g/L peptone, 10 g/L yeast extract) for 14 to 16 h. The cells were then transferred (initial OD_{600}=0.05) to 5 ml of SDC-A,U medium (20 g/L dextrose, 5 g/L casamino acids, 5 g/L ammonium sulfate, 1.7 g/L yeast nitrogen base without amino acids, 50 mg/L adenine, 20 mg/L uracil) and incubated for 10-12 h. This culture was used as the inoculum for the subsequent tube and flask cultures (initial OD_{600}=0.05) in SDC-A,U supplemented with either octanoic acid (from a 100 mM stock in 70 % ethanol, as previously described (Liu et al., 2013)) or an equivalent amount of 70 % ethanol for the control cultures. Maximum concentration of ethanol did not exceed 1% (v/v), a concentration at which there is no detectable effect on yeast growth (Cabral et al., 2001). For all conditions studied, media was buffered using 100 mM MES (2-(4-Morpholino)ethane Sulfonic Acid) and pH was adjusted to 5.5 before inoculation in order to prevent the effect of pH variations due to addition of octanoic acid. All cells were cultivated at 30°C in a 250 rpm agitated shaker, and growth was monitored by optical density measurements at 600 nm in a Shimadzu UV-2450 spectrophotometer (Columbia, MD). For the other stressors, buffered media was supplemented with

hexanoic or decanoic acid using 2 M and 100 mM stock solutions in 70% ethanol, respectively. Ethanol, *n*-butanol and 2-propanol were directly supplemented to the media.

Determination of octanoic acid Minimal Inhibitory Concentration (MIC) was performed using the broth macrodilution method described by Narendranath et al. (2001). Biological triplicates were exposed to concentrations of 3 mM, 2.5 mM, 1.5 mM, 1.25 mM and 0.75 mM at pH=5.5. The octanoic acid stock in 70% ethanol was prepared at 300 mM to maintain final ethanol concentration below 1%. MIC was defined as the smallest concentration of acid that inhibited growth for a period of 72 h.

Fatty acid extraction analysis

Yeast cells were grown in 250 ml of media buffered with 100 mM MES in the absence or presence of 0.7 mM and 1.5 mM octanoic acid at pH=5.50. At OD_{600}=1, the cells were harvested by centrifugation (2000g, 4°C, 10 min) to obtain the same cell mass for each culture. Fatty acid extraction, methylation, and quantification via GC-MS were performed as described by Leber et al. (2015) using nonadecanoic acid as an internal standard. Fatty acid percentages are given on a mass basis.

Membrane leakage quantification

The membrane leakage protocol was based on previously described work by Prashar et al. (2003) and Liu et al. (2013). Cells were grown in 25 ml of SDC-A,U medium in 150 mL Erlenmeyer flasks at 30°C and 250 rpm. When OD_{600} reached 1.0, cells were harvested by centrifugation and washed twice with NaCl solution (0.9% w/v, pH=5.5). The cells were then resuspended in 4 ml of the same NaCl solution and supplemented with either 1 ml of a 7.5 mM octanoic acid solution (prepared from the 100 mM octanoic acid stock solution in 70% ethanol) to achieve a final concentration of 1.5 mM while maintaining pH at 5.5, or the equivalent amount of ethanol solution (for the controls). In

all cases, ethanol concentration was kept at <1% (v/v). After incubation in the presence of the acid for 25 min at 30°C, the cells were centrifuged at 17,000g for 5 min at 4°C and the magnesium content of the supernatant was determined via fluorescence using the cell impermeable pentapotassium salt of the molecular probe Magnesium Green™ (M3733, ThermoFisher Scientific, Eugene, OR). Fluorescence at λ_{em}=531 nm was measured by excitation at λ_{ex}=506 nm in Costar Black 384-well or Nunc 96-well plates using a fluorometer with temperature control at 25°C (SpectraMax M2, Molecular Devices, Sunnyvale, CA). The concentration of magnesium was determined using the equation below (Szmacinski and Lakowicz, 1996) by running standards for calculation of K_D and F_{max} at each experimental condition (0.9% NaCl pH=5.50 with and without 1.5 mM octanoic acid). Reported values correspond to the difference in measured Mg^{2+} concentration per cell between exposed and unexposed, as previously reported by Prashar et al. (2003). Mg^{2+} concentration was determined by the change in fluorescence observed at t=25 min with respect to t=0 min upon exposure to octanoic acid. A minimum of 6 biological replicates (with 3 readings per replicate) were performed for each condition.

$$[A] = K_D \frac{F - F_{min}}{F_{max} - F}$$

Statistical analysis

Statistical analysis consisted of either t-test or a two-way ANOVA followed by t-test using Bonferroni corrections to account for multiple comparisons (Rice, 1989).

RESULTS AND DISCUSSION

Effects of expression of Acc1 mutant on cell lipid composition

The enzyme Acc1 catalyzes the conversion of acetyl-CoA to malonyl-CoA, a key step in the synthesis of polyketides and fatty acids. In prior work, we engineered the yeast Acc1 to prevent deactivation via phosphorylation of the serine residue 1157 (Choi and Da Silva, 2014). The prolonged and higher activity of the $Acc1^{S1157A}$ enzyme led to approximately 3-fold increases in both the polyketide 6-methylsalicylic acid and native fatty acids, with the most dramatic change in fatty acid composition observed for oleic acid. We thus integrated a copy of this mutant *ACC1* gene into *S. cerevisiae* strain BY4741 (BY4741-ACC1m) and the fatty acid composition was compared to the original baseline strain.

Strains BY4741 and BY4741-ACC1m were cultivated in buffered SDC-AU medium, and collected in early exponential phase. Intracellular fatty acid levels were then measured via GC-MS following methylation. The most abundant fatty acids in *S. cerevisiae* are palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0) and oleic (C18:1) acids, comprising more than 96% of total fatty acids (Augustyn, 1989; Liu et al., 2013; Prashar et al., 2003). The fatty acid profile of the baseline BY4741 strain was consistent with these reports, with 57% palmitoleic acid, 15% palmitic acid, 26% oleic acid and 2% stearic acid (Figure A.1-A). The engineered BY4741-ACC1m strain had a significantly different lipid profile, consisting of 8% palmitoleic acid, 3% palmitic acid, 79% oleic acid and 10% stearic acid. Comparison of the two shows a 3.1-fold increase in the oleic acid fraction for strain BY4741-ACC1m, with a simultaneous 7.6-fold decrease in the palmitoleic acid fraction. This trend is also observed for saturated palmitic and stearic acids, where C16 fraction decreases while C18 increases. The increase in the percentage of oleic acid was significantly greater than that observed for the strain used in our

initial Acc1^{S1157A} study. Overall, the changes in fatty acid composition resulted in a 7.8% increase in the average length of the fatty acid chains (Figure A.1-B). This has been linked to increased stability and rigidity of the membrane in bacteria (Sandoval and Papoutsakis, 2016) and has been reported in octanoic-acid adapted *S. cerevisiae* cells (Liu et al., 2013). This increase in average lipid length was accompanied by an increase in total fatty acid content, as previously observed (Choi and Da Silva, 2014).

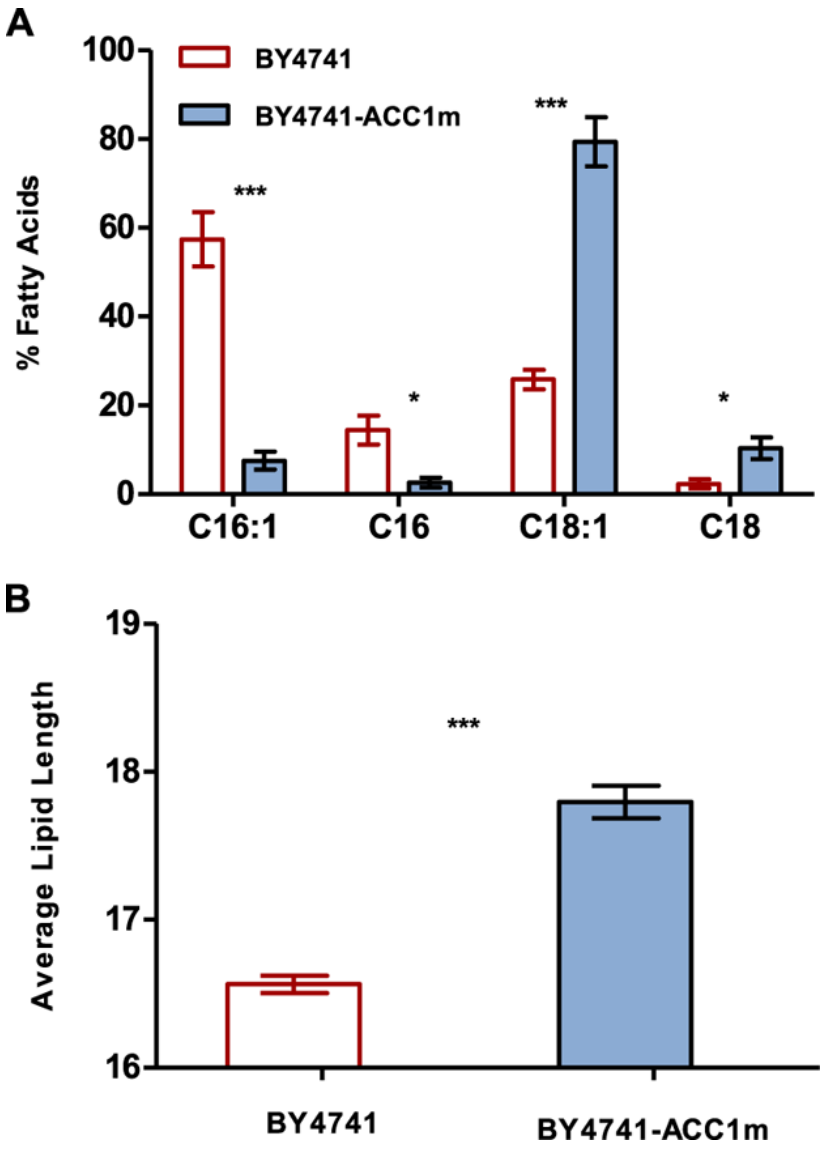

Figure A.1. Fatty acid profiles. (A) Fatty acid percentages of C16:1, C16, C18:1 and C18 for *S. cerevisiae* strains BY4741 (open bars) and BY4741-ACC1m (filled bars) and (B) average lipid length of both strains. Results show average and SEM of biological triplicates (*p <0.05 and ***p <0.001, relative to baseline strain).

Longer average fatty acid chain length has also been observed in other studies expressing $Acc1^{S1157A}$. Hofbauer et al. (2014) showed that a higher activity resulting from the serine mutation leads to an increased production of malonyl-CoA and higher ratio of C18 to C16 fatty acids. Interestingly, when the Acc1-specific inhibitor Soraphen A was added, this trend was reversed. Moreover, Zhou et. al (2016) observed increased synthesis of longer chain fatty acids when $Acc1^{S1157A,S659A}$ was overexpressed. In *in vitro* studies on the *S. cerevisiae* fatty acid synthase (Hori et al., 1987), fatty acids with longer average lipid lengths were obtained when acetyl-CoA is limiting and there is an excess of malonyl-CoA.

Resistance against octanoic acid toxicity

Supplementation of oleic acid to the media has been reported by Liu et al. (2013) to alleviate octanoic acid toxicity in *S. cerevisiae* by incorporation into the cell's membrane as a defense mechanism against this membrane-damaging compound. However, engineering the yeast to increase endogenous oleic acid levels (by expressing TniNPVE) only increased the C18:1 fraction by 9%, insufficient to mitigate membrane damage. Our strain BY4741-ACC1m expressing the modified Acc1 enzyme substantially altered the fatty acid profile in the cell and increased the percentage of oleic acid by more than 3-fold to 79% percent of total fatty acids. Thus, we examined whether this increase is able to provide resistance against octanoic acid toxicity.

Strains BY4741-ACC1m and BY4741 (control) were cultured in buffered SDC-A,U medium supplemented with increasing concentrations of octanoic acid. Cell optical density (OD) was measured at 24 h and 36 h (Figure A.2). At 24 h, the greatest difference in cell growth was observed in the medium containing 0.9 mM C8, with a 10-fold higher OD when $Acc1^{S1157A}$ was expressed. The concentration of 0.7 mM C8 was not completely inhibitory for the base strain, and a 3.2-fold increase

in cell growth was observed for the engineered BY4741-ACC1m. The highest concentration screened (1.5 mM C8) showed similar inhibitory effects for both strains, with no significant difference in growth. At 36 h (Figure A.2-B), the greatest difference in cell density was observed at 1.2 mM C8, with a 19-fold improvement for the strain expressing $Acc1^{S1157A}$. At the highest concentration screened (1.5 mM), cell density was augmented 3.7-fold.

These results demonstrate that the strain expressing $Acc1^{S1157A}$ can survive at concentrations up to 1.5 mM (Figure A.2-C), while the maximum tolerance of the baseline strain is 0.7-0.9 mM. Exposing the cells to higher concentration of octanoic acid allowed determination of a Minimal Inhibitory Concentration (MIC). The resulting MIC for the baseline strain was 1.25 mM, while for the engineered strain it was 2.5 mM (where MIC was defined as the smallest concentration of acid that inhibited growth for a period of 72 h). Liu *et. al* (2013) reported growth rates lower than 0.025 h^{-1} at 0.8 mM and 1 mM for BY4741, an observation that is consistent with these results. Moreover, Figure A.2 shows that expression of $Acc1^{S1157A}$ does not have a detrimental effect on cell growth, with cell density of the engineered strain only 10% lower than the base strain at 24h in C8-free medium and with no statistically significant difference at 36 h. This indicates that there is no significant metabolic burden with our approach.

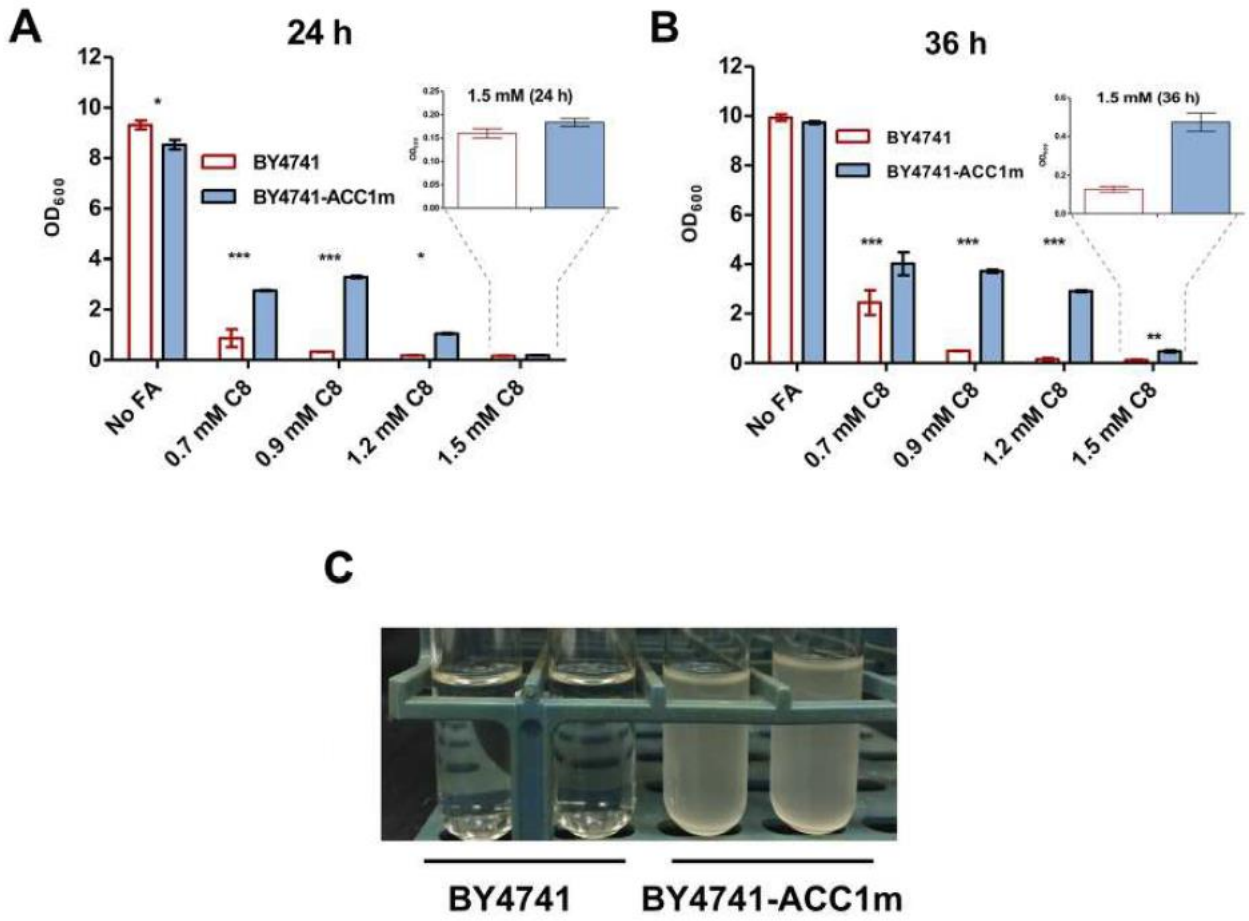

Figure A.2. Effect of 0.7 mM, 0.9 mM, 1.2 mM and 1.5 mM octanoic acid on cell density (OD_{600}) of BY4741 (open bars) and BY4741-ACC1m (filled bars) at (A) 24 h and (B) 36 h. Results represent average and SEM of three biological replicates. C) Tube cultures (after 80 h) of BY4741 and BY4741-ACC1m exposed to 1.5 mM octanoic acid (**p* <0.05, ***p* <0.01, ****p* <0.001, relative to baseline strain).

Fatty acid composition and cell growth for cells exposed to octanoic acid

Expression of the enzyme $Acc1^{S1157A}$ resulted in a 3.1-fold increase in the percentage of oleic acid relative to the baseline strain in the absence of an octanoic challenge (Figure A.1-A). It has been reported that *S. cerevisiae* modifies its membrane composition upon exposure to octanoic acid. Thus, we compared the lipid profile and cell growth rate for BY4741 and BY4741-ACC1m in the presence of octanoic acid. We chose the level of supplemented C8 to have a significant effect on cell survival, but still allow enough growth for sufficient cell harvesting for fatty acid analysis. Using this rationale, we

exposed the cells to 0.7 mM octanoic acid. Three independent colonies of the baseline and engineered strains were grown in buffered SDC-A,U medium containing 0.7 mM octanoic acid. Cells were collected by centrifugation in early exponential phase, intracellular fatty acids were extracted and methylated, and fatty acid content was analyzed via GC-MS.

The fatty acid profiles in the presence of 0.7 mM octanoic acid are shown in Figure A.3-A. Comparison of the two strains shows that the C18:1 fraction is 1.6-fold higher in BY4741-ACC1m, while the C16:1 fraction is 5.6-fold greater in BY4741. For saturated fatty acids, the percentage of C18 is 9.6 times higher in the engineered strain, while differences in C16 are not significant. A comparison of Figure A.3-A with Figure A.1-A shows that for the base strain BY4741, upon exposure to 0.7 mM of octanoic acid, the percentage of palmitoleic acid decreased by 17% ($p<0.01$), while the oleic acid increased by 14% ($p<0.01$), reaching 40% of total fatty acids. Changes in individual saturated fatty acid fractions were not significant. This is in accordance with Liu *et. al* (2013), where cells pre-exposed to 0.8 mM octanoic acid and then challenged again with 0.8 mM C8 showed a significant drop in palmitoleic content, while oleic acid levels increased. These adapted cells showed improved membrane integrity, indicating that this change in membrane composition could be an adaptation mechanism to cope with membrane-associated octanoic acid stress. For the engineered strain BY4741-ACC1m, there is a 15% reduction in the fraction of oleic acid upon exposure ($p<0.001$) and a higher proportion of palmitic and stearic acids, shifting from 2% to 11% and from 10% to 18% ($p<0.05$) respectively.

We measured growth rates for the two strains to assess the effect of the changes in membrane composition on cell resistance to C8 toxicity. In the absence of C8, cell growth rate for BY4741-ACC1m was only 7% lower than that for BY4741, indicating that there is no significant metabolic burden associated with expression of $Acc1^{S1157A}$ (Figure A.3-B). During cultivation in the

presence of 0.7 mM C8, growth rate was 84% higher for the engineered strain, demonstrating increased tolerance to the octanoic acid.

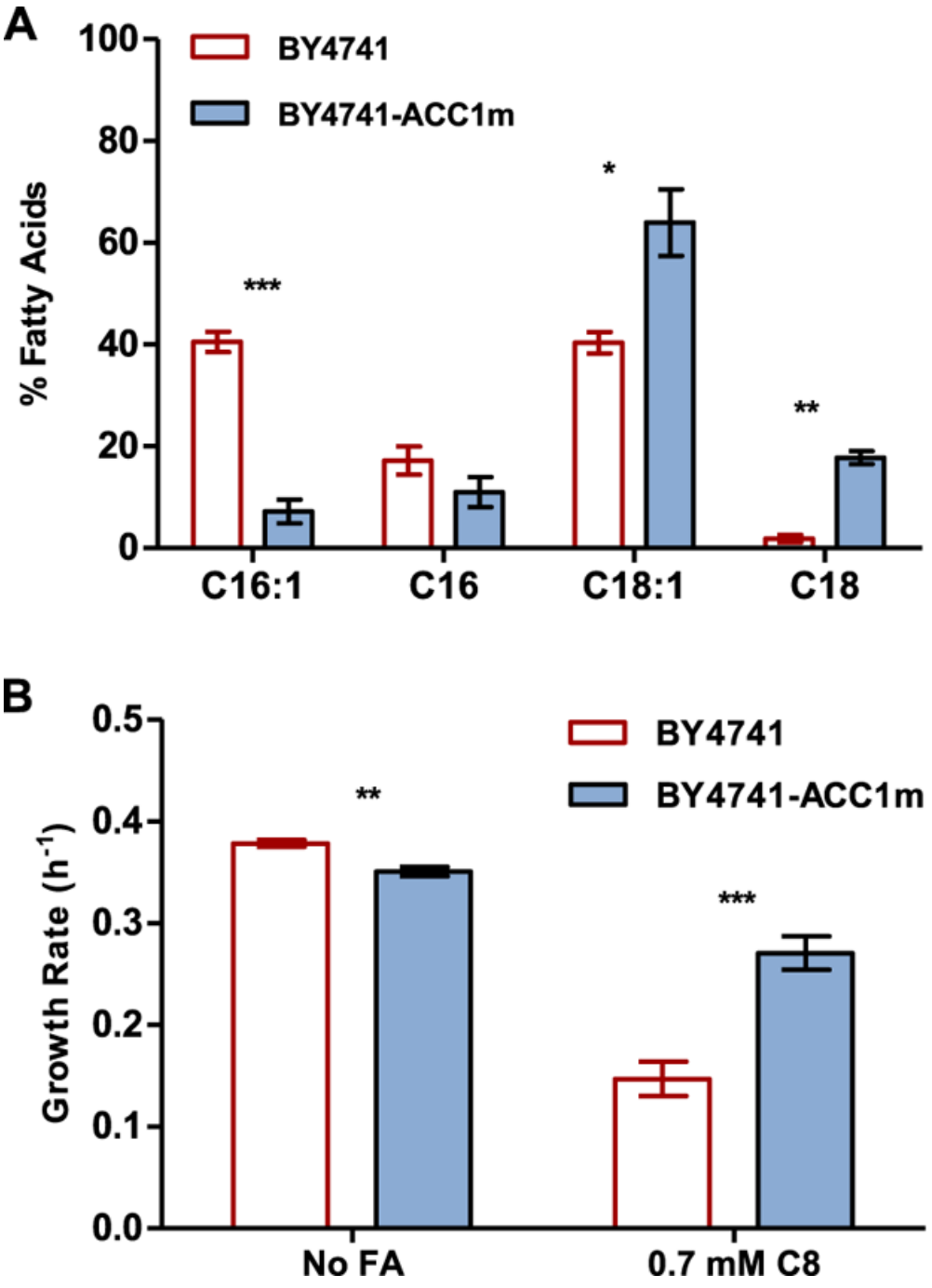

Figure A.3. Fatty acid percentages and growth rates for BY4741 and BY4741-ACC1m in the presence of 0.7 mM octanoic acid. (A) Fatty acid fractions in early exponential phase and (B) Cell growth rates for BY4741 and BY4741-ACC1m in the presence of 0.7 mM octanoic acid. Results show average for biological replicates (n=3 for fatty acid profile and n=4 for growth rate). Error bars represent SEM (*p <0.05, **p <0.01, ***p <0.001, relative to baseline strain).

As seen in Figure A.2, cells expressing Acc1^{S1157A} were able to grow (albeit poorly) in octanoic acid at concentrations of up to 1.5 mM. To determine whether there is further modification of cell lipid composition at increasing levels of C8, BY4741-ACC1m were grown in the presence of 1.5 mM octanoic acid and the fatty acid profile was analyzed (Figure A.4). Membrane composition remained constant with increasing C8 concentration. This finding, along with the decreased viability of cells at 1.5 mM C8, shows that our approach to control the homeoviscous response to counteract membrane damage is limited, processes such as oxidative stress (Legras et al., 2010) are simultaneously taking place and inducing octanoic acid toxicity.

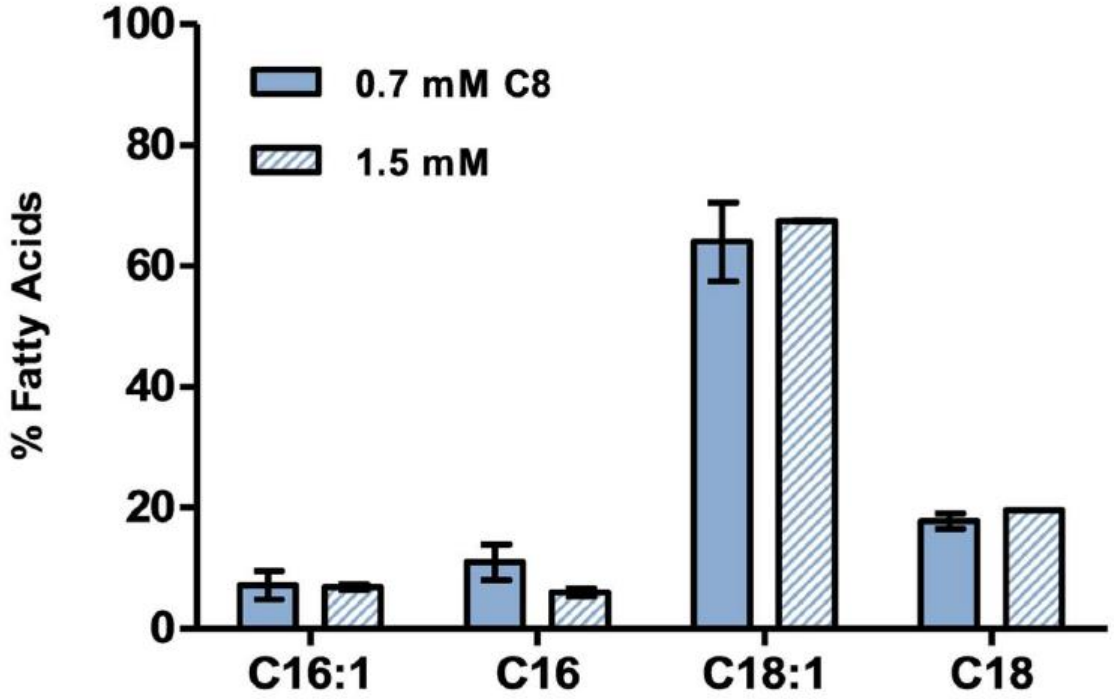

Figure A.4. Fatty acid composition of BY4741-ACC1m at OD_{600}=1 during cultivation in medium containing 0.7 mM C8 (n=3 biological replicates, filled) and 1.5 mM C8 (n=2, hatched bars). Error bars represent SEM.

Analysis of changes in average lipid length and percentage of saturated fatty acids

The biophysical properties of the membrane bilayer depend on the structure of the fatty acid chains (Zhang and Rock, 2008) and their interactions (Marguet et al., 2006), and analysis of average lipid length and percentage of saturated fatty acids provides a general assessment of membrane properties. Our experiments show that the average lipid length of the baseline strain increased 1.6% when grown in 0.7 mM octanoic acid, shifting from 16.5 to 16.8 (Figure A.5-A). Changes in BY4741 are in accordance with Liu *et al.* (2013), where increasing doses of octanoic acid resulted in increased average lipid length, indicating that this is one of the cell's mechanisms to cope with C8 stress. In contrast, no significant change was observed for BY4741-ACC1m in the C8-containing medium; a higher average lipid length of 17.6 was observed in both media relative to the baseline strain.

Exposure to octanoic acid also resulted in no significant change in the percentage of saturated fatty acids for BY4741 (Figure A.5-B). However, addition of 0.7 mM C8 increased the proportion of saturated acyl chains by 16% in BY4741-ACC1m. This was due to an increase in levels of saturated stearic acid and palmitic acid with a simultaneous decrease in oleic acid (palmitoleic acid levels remain constant). In the absence of the octanoic acid, the engineered strain had 3.8% less saturated fatty acids than the baseline, while exposure to C8 reversed this trend, making the saturated content 9.7% higher for BY4741-ACC1 relative to BY4741.

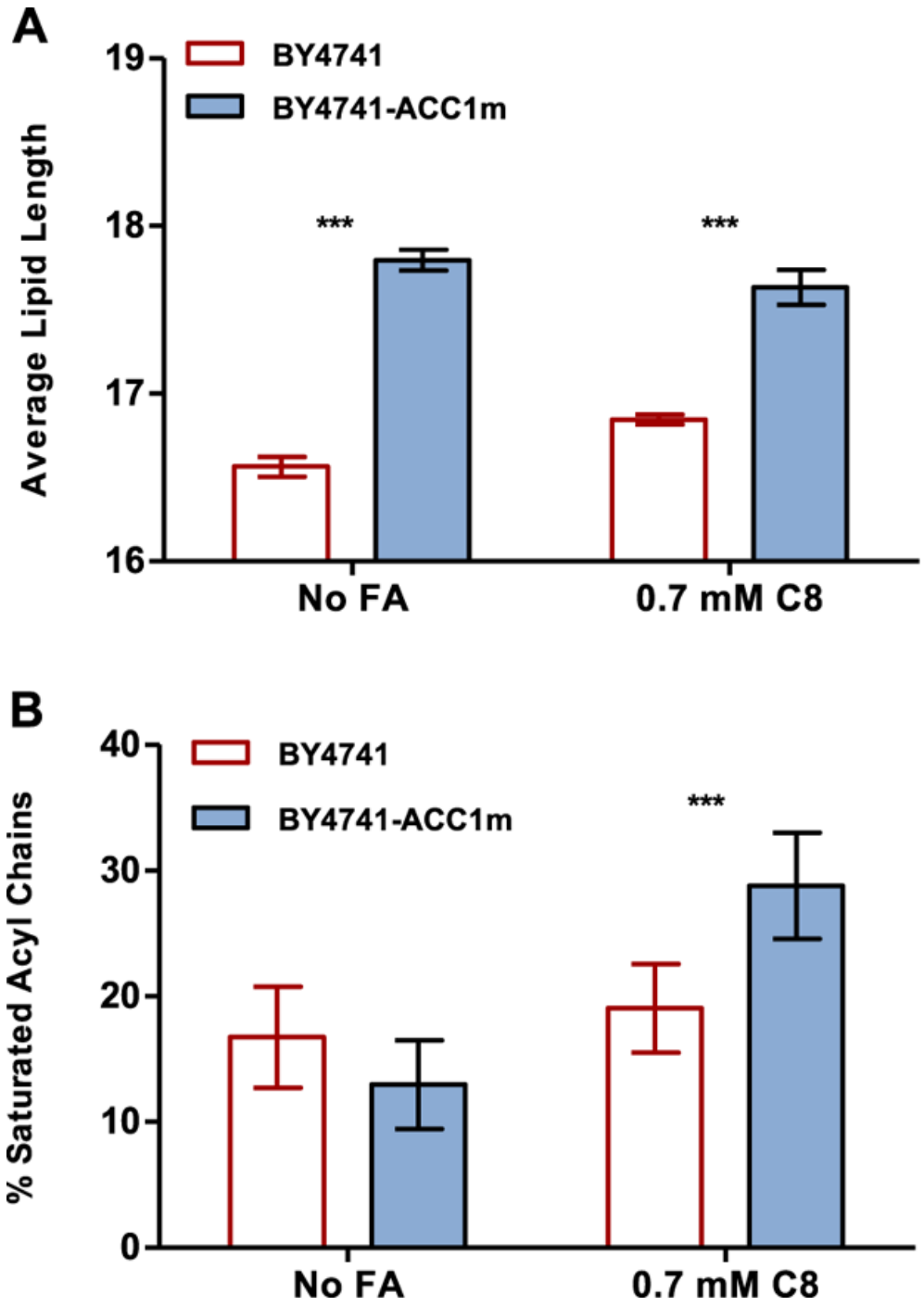

Figure A.5. Average lipid length and percent of saturated fatty acid chains in absence and presence of 0.7 mM octanoic acid for *S. cerevisiae* strains BY4741 (open bars) and BY4741-ACC1m (filled bars). Results show average and SEM of biological triplicates (**p <0.01, ***p <0.001, relative to baseline strain).

Overall, overexpression of Acc1^{S1157A} increased average lipid length and percent of saturated fatty acids upon exposure to 0.7 mM of octanoic acid. Generally, short acyl chains provide higher fluidity to the membrane, while an increasing proportion of longer chains results in stability and rigidity by increasing the tendency of the aliphatic tails to interact with each other (Sandoval and Papoutsakis, 2016). Introduction of a *cis*-unsaturation results in local decrease of membrane order at the vicinity of the double bond. This decrease in order is accompanied by the insertion of a rigid element that diminishes the range of conformations available to an otherwise flexible chain, resulting in a bend in the aliphatic chain (Hazel and Williams, 1990; Marguet et al., 2006). In contrast, saturated fatty acids tend to form ordered structures with low permeability by packing tightly together through van der Waals interactions (Sandoval and Papoutsakis, 2016; Zhang and Rock, 2008). Therefore, an increase in the proportion of saturated fatty acid is expected to lead to higher stability and lower permeability of the membrane in the presence of octanoic acid. Based on the increased membrane stability and lower permeability derived from the combination of these changes, we speculate that they are responsible for mitigation of octanoic acid toxicity in the present approach. This is attributable not only to higher levels of oleic acid but also stearic acid. Increased stearic acid levels in *S. cerevisiae* have been observed in previous studies that aimed to increase oleic acid levels to cope with membrane stress caused by with aliphatic alcohols (Yazawa et al., 2011; You et al., 2003), as well as exposure to 0.5 mM and 0.8 mM octanoic acid (Liu et al., 2013) and 35 µM of decanoic acid (Alexandre et al., 1996). However, it is important to note that this is a simplified analysis of lipid composition effects on the cell that does not consider the impact of lipids on membrane proteins, the presence of microdomains on cell membrane organization and dynamics (Marguet et al., 2006; Rest et al., 1995), as well as other regulatory mechanisms involved in the cell response to stress (Fang et al., 2017; Legras et al., 2010; Nasutution et al., 2017).

Analysis of membrane integrity

In prior work, octanoic aid was shown to increase membrane permeability (Liu et al., 2013) due to its retention in the cell membrane (Borrull et al., 2015). Liu et al. (2013) used magnesium leakage as a measure of membrane permeability. Magnesium plays an essential role in many physiological cell functions such as cell growth, cell division, enzyme activity, repression of stress protein biosynthesis and stabilization of the plasma membrane via interaction with phospholipids (Birch and Walker, 2000). It is thus a suitable model ion to evaluate membrane integrity.

To investigate whether the increased proportion of oleic acid resulting from $Acc1^{S1157A}$ expression reduces membrane permeability, we performed a magnesium leakage assay. Strains BY4741 and BY4741-ACC1m were cultivated in SDC-AU medium to OD_{600}=1, and then exposed to 1.5 mM octanoic acid for 25 minutes at 30°C. Extracellular magnesium was then measured via fluorescence by addition of a molecular probe. Reported values correspond to the difference in measured magnesium concentration between exposed and unexposed conditions, as previously reported (Liu et al., 2013; Prashar et al., 2003) and normalized by cell density prior to exposure. As seen in Figure A.6, the strain expressing $Acc1^{S1157A}$ had an 11-fold decrease in extracellular magnesium concentration relative to baseline BY4741. This result supports that the observed changes in fatty acid profile are linked to improved membrane integrity, and thus reduced C8 toxicity.

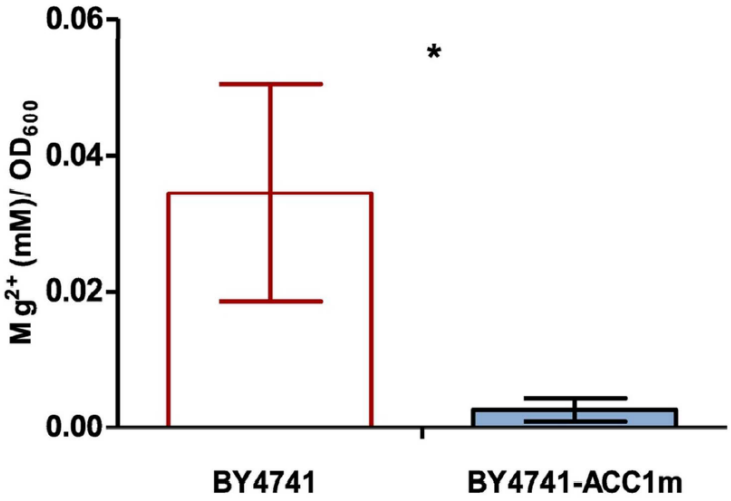

Figure A.6. Concentration of extracellular magnesium (mM Mg^{2+} per OD_{600}) for BY4741 (n=6, open bar) and BY4741-ACC1m (n=6, filled bar) after exposure to 1.5 mM octanoic acid. Error bars represent SEM (*p <0.05, relative to baseline strain).

Resistance against other stressors

Increased synthesis of oleic acid has been associated with enhanced tolerance to short-chain alcohols such as ethanol, *n*-butanol and 2-propanol (Yazawa et al., 2011; You et al., 2003). This increase has also been observed as a response to exogenous addition of decanoic acid in complex media, where oleic acid levels shifted from 15.8% to 44.1% (Alexandre et al., 1996). Moreover, early studies have shown that exogenous addition of oleic acid partially alleviated toxicity of hexanoic acid (Nordström, 1964).

To determine whether the increase in oleic acid observed due to the $Acc1^{S1157A}$ mutant is capable of alleviating toxicity of these stressors, strains BY4741-ACC1m and BY4741 (control) were cultured in buffered SDC-A,U medium supplemented with either 8.75 mM hexanoic acid (C6), 0.3 mM decanoic acid (C10), 8% ethanol, 1.3% *n*-butanol, or 6% 2-propanol. Alcohol concentrations and time intervals were chosen based on values reported by Yazawa et al. (2011). Carboxylic acid

concentrations were determined by screening and selecting the most inhibitory concentrations at 24 h and 48 h.

Following cultivation for 48h in the presence of the various stressors, cell density (OD) was measured and compared for BY4741-ACC1m and BY4741 (Figure A.7). Expression of $Acc1^{S1157A}$ and the higher oleic acid content in the membrane increased resistance to hexanoic acid, 2-propanol, and *n*-butanol. This resulted in cell densities 29-fold, 1.8-fold, and 3.2-fold higher than the baseline strain, respectively. The results for octanoic acid at 1.25mM (MIC) are shown for comparison. That no effect was observed for decanoic acid is likely a consequence of the different mechanisms that yeast employs to overcome octanoic and decanoic acids stress. While decanoic acid penetrates the cell and is esterified, octanoic acid remains in the membrane (Borrull et al., 2015; Legras et al., 2010). Interestingly, there was no improvement in ethanol resistance and significant drops in cell growth were observed at 24 h and 36 h; this contrasts with prior reports in the literature at lower oleic acid levels (Yazawa et al., 2011; You et al., 2003; Zheng et al., 2013). Even so, the improved resistance to hexanoic acid, octanoic acid, 2-propanol, and *n*-butanol demonstrates the general applicability of increasing oleic acid content via $Acc1^{S1157A}$ expression.

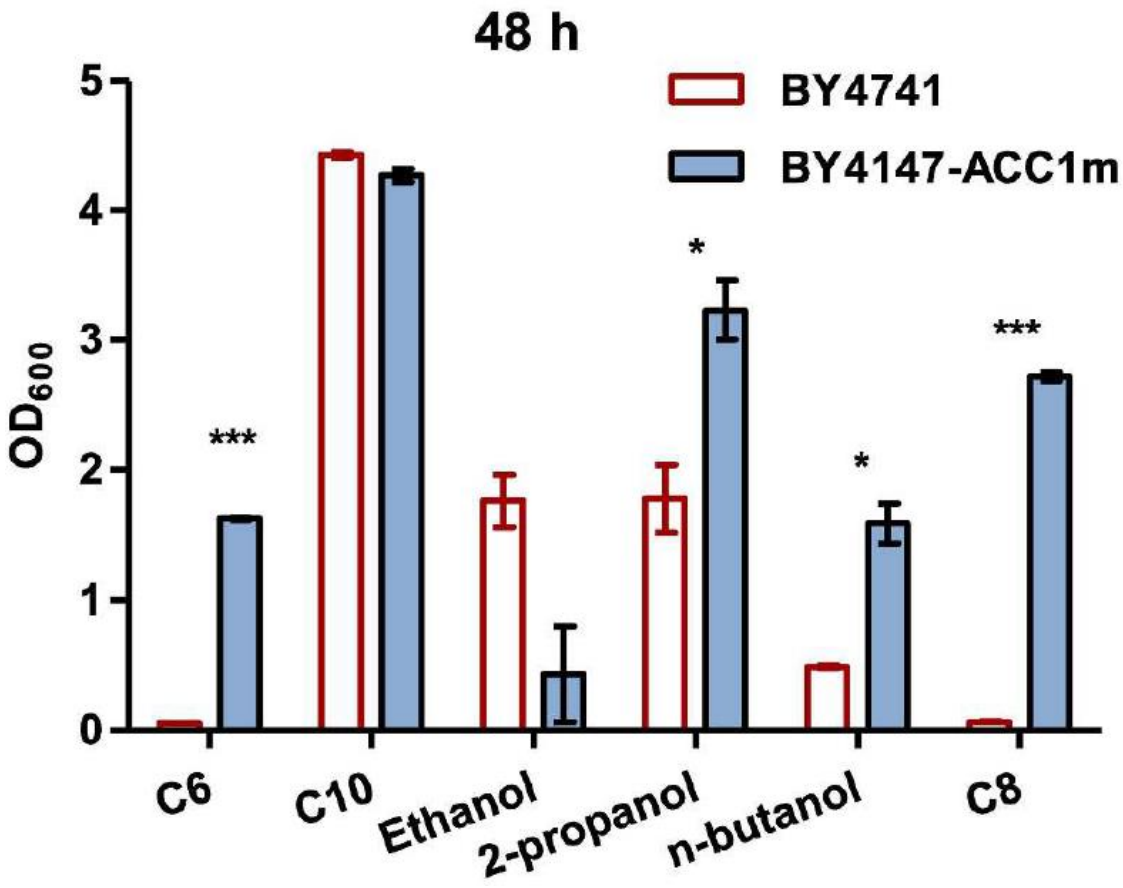

Figure 7. Cell density (OD_{600}) at 48 h for baseline strain (BY4741) and engineered strain (BY4741-ACC1m) upon exposure to 8.75 mM hexanoic acid (C6), 0.3 mM decanoic acid (C10), 8% ethanol, 6% 2-propanol and 1.3% *n*-butanol. The results for 1.25 mM octanoic acid (C8) have been included for comparison. Results show average and SEM of biological duplicates (*p <0.05, *** p <0.001, relative to baseline strain).

CONCLUSIONS

We have ameliorated the toxicity of octanoic acid on *Saccharomyces cerevisiae* by increasing the fraction of oleic acid through expression of the $Acc1^{S1157A}$ mutant of the native acetyl-CoA carboxylase. To our knowledge, this is the highest fraction of oleic acid (79 % without C8 challenge, 64 % with C8 challenge) reported to date. The engineered strain showed an optical density 19-fold higher upon exposure to 1.2 mM octanoic acid for 36 h and an increase in growth rate of 84% when cells were exposed to 0.7 mM C8. The minimal inhibitory concentration was 2.5 mM for the engineered strain and 1.25 mM for the baseline strain. We attribute this decrease in toxicity to changes in membrane composition, as we observed higher average lipid chain length and percentage of saturated fatty acids in the $Acc1^{S1157A}$ expressing cells exposed to 0.7 mM C8, indicators of increased membrane stability and lowered permeability. These changes resulted in enhanced membrane integrity, based on the 11-fold decrease in magnesium leakage observed. We have also shown that our approach can be applicable to other stressors and increases the tolerance to hexanoic acid, *n*-butanol and 2-propanol.

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REFERENCES

Alexandre H, Mathieu B, Charpentier C. 1996. Alteration in membrane fluidity and lipid composition, and modulation of H+-ATPase activity in Saccharomyces cerevisiae caused by decanoic acid. *Microbiology* **142**:469–475.

Arneborg N, Hoy CE, Jorgensen OB. 1995. The effect of ethanol and specific growth rate on the lipid content and composition of Saccharomyces cerevisiae grown anaerobically in a chemostat. *Yeast* **11**:953–959.

Augustyn OPH. 1989. Differentiation between Yeast Species, and Strains within a Species, by Cellular Fatty Acid Analysis. Saccharomyces cerevisiae. *South African J. Enol. Vitic.* **10**:8–17.

Birch RM, Walker GM. 2000. Influence of magnesium ions on heat shock and ethanol stress responses of Saccharomyces cerevisiae. *Enzyme Microb. Technol.* **26**:678–687.

Borrull A, López-Martínez G, Poblet M, Cordero-Otero R, Rozès N. 2015. New insights into the toxicity mechanism of octanoic and decanoic acids on Saccharomyces cerevisiae. *Yeast* **32**:451–460.

Brachmann CB, Davies A, Cost GJ, Caputo E. 1998. Designer Deletion Strains derived from Saccharomyces cerevisiae S288C : a Useful set of Strains and Plasmids for PCR-mediated Gene Disruption and Other Applications. *Yeast* **14**:115–132.

Bui LM, Lee JY, Geraldi A, Rahman Z, Lee JH, Kim SC. 2015. Improved n-butanol tolerance in Escherichia coli by controlling membrane related functions. *J. Biotechnol.* **204**:33–44.

Cabral MG, Viegas C a., Sá-Correia I. 2001. Mechanisms underlying the acquisition of resistance to octanoic-acid-induced-death following exposure of Saccharomyces cerevisiae to mild stress imposed by octanoic acid or ethanol. *Arch. Microbiol.* **175**:301–307.

Carlson WB. 2011. The Modeling of World Oil Production Using Sigmoidal Functions—Update 2010. *Energy Sources, Part B Econ. Planning, Policy* **6**:178–186.

Casal M, Paiva S, Queirós O, Soares-Silva I. 2008. Transport of carboxylic acids in yeasts. *FEMS Microbiol. Rev.* **32**:974–994.

Chen M, Smith PM, Wolcott MP. 2015. U . S . Biofuels Industry : A Critical Review of Opportunities and Challenges. *Bioprod. Bussinessiness* **2025**:42–57.

Choi JW, Da Silva NA. 2014. Improving polyketide and fatty acid synthesis by engineering of the yeast acetyl-CoA carboxylase. *J. Biotechnol.* **187**:56–59..

Demski C, Poortinga W, Pidgeon N. 2014. Exploring public perceptions of energy security risks in the UK. *Energy Policy* **66**:369–378.

Fang Z, Chen Z, Wang S, Shi P, Shen Y, Zhang Y, Xiao J, Huang Z. 2017. Overexpression of OLE1 enhances cytoplasmic membrane stability and confers resistance to cadmium in Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* **83**:e02319-16.

Gunukula S, Keeling PL, Anex R. 2016. Risk advantages of platform technologies for biorenewable

chemical production. *Chem. Eng. Res. Des.* **107**:24–33.

Hazel JR, Williams EE. 1990. The role of alteration in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog. Lipid Res.* **29**:167–227.

Hazel JR, Eugene Williams E. 1990. The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog. Lipid Res.* **29**:167–227.

Hofbauer HF, Schopf FH, Schleifer H, Knittelfelder OL, Pieber B, Rechberger GN, Wolinski H, Gaspar ML, Kappe CO, Stadlmann J, Mechtler K, Zenz A, Lohner K, Tehlivets O, Henry SA, Kohlwein SD. 2014. Regulation of gene expression through a transcriptional repressor that senses acyl-chain length in membrane phospholipids. *Dev. Cell* 29:729–739.

Hori T, Nakamura N, Okuyama H. 1987. Possible involvement of acetyl coenzyme A carboxylase as well as fatty acid synthetase in the temperature-controlled synthesis of fatty acids in Saccharomyces cerevisiae. *J. Biochem.* **101**:949–956.

Jarboe LR, Royce L a, Liu P. 2013. Understanding biocatalyst inhibition by carboxylic acids. *Front. Microbiol.* **4**:272.

Leber C, Polson B, Fernandez-Moya R, Da Silva NA. 2015. Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in Saccharomyces cerevisiae. *Metab. Eng.* **28**:54–62.

Legras JL, Erny C, Le Jeune C, Lollier M, Adolphe Y, Demuyter C, Delobel P, Blondin B, Karst F. 2010. Activation of two different resistance mechanisms in Saccharomyces cerevisiae upon exposure to octanoic and decanoic acids. *Appl. Environ. Microbiol.* **76**:7526–35.

Liao JC, Mi L, Pontrelli S, Luo S. 2016. Fuelling the future: microbial engineering for the production of sustainable biofuels. *Nat. Rev. Microbiol.* **14**:288–304.

Liu P, Chernyshov A, Najdi T, Fu Y, Dickerson J, Sandmeyer S, Jarboe L. 2013. Membrane stress caused by octanoic acid in Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* **97**:3239–3251.

Lopes MSG. 2015. Engineering biological systems toward a sustainable bioeconomy. *J. Ind. Microbiol. Biotechnol.* **42**:813–38.

Marguet D, Lenne P-F, Rigneault H, He H-T. 2006. Dynamics in the plasma membrane: how to combine fluidity and order. *EMBO J.* **25**:3446–57.

Matsufuji Y, Fujimora S, Ito T, Nishizawa M, Miyaji T, Nakagawa J, Ohyama T, Tomizuka N, Nakagawa T. 2008. Acetaldehyde tolerance in Saccharomyces cerevisiae involves pentose phosphate pathway and oleic acid biosynthesis. *Yeast* **25**:825–833.

Mira NP, Teixeira MC, Sá-Correia I. 2010. Adaptive response and tolerance to weak acids in Saccharomyces cerevisiae: a genome-wide view. *OMICS* **14**:525–40.

Narendranath N V, Thomas KC, Ingledew WM. 2001. Effects of acetic acid and lactic acid on the growth of Saccharomyces cerevisiae in a minimal medium. *J. Ind. Microbiol. Biotechnol.* **26**:171–177.

Nasutution O, Lee YM, Kim E, Lee Y, Kim W, Choi W. 2017. Overexpression of OLE1 enhances stress

tolerance and constituvely activates MAPK HOG pathway in Saccharomyces cerevisiae. *Biotechnol. Bioeng.* **114**:620–31.

Nordström K. 1964. Effect of higher fatty acids and toxicity of lower fatty acids. *J. Inst. Brew.* **70**:223–42.

Perlack RD, Wright LL, Turhollow AF, Graham RL, Strokes BJ, Erbach DC. 2005. Biomass as feedstock for a bioenergy and bioproducts industry: The technical feasibility of a billion-ton annual supply. U.S. Department of Energy and U.S. Department of Agriculture, Oak Ridge, Tenessee.

Piper P, Calderon CO, Hatzixanthis K, Mollapour M. 2001. Weak acid adaptation: The stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology* **147**:2635–2642.

Prashar A, Hili P, Veness RG, Evans CS. 2003. Antimicrobial action of palmarosa oil (Cymbopogon martinii) on Saccharomyces cerevisiae. *Phytochemistry* **63**:569–575.

Rest M, Kamminga AH, Nakano A, Anraku Y, Poolman B, Konings WILN. 1995. The Plasma Membrane of Saccharomyces cerevisiae: Structure, Function, and Biogenesis. *Microbiol. Rev.* **59**:304–322.

Rice WR. 1989. Analyzing Tables of Statistical. *Evolution (N. Y).* **43**:223–225.

Rodríguez-Vargas S, Sánchez-García A, Martínez-Rivas JM, Prieto JA, Randez-Gil F. 2007. Fluidization of membrane lipids enhances the tolerance of Saccharomyces cerevisiae to freezing and salt stress. *Appl. Environ. Microbiol.* **73**:110–116.

Sandoval NR, Papoutsakis ET. 2016. Engineering membrane and cell-wall programs for tolerance to toxic chemicals: Beyond solo genes. *Curr. Opin. Microbiol.* **33**:56–66.

Schwartz TJ, Shanks BH, Dumesic JA. 2016. Coupling chemical and biological catalysis: A flexible paradigm for producing biobased chemicals. *Curr. Opin. Biotechnol.* **38**:54–62.

Szmacinski H, Lakowicz JR. 1996. Fluorescence lifetime characterization of magnesium probes: Improvement of Mg(2+) dynamic range and sensitivity using phase-modulation fluorometry. *J. Fluoresc.* **6**:83–95.

Wojtczak L, Więckowski MR. 1999. The mechanisms of fatty acid-induced proton permeability of the inner mitochondrial membrane. *J. Bioenerg. Biomembr.* **31**:447–455.

Yazawa H, Kamisaka Y, Kimura K, Yamaoka M, Uemura H. 2011. Efficient accumulation of oleic acid in Saccharomyces cerevisiae caused by expression of rat elongase 2 gene (rELO2) and its contribution to tolerance to alcohols. *Appl. Microbiol. Biotechnol.* **91**:1593–1600.

You KM, Rosenfield C, Knipple DC. 2003. Ethanol tolerance in the yeast Saccharomyces cerevisiae is dependent on cellular oleic acid content. *Appl. Environ. Microbiol.* **69**:1499.

Zhang Y-M, Rock CO. 2008. Membrane lipid homeostasis in bacteria. *Nat. Rev. Microbiol.* **6**:222–233.

Zheng DQ, Liu TZ, Chen J, Zhang K, Li O, Zhu L, Zhao YH, Wu XC, Wang PM. 2013. Comparative functional genomics to reveal the molecular basis of phenotypic diversities and guide the genetic

breeding of industrial yeast strains. *Appl. Microbiol. Biotechnol.* **97**:2067–2076.

Zhou YJ, Buijs NA, Zhu Z, Qin J, Siewers V, Nielsen J. 2016. Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. *Nat. Commun.* **7**:11709.