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Engineering *Saccharomyces cerevisiae* Fatty Acid Composition for Increased Tolerance to Octanoic Acid

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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- 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-, 1.8-, and 29-fold higher than the baseline strain, respectively.

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KEYWORDS: *Saccharomyces cerevisiae*; octanoic acid; membrane damage; toxicity; oleic acid; acetyl-CoA carboxylase

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 transmembrane proton motive force, triggering activation of H^+-ATPase

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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 3 h 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 threefold 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

S. 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 14–16 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, 2.5, 1.5, 1.25, 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 = 5.5. At $OD_{600} = 1$, the cells were harvested by centrifugation (2000*g*, 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 17,000*g* 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, Thermo Fisher Scientific, Eugene, OR). Fluorescence at $\lambda_{em} = 531$ nm was measured by excitation at $\lambda_{ex} = 506$ 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 = 5.5 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 six biological replicates (with three 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 threefold 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 (Fig. 1A). 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,

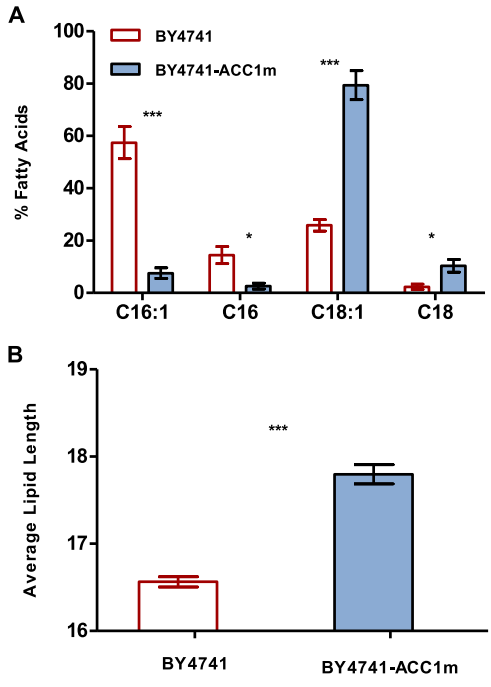

Figure 1. Fatty acid profiles. (**A**) Fatty acid percentages of C16:1, C16, C18:1, and C18 for *S. cerevisiae* strains BY4741 (open bars) and BY4741-ACC1m (filled bars) and (**B**) average lipid length of both strains. Results show average and SEM of biological triplicates (* $P < 0.05$ and *** $P 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

the changes in fatty acid composition resulted in a 7.8% increase in the average length of the fatty acid chains (Fig. 1B). 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).

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 threefold to 79% 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 and 36 h (Fig. 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 (Fig. 2B), 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 (Fig. 2C), 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 and 1 mM for BY4741, an observation that is consistent with these results. Moreover, Figure 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 24 h 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.

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 (Fig. 1A). 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

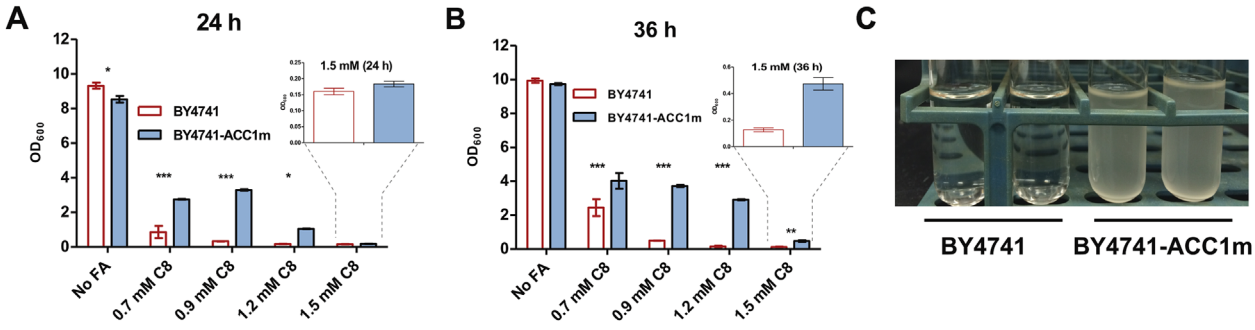

Figure 2. Effect of 0.7, 0.9, 1.2, and 1.5 mM octanoic acid on cell density (OD_{600}) of BY4741 (open bars) and BY4741-ACC1m (filled bars) at (**A**) 24 h and (**B**) 36 h. Results represent average and SEM of three biological replicates. (**C**) Tube cultures (after 80 h) of BY4741 and BY4741-ACC1m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

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 3A. 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 Figures 3A with 1A 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.

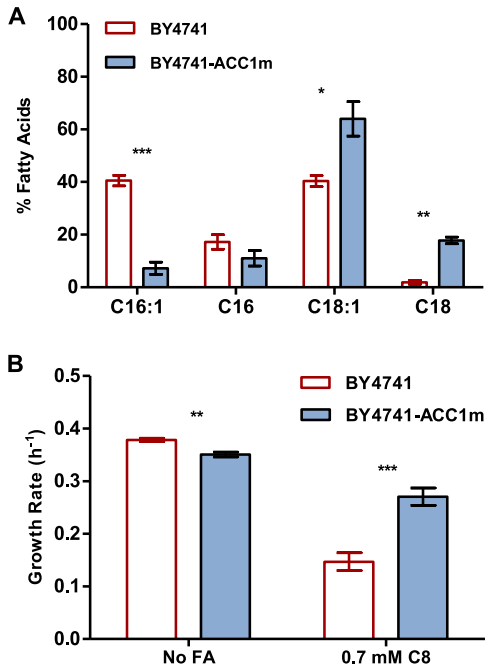

Figure 3. Fatty acid percentages and growth rates for BY4741 and BY4741-ACC1m in the presence of 0.7 mM octanoic acid. (**A**) Fatty acid fractions in early exponential phase and (**B**) Cell growth rates for BY4741 and BY4741-ACC1m in the presence of 0.7 mM octanoic acid. Results show average for biological 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).

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 $Acc1^{S1157A}$ (Fig. 3B). 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.

As seen in Figure 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-ACC 1m was grown in the presence of 1.5 mM octanoic acid and the fatty acid profile was analyzed (Fig. 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.

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 (Fig. 5A). 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 resulted in no significant change in the percentage of saturated fatty acids for BY4741 (Fig. 5B). However,

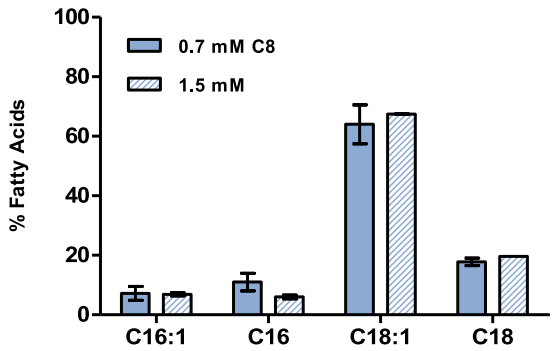

Figure 4. Fatty acid composition of BY4741-ACC1m at $OD_{600} = 1$ during cultivation in medium containing 0.7 mM C8 ($n = 3$ biological replicates, filled) and 1.5 mM C8 ($n = 2$, hatched bars). Error bars represent SEM.

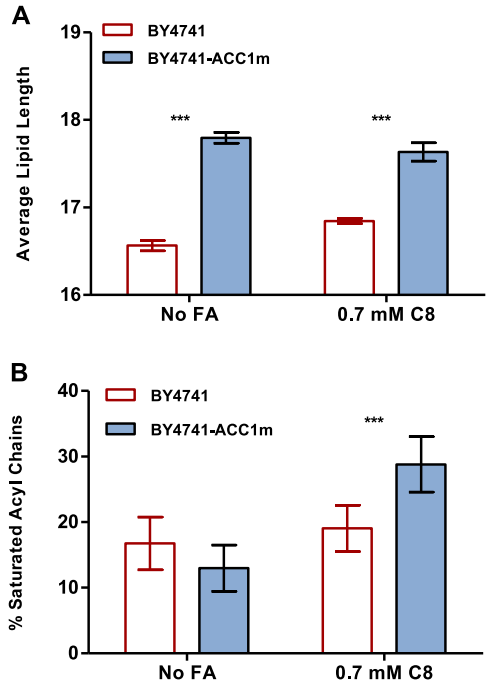

Figure 5. Average lipid length and percent of saturated fatty acid chains in absence and presence of 0.7 mM octanoic acid for *S. cerevisiae* strains BY4741 (open bars) and BY4741-ACC1m (filled bars). Results show average and SEM of biological triplicates (** $P < 0.01$, *** $P < 0.001$, relative to baseline strain).

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.

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 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 min 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 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.

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

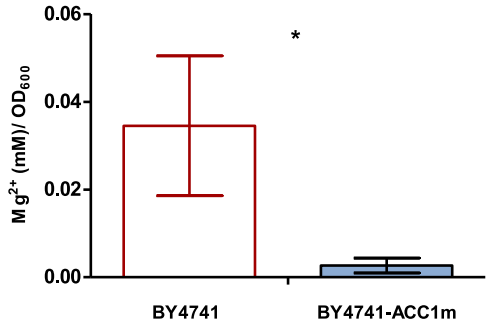

Figure 6. Concentration of extracellular magnesium (mM Mg^{2+} per OD_{600}) for BY4741 ($n = 6$, open bar) and BY4741-ACC1m ($n = 6$, filled bar) after exposure to 1.5 mM octanoic acid. Error bars represent SEM ($^{*}P < 0.05$, relative to baseline strain).

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 and 48 h.

Following cultivation for 48 h in the presence of the various stressors, cell density (OD) was measured and compared for BY4741-ACC1m and BY4741 (Fig. 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-, 1.8-, and 3.2-fold higher than the baseline strain, respectively. The results for octanoic acid at 1.25 mM (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 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.

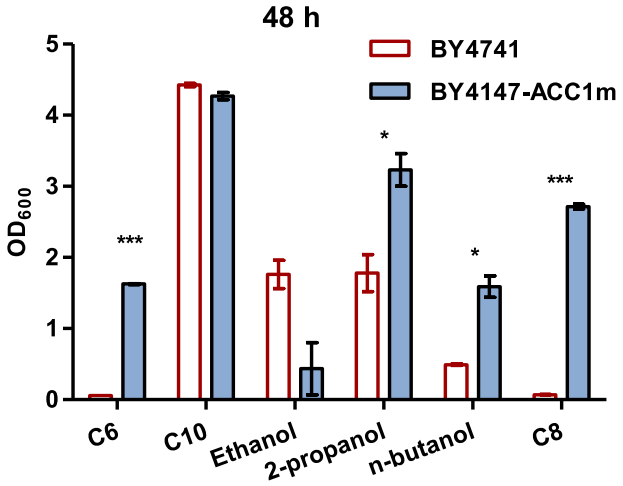

Figure 7. Cell density (OD_{600}) at 48 h for baseline strain (BY4741) and engineered strain (BY4741-ACC1m) upon exposure to 8.75 mM hexanoic acid (C6), 0.3 mM decanoic acid (C10), 8% ethanol, 6% 2-propanol, and 1.3% *n*-butanol. The results for 1.25 mM octanoic acid (C8) have been included for comparison. 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 *S. 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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