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BIOENERGY AND BIOFUELS

Membrane stress caused by octanoic acid in *Saccharomyces cerevisiae*

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Abstract In order to compete with petroleum-based fuel and chemicals, engineering a robust biocatalyst that can convert renewable feedstocks into biorenewable chemicals, such as carboxylic acids, is increasingly important. However, product toxicity is often problematic. In this study, the toxicity of the carboxylic acids hexanoic, octanoic, and decanoic acid on *Saccharomyces cerevisiae* was investigated, with a focus on octanoic acid. These compounds are completely inhibitory at concentrations of magnitude 1 mM, and the toxicity increases as chain length increases and as media pH decreases. Transciptome analysis, reconstruction of gene regulatory network, and network component analysis suggested decreased membrane integrity during challenge with octanoic acid. This was confirmed by quantification of dose-dependent and chain length-dependent induction of membrane leakage, though membrane fluidity was not affected. This induction of membrane leakage could be significantly decreased by a period of pre-adaptation, and this pre-adaptation was accompanied by increased oleic acid content in the membrane, significantly increased production of saturated lipids relative to unsaturated lipids, and a significant increase in the average lipid chain length in the membrane. However, during adaptation cell surface hydrophobicity was not altered. The supplementation of oleic acid to the medium not only elevated the tolerance of yeast cells to octanoic acid but also attenuated the membrane leakiness. However, while attempts to mimic the oleic acid supplementation effects through expression of the *Trichoplusia ni* acyl-CoA Δ9 desaturase OLE1(TniNPVE desaturase) were able to increase the oleic acid content, the magnitude of the increase was not sufficient to reproduce the supplementation effect and increase octanoic acid tolerance. Similarly, introduction of cyclopropanated fatty acids through expression of the *Escherichia coli cfa* gene was not helpful for tolerance. Thus, we have provided quantitative evidence that carboxylic acids damage the yeast membrane and that manipulation of the lipid content of the membrane can increase tolerance, and possibly production, of these valuable products.

Keywords *S. cerevisiae* · Carboxylic acid · Membrane leakage · Membrane lipid composition

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Introduction

The increasing cost and environmental pollutions from fossil carbon feedstocks are increasing the demand for biorenewable production of fuels and chemicals. Fermentation of renewable feed stocks by biocatalysts to produce such fuels and chemicals has the potential to replace petrochemical-based production (Nikolau et al. 2008; Steen et al. 2010; Hermann and Patel 2007; Erturk 2011). Carboxylic acids derived from microbial fermentation can be used as a platform to generate primary building blocks of industrial chemicals. For example, free fatty acids can be extracted from the medium and catalytically converted into esters or alkanes (Mäki-Arvela et al. 2007; Lennen et al. 2010; Lennen and Pfleger 2012) or

biocatalyzed into alcohols through syngas fermentation (Perez et al. 2012). Generally speaking, a robust strain with high product yield and titer is desirable in order to increase the economic viability of the process.

Saccharomyces cerevisiae grows well at low pH and in simple media, and the extensive knowledge of genetics and physiology makes it a promising platform for industrial production of carboxylic acids (Abbott et al. 2009; Liu and Jarboe 2012). However, carboxylic acids, such as octanoic acid and decanoic acid, have been reported to cause inhibition of growth, even death, of yeast cells (Cabral et al. 2001; Kabara and Eklund 1991). These lipophilic weak acids can cross the plasma membrane by passive diffusion and dissociate their liposoluble form in the neutral cytosol, leading to a decrease of the intracellular pH and accumulation of toxic anion (Legras et al. 2010; Cabral et al. 2001; Alexandre et al. 1994).

Additionally, these highly liposoluble carboxylic acids can possibly perturb the organization of the plasma membrane, affecting its function as a matrix of enzymes and a selective barrier (Legras et al. 2010; Cabral et al. 2001; Viegas et al. 1998). When yeast cells are challenged with decanoic acid and octanoic acid, the activity of plasma membrane H^+–ATPase was stimulated to counteract increased cytoplasmic acidification; presumably, this increases the ATP burden (Cabral et al. 2001; Viegas et al. 1998; Alexandre et al. 1996). Moreover, yeast cells under decanoic acid stress exhibited a significant change in membrane fluidity, indicating a perturbation of membrane integrity (Alexandre et al. 1996). Loss of membrane integrity could affect some transportation systems and decrease the cell's ability to maintain appropriate concentration gradients across the plasma membrane (Salgueiro et al. 1988). It has also been reported that in *Escherichia coli* a loss of membrane integrity is induced by exposure to or production of free fatty acids, along with decreased cell viability (Lennen et al. 2011).

Understanding of the underlying inhibitory mechanism is key to rational engineering to mitigate the toxicity of these carboxylic acids (Jarboe et al. 2011). The genome-wide view provided by transcriptome analysis yields insight about which genes and pathways are involved in the carboxylic acid response. Transcriptome analysis has previously been used to compare the response of yeast to the presence of four weak acids (benzoate, sorbate, acetate, and propionate). Fourteen genes related to the cell wall, superoxide dismutase, and DNA synthesis and repair were identified as upregulated to all four acids and defined as a conserved carboxylic acid response (Abbott et al. 2007). Moreover, transcriptome analysis of yeast exposed to medium-chain carboxylic acids (octanoic acid and decanoic acid) has revealed shared resistance genes, like oxidative stress, to both acids. Specifically, the resistance to octanoic acid involved two membrane transporters, Pdr12p and Tpo1p, which counter the stress by exporting the acids from the cell (Legras et al. 2010). However, whether the stress from carboxylic acid will affect the function of the membrane is not yet known.

In this paper, we carried out an investigation of yeast response to octanoic acid exposure. Transcriptome analysis revealed a possible membrane disruption, which was then verified through quantitative analysis of membrane leakage induced by octanoic acid stress. Modulating membrane lipids by supplying external oleic acid can attenuate this effect and increase yeast tolerance, indicating that membrane leakage induced by octanoic acid is a major factor cause of growth inhibition by this important biorenewable product.

Materials and methods

Strains, media, and culture conditions

S. cerevisiae BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann et al. 1998) was grown in synthetic defined (SD) medium with 2 % glucose (Fisher Scientific, Fair Lawn, NJ, USA) at 30 °C with orbital shaking at 150 rpm. SD medium contained 0.67 % (*w*/*v*) Bacto-yeast nitrogen base without amino acids (Becton, Dickinson and Company, Sparks, MD, USA), 0.5 % (*w*/*v*) Casamino acids (Acros Organics, Fair Lawn, NJ, USA), and 0.02 mg/ml uracil (Acros Organics, Fair Lawn, NJ, USA). Growth was monitored by optical density at 600 nm. The pH was adjusted with HCl to 5.0 before inoculation. Fatty acids (Acros Organics, Fair Lawn, NJ, USA) were provided in 100 mM stock solutions in 70 % ethanol. For fatty acid tolerance experiments, a comparable amount of ethanol was added to control cultures. To solubilize oleic acid (Sigma-Aldrich, St. Louis. MO, USA), we added 1 % (*v*/*v*) Tergitol NP-40 (Sigma-Aldrich, St. Louis. MO, USA) to the media (You et al. 2003). Moreover, 0.5 M ferric chloride, 0.1 M ferrous chloride, and 100 mM KH_2PO_4 (Fisher Scientific, Fair Lawn, NJ, USA) were dissolved in nanopure water and sterilized.

For growth rate studies, each culture was started by inoculating 25 ml of medium from an overnight culture to OD_{600}=0.05 in a 250-ml Erlenmeyer flask. The specific growth rate was determined by the growth rate in the exponential phase.

Microarray analysis

We performed transcriptome analysis of *S. cerevisiae* BY4741 during mid-log growth in SD media with 2 %

glucose, pH5.0, 30 °C with and without 0.3 mM octanoic acid present in the media.

Transcriptome analysis was performed on three biological replicates, using Affymetrix arrays (Affymetrix, Inc., Santa Clara, CA, USA) and Cyber T statistical analysis (http://cybert.ics.uci.edu/). Sample processing was performed at the UCI DNA & Protein MicroArray Facility, University of California, Irvine, CA, USA. Isolated total RNA samples were processed as recommended by Affymetrix, Inc. (Affymetrix GeneChip® Expression Analysis Technical Manual, Affymetrix, Inc., Santa Clara, CA, USA). In brief, total RNA was initially isolated using TRIzol Reagent (Gibco BRL Life Technologies, Rockville, MD, USA), and passed through an RNeasy spin column (Qiagen, Chatsworth, CA, USA) for further cleanup. Eluted total RNAs were quantified with a portion of the recovered total RNA adjusted to a final concentration of 1.25 μg/μl. The quality of all starting total RNA samples was assessed prior to beginning the target preparation and processing steps by running out a small amount of each sample (typically 25–250 ng/well) onto a RNA Lab-On-A-Chip (Caliper Technologies Corp., Mountain View, CA, USA) that was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Single-stranded, then double-stranded cDNA, was synthesized from the poly(A)+ mRNA present in the isolated total RNA (typically 10 μg total RNA starting material for each sample reaction) using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen Corp., Carlsbad, CA, USA) and poly (T)-nucleotide primers that contained a sequence recognized by T7 RNA polymerase. A portion of the resulting ds cDNA was used as a template to generate biotin-tagged cRNA from an in vitro transcription reaction using the BioArray High-Yield RNA Transcript Labeling Kit (T7) (Enzo Diagnostics, Inc., Farmingdale, NY, USA). A total of 15 μg of the resulting biotin-tagged cRNA was fragmented to an average strand length of 100 bases (range 35–200 bases) following prescribed protocols (Affymetrix GeneChip® Expression Analysis Technical Manual). Subsequently, 10 μg of this fragmented target cRNA was hybridized at 45 °C with rotation for 16 h (Affymetrix GeneChip® Hybridization Oven 640, Affymetrix, Inc., Santa Clara, CA, USA) to probe sets present on an Affymetrix GeneChip Yeast Genome 2.0 array. The GeneChip® arrays were washed and then stained (streptavidin–phycoerythrin) on an Affymetrix Fluidics Station 450, followed by scanning on a GeneChip® Scanner 3000. The results were quantified and analyzed using GCOS 1.2 software (Affymetrix, Inc., Santa Clara, CA, USA) using default values (scaling, target signal intensity=500; normalization, all probe sets; parameters, all set at default values).

Background subtraction, normalization, and summarizing probe sets from Affymetrix expression microarrays were performed using the Probe Logarithmic Intensity ERror algorithm developed by Affymetrix. Statistical analysis was conducted using the Cyber-T software described by Salmon et al. (2003, 2005). The Cyber-T software package is available for online use at the website for the Institute for Genomics and Bioinformatics at the University of California, Irvine, CA, USA (http://cybert.ics.uci.edu/). The Cyber-T software package was used to perform a regularized *t*-test based on a Bayesian statistical framework and to calculate the posterior probability of differential expression values (Baldi and Hatfield 2002; Hung et al. 2002). Raw data and processed data are available in the "Electronic supplementary materials".

Reconstruction of gene regulatory network

Log ratios of transcription factor activities (TFAs) can be estimated based on transcriptome data and known gene regulatory topology by using Network Component Analysis (Liao et al. 2003). GTRNetwork (Fu et al. 2011) compares the estimated TFA log ratios and transcriptome data to reconstruct gene regulatory networks (GRNs) and identify potential new regulatory links from already known gene regulatory topologies. In this study, yeast gene regulatory networks were reconstructed using GTRNetwork algorithm with inputs of transcriptome data from Many Microbe Microarrays (M3D) database (Faith et al. 2008) and initial gene regulatory topology from Yeastract database (Abdulrehman et al. 2011). M3D database collected and normalized 247 yeast microarray experiments. The gene regulatory topology obtained from Yeastract database (updated on 2010-12-13) contains 48,082 regulatory links between 183 TFs and 6,403 genes. The predicted regulatory links were included in the initial gene regulatory network for the estimation of TFA perturbations under C8 stress condition; this information is available as "Electronic supplementary material 1".

Estimation of TFA changes under C8 stress conditions

TFA $\log_2$ ratios between the C8 stress and the control condition were estimated using FastNCA (Chang et al. 2008) algorithm with the input of $\log_2$ expression ratios between treatment conditions and control conditions from the C8 stress transcriptome data. Three different initial gene regulatory networks were used as FastNCA initial gene regulatory topology inputs: the original Yeastract regulatory data, the original Yeastract data plus the 28 predicted new regulatory links, and the original Yeastract data plus 93 predicted new regulatory links. Each specified initial GRN would generate a different estimate of TFA ratios. Two-sample *t*-tests were performed on the estimated TFA ratios between the C8 stress conditions and the control conditions. *P*-values were calculated to identify significant change of TFA $\log_2$ ratios. ("Electronic supplementary material 1").

The GTRNetwork Matlab package and data of this study are available at: http://vrac.iastate.edu/~afu/GTRNetwork/GTRNetwork_1.2.1_adjustedForYeast.rar.

Membrane fluidity measurement

The measurement of plasma membrane fluidity was performed on yeast protoplasts containing 0.6 mM diphenylhexatriene (DPH) as described previously (Namiki et al. 2011; Alexandre et al. 1994). Octanoic acid was added to the protoplast suspension before measurements. Membrane fluidity was assessed by measuring the fluorescence polarization of DPH using a Synergy 2 Multi-Mode microplate reader from BioTek (Winooski, VT, USA) and a sterile black-bottom Nunclon delta surface 96-well plate (Sigma Aldrich, St. Louis, MO, USA) over the course of 60 min.

Membrane leakage quantification

The membrane leakage protocol was based on that described by Prashar et al. (2003). Yeast cells were grown in 25 ml SD medium with 2 % glucose at 30 °C, 150 rpm, in 250-ml Erlenmeyer flask flasks and harvested at OD_{600}=1.0. Cells were centrifuged at 5,000*g*, 4 °C for 20 min, washed twice with 0.9 % sodium chloride (*w*/*v*), and resuspended in 0.9 % sodium chloride. The cells were then treated with octanoic acid, mixed well, and incubated at 30 °C for 30 min. Then, the cells were centrifuged at 16,873*g*, 4 °C for 5 min, and magnesium in the supernatant was measured by infinity magnesium reagent (Thermo Scientific, Fair Lawn, NJ, USA) and spectrophotometer with temperature control (Varian Cary 50 Series, Varian Australia Pty Ltd, Mulgrave, VIC 3170, Australia) at 37 °C and 570 nm. The concentration of magnesium was calculated from a standard curve generated using standard solutions. Six replicates were performed for each treatment, and all error bars correspond to one standard deviation.

For oleic acid supplementation, the yeast cells were grown in SD medium plus oleic acid until harvest. The pre-exposure experiment was conducted by exposing yeast cells to 0.3 and 0.5 mM octanoic acid from the time of inoculation until log phase (OD_{600}=1.0) was reached; then, the cells were collected and the membrane leakage induced by 0.3 or 0.5 mM, respectively, of octanoic acid was measured as described earlier.

Fatty acid analysis

Yeast cells grown to mid-log in SD medium with 2 % glucose and different concentrations of octanoic acid were harvested by centrifugation at 5,000*g*, 4 °C for 20 min, and the pellets were washed twice with ice-cold sterile water before storing at −80 °C.

Lipid extraction was performed according to Bligh and Dyer's (Dyer 1959) methods. Methylation of fatty acids was performed by adding 2 ml of 1 N HCl in methanol (1.6 ml HCl in 8.4 ml methanol) with vortexing, and holding at 80 °C for 30 min, then addition of 2 ml 0.9 % NaCl (*w*/*v*) and 500 μL of hexane, then vortexing for 1 min. The hexane layer was recovered twice by centrifugation at 5,000*g*, 4 °C for 5 min. The fatty acid content was analyzed using an Agilent GC-FID/MS system equipped with the DB-5MS column (30 m, 0.25 mm i.d., 0.25 μm; Agilent, Wilmington, DE, USA), and a MS detector was used for sample analysis. Three replicates were analyzed; error bars indicate one standard derivation.

Plasmid construction and transformation

The coding sequence of TniNPVE desaturase (Genbank AF038050) was synthesized chemically by GENEWIZ, Inc (South Plainfield, NJ, USA). A 1.07-kb *Spe*I–*Xho*I DNA fragment of TniNPVE desaturase was inserted between *Spe*I and *Xho*I (New England Biolabs, Ipswich, MA, USA) of a constitutive plasmid pXP 418 with *TEF1* promoter and *URA3* selective marker (Fang et al. 2011). A 1.14-kb *Hin*dIII–*Bam*H1 gene fragment of cyclopropane fatty acyl phospholipid synthase (*cfa*) (EcoCyc, EG11531) was amplified from the *E. coli* genome using a forward primer (TAAAGCTTA TGAGTTCATCGTGTATAGAAGAAGTC) and a reverse primer (CGGATCCTTAGCGAGCCACTCGAAGG). Twenty-microliter polymerase chain reactions (PCR) were performed with the following components: 0.5 μl bacterial DNA, 10 μl 2×Taq PCR Master Mix (Qiagen, Valencia, CA, USA), 0.2 μl each primer, and 9.1 μl RNase-free water. Amplification was conducted in a BioRad iCycler Thermocycler (BioRad, Hercules, CA, USA) under the following conditions: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s, extension at 72 °C for 3 min, and a final extension at 72 °C for 7 min. The PCR product was directly ligated to the linearized vector pYES2 (Invitrogen, Carlsbad, CA, USA) with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) following a transformation into the competent cells of Top10 *E. coli* (Invitrogen, Carlsbad, CA, USA) by heat shock. pYES2 vector has the selective marker AMP for selection in *E. coli* and *URA3* for yeast. The positive colonies were selected on Luria broth with ampicillin and confirmed by PCR. Then, plasmids were extracted from these colonies using Miniprep kit from Qiagen Sciences (Germantown, MD, USA) and transformed into the yeast strains using the lithium acetate method following the instructions from Invitrogen (Carlsbad, CA, USA).

Hydrophobicity test

Cells grown with or without 0.3 mM octanoic acid were harvested at mid-log, centrifuged at 5,000*g* for 20 min, and resuspended in 0.9 % sodium chloride (*w*/*v*) to OD_{600}=1.

1.2 ml cells were added to 0.6, 1.2, and 2.4 ml hexane as previously described (Rosenberg et al. 1980; Zhang et al. 2007). Three replicates were included in the analysis. The microbial adhesion to hydrocarbons values were calculated using the 2.4 ml hydrocarbon samples as described by Aono and Kobayashi (1997).

Statistical analysis

T-test was applied for statistical analysis.

Results

Toxicity of medium-chain fatty acids is a function of dose, chain length, and pH

In order to characterize the effect of fatty acid on yeast growth, *S. cerevisiae* was grown in SD medium plus 2 % glucose with various concentrations of hexanoic acid (C6), octanoic acid (C8), and decanoic acid (C10) (Fig. 1a). All cultures were adjusted to pH5.0 before inoculation. The presence of these fatty acids caused a dose-dependent inhibition of growth rate, and this inhibitory effect is also a function of carboxylic acid chain length. As the chain length increases, the toxicity to yeast cells was increased.

The toxicity of octanoic acid and decanoic acid on yeast cells in the presence of 6 % (*v*/*v*) ethanol was previously described to be affected by the medium pH between 3.0 and 5.4 (Viegas et al. 1989). Here we characterized the broad effect of medium pH on yeast growth in the presence of only octanoic acid (Fig. 1b). The pK_a of octanoic acid is 4.89. As the medium pH approached the pK_a, the C8 toxicity increased. Because the abundance of the undissociated form will increase as the media pH approaches the pK_a, these growth results strongly support the assertion that the undissociated form of octanoic acid is the toxic form.

Thus, if we wish to take advantage of yeast's ability to grow at a low pH in a carboxylic acid production platform, carboxylic acid toxicity is a concerning problem.

The indication of membrane leakage by microarray analysis

Previous studies have shown that knowledge of the mechanism of toxicity of an inhibitory molecule can enable rational

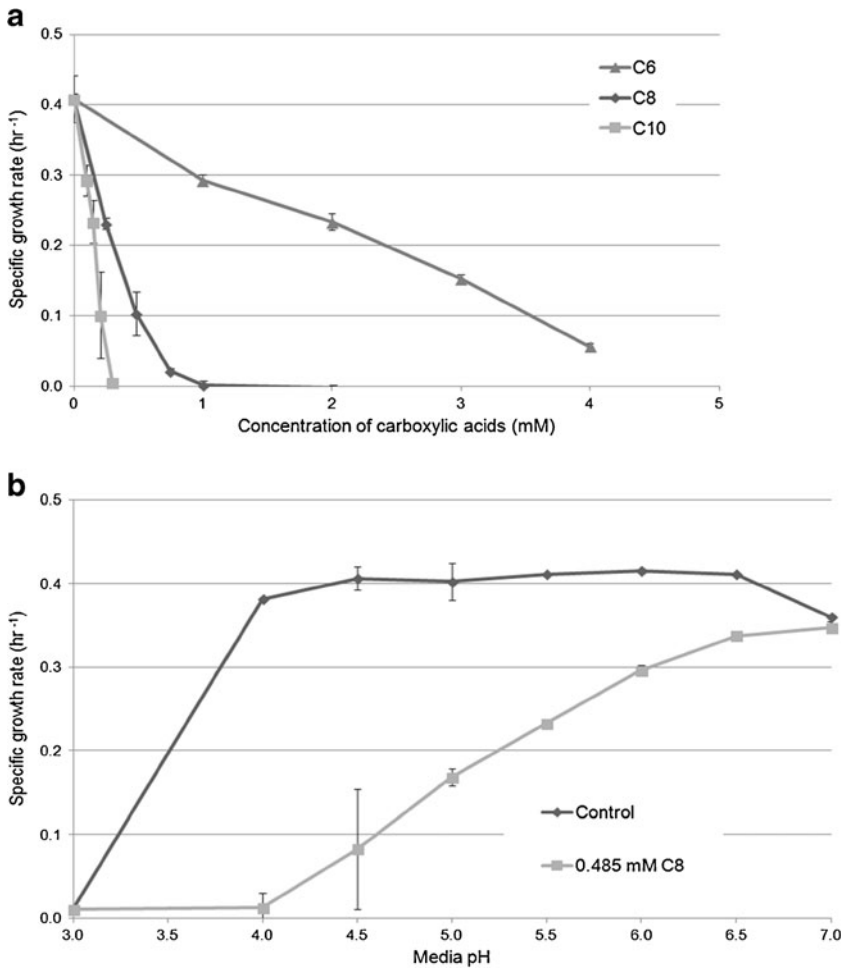

Fig. 1 Short-chain fatty acids are inhibitory, with toxicity increasing with chain length and acidic growth condition. Cells were grown in 25 ml SD with 2 % glucose in 250 ml baffled shakes shaking at 150 rpm at 30 °C. **a** Sensitivity of yeast BY4741 to hexanoic acid (*C6*), octanoic acid (*C8*), and decanoic acid (*C10*), respectively, at pH5.0. **b** The effect of medium pH on octanoic acid toxicity. Three replicates were conducted

engineering to increase tolerance (Jarboe et al. 2011). Omics analysis, such as transcriptome analysis, is one way to gain insight into the mechanism of toxicity.

We performed transcriptome analysis of *S. cerevisiae* BY4741 during mid-log growth in SD media with 2 % glucose at pH5.0, with and without exposure to 0.3 mM C8. A dose of 0.3 mM C8 is sufficient to decrease the specific growth rate by 25 %. This analysis identified 937 genes with significantly (p <0.01) perturbed expression in the+C8 condition relative to the control; 136 of these genes have expression that is perturbed more than two fold ("Electronic supplementary material 2"). Note that the genes related to iron starvation, such as *FIT2*, *FIT3*, and *TIS11*, were each activated more than five fold. We did not observe perturbation of any of the 14 genes identified by Abbott et al. (2007) as part of the conserved carboxylic acid response (data not shown). This could be due to differences in experimental conditions.

Reconstruction of the gene regulatory networks resulted in 28 new potential links within the top 100 links with highest confidence and 93 new links within the top 200 links. These new connections are listed in "Electronic supplementary material 1". Network component analysis was performed with and without these new regulatory links; regulators that were significantly perturbed in all three analyses are listed in Table 1. Of particular interest here is the perturbation of Hap5 and Pho2. Hap5 has been reported as involved in the regulation of iron homeostasis in *Candida albicans* (Singh et al. 2011); Pho2 involves in the regulation of genes related to phosphate metabolism and starvation (Bhoite et al. 2002). The fact that these iron and phosphate starvation sensing regulators were activated under C8 stress suggests that these regulators are sensing a lack of these important compounds. Therefore, we supplemented the medium with 20 μM ferrous chloride (Fe^{2+}), 100 μM ferric chloride (Fe^{3+}), or 100 mM KH_2PO_4. However, no increase in C8 tolerance was observed (Fig. 2). The fact that increasing the abundance of these compounds in the growth media did not alleviate the growth inhibition leads to the proposition that octanoic acid-mediated disruption of the cell membrane indirectly perturbs the intracellular concentration of free iron and phosphate, resulting in the observed transcriptional perturbations. If this hypothesis is true, then the observed perturbation of iron- and phosphate-related genes is a symptom of membrane disruption and not directly related to growth inhibition. This implies that the altered activity of these two regulators was possibly due to membrane damage instead of nutrient starvation.

Membrane leakage induced by octanoic acid is less severe in adapted cells

Because of their lipophilic features, fatty acids like octanoic acid can possibly change the membrane organization and affect its integrity (Alexandre et al. 1996). The leakage of internal material from yeast cells has been previously detected during ethanol stress (Mizoguchi and Hara 1998; Osman and Ingram 1985; Salgueiro et al. 1988), and the release of intracellular magnesium has already been detected in studies with other chemicals, such as palmarosa oil and aldehydes (Prashar et al. 2003; Zaldivar et al. 1999). Here we use magnesium as a model molecule to report leakage of intracellular contents into the extracellular medium in response to carboxylic acids, such as octanoic acid.

Table 1 Transcription factors (TFs) significantly perturbed by 0.3 mM C8 exposure as determined by network component analysis

TFs		Functions
Hap5	+	Subunit of the heme-activated, glucose-repressed Hap2/3/4/5 CCAAT-binding complex; required for assembly and DNA binding activity of the complex. In *C. albicans*, *HAP5* was activated in iron deprivation in Cap2-dependent manner. In iron deprivation, Cap2 associates with Hap5, Hap2, and Hap32 complex to activate genes for iron uptake (Singh et al. 2011)
Pho2	+	Regulatory targets include genes involved in phosphate metabolism; binds cooperatively with Pho4p to the *PHO5* promoter
Cst6	n/a	Mediates transcriptional activation of *NCE103* (encoding carbonic anhydrase) in response to low CO_2 levels; involved in utilization of non-optimal carbon sources and chromosome stability
Haa1	+	Involved in genes putatively encoding membrane stress proteins; involved in adaptation to weak acid stress
Hac1	n/a	Involved in endoplasmic reticulum stress
Met32	n/a	Involved in transcriptional regulation of the methionine biosynthetic genes
Ppr1	n/a	Activates transcription of the genes (*URA1*, *URA3*, *URA4*), which encode enzymes involved in the regulation of pyrimidine levels
Stb5	+	Involved in regulating multidrug resistance and oxidative stress responses
Usv1	n/a	Mutation affects transcriptional regulation of genes involved in growth on non-fermentable carbon sources, response to salt stress, and cell wall biosynthesis

The p-value criterion for significance was 0.05

+ increased activity, *n/a* not determined

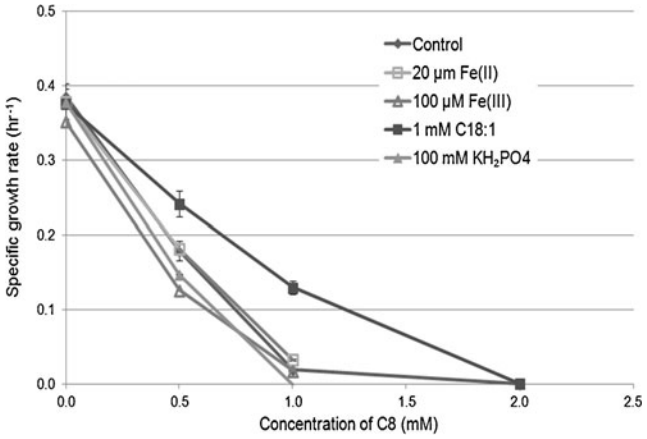

Fig. 2 Growth rate of yeast cells in SD medium at pH5.0 containing octanoic acid was increased by the supplementation of oleic acid, but not with iron or phosphate. Three replicates were conducted

Yeast cells were grown to mid-log phase, exposed to octanoic acid, and the resulting leakage of magnesium into the supernatant was measured. The effect of octanoic acid on membrane leakage is shown in Fig. 3a. A dose-dependent increase in leakage of magnesium was induced by octanoic acid, with the highest leakage observed at 1 mM. Note that 1 mM octanoic acid induces total growth inhibition in this condition (Fig. 1a). Additionally, when yeast cells were exposed to 0.3 mM C6, C8, and C10, respectively, the leakage of magnesium increased as chain length increased (Fig. 3b), similar to the trends observed with growth inhibition.

However, this leakage was somewhat mitigated when yeast cells were pre-exposed to moderate amounts of octanoic acid until log phase and then harvested to examine leakage at the corresponding concentrations (Fig. 3c). For example, the release of magnesium was decreased by 50 % for 0.3 mM (p=0.0026) and 0.5 mM (p=0.054). This result indicates that some critical cell property, possibly membrane organization, is altered during adaptation to octanoic acid, decreasing the release of magnesium and other critical compounds.

We also measured membrane fluidity and cell hydrophobicity under octanoic acid stress, but no change was detected for either property in the presence of either 0.3 or 1 mM octanoic acid (Figs. S1 and S4 in "Electronic supplementary material 3").

Modification of membrane lipid composition with octanoic acid challenge

The membrane lipids play an important role in maintaining the normal function of plasma membrane (Vanderrest et al. 1995). Yeast cells adapted to herbicide (2,4-dichlorophenoxyacetic acid) showed decreased plasma permeability relative to cells without adaptation, and the membrane lipid distribution was modified in these adapted cells (Viegas et al. 2005). Given that we also saw adaptation of membrane permeability, as evidenced by magnesium leakage, we aimed to determine if there were any changes in membrane lipid composition during adaptation to octanoic acid.

The major fatty acid components of the yeast membrane were C16:1, C16:0, C18:1, and C18:0, consistent with previous reports (Prashar et al. 2003). Figure 4a shows the effect of octanoic acid on membrane composition. We investigated adaptation to three concentrations of C8: 0.3, 0.5, and 0.8 mM. Note that there was insufficient growth to investigate cells exposed to 1 mM octanoic acid. Compared to cells grown without octanoic acid, adapted cells in all conditions showed increased relative abundance of C18:1 and decreased C16:1. Additionally, C16:0 was increased in 0.3 and 0.5 mM C8 treatment, respectively, but decreased in 0.8 mM C8 treatment; C18:0 was elevated under 0.5 and 0.8 mM C8, respectively (Fig. 4a). The ratio of saturated to unsaturated fatty acids was significantly increased by adaptation to 0.3 and 0.5 mM C8, respectively (Fig. 4b). Additionally, the average chain length was increased in response to increasing concentrations of octanoic acid (Fig. 4c).

Oleic acid supplementation increased octanoic acid tolerance and reduced leakage

Since oleic acid was reported to be helpful for ethanol tolerance and fermentation by yeast (Kajiwara et al. 2000; You et al. 2003), and our results from membrane lipid analysis also showed an increased proportion of oleic acid under C8 stress (Fig. 4a), we supplemented our growth medium with 1 mM oleic acid and tested the resulting impact on octanoic acid tolerance. Cells were grown in medium containing octanoic acid (0.5 and 1 mM) with and without 1 mM oleic acid. With the supplementation of oleic acid, cells grew faster than that without oleic acid addition (Fig. 2), with the specific growth rate 1.4-fold higher in 0.5 mM C8 and 6.9-fold higher in 1 mM C8. Fatty acid analysis between these two different growth conditions showed that in the presence of 0.5 mM C8, the membrane oleic acid content increased from 35 to 54 % (p <0.05; Fig. S2 in "Electronic supplementary material 3").

We also examined the effect of oleic acid addition on the membrane leakage induced by C8. Cells were grown in medium supplemented with 1 mM oleic acid before challenge with C8. The release of magnesium from cells supplied with 1 mM oleic acid was significantly reduced in the presence of 0.3 and 0.5 mM (p<0.05) (Fig. 3a). For the cultures supplemented with oleic acid,

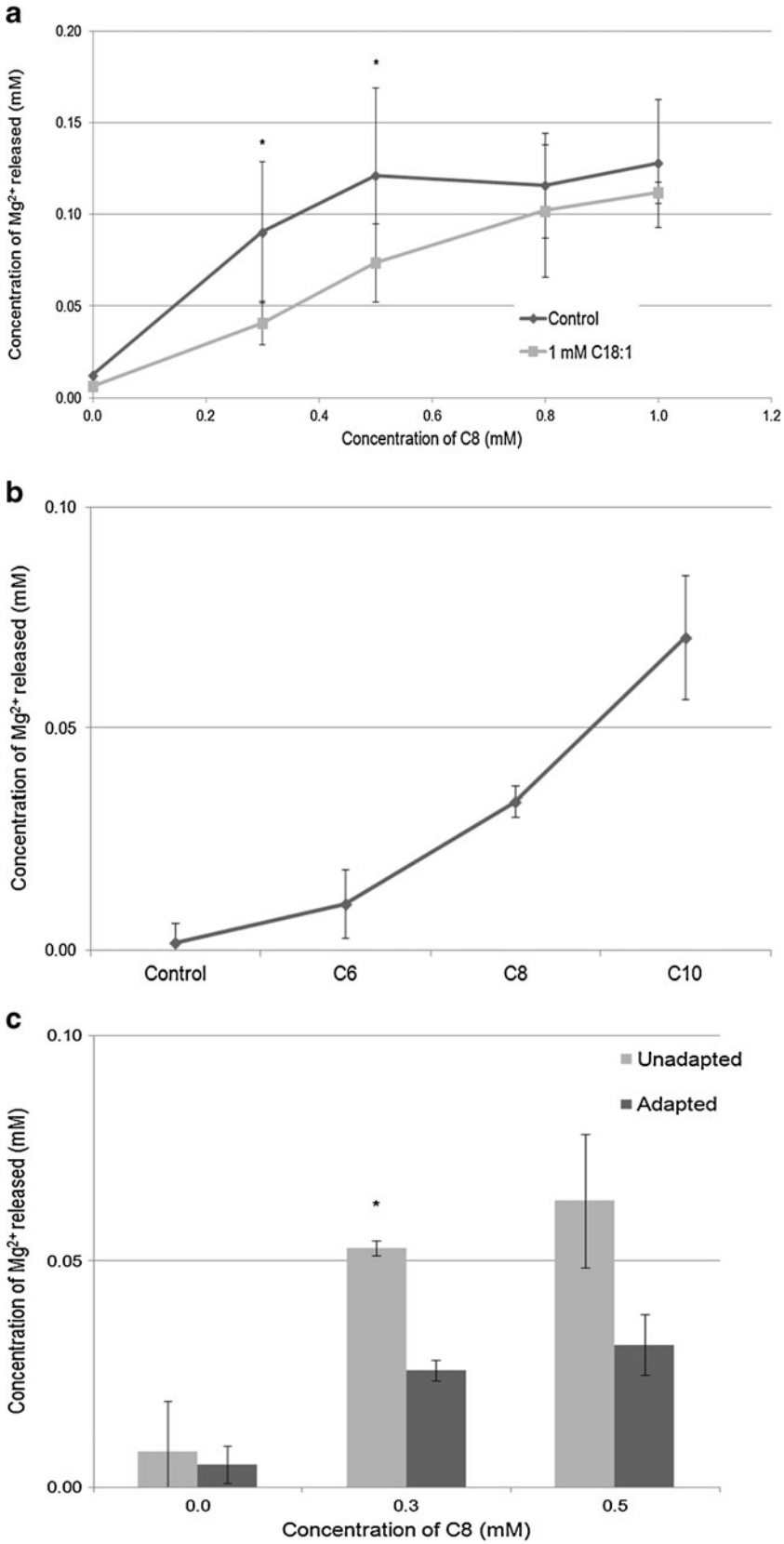

Fig. 3 Membrane leakage induced by octanoic acid was decreased by oleic acid supplementation or pre-adaptation until log phase to octanoic acid at the indicated concentration. **a** Magnesium leakage induced by octanoic acid with and without 1 mM oleic acid supplementation. **b** The leakage of magnesium in yeast cells exposed to 0.3 mM C6, C8, and C10, respectively. **c** The leakage of magnesium in yeast cells either adapted or not adapted to 0.3 and 0.5 mM C8. The two different groups were tested in the presence or absence of 0.3 and 0.5 mM C8, respectively. Six replicates were conducted (*p <0.05 relative to unadapted cells)

the ratio of saturated/unsaturated fatty acids was 23.33±1.19 and 31.76±1.02 % for the control and 1 mM C8, respectively. Similarly, the average change lengths were 17.34±0.17 and 17.25±0.06 (data not shown).

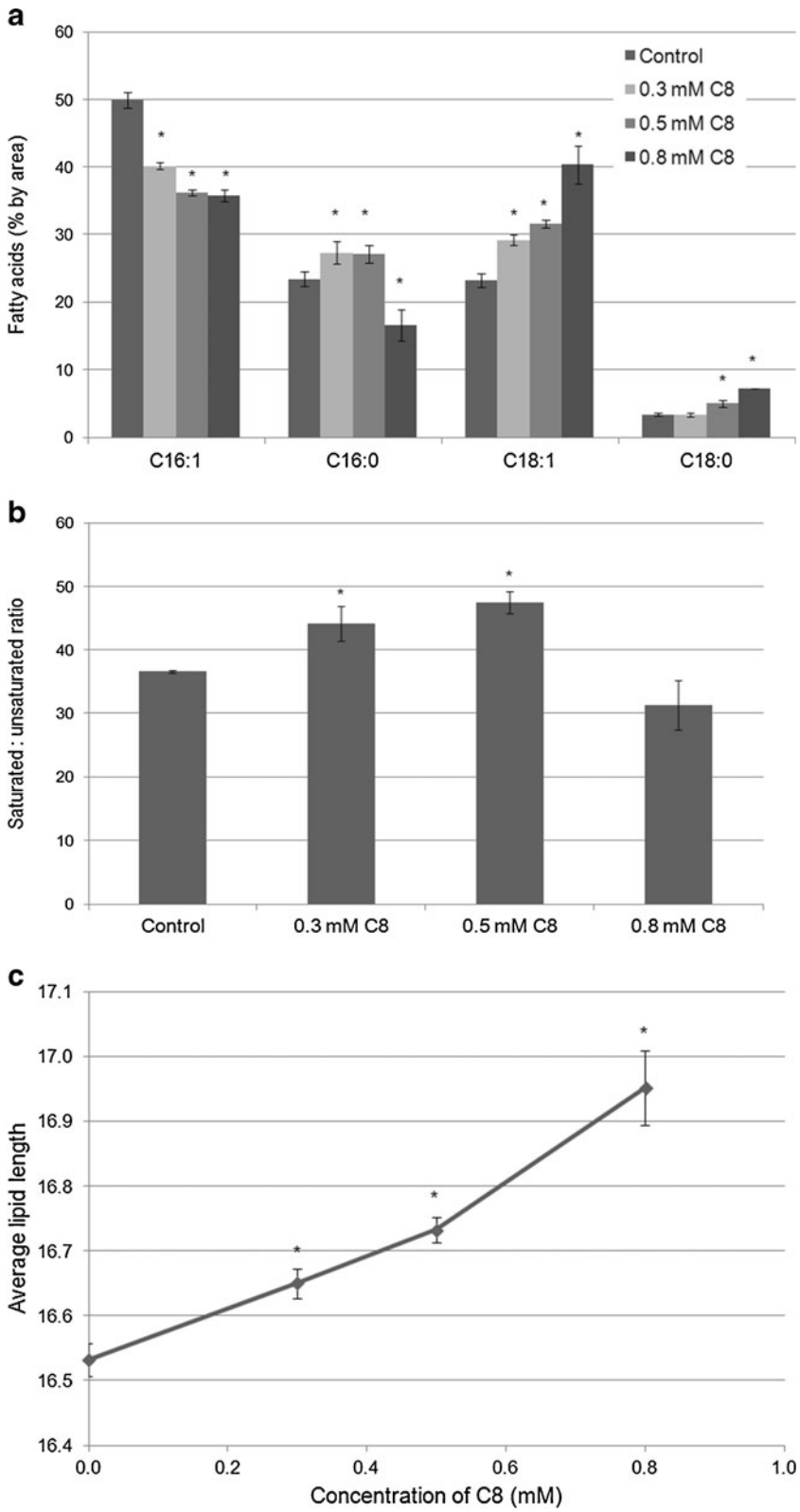

Fig. 4 Increasing concentrations of octanoic acid result in an increase in oleic acid content. **a** Membrane lipid composition of yeast cells adapted to increasing concentrations of octanoic acid until log phase. The percentages of palmitoleic acid (*C16*:*1*), palmitic acid (*C16*:*0*), oleic acid (*C18*:*1*), and stearic acid (*C18*:*0*) are shown. **b** Ratio of saturated to unsaturated fatty acids under increasing concentrations of octanoic acid (*p <0.05 relative to 0 mM control). **c** The average chain length was increased in a dose-dependent manner. Three replicates were conducted (*p <0.05 relative to 0 mM control)

These results indicate that the tolerance to octanoic acid can be elevated by increasing the content of oleic acid in the cell membrane, possibly by increasing the membrane integrity and preventing the leakage of valuable metabolites and cofactors.

Metabolic engineering to change membrane composition does not increase octanoic acid tolerance

Our results show that increasing oleic acid content in the cell membrane increases tolerance to octanoic acid, consistent with other reports describing increased tolerance to ethanol. For example, acyl-CoA Δ-9 desaturase encoded by *OLE1* can convert saturated fatty acids to unsaturated fatty acids, and overexpression of this gene has been found to contribute to ethanol tolerance (You et al. 2003; Kajiwara et al. 2000). Here we over-expressed TniNPVE desaturase. This enzyme was selected due to reports that it enables increased oleic acid content relative to the native yeast desaturase (You et al. 2003). However, no increase in tolerance was detected when comparing the TniNPVE desaturase overexpression strain and wild type (Fig. S3 in "Electronic supplementary material 3"). We analyzed the fatty acid profile in the TniNPVE desaturase overexpression strain with and without octanoic acid to assess the increase of oleic acid content. The content of oleic acid in the TniNPVE desaturase overexpression strain showed 9 % increase relative to the control strain (Fig. 5); however, when cells were grown with 0.5 mM octanoic acid, oleic acid content was increased only 3 % in TniNPVE desaturase-expressing cells relative to the control. For the TniNPVE desaturase-expressing cells in the presence of 0.5 mM C8, the saturated/unsaturated ratio was 52.21±0.03 % and the average chain length was 17.050± 0.006. For 0.5 mM C8 treatment supplemented with oleic acid, the saturated/unsaturated ratio and the average chain length was 28 ±1 % and 17.12±0.04, respectively (data not shown). This inability to sufficiently increase oleic acid content is possibly due to the tight regulation of membrane content or use of the oleic acid in other metabolic pathways.

Cyclopropanated fatty acids have been reported to be a major factor in acid resistance in *E. coli* (Chang and Cronan 1999). We expressed the *cfa* gene from *E. coli* in our yeast strain to test whether it can be helpful for increasing octanoic acid tolerance. Although the expression of *CFA* and production of C17 cyc as more than 10 % of the fatty acid content was confirmed (Fig. S5 in "Electronic supplementary material 3"), no improvement in yeast octanoic acid tolerance was observed (data not shown).

Discussion

Carboxylic acids produced by fermentation of renewable feedstocks are potential intermediates for biorenewable fuels and chemicals. However, biocatalyst inhibition by these products is becoming more problematic as we expand production to next-generation fuels and chemicals (Jarboe et al. 2011). The toxicity of organic acids is correlated with their lipophilicity, which possibly affects membrane organization (Legras et al. 2010; Alexandre et al. 1996). In the present work, we investigated the toxicity mechanism of octanoic acid in yeast. Analysis of transcriptome and gene regulatory networks indicated starvation of iron and phosphate, but it was not helpful for growth to supply these items externally, suggesting that membrane disruption may occur during octanoic acid challenge. Consistent with this lipophilicity trend, our results showed that the inhibition of yeast cells by C8 was related to C8-induced membrane leakage but not a change in membrane fluidity or hydrophobicity. Furthermore, we saw that modification of the membrane lipid profile by increasing the content of oleic acid was accompanied by a decrease in the leakage induced by C8 and also an increase in tolerance. Having identified membrane leakiness as a probable mechanism of inhibition and tolerance, future metabolic engineering efforts can directly target membrane composition.

It is interesting to note that similar studies of carboxylic acid toxicity in *E. coli* found that changes in membrane

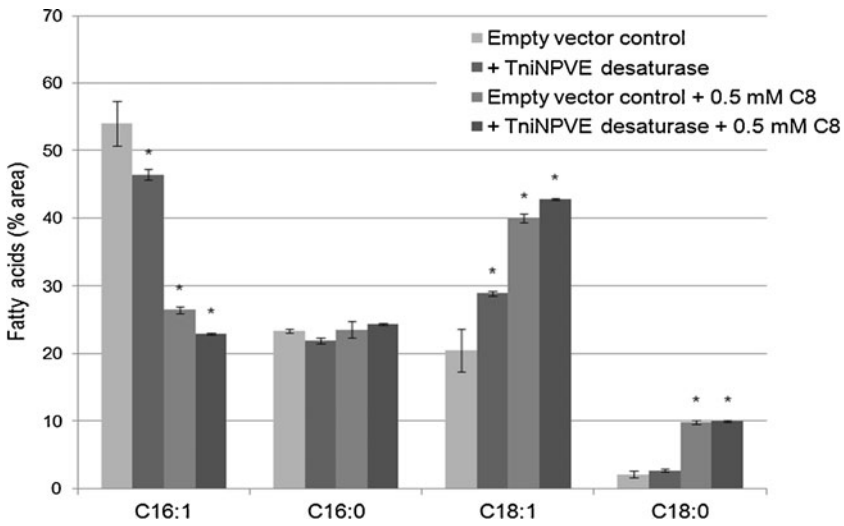

Fig. 5 Membrane lipid composition of TniNPVE desaturase overexpression strain and the control strain (carrying empty plasmid pxp418) with or without 0.5 mM octanoic acid stress. Increased expression of TniNPVE desaturase did increase oleic acid content but not to the same degree observed with oleic acid supplementation. Three replicates were conducted (*p <0.05 relative to empty plasmid)

fluidity and hydrophobicity, and not leakage, are possible mechanisms for growth inhibition (Royce et al., manuscript in preparation).

The plasma membrane is the major target for many biological processes that are vital for yeast cells (Viegas et al. 2005). Organic solvents are known to be toxic to cells because they impair vital membrane functions (Ramos et al. 2002; Segura et al. 2012). Studies on cell performance under carboxylic acid stress have shown effects on the plasma membrane. For example, the activity of plasma membrane H^+–ATPase was affected when yeast cells were grown with decanoic acid and octanoic acid (Viegas et al. 1998; Alexandre et al. 1996). Moreover, yeast cells under decanoic acid stress exhibited higher membrane fluidity than cells without acid stress (Alexandre et al. 1996), though we did not observe this effect in our analysis (Fig. S1 in "Electronic supplementary material 3"), possibly due to the different strain we used or a different mechanism for octanoic acid and decanoic acid. Decreased integrity of the plasma membrane can enable leakage of intracellular materials, leading to decreased availability of important cofactors for biological process (Osman and Ingram 1985). Our hypothesis, based on perturbed activity of regulators for iron and phosphate starvation and lack of increased tolerance despite supplementation (Table 1; Fig. 2), is that octanoic acid disrupts the plasma membrane, leading to leakage of iron, phosphate, and other valuable compounds. Note that the *leu2* mutation in our strain could possibly contribute to the growth inhibition due to limitation of leucine uptake (Cohen and Engelberg 2007). This disruption of the membrane may result in growth inhibition. This hypothesis is supported by our observation of both intracellular magnesium leakage after adding carboxylic acids to yeast cells (Fig. 3a) and an increase in leakage with increasing chain length, further strengthening this assumption (Fig. 3b). Carboxylic acids can possibly change the membrane organization and affect its integrity due to their partial solubility within the membrane (Alexandre et al. 1996). Octanoic acid and decanoic acid have been reported to stimulate the membrane leakage induced by ethanol (Sá-Correia et al. 1989). Moreover, membrane leakage has been related with toxicity of some chemicals, especially ethanol. The ethanol-inhibited fermentation in *Zymomonas mobilis* and yeast was related to ethanol-induced membrane leakage. As the rate of fermentation decreased, the efflux of magnesium and nucleotides was increased, which leads to the loss of intracellular cofactors and coenzymes essential for the enzymes involved in alcohol production (Osman and Ingram 1985; Salgueiro et al. 1988). We detected a similar relationship between the toxicity of octanoic acid and magnesium leakage in this work. The magnesium leakage induced by octanoic acid was increased in a dose-dependent manner; for example, the highest leakage occurred at 1 mM, a concentration which totally inhibits yeast growth.

The ability of microbes to cope with environmental stresses that affect plasma membrane organization and function depends upon the membrane composition (Vanderrest et al. 1995). Cells can increase resistance to toxic compounds by fine-tuning the membrane composition. The related studies reported that the main adaptive responses of bacteria to organic solvents are alterations in the membrane, such as fatty acid composition, phospholipid headgroups, and proteins (Keweloh et al. 1991; Heipieper et al. 1994). We observed that magnesium leakage induced by octanoic acid was decreased when cells were pre-adapted to octanoic acid (Fig. 3c), suggesting that some protective changes in the membrane occurred during adaptation. A similar phenomenon was reported previously when yeast cells were challenged with 2,4-dichlorophenoxyacetic acid; cells adapted to this compound were more resistant to membrane permeabilization by ethidium bromide than the unadapted cells, possibly due to increased saturation degree of membrane fatty acid (Viegas et al. 2005). A similar trend found in our experiment was that the ratio of saturated to unsaturated was increased under the stress of 0.3 C8 and 0.5 mM C8 (Fig. 4b). Additionally, alterations of membrane composition were also reported in organic acid-stressed cells. When yeast cells were exposed to decanoic acids, the proportion of C18:1 (oleic acid) increased and that of C16:1 (palmitoleic acid) decreased (Alexandre et al. 1996). We also observed the same trend when yeast cells were grown in the presence of octanoic acid (Fig. 4a). Previous studies of other organisms concluded that ethanol tolerance was mediated by altering membrane composition through increasing the chain length of fatty acid or elevating the degree of *cis*-mono-unsaturated fatty acids (Ingram 1986; Ingram and Buttke 1984). Here we found that under the elevated concentrations of octanoic acid, the average chain length was also increased (Fig. 4c). Moreover, ethanol tolerance and yield in yeast cells can be increased by increasing the amount of oleic acid in the membrane (You et al. 2003; Zaldivar et al. 1999). Here we investigate it further by changing the lipid profile artificially to see whether yeast tolerance to octanoic acid is elevated. Based on Fig. 4a and literature data about ethanol tolerance cited earlier, we supplied external oleic acid to the growth medium. Yeast cells supplied with oleic acid had increased tolerance of octanoic acid (Fig. 2), drastically increased content of oleic acid in the membrane (Fig. S2 in "Electronic supplementary material 3"), and significantly decreased magnesium leakage (Fig. 3a). So, reducing membrane leakage by rationally altering the membrane composition is one possible way to increase the tolerance to these carboxylic acids. However, metabolic engineering efforts to increase oleic acid content by exogenous expression of TniNPVE desaturase in the presence of yeast native desaturase were unable to implement the drastic increase needed for increased tolerance

(Fig. 5). Thus, future efforts to understand and circumvent the control of membrane composition could be useful.

In conclusion, we have shown that the membrane damage induced by carboxylic acids can be partially alleviated by changing the membrane composition. Resolving the challenges associated with rational modification of the membrane composition, structure, and integrity could enable further progress in this area.

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