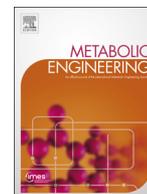




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Membrane engineering via trans unsaturated fatty acids production improves *Escherichia coli* robustness and production of biorenewables


 Zaigao Tan ^a, Jong Moon Yoon ^a, David R. Nielsen ^b, Jacqueline V. Shanks ^a, Laura R. Jarboe ^{a,*}
^a Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011, USA

^b Chemical Engineering, School for Engineering of Matter, Transport, and Energy, Arizona State University, Tempe, AZ 85287-6106, USA

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ABSTRACT

Constructing microbial biocatalysts that produce biorenewables at economically viable yields and titers is often hampered by product toxicity. For production of short chain fatty acids, membrane damage is considered the primary mechanism of toxicity, particularly in regards to membrane integrity. Previous engineering efforts in *Escherichia coli* to increase membrane integrity, with the goal of increasing fatty acid tolerance and production, have had mixed results. Herein, a novel approach was used to reconstruct the *E. coli* membrane by enabling production of a novel membrane component. Specifically, trans unsaturated fatty acids (TUFA) were produced and incorporated into the membrane of *E. coli* MG1655 by expression of cis-trans isomerase (Cti) from *Pseudomonas aeruginosa*. While the engineered strain was found to have no increase in membrane integrity, a significant decrease in membrane fluidity was observed, meaning that membrane polarization and rigidity were increased by TUFA incorporation. As a result, tolerance to exogenously added octanoic acid and production of octanoic acid were both increased relative to the wild-type strain. This membrane engineering strategy to improve octanoic acid tolerance was found to require fine-tuning of TUFA abundance. Besides improving tolerance and production of carboxylic acids, TUFA production also enabled increased tolerance in *E. coli* to other bio-products, e.g. alcohols, organic acids, aromatic compounds, a variety of adverse industrial conditions, e.g. low pH, high temperature, and also elevated styrene production, another versatile bio-chemical product. TUFA permitted enhanced growth due to alleviation of bio-product toxicity, demonstrating the general effectiveness of this membrane engineering strategy towards improving strain robustness.

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1. Introduction

Engineering of microbial catalysts for the production of fuels and chemicals is a promising, biorenewable alternative to current petroleum-based methods (Gallezot, 2007; Larson, 2006). A variety of microorganisms have been engineered for production of biofuels, bulk chemicals, and value added chemicals (Thakker et al., 2012; Park et al., 2012; McKenna and Nielsen, 2011; Zhu et al., 2014; Atsumi et al., 2008). However, toxicity of biorenewable products to the host strain often limits the strain performance (Dunlop, 2011; Huffer et al., 2012). For instance, ethanol-producing *Escherichia coli* grows poorly in the presence of 35 g L⁻¹ ethanol and less than 10% of cells survive when exposed to 100 g L⁻¹ of ethanol (Yomano et al., 1998). *Clostridia* produces n-butanol during ABE fermentation, but does not grow when challenged with 2%

(v/v) n-butanol (Knoshaug and Zhang, 2009). The bulk industrial chemical styrene can be produced by engineered microbes, but significantly inhibits the growth of the producer strain at a concentration of only 300 mg L⁻¹ (McKenna and Nielsen, 2011).

Inhibitory concentrations of products can cause a variety of detrimental effects to production strains. Arguably, the most frequently described effect is membrane damage, often considered as a general mechanism of toxicity (Huffer et al., 2011; Lennen et al., 2011; Liu et al., 2013; Royce et al., 2013; Zaldivar and Ingram, 1999). As the primary architecture of the cell, the membrane plays important roles in transport, energy exchange and protection from infection (Spector and Yorek, 1985). Alcohols, such as ethanol, can inhibit cell growth by causing membrane leakage (Zaldivar and Ingram, 1999) and longer chain alcohols, such as butanol, also cause leakage of important metabolites and fluidize the cell membrane (Huffer et al., 2011). Some organic acids, e.g. acetic acid, also induce membrane leakage (Trcek et al., 2015).

Carboxylic acids, which can serve as catalytic precursors for a variety of chemicals (Korstanje et al., 2015), are broadly useful as

* Correspondence to: Department of Chemical and Biological Engineering, 4134 Biorenewables Research Laboratory, Iowa State University, Ames, IA 50011, USA.

E-mail address: ljjarboe@iastate.edu (L.R. Jarboe).

lubricants, preservatives, fuels and other applications (Lennen et al., 2010; Lopez-Ruiz and Davis, 2014). In recent years, *E. coli* and *Saccharomyces cerevisiae* have both been engineered to produce carboxylic acids at high titer, productivity and yield (Park et al., 2012; Lennen et al., 2010; Steen et al., 2010; Zhang et al., 2012; Lennen and Pfleger, 2012; Choi and Da Silva, 2014; Leber and Da Silva, 2014; Tee et al., 2014; Wu et al., 2014; Thakker et al., 2015). However, as with other biorenewable chemicals, high concentrations of carboxylic acids inhibit the growth of the host strain and decrease the performance of engineered strains (Liu et al., 2013; Lennen et al., 2011; Jarboe et al., 2013). Carboxylic acids can cause a variety of detrimental effects, including membrane damage, cellular expansion, intracellular acidification, and disruption of amino acid pools (Lennen et al., 2011; Liu et al., 2013; Royce et al., 2013; Lennen et al., 2010; Lennen and Pfleger, 2012; Jarboe et al., 2013; Lennen and Pfleger, 2013; Royce et al., 2014). Among these various effects, membrane damage is typically considered as the primary cause of carboxylic acid toxicity (Lennen et al., 2011; Royce et al., 2013; Lennen and Pfleger, 2013; Royce et al., 2015; Sherkhonov et al., 2014). Multiple studies have reported membrane leakage of *E. coli* during fatty acid production. Lennen et al. (2011) reported that the membrane leakage significantly increased after induction of fatty acid production and cell viability decreased by 85% relative to the control strain. Lennen's transcriptome analysis of strains during fatty acid production showed that even early in the production phase when titers were still below 100 mg/L, genes known to be activated in response to membrane damage had increased expression relative to the non-producing control (Lennen et al., 2011). Our own prior results also showed that both exogenously-added octanoic acid (C8) and endogenously-produced long-chain fatty acids (C14+C16) significantly increased membrane leakage (Royce et al., 2013).

To this end, construction of a “stronger” membrane may increase the tolerance of microbial biocatalysts to carboxylic acids, with the goal of further increasing carboxylic acids production. Lennen and Pfleger (2013) showed that expressing a thioesterase, which prevents medium chain unsaturated acyl-ACPs from being incorporated into the membrane, decreased unsaturated fatty acid content in the membrane and thus increased cell integrity during fatty acid production. Although this was effective in decreasing membrane leakage, no increase in production was observed (Lennen and Pfleger, 2013). Sherkhonov et al. (2014) found that deletion of the pathway responsible for incorporating

medium chain fatty acids into the membrane increased the average length of membrane lipids, alleviated the toxicity of fatty acids and improved fatty acid production by 20%. In our prior study, *E. coli* was evolved for tolerance of exogenous octanoic acid. The evolved strain not only showed increased tolerance of fatty acids, but also improved fatty acid production and even increased butanol tolerance. These changes in tolerance and production were accompanied by an increase in the average membrane lipid length and decreased membrane leakage (Royce et al., 2015).

These results demonstrate the possibility of improving carboxylic acids tolerance and/or production by modification of the microbial cell membrane, specifically by altering the chain length or degree of saturation of the membrane components in order to increase membrane integrity and mitigate leakage (Lennen et al., 2011; Royce et al., 2013; Lennen and Pfleger, 2013; Royce et al., 2015; Sherkhonov et al., 2014). However, some intriguing possibilities remain. First, are there other routes for changing membrane lipid composition? Second, can other membrane characteristics be addressed to improve tolerance? Finally, besides carboxylic acids, could membrane engineering exhibit general effects in increasing microbial robustness in the context of other bio-products and/or severe growth conditions?

In order to answer these questions, we introduced a non-native component into the *E. coli* membrane. Specifically, trans unsaturated fatty acids (TUFA) were synthesized and incorporated into the membrane of *E. coli* via expression of the Cti enzyme from *Pseudomonas aeruginosa*. In contrast to other engineering strategies (Lennen and Pfleger, 2013; Sherkhonov et al., 2014), our engineered strain showed no change in the membrane phospholipid length or integrity. Instead, there was a decrease in membrane fluidity. This engineered strain displayed increased tolerance to octanoic acid and increased octanoic acid titer. In addition, we found that TUFA production also enabled increased tolerance to other biorenewables as well as a variety of adverse environmental conditions of industrial relevance.

2. Materials and methods

2.1. Strains and plasmids

All plasmids and strains constructed in this study are listed in Table 1. Two forms of *cti*, intact *cti* with the native signal peptide

Table 1
Plasmids and strains used in this study.

Plasmids/Strains	Genetic characteristics	Resource
Plasmids		
pJMYEEI82564	pTrc-EEI82564 thioesterase (TE10) from <i>Anaerococcus tetradicus</i> , Amp ^r	(Royce et al., 2015)
pKD3	Containing FRT-Cat-FRT fragment, Cm ^r	(Datsenko and Wanner, 2000)
pET22b	Expression vector with <i>pelB</i> coding sequence, Amp ^r	Novagen
pET22b-cti	pET22b, FRT-Cat-FRT DNA fragment from pKD3 and <i>cti</i> gene from <i>P. aeruginosa</i> cloned into pET22b, Amp ^r Cm ^r	This study
pTpal-fdc	PAL of <i>A. thaliana</i> and FDC of <i>S. cerevisiae</i> inserted into pTrc99a plasmid, Amp ^r	(McKenna et al., 2013)
Strains		
<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i> wild type	Shao lab, ISU
MG1655	<i>E. coli</i> K-12 wild type	This lab
Control	MG1655, <i>ldhA</i> : FRT-Cat-FRT	This study
M1-12-Pacti	MG1655, <i>ldhA</i> : FRT-Cat-FRT, M1-12-Pacti	This study
M1-37-Pacti	MG1655, <i>ldhA</i> : FRT-Cat-FRT, M1-37-Pacti	This study
M1-93-Pacti	MG1655, <i>ldhA</i> : FRT-Cat-FRT, M1-93-Pacti	This study
M1-12-PeB-Pacti	MG1655, <i>ldhA</i> : FRT-Cat-FRT, M1-12-PeB-Pacti	This study
M1-37-PeB-Pacti	MG1655, <i>ldhA</i> : FRT-Cat-FRT, M1-37-PeB-Pacti	This study
M1-93-PeB-Pacti	MG1655, <i>ldhA</i> : FRT-Cat-FRT, M1-93-PeB-Pacti	This study
ldhAD+TE10	Control, Δ <i>fadD</i> , pJMYEEI82564	This study
M1-12-PactiD+TE10	M1-12-Pacti, Δ <i>fadD</i> , pJMYEEI82564	This study
ldhA-Sty	Control, pTpal-fdc1	This study
M1-12-Pacti-Sty	M1-12-Pacti, pTpal-fdc	This study

sequence and a truncated *cti* with the PelB signal peptide sequence, were regulated by three different promoters with varying strengths (M1-12, M1-37, M1-93) (Lu et al., 2012; Tan et al., 2013) and then inserted individually into the *E. coli* MG1655 genome. For octanoic acid production the *fadD* gene was deleted to prevent fatty acid degradation and strains were transformed with the pJMYEEI82564 plasmid (Royce et al., 2015) harboring thioesterase (TE10) from *Anaerococcus tetradius* (Jing et al., 2011). One-step recombination method (FLP-FRT) was employed in this study (Datsenko and Wanner, 2000).

2.2. Growth rate characterization

Specific growth rate μ (/hr) was calculated by fitting the equation $OD = OD_0 e^{\mu t}$ over the duration of the exponential growth phase. OD was measured at 550 nm and all estimated μ values had an R^2 of at least 0.95.

2.3. Strain tolerance characterization

All tolerance experiments were implemented in 50 mL MOPS 2.0% (w/v) dextrose medium (Neidhard. et al., 1974) in 250 mL baffled flasks at 200 rpm and initial pH of 7.0. Tolerance to octanoic acid was assessed at 30 °C, high temperature tolerance was assessed at 42 °C, and all other tolerance experiments were performed at 37 °C.

2.4. Membrane characterization

Membrane integrity was analyzed by SYTOX Green staining and membrane fluidity was measured by using 1,6-diphenyl-1,3,5-hexatriene (DPH). Membrane composition was measured by a modified Bligh and Dyer method. All of these analyses were performed as previously described (Royce et al., 2013). The two-tailed *t*-test method was employed to analyze the statistical significance of all data in this study.

2.5. Batch fermentation for carboxylic acid production

Individual colonies were selected from Luria Broth (LB) plates with 100 mg/L ampicillin, inoculated into 3 ml of LB liquid medium with 100 mg/L of ampicillin for 4 h. Then, 1 ml of culture was added to 50 ml MOPS 2.0% dextrose medium with 100 mg/L of

ampicillin at 30 °C, 200 rpm overnight for seed culture preparation. Mid-log phase seed culture was collected, resuspended with fresh MOPS 2.0% dextrose medium and transferred into 300 ml MOPS 2.0% dextrose medium with 100 mg/L of ampicillin and 1 mM of isopropyl- β -D-thiogalactopyranoside (IPTG) in 500 ml fermentor (INFORS HT) at the final concentration of $OD_{550} \sim 0.1$. Cultures were grown at 30 °C, and the pH was maintained at 7.0 by addition of 2 M KOH. Air flow rate was maintained at 0.3 L/min initially. The dissolved oxygen (DO) level was set over 40% and controlled by changing the stirring speed to a maximum of 600 rpm.

2.6. Determination of carboxylic acid titers

For total fatty acids titer measurement, 400 μ L of culture was extracted and quantified as previously described (Torella et al., 2013). For free fatty acids analysis, the fermentation broth was centrifuged and same volume of supernatant was quantified (Torella et al., 2013).

2.7. Styrene production

The pTpal-fdc plasmid (McKenna et al., 2013) harboring *PAL2* from *Arabidopsis thaliana* and *FDC1* from *S. cerevisiae* was transformed into *E. coli*. A single colony was selected and inoculated into 5 ml of LB liquid medium with 100 mg/L of ampicillin. After incubation at 32 °C, 220 rpm overnight, this seed culture was added to 50 ml of MOPS 2.0% dextrose with 100 mg/L of ampicillin, 1 g/L L-phenylalanine, 0.5 mM of IPTG at the final concentration of $OD_{550} \sim 0.1$ in 250 ml shake flasks. This culture was incubated at 32 °C, 220 rpm for 72 h. Styrene was quantified as described by McKenna and Nielsen (2011).

3. Results

3.1. Enabling TUFA production and incorporation in the *E. coli* membrane

Phospholipids are the primary structural constituent of the cell membrane (Villanueva et al., 2013). A phospholipid molecule generally consists of a hydrophilic phosphate head and two hydrophobic fatty acids tails (Mashaghi et al., 2013). There are two

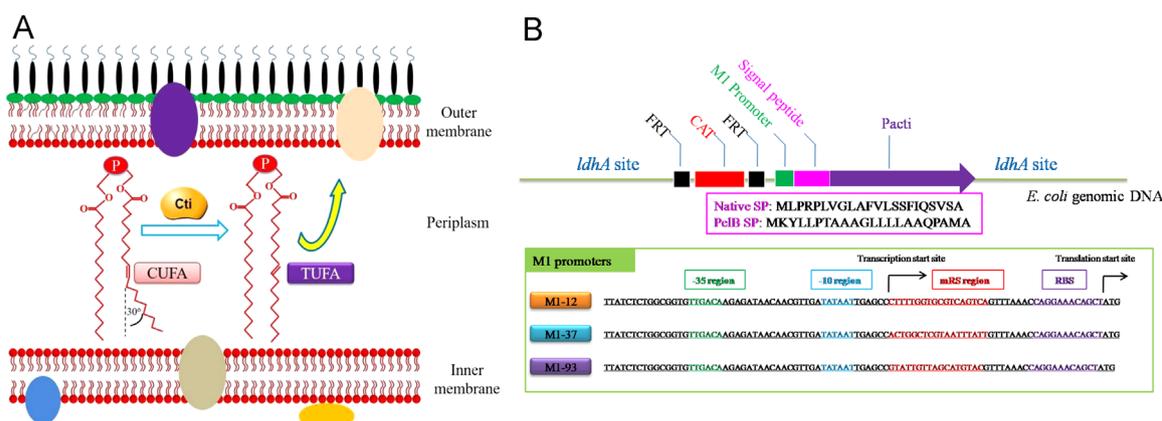


Fig. 1. Functional characteristics and expression of cis-trans isomerase (Cti) in *E. coli*. (A) Conversion of cis unsaturated phospholipid fatty acids (CUFA) to trans unsaturated phospholipid fatty acids (TUFA) by Cti. Mature Cti is located in the periplasmic space of *Pseudomonas* and *Vibrio* strains. CUFA will form a kink with an angle of 30° at double bond position ($\Delta 9$). Cti changes the configuration of CUFA at the $\Delta 9$ position to form the corresponding TUFA without a kink. (B) Integration of *cti* from *Pseudomonas aeruginosa* (Pachi) into genomic DNA of *E. coli* MG1655 at the *ldhA* (lactate dehydrogenase) site using FRT-CAT-FRT as selection marker. Two kinds of signal peptide (SP), native SP and PelB SP were used to transport Cti across the inner membrane. Three different promoters (M1-12, M1-37, M1-93) were employed to regulate the expression of *cti*. The three promoters have the same -35 region (green), -10 region (cyan) and RBS region (purple), while the mRS (mRNA stabilizing) regions (red) are distinct. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

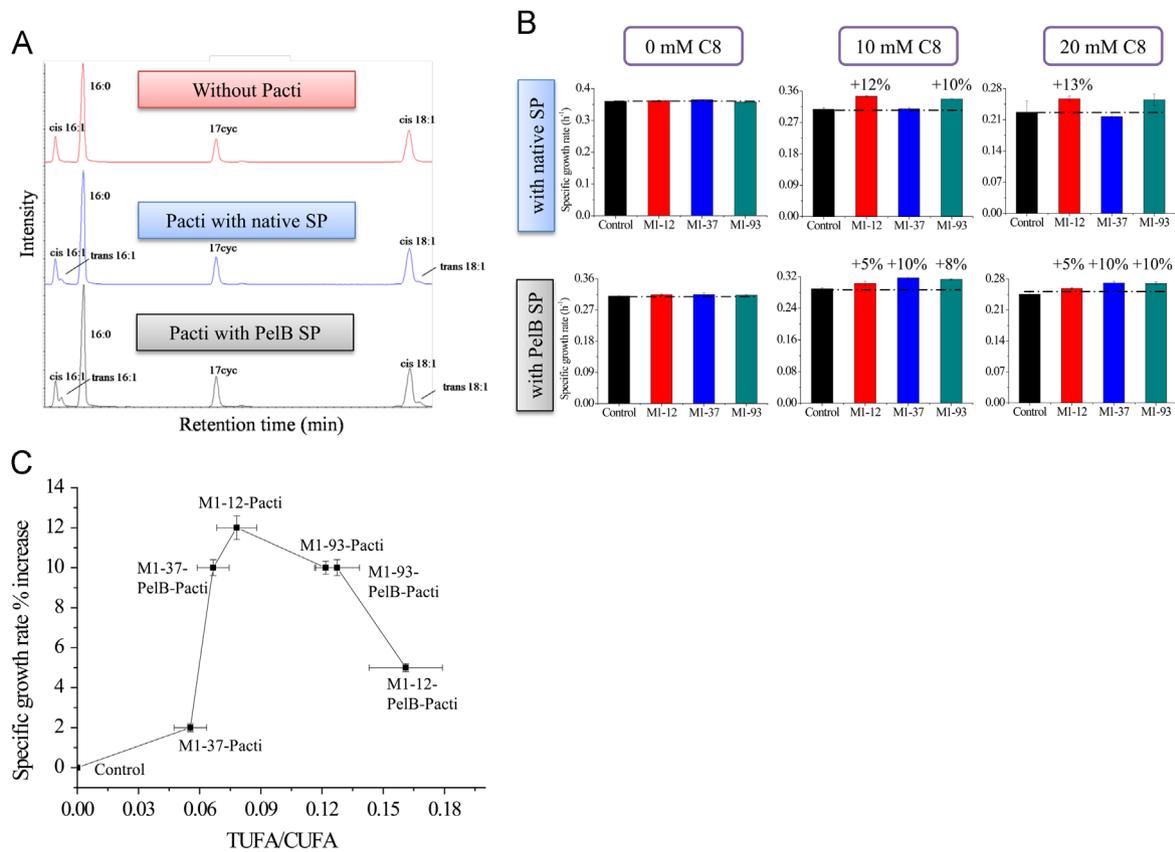


Fig. 2. Appropriately tuned production of TUFA enables increased octanoic acid tolerance. (A) Membrane fatty acids profile of strains without Pacti (upper), Pacti with the native signal peptide (SP, middle), and a truncated Pacti with the PelB SP (lower). Either using native SP or PelB, Pacti could be transported into periplasm of *E. coli* and catalyze TUFA formation (e.g. trans 16:1, trans 18:1). (B) Specific growth rate changes of cti engineered strains with different promoters (M1-12, M1-37, M1-93) under varying concentrations of octanoic acid (C8) conditions. Strains were grown in MOPS + 2% dextrose shake flasks at 200 rpm 30 °C with an initial pH of 7.0. (C) Relationship between ratio of TUFA/CUFA and specific growth rate % increase under 10 mM C8 condition. Values are the average of three biological replicates with error bars indicating one standard deviation. % increase values are only shown for differences that were deemed statistically significant ($P < 0.05$) using a two-tailed t-test.

different forms of fatty acids in phospholipids: saturated fatty acids (SFA), e.g. C16:0, and unsaturated fatty acids (UFA), e.g. C16:1. UFAs can be further divided into cis unsaturated fatty acids (CUFA) and TUFA. Although CUFA and TUFA with same carbon numbers have identical average chain length, their conformations are distinct. CUFAs bend at an angle of $\sim 30^\circ$ at the $\Delta 9$ double bond position while TUFAs are straight, resembling the structure of SFA (Fig. 1A) (Heipieper et al., 2003).

Packing of CUFAs in the membrane will form a disorderly membrane and increase its fluidity (Holtwick et al., 1997). CUFAs can, however, be converted to the corresponding TUFAs, as demonstrated by several *Pseudomonas* and *Vibrio* strains (Diefenbach et al., 1992). The conformational change enabled by this process, known as homeoviscous adaptation (Heipieper et al., 2003; Diefenbach et al., 1992; Weber et al., 1993; von Wallbrunn et al., 2003), increases the membrane phase transition temperature, thereby improving survival in severe environments, including exposure to toxic organic solvents. However, model biorenewables-producing microorganisms, such as *E. coli* and *S. cerevisiae*, have not been reported to naturally produce TUFAs. The enzyme responsible for catalyzing the conversion of CUFAs to TUFAs is Cis-trans isomerase (Cti). Therefore, we proposed that enabling cis-trans isomerization in model strains via expression of the Cti enzyme might increase robustness in adverse conditions typically faced in a bioproduction environment.

Mature Cti isomerase is located in the periplasmic space of *Pseudomonas* and *Vibrio* sp and a signal peptide is required for its transport across the cytoplasmic membrane (Pedrotta and Witholt, 1999). Although Cti contains a native signal peptide, we postulated that it might not be correctly transported in *E. coli*.

Thus, two different strategies for expression of *P. aeruginosa* cis-trans isomerase (Pacti) in *E. coli* were employed: (1) Expression of intact Pacti with its native signal peptide; (2) Substitution of the native signal peptide with the PelB signal peptide (Fig. 1B). In addition, for the sake of tunable expression of Pacti, three different constitutive promoters, M1-12, M1-37, M1-93 (Lu et al., 2012; Tan et al., 2013), of which the mRNA stabilizing region (mRS) sequences are different, were employed. Thus, six Pacti engineered *E. coli* strains with different combinations of promoters and signal peptides were constructed (Table 1).

Strains expressing Pacti with either its native signal peptide or the PelB signal peptide were found to contain TUFA in the membrane (Fig. 2A), meaning that in both cases, Cti was able to be transported to the periplasmic space.

3.2. Production of TUFA increases *E. coli* octanoic acid tolerance

In order to investigate the effect of TUFA production on *E. coli* tolerance of octanoic acid, the growth of our six engineered strains was assessed in the presence of three different concentrations of octanoic acid. Note that there was no significant difference ($P < 0.05$) in the specific growth rate between all Pacti engineered strains and the control strain in the absence of octanoic acid (Fig. 2B). Consistent with our hypothesis, most of the Pacti engineered strains were found to have increased tolerance to octanoic acid compared with the control strain (Fig. 2B). For instance, in the presence of 10 mM octanoic acid, the specific growth rate of M1-12-Pacti and M1-93-Pacti increased 12% ($P = 0.007$) and 10% ($P = 0.008$) over the control strain (0.308 h^{-1}), respectively. This

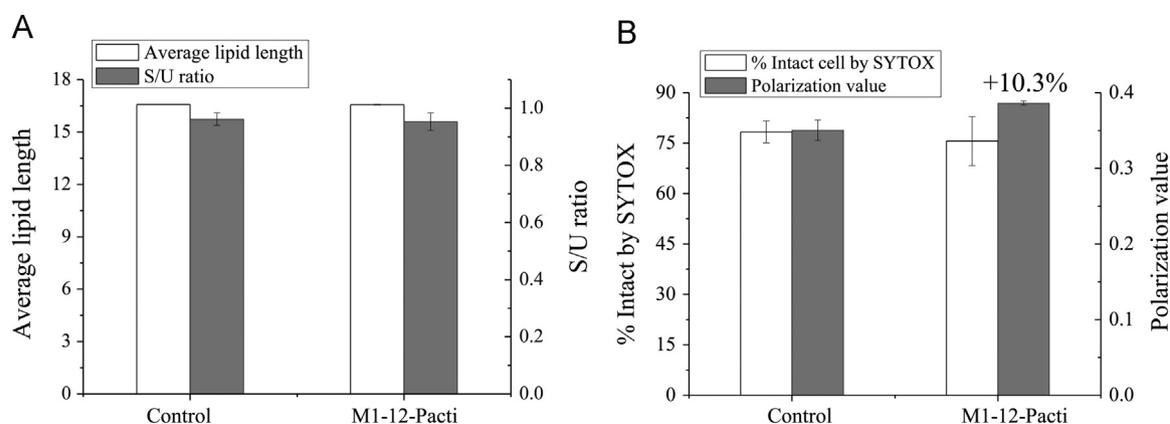


Fig. 3. TUFA production primarily alters membrane rigidity, not integrity. All characteristics were assessed after growth in the presence of 10 mM C8 at 30 °C, initial pH 7.0. (A) Average lipid length and saturated/unsaturated (S/U) ratio of M1-12-Pacti engineered strain and control strain. No significant changes of either metric was observed. (B) Percentage of intact cells, assessed using SYTOX Green, and membrane polarization, assessed using DPH, of M1-12-Pacti and control strain. % increase values are only shown for differences that were deemed statistically significant ($P < 0.05$) using a two-tailed t -test. Values are the average of three biological replicates with error bars indicating one standard deviation.

trend continues under higher concentrations of octanoic acid. In the presence of 20 mM octanoic acid, the specific growth rate of M1-12-Pacti and M1-93-Pacti strains were 13% ($P=0.048$) and 12% ($P=0.078$) higher than the control strain (0.227 h^{-1}).

However, not all Pacti engineered strains had significantly increased octanoic acid tolerance. Strain M1-37-Pacti had specific growth rates similar to the control strain in the presence of 10 or 20 mM octanoic acid (Fig. 2B). Further characterization showed that the molar ratio of TUFA/CUFA in M1-37-Pacti is only 0.055 ± 0.008 , which although higher than that in the control strain (~ 0), is lower than that in the other engineered strains (Fig. 2C). This suggests that the lack of increased octanoic acid tolerance of M1-37-Pacti results from the lower TUFA/CUFA ratio.

Consistent with these findings, we found that there is a dual relationship between the TUFA/CUFA ratio and the specific growth rate increases in the presence of 10 mM octanoic acid (Fig. 2C). For instance, the TUFA/CUFA ratio in M1-12-Pacti is 0.078 ± 0.01 , and there was a 12% increase in specific growth rate relative to the control strain. However, ratios larger than 0.09 were associated with decreased specific growth rate. For instance, the TUFA/CUFA ratio in M1-12-PelB-Pacti is 0.16 ± 0.02 , which is about 2-fold higher than the ratio of M1-12-Pacti, but the specific growth rate of M1-12-PelB-Pacti strain only increased by 5% relative to control strain. These findings suggest that careful tuning of the relative abundance of the TUFA is required in order to positively impact strain robustness.

3.3. TUFA-producing strains have increased membrane rigidity

The composition of the membrane contributes to its overall physical properties, such as membrane integrity and fluidity. Given that the M1-12-Pacti strain exhibits the most prominent increase in octanoic acid tolerance, we chose this strain for membrane characterization under 10 mM C8 stress.

In previous studies, altering the saturated/unsaturated (S/U) ratio in the membrane or decreasing the incorporation of shorter lipids into the membrane have been the main routes used to address membrane damage (Lennen and Pfleger, 2013; Sherkhonov et al., 2014; Luo et al., 2009). However, here we found that membrane S/U ratio was not changed in our TUFA-producing strains. For instance, the S/U ratio in M1-12-Pacti was not significantly different from the non-TUFA producing control strain (Fig. 3A). In addition, the average lipid length in M1-12-Pacti was almost identical to that of the control strain (Fig. 3A), suggesting

that the membrane “thickness” was not affected by TUFA production and incorporation.

Membrane leakage has previously been observed in fatty acid producing strains, and efforts to improve membrane integrity have been effective in increasing viability (Lennen et al., 2011; Lennen and Pfleger, 2013). However, membrane integrity of M1-12-Pacti was not enhanced by TUFA production and incorporation (Fig. 3B). The fact that carboxylic acid tolerance was increased without increasing membrane integrity is distinct from previously-described strains with increased carboxylic acid tolerance (Lennen and Pfleger, 2013; Royce et al., 2015).

In contrast to the lack of change in membrane integrity, we found the membrane polarization value of M1-12-Pacti (0.386 ± 0.003) to be increased by 10.3% ($P < 0.01$) relative to the control strain (0.35 ± 0.01) (Fig. 3B). This means that the membrane fluidity of the engineered strain was decreased when TUFA production was enabled.

3.4. TUFA production increases octanoic acid production

Since the engineered TUFA-producing strains demonstrated increased specific growth rate when challenged with exogenously supplied octanoic acid, we tested whether this increased tolerance also enabled improved octanoic acid production. Specifically, the M1-12-Pacti strain was engineered for octanoic acid production by deletion of *fadD* and expression of a plasmid-encoded thioesterase, resulting in strain M1-12-PactiD+TE10. Analogous manipulations were also performed on the control strain to obtain the corresponding non-TUFA-producing *ldhAD*+TE10 strain.

When characterized in minimal medium, the engineered strain had a shorter lag than the control strain, both in terms of growth and glucose consumption (Fig. 4A). By 72 h, the two strains had reached similar optical density values. Starting at the 48 h time-point, the TUFA-producing strain had increased titers of octanoic acid and total fatty acids relative to the control strain (Fig. 4B). The final octanoic acid titer of the engineered strain was 41% higher (43.7 mg/L) than the control (31.0 mg/L) and total fatty acids were 35% higher (82.1 mg/L) than the control (61.0 mg/L, $P < 0.05$) (Fig. 4B). For both strains, approximately 90% of the fatty acids were secreted into the media. Specifically, the engineered strain produced 40 mg/L of free octanoic acid and 74.9 mg/L free total fatty acids, while the control strain produced 27.7 and 55.4 mg/L of free octanoic acid and free total fatty acids, respectively. Octanoic acid accounts for about 65% (by mol) of total fatty acids with the second most abundant fatty acid being C16, at 16–18% (Fig. S1).

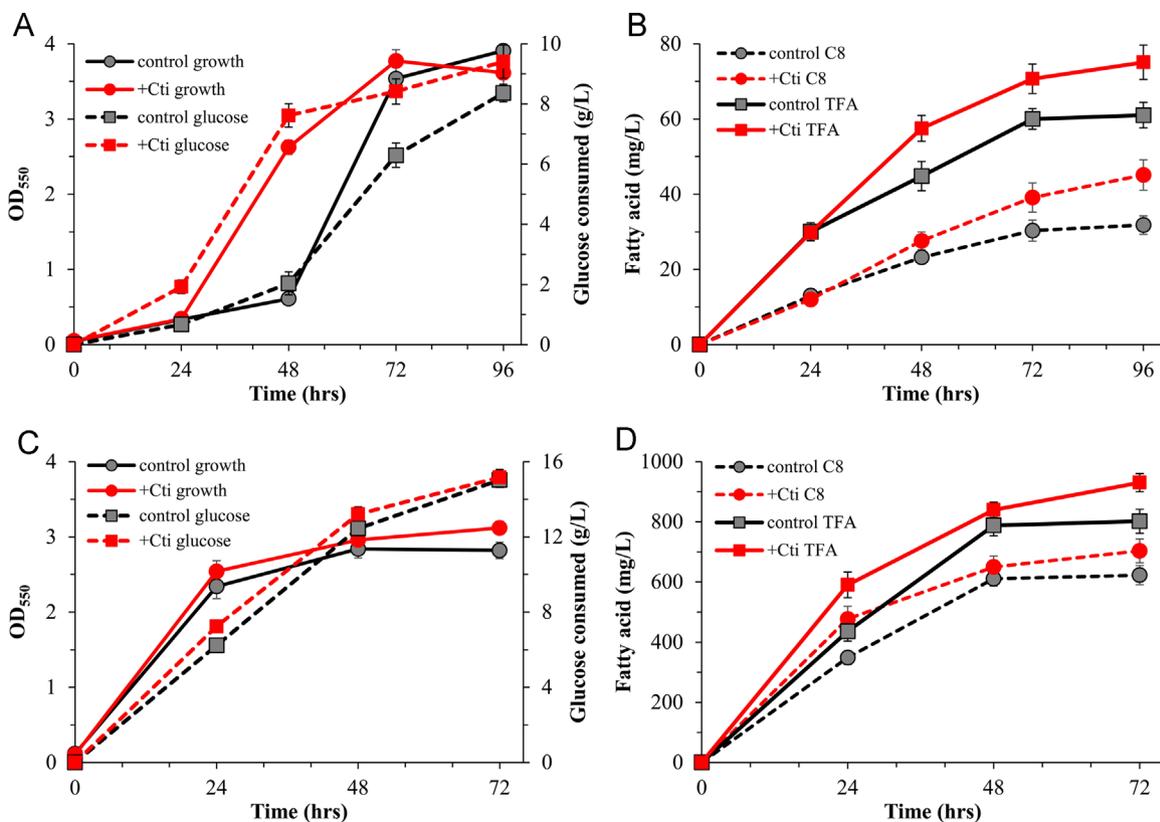


Fig. 4. TUFA production enables increased carboxylic acid titers. Production of octanoic acid (C8) and total fatty acids by TUFA-producing strains in (A and B) mineral salts medium (MOPS+2% (w/v) dextrose) at 30 °C, initial pH 7.0, 120 h, and in (C and D) rich medium (LB+1.5% (w/v) dextrose) at 30 °C, initial pH 7.0, 72 h. Both the engineered and control strain were subjected to deletion of *fadD* to prevent fatty acid degradation and both carry the pJMYEEI82564 plasmid, which harbors thioesterase 10 (TE10). Values are the average of three biological replicates with error bars indicating one standard deviation. Control, *ldhAD*+TE10. Pacti, M1-12-+Cti+TE10.

In rich media, growth and glucose consumption were similar for the engineered TUFA-producing strain and the control strain (Fig. 4C). The TUFA-producing strain produced a final titer of 730 ± 40 mg/L octanoic acid and 930 ± 30 mg/L total fatty acids, which are 29% and 16% higher than that in control strain (620 ± 30 mg/L, 800 ± 40 mg/L, $P < 0.05$) (Fig. 4D). The percent difference in the fatty acid titer between the TUFA-producing strain and control strain in rich medium is not as drastic as the difference in minimal medium. This might be due to the nutrients provided by the rich medium compensating for the damage caused by the fatty acids. Octanoic acid accounts for 76–80% (by mol) of total fatty acids, with the second most abundant fatty acid being C6, at 6–10% (Fig. S1).

In conclusion, these results demonstrate the effectiveness of TUFA production in increasing octanoic acid and total fatty acids production.

3.5. TUFA production increases tolerance to other bio-products and adverse conditions

Given that decreased fluidity is a general membrane characteristic change, we deemed that it should not be restricted to only improving tolerance of fatty acids. To this end, we investigated the tolerance of the M1-12-Pacti engineered strain to other inhibitory compounds and processing conditions. Four general classes of compounds were considered: other short-chain fatty acids (SCFA), alcohols, organic acids and aromatics, as well as three classes of adverse processing conditions: high temperature, low pH and high osmotic pressure (Fig. 5). Each of the investigated compounds is an attractive biorenewable chemical and each of the processing conditions can impact the process cost. Concentrations for these inhibitors were selected based on previous reports, so

that growth was at least moderately, but not completely, inhibited. For each of the compounds tested here, the specific growth rate of the control strain was decreased at least 13% but not more than 57% relative to the no-inhibitor control condition. Values for the specific growth rates are given in Table S1.

We found that besides octanoic acid (C8), the engineered TUFA-producing strain also showed increased tolerance to even shorter chain fatty acids. Specifically, the specific growth rate was increased by 15.0% during challenge with hexanoic acid (C6). However, TUFA production had a limited impact on tolerance to decanoic acid (C10).

For alcohols, although the TUFA-producing strain exhibited decreased specific growth rate during challenge with either ethanol or isobutanol, it did show 16% and 8.0% increased specific growth rate during challenge with n-butanol and hexanol relative to the control, respectively. Each of these four alcohols has previously been shown to inhibit the growth of *E. coli*, but with distinct modes of toxicity (Ingram and Vreeland, 1980; Brynildsen and Liao, 2009; Konas et al., 2015). In general, it has been reported that the degree of membrane damage will vary due to the structure of the focal alcohol, such as the chain length and whether the alcohol is straight-chained or branched (Huffer et al., 2011; Ingram and Vreeland, 1980).

For organic acids, there was a 5.6% increase of specific growth rate in the presence of acetate and a 5.5% increase when grown with succinate. Some hydrophobic aromatics have previously been shown to disrupt the cell membrane and are toxic to microbes (de Smet et al., 1978; Sikkema et al., 1995; Heipieper et al., 1991). Herein, we found that although there was a limited increase in tolerance of phenol, the engineered strain had significantly enhanced tolerance to styrene (17%) and toluene (20%) ($P < 0.05$).

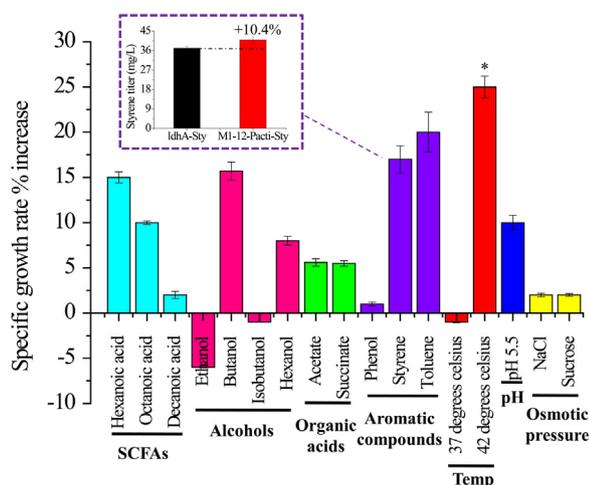


Fig. 5. TUFA production enables increased tolerance to a variety of inhibitory compounds and environmental stressors. Specific growth rate changes in the engineered TUFA-producing M1-12-Pacti strain relative to the control strain under a variety of chemical and environmental challenges. The final concentrations of each chemical are as follows. SCFA, hexanoic acid, 10 mM; octanoic acid, 10 mM; decanoic acid, 10 mM. Alcohols: ethanol, 2% (v/v), 343 mM; butanol, 0.6% (v/v), 66 mM; isobutanol, 0.6% (v/v), 66 mM; hexanol, 0.1% (v/v), 8 mM. Organic acids: acetate, 30 mM; succinate, 200 mM. Aromatic compounds: phenol, 11 mM; styrene, 2 mM; toluene, 10 mM. Acid condition: initial pH 5.5. Osmotic pressure: sodium chloride, 600 mM; sucrose, 480 mM. Values are the average of at least three biological replicates with error bars indicating one standard deviation. The inset shows the styrene titer of cti engineered strain and control strain. For styrene production, M1-12-Pacti and ldhA control strains were transformed with pTpal-fdc plasmid, and styrene titer data has a P -value < 0.05 using a two-tailed t -test. All tolerance data except under decanoic acid, isobutanol, phenol, 37 °C conditions have a P -value < 0.05 . Star (*), cell mass (g/L) increase. SCFA, short chain fatty acids. Temp, Temperature.

In addition to these inhibitory products, the TUFA-producing strain also had increased tolerance to harsh growth conditions. For instance, the specific growth rate in media that was at an initial pH of 5.5 was increased by 10%. This increased acid resistance could save processing costs by decreasing the base usage to maintain a near-neutral pH, and also decrease the vulnerability to contamination. Similarly, the engineered strain's specific growth rate at 42 °C improved by 15% relative to the non-TUFA producing control and its final cell mass increased by 25%. This increased thermotolerance could be useful in industrial applications by reducing cooling water usage and decreasing the probability of contamination. Note that TUFA production showed limited impact on tolerance to high osmotic pressure conditions (Fig. 5).

In conclusion, TUFA production showed diverse effectiveness in improving tolerance to a variety of inhibitory bio-products and conditions. As with the carboxylic acid tolerance, we furthermore verified that increased tolerance of styrene enabled increased styrene production. Specifically, our TUFA-producing strain was engineered to express a previously-described pathway that converts phenylalanine to styrene (McKenna et al., 2013). The control strain only contained the styrene production pathway and did not produce TUFA. We observed that in minimal medium containing phenylalanine, the TUFA-producing strain produced 10.4% more styrene than the control strain ($P < 0.05$) (Fig. 5, inset). These results again demonstrate the effectiveness of TUFA production on increasing both tolerance and production of biorenewable chemicals.

4. Discussion and conclusions

Product toxicity is a common obstacle for attaining sufficiently high titers of biorenewable fuels and chemicals (Nicolau et al.,

2010; Mukhopadhyay, 2015). Therefore, constructing robust organisms with high tolerance to these products is indispensable for industrial applications and has attracted extensive attention in recent years (Lennen et al., 2011, 2012; Lennen and Pflieger, 2013; Royce et al., 2015). The typical approach for improving tolerance is to use evolutionary methods (Jin et al., 2016), relying on Orgel's Second Rule that "evolution is cleverer than you are". Such studies have often confirmed the fact that the membrane is an effective target for increasing robustness. For example, our own evolution of an *E. coli* strain with improved carboxylic acid tolerance produced a strain with significantly altered membrane integrity, fluidity and composition (Royce et al., 2015). Similarly, evolutionary work in *S. cerevisiae* that aimed to improve ethanol production at 40 °C also identified critical changes in membrane composition (Caspeta et al., 2014).

Given this importance of the cell membrane for supporting overall metabolic behavior, the membrane has become a popular engineering target for increasing tolerance to some toxic products, including carboxylic acids or fatty acids (Liu et al., 2013; Lennen and Pflieger, 2013; Royce et al., 2014, 2015). Among these previous reports, many projects have focused on increasing the length of fatty acids in order to construct a "thicker" membrane with the goal of improving membrane integrity or decreasing leakage and this strategy did work in some cases (Lennen and Pflieger, 2013). However, except for membrane integrity, we know little about the impact of changes in other membrane characteristics on *E. coli* tolerance of inhibitory compounds.

Here, we rationally sought to improve the membrane robustness by enabling homeoviscous adaptation in *E. coli*. Specifically, we expressed the Cti enzyme from *P. aeruginosa* in order to convert some of the existing CUFA to TUFA. This production of TUFA is a novel behavior for this common fermentation organism. When expression of Cti was carefully tuned, increased tolerance and production of membrane-damaging compounds was observed. The TUFA appears to primarily impact membrane function via a decrease in membrane fluidity, as opposed to increased membrane integrity that has been observed for other engineering strategies (Lennen and Pflieger, 2013; Luo et al., 2009). This altered membrane fluidity is consistent with the structural advantages of TUFA over CUFA and may support appropriate functioning of membrane-associated proteins during fatty acid production. Given the presence of a 30° angle at the double bond in CUFA, the straight-chain TUFA will pack more tightly than the CUFA and thus increase membrane rigidity (Heipieper et al., 2003; Zhang and Rock, 2008). Presumably, a similar effect could also be seen with cis and trans forms of oleic acid (C18:1), though this has not been explored here.

We showed that the engineered TUFA producing strain had increased tolerance of a variety of compounds, including our primary target of octanoic acid, but also hexanoic acid, butanol, hexanol, acetate, succinate, styrene, and toluene. Robustness in industrially-attractive harsh conditions, such as high temperature (42 °C) and low pH (5.5) further demonstrated the effectiveness of this membrane engineering strategy. However, despite the effectiveness of TUFA production on increasing tolerance to these adverse conditions, excessive TUFA formation was found to be counter-productive. Specifically, we found that there is a positive relationship between ratio of TUFA/CUFA and tolerance to octanoic acid when the ratio is below 0.078, while excessive TUFA formation decreased tolerance. The optimum TUFA/CUFA ratio probably differs according to the chemistry of the inhibitory molecule.

The relationship between the chemistry of a molecule, its toxicity and the effectiveness of TUFA production may be related to the hydrophobicity of the molecule. For example, when considering the octanol/water partition coefficients for each of our focal compounds, an optimal hydrophobicity appears to exist, on

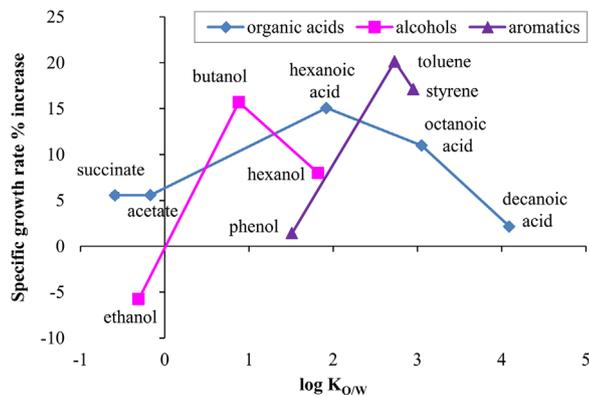


Fig. 6. The protective effect of TUFA production varies according to the molecule class and octanol/water partition coefficient $K_{O/W}$.

either side of which the efficacy of the TUFA incorporation strategy gradually diminishes (Fig. 6). For example, among the organic acids tested here, TUFA production was most helpful in providing protection against hexanoic and octanoic acid (Fig. 5). These two acids have $\log K_{O/W}$ values of approximately 2 and 3, respectively. TUFA production was not protective against decanoic acid, which has a $\log K_{O/W}$ value of 4, or acetic acid, which has a $\log K_{O/W}$ value of -0.2 . Said optima, however, appear to differ depending on the class of compounds investigated. For organic acids, this optima is around 2; alcohols, ~ 1 ; and aromatics ~ 2.5 . This perhaps highlights the different mechanisms of inhibition that they elicit.

Although product toxicity is often identified as a primary reason for limiting further improvements in strain performance, increased tolerance does not always lead to improved production. In this study, our rational membrane engineering approach of enabling TUFA production not only increased tolerance to exogenously added octanoic acid but also significantly increased the octanoic acid titer ($P < 0.05$). Similar results were observed in regards to styrene tolerance and styrene production. Thus, these two examples demonstrate the effectiveness of enabling TUFA production in improving biocatalyst performance.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ymben.2016.02.004>.

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