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

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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.

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

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