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Evolution for exogenous octanoic acid tolerance improves carboxylic acid production and membrane integrity

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ABSTRACT

Carboxylic acids are an attractive biorenewable chemical, but as with many biorenewables, their toxicity to microbial biocatalysts limits their fermentative production. While it is generally accepted that membrane damage is the main mechanism of fatty acid toxicity, previous metabolic engineering efforts that increased membrane integrity did not enable increased carboxylic acid production. Here we used an evolutionary approach to improve tolerance to exogenous octanoic acid, with the goal of learning design strategies from this evolved strain. This evolution of an *Escherichia coli* MG1655 derivative at neutral pH in minimal media produced a strain with increased tolerance not only to octanoic acid, but also to hexanoic acid, decanoic acid, n-butanol and isobutanol. This evolved strain also produced carboxylic acids at a 5-fold higher titer than its parent strain when expressing the *Anaerococcus tetradius* thioesterase. While it has been previously suggested that intracellular acidification may contribute to carboxylic acid toxicity, we saw no evidence that the evolved strain has increased resistance to this acidification. Characterization of the evolved strain membrane showed that it had significantly altered membrane polarization (fluidity), integrity (leakage) and composition relative to its parent. The changes in membrane composition included a significant increase in average lipid length in a variety of growth conditions, including 30 °C, 42 °C, carboxylic acid challenge and ethanol challenge. The evolved strain has a more dynamic membrane composition, showing both a larger number of significant changes and larger fold changes in the relative abundance of membrane lipids. These results highlight the importance of the cell membrane in increasing microbial tolerance and production of biorenewable fuels and chemicals.

1. Introduction

Carboxylic acids are an attractive biorenewable chemical, due in part to their ability to be catalytically converted to a variety of industrial chemicals, such as alkanes (Lennen et al., 2010) and olefins (Lopez-Ruiz and Davis, 2014). Significant strides have been made in engineering *Escherichia coli* and *Saccharomyces cerevisiae* for production of these compounds (Lennen and Pfleger, 2012; Choi and Da Silva, 2014; Leber and Da Silva, 2014; Tee et al., 2014). Unfortunately, the toxicity of these carboxylic acids to the microbial biocatalyst appears to be limiting biocatalyst performance. There is strong evidence that membrane damage is the main mechanism of toxicity (Lennen et al., 2011; Jarboe et al., 2013; Liu et al., 2013), including the observation that membrane leakage sharply increases near the end of the production phase (Royce et al., 2013). Previous metabolic engineering efforts that were successful in increasing the membrane integrity were not successful in increasing carboxylic acid titers (Lennen and Pfleger, 2013), though other efforts that increased tolerance by preventing medium-chain fatty acid incorporation into the cell membrane did enable increased production (Sherkhanov et al., 2014).

Product toxicity is frequently encountered in metabolic engineering projects (Nicolaou et al., 2010; Jarboe et al., 2011). There are a variety of strategies for mitigating this toxicity, including *in situ* removal of the product during the course of the fermentation (Dafoe and Daugulis, 2014; Lopez-Garzon and Straathof, 2014; McKenna et al., 2014) and increasing the robustness of the microbial biocatalyst. Biocatalyst tolerance can be increased either through an evolutionary approach (Portnoy et al., 2011; Abatemarco et al., 2013) or through rational engineering that addresses known mechanisms of toxicity (Luo et al., 2009; Jarboe et al., 2011). The evolutionary approach has been quite successful in improving strain performance when production of the target compound can be linked to growth (Jarboe et al.,

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2010). This linkage between growth and production generally requires redox balance and a net ATP production by the pathway. Evolution for tolerance to an exogenously-supplied inhibitor with the goal of increasing production of that same inhibitory compound has met with mixed results (Yomano et al., 1998; Atsumi et al., 2010; Wang et al., 2011).

The use of evolutionary-based strain improvement relies on Orgel's Second Rule that "evolution is cleverer than you are" (Dennett, 1996). In order to learn from this cleverness and increase our arsenal of rational strategies for strain improvement, it is important to reverse engineer these evolved strains. The outcome of such reverse engineering efforts have included the identification of improved succinate production pathways (Zhang et al., 2009), the identification of the major *E. coli* furfural reductase (Miller et al., 2009), methods of enabling ethanol resistance in *E. coli* (Haft et al., 2014) and methods for improving the yeast membrane for thermotolerance (Caspeta et al., 2014).

Here we describe the evolution of *E. coli* for tolerance to exogenously supplied octanoic acid, with a resulting 5-fold increase in carboxylic acid production and increased tolerance not just of carboxylic acids, but also of butanol isomers. This work describes the phenotypic characterization of this strain, with a particular focus on the membrane.

2. Methods

2.1. Strains and growth conditions

E. coli strains used in this study are listed in Table 1. Carboxylic acid stock solutions were prepared in 100% ethanol. The concentration of ethanol used in these stock solutions had no significant impact on growth (*data not shown*). Media were adjusted to pH 7.0 with 2.0 M potassium hydroxide.

2.2. Directed evolution and strain selection

Strain ML115 was subjected to 17 serial transfers in 350 mL MOPS medium (Wanner, 1994) with 2.0% dextrose and 350 mg/L chloramphenicol in 500 mL fleakers. The temperature was controlled at 37 °C and mixing was maintained at 150 rpm with magnetic stir bars. The pH was maintained at 7.0 using gravity-fed 2.0 M potassium hydroxide. The octanoic acid concentration was periodically increased in 10 mM increments. The final culture was plated onto solid media and individual colonies were selected and characterized by measuring the specific growth rate in closed fermentation vessels containing MOPS 2.0 wt% dextrose with 10 mM C8, pH 7.0, 150 rpm, 37° C. Colonies were first analyzed as mixed cultures of 5–6 strains. The strains populating the mixed population showing the highest specific growth rate were then characterized in pure culture. This work describes the characterization of the strain with the highest growth rate, LAR1.

2.3. Strain characterization

Tolerance to hexanoic, octanoic and decanoic acid was performed in 500 mL fleakers automatically controlled at 37 °C, 150 rpm and pH 7.0 in 350 mL MOPS 2.0% dextrose. Tolerance to n-butanol and isobutanol was assessed in 250 mL baffled flasks with 25 mL MOPS 2.0 wt% dextrose, 37 °C, 200 rpm, with an initial media pH of 7.0.

2.4. Plasmid construction

The acyl–acyl carrier protein thioesterase (acyl-ACP TE) from *Anaerococcus tetradius* (GenBank: EEI82564) which primarily produces octanoic acid (Jing et al., 2011) was provided by Dr. Nikolau at Iowa State University. The acyl-ACP TE originally cloned in pUC57 was digested with *BamHI* and *EcoRI* at 5′ and 3′ ends and cloned into pTrcHisB plasmid (Life Technologies) to produce plasmid pJMY-EEI82564.

2.5. Characterization of carboxylic acid production

Strains transformed with pJMY-EEI82564 were spread onto Luria Broth (LB) plates with 100 mg/L ampicillin and incubated at 30 °C overnight. Individual colonies were precultured in 5 mL LB media with 100 mg/L of ampicillin in 50 mL centrifuge tubes overnight, and then were inoculated into 250 mL shake flasks containing fresh 50 mL of LB media with 1.5% dextrose, 100 mg/L ampicillin, and 1.0 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were grown at 30 °C on a rotary shaker at 200 rpm. The initial ODs were approximately 0.07 and the cells were harvested after 72 h.

2.6. Determination of carboxylic acid titers

Carboxylic acid production was quantified by an Agilent 7890 gas chromatograph equipped with an Agilent 5975 mass spectroscope using flame ionization detector and mass spectrometer (GC-FID/MS) after carboxylic acid extraction as described in (Zhang et al. 2012). Briefly, 1 mL of whole liquid media was taken and extracted by 9 mL of a mixture of chloroform/methanol/water (1.5:1.0:1.0 vol) with 100 µl acetic acid and 20 µL of a mixture of internal standards (1.0 mg/mL for each). The organic phase (bottom) was filtered through a glass column filled with 1.0 g of magnesium sulfate, and then dried under nitrogen flow. After methylation with 4.0 mL of 5.0% sulfuric acid in methanol at 90 °C for 2 h, 2.0 mL of hexane/chloroform (4.0:1.0 vol) and 2.0 mL of 0.90% sodium chloride solution were added and vortexed. The top organic layer was filtered through a glass column filled with 0.20 g sodium bicarbonate and 1.0 g of magnesium sulfate, dried again with nitrogen gas, and then resuspended in 500 µL of hexane before injection to GC-FID/MS. The temperature for GC-FID/MS analysis was held at 50 °C for 1 min, ramped to 140 °C at 20 °C/min, and then to 220 °C at 4 °C/min before it was raised to 280 °C at 15 °C/min. Helium was used as a carrier gas and the flow rate was 1 mL/min through a DB-5MS separation column (30 m,

Table 1
Strains and plasmids.

Strain	Genotype	References
ML115 "parent"	MG1655 *fadD poxB ackA-pta: cmR*	(Zhang et al., 2012)
LAR1 "evolved"	ML115 evolved for C8 tolerance	This work
Plasmid		
pJMY-EEI82564	pTrc-EEI82564 thioesterase from *Anaerococcus tetradius* (Jing et al., 2011)	This work
pJTD1	pBad24-TorA-GFPmut3*	(Thomas et al., 2001)