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Engineering *Escherichia coli* membrane phospholipid head distribution improves tolerance and production of biorenewables☆

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ABSTRACT

Economically competitive microbial production of biorenewable fuels and chemicals is often impeded by toxicity of the product to the microbe. Membrane damage is often identified as a major mechanism of this toxicity. Prior efforts to strengthen the microbial membrane by changing the phospholipid distribution have largely focused on the fatty acid tails. Herein, a novel strategy of phospholipid head engineering is demonstrated in *Escherichia coli*. Specifically, increasing the expression of phosphatidylserine synthase (+*pssA*) was found to significantly increase both the tolerance and production of octanoic acid, a representative membrane-damaging solvent. Tolerance of other industrially-relevant inhibitors, such as furfural, acetate, toluene, ethanol and low pH was also increased. In addition to the increase in the relative abundance of the phosphoethanolamine (PE) head group in the +*pssA* strain, there were also changes in the fatty acid tail composition, resulting in an increase in average length, percent unsaturation and decreased abundance of cyclic rings. This +*pssA* strain had significant changes in: membrane integrity, surface potential, electrochemical potential and hydrophobicity; sensitivity to intracellular acidification; and distribution of the phospholipid tails, including an increase in average length and percent unsaturation and decreased abundance of cyclic rings. Molecular dynamics simulations demonstrated that the +PE membrane had increased resistance to penetration of ethanol into the hydrophobic core and also the membrane thickness. Further hybrid models in which only the head group distribution or fatty acid tail distribution was altered showed that the increase in PE content is responsible for the increase in bilayer thickness, but the increased hydrophobic core thickness is due to altered distribution of both the head groups and fatty acid tails. This work demonstrates the importance of consideration of the membrane head groups, as well as a modeling approach, in membrane engineering efforts.

1. Introduction

Construction of microbial cell factories for synthesis of bio-products using cheap and renewable feedstocks is an attractive alternative to current petroleum-based production methods (Larson, 2006; Energy, 2016; Kircher, 2015; Dale, 2011). A variety of microbes have been genetically engineered for production of biofuels, bulk chemicals, and high-value fine chemicals (Zhu et al., 2014; Park et al., 2012; McKenna and Nielsen, 2011; Atsumi et al., 2008; Galanie et al., 2015). Although many high-performing strains have been described, strain performance is often still limited by inhibition of microbial activity by components of the feedstock and the bio-products (Dunlop et al., 2011; Jarboe et al., 2011; Chen and Dou, 2016).

Arguably, membrane damage has been deemed as a fundamental mechanism of inhibitor toxicity due to the membrane's role as a protective barrier (Lennen et al., 2011; Liu et al., 2013; Royce et al., 2013; Zaldivar and Ingram, 1999). Ethanol was observed to fluidize the cell membrane, leading to leakage of important ions and arbitrary transport of solutes, which decreased the transmembrane potential and proton gradient (Huffer et al., 2011). Transcriptome-based analysis led to the conclusion that membrane damage is a key component of isobutanol toxicity, possibly due to disruption of the electron transport chain

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(Brynildsen and Liao, 2009). Both membrane integrity and cell viability decrease markedly during fatty acid production (Lennen et al., 2011). Our prior studies also showed that either exogenously-added or endogenously-produced fatty acids or styrene increased membrane leakage of *E. coli* (Royce et al., 2013; Lian et al., 2016). If one envisions each microbial biocatalyst as a reactor, this membrane damage is analogous to a reaction vessel being corroded by its contents. The standard approach in this scenario would be to change the composition of the vessel so that it is resistant to this damage. This approach can also be applied to the microbial cell membrane (Sandoval and Papoutsakis, 2016), with the goal of increasing tolerance (Lennen and Pfleger, 2013; Luo et al., 2009; Tan et al., 2016; Besada-Lombana et al., 2017) and production (Tan et al., 2016; Sherkhanov et al., 2014) of membrane-damaging compounds.

Fatty acids, which can serve as catalytic precursors for a variety of chemicals (Korstanje et al., 2015), are widely used in production of lubricants, preservatives, and fuels and thus are an attractive fermentation product. However, as stated above, these compounds have also been reported to cause microbial membrane damage during production (Lennen et al., 2011; Royce et al., 2013). The fatty acid tails of the membrane phospholipids have been a previous engineering target, with the intention of alleviating toxicity. For example, increasing the average length of membrane lipids partially alleviated the toxicity of fatty acids and increased fatty acids titers by 20% (Sherkhanov et al., 2014). Altering the relative distribution of the saturated and unsaturated fatty acids tails was effective in alleviating membrane leakage during fatty acid production, although fatty acids production was not increased (Lennen and Pfleger, 2013). Our prior study showed that production of non-native trans unsaturated fatty acids (TUFA) significantly decreased membrane fluidity and increased fatty acid tolerance and production (Tan et al., 2016). This TUFA production in *E. coli* also increased tolerance and production of other membrane-damaging biorenewables (Tan et al., 2016).

These previous results demonstrate that engineering of the length, degree of saturation and conformation of the phospholipid fatty acids can improve tolerance and/or production of membrane-damaging compounds (Lennen and Pfleger, 2013; Luo et al., 2009; Tan et al., 2016; Besada-Lombana et al., 2017; Sherkhanov et al., 2014). However, by focusing on the fatty acids, these studies addressed the tails of the phospholipid molecules, and we know relatively little about the role of the phospholipid head group in tolerance and production of membrane-damaging compounds. Inspired by our previous computational results which indicated that the degree of membrane disruption imposed by ethanol differed according to the phospholipid head group identity (Konas et al., 2015), here we postulated that engineering the phospholipid head group distribution might be an effective strategy for engineering robustness To this end, a proof-of-concept design was conceived and performed to alter the phospholipid head distribution. Specifically, the abundance of three different phospholipids (phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL)) with distinct head groups was modulated by altering the expression of key phospholipid biosynthesis enzymes PssA, PgsA, and ClsA. While other studies have concluded that alteration of the phospholipid head distribution is detrimental to cellular function and resistance to environmental stressors (Rowlett et al., 2017), here we report that increasing the relative abundance of the PE head group (+PE) increased tolerance and production of representative membrane-damaging short-chain fatty acids and also increased tolerance of the model biomass-derived inhibitors furfural and acetate. Computational analysis of this membrane engineering strategy indicates that this increased tolerance to membrane-damaging compounds is due to decreased penetration of membrane-damaging compounds into the membrane hydrophobic core and increased membrane thickness.

2. Materials and methods

Detailed materials and methods can be found in the online supporting material.

2.1. Strains and plasmids

All plasmids and strains used in this paper are listed in Table S1. All strains are derivatives of *E. coli* MG1655. One-step recombination (FLP-FRT) was used for chromosomal editing (Datsenko and Wanner, 2000). Gene expression was increased by chromosomal insertion of a second gene copy and regulation usually by strong promoter M1-93, or other promoters as specified (Zhu et al., 2014; Tan et al., 2013; Zhang et al., 2009; Lu et al., 2012). Copies of *pssA*, *clsA* and *pgsA* were inserted at *ldhA*; *acrAB* was integrated at *mgsA* site, and *tolC* was integrated at *maeB*. For octanoic acid production, the pJMYEEI82564 plasmid harboring thioesterase of *Anaerococcus tetradius* was used (San et al., 2011).

2.2. Growth conditions and characterization

All tolerance experiments were performed in 250 mL baffled flasks containing 50 mL MOPS with 2.0% (w/v) dextrose (Neidhard et al., 1974) at 220 rpm and initial pH 7.0. Tolerance to octanoic acid was assessed at 30 °C or 37 °C, high temperature tolerance was assessed at 42 °C, all other tolerance experiments were performed at 37 °C. Specific growth rate μ (h^{-1}) was calculated by fitting the equation $OD_{550,t} = OD_{550,0}e^{\mu t}$ to the exponential growth phase. All estimated μ values had an R^2 of at least 0.95.

2.3. Membrane and cell surface characterization

Membrane integrity was analyzed by SYTOX green (Invitrogen) staining (Lennen and Pfleger, 2013), membrane fluidity by 1,6-diphenyl-1,3,5-hexatriene (DPH) (Invitrogen) (Royce et al., 2013), membrane surface potential by monovalent cationic florescence dye 9-aminoacridine (9-AA) (Cerbon and Luna, 1991), and membrane electrochemical potential by $DiOC_2(3)$ dye (Thermo Fisher Scientific). Intracellular pH was measured by pTorA-GFPmut3* (pJDT1) plasmid (Royce et al., 2014). Cellular hydrophobicity was analyzed by addition of n-hexane (Royce et al., 2013). Membrane-bound fatty acids were extracted by a modified Bligh and Dyer method (Bligh and Dyer, 1959) and analyzed by GC-MS (Royce et al., 2013). Phospholipids distribution was analyzed by HPLC-ELSD (Agilent) (Becart et al., 1990).

2.4. Preparation of the corn stover hydrolysate

Corn stover hydrolysate was kindly provided by Nancy Nichols and Sarah Frazer from National Center for Agricultural Utilization Research, U.S. Department of Agriculture. Ten grams of corn stover were milled and passed through a 4 mm screen, mixed with 100 mL of 0.5% (v/v) sulfuric acid and heated to 160 °C for 10 min. The reaction product was filtered and the solids were washed. Calcium hydroxide was added, with stirring, to pH 6.5. The hydrolysate was filtered again and stored at or below 4 °C until use. The hydrolysate composition is given in Table S7.

2.5. Carboxylic acid production

Individual colonies from Luria Broth (LB) plates with ampicillin were inoculated into LB liquid medium with ampicillin and grown for 4 h. Then, 0.5 mL of culture was added to 20 mL MOPS 2.0% dextrose with ampicillin at 30 °C, 220 rpm and grown overnight as seed culture. Fermentations were performed in 50 mL MOPS 2.0% dextrose with ampicillin and an initial pH of 7.0 at an initial OD_{550} of 0.1. Where indicated, furfural, acetate and vanillic acid (FAV) were added at final