

Engineering 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 *E. coli*. Specifically, increasing the expression of phosphatidylserine synthase (+PE) 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 +PE 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 +PE 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 Ca^{2+} (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. Plasmids and strains

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 $\text{p}A_{\text{M1-93}}$ and $\text{p}A_{\text{M1-93}}$ were inserted at $\text{p}A_{\text{M1-93}}$ was integrated at $\text{p}A_{\text{M1-93}}$ site, and $\text{p}A_{\text{M1-93}}$ was integrated at $\text{p}A_{\text{M1-93}}$. For octanoic acid production, the pJMYEEI82564 plasmid harboring thioesterase of *C. glutamicum* was used (San et al., 2011).

2.2. Tolerance and production experiments

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 $\text{OD}_{550,t} = \text{OD}_{550,0}e^{\mu t}$ to the exponential growth phase. All estimated μ values had an R^2 of at least 0.95.

2.3. Membrane integrity and membrane fluidity

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 fluorescence dye 9-aminoacridine (9-AA) (Carbon and Luna, 1991), and membrane electrochemical potential by DiOC₂(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. Hydrolysis of corn stover

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. Furfural, acetate and vanillic 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

concentrations of 0.8, 1.2 and 0.5 g/L, respectively. When OD₅₅₀ ~ 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1.0 mM to induce thioesterase expression. Cultures were grown at 30 °C, 220 rpm for 72 or 96 h. Carboxylic acids were extracted from the fermentation broth and quantified by an Agilent 7890 gas chromatograph equipped with an Agilent 5975 mass spectroscope using flame ionization detector and mass spectrometer (GC-FID/MS). C7:0, C11:0, C13:0, C15:0 and C17:0 were used as internal standards.

2. Model lipid bilayers

Model lipid bilayers contained a total of 160 lipids maintained at 310.15 K and 1 bar. Systems were fully hydrated and the hydration numbers for systems with and without ethanol were specified as 50 and 35, respectively. All-atom molecular dynamics (MD) simulations using explicit solvent were carried out for all systems. Initial membrane conformations were built by CHARMM-GUI (Chang et al., 2012) (Jo et al., 2007, 2008, 2009; Wu et al., 2014). Minimization, equilibration, and isobaric-isothermic ensemble (NPT) MD simulations were performed using NAMD simulation package. Lennard-Jones potential was used to describe van der Waals interactions, with a force-based switching function in the range of 8–12 Å. After equilibration, each simulation was run for 300 ns using a time step of 2 fs. The Particle Mesh Ewald method was used for long-range electrostatics interactions. Hydrogen atoms were constrained by the RATTLE algorithm. The CHARMM36 (C36) force field was used (Klauda et al., 2010; Pandit and Klauda, 2012). Langevin dynamics maintained the temperature and the Nose-Hoover Langevin-piston algorithm was used to maintain the pressure. Three replicates were carried out for each system. The last 100 ns of each simulation were used for the analysis.

2.1. Simulation setup

The two-tailed *t*-test method was employed to analyze the statistical significance of all the data in this study.

3. Results

3.1. Phospholipid biosynthesis and genetic deletion

Phospholipids are the primary structural constituent of the cell membrane. A phospholipid molecule generally consists of a hydrophilic phosphate head and two hydrophobic fatty acid tails. In *E. coli*, there are three different phospholipids: PE, PG, and CL (Fig. 1) (Oliver et al., 2014). Head group diversity of the three phospholipids can be seen in Fig. 1. For PE, PG and CL, the specific head group is -CH₂-CH₂-NH₃⁺, -CH₂-CHOH-CH₂-OH and -CH₂-CHOH-CH₂-O-PG, respectively. The size, polarity and charge of these head groups are distinct. PE is neutral and typically accounts for nearly 70–90% of total phospholipids. PG is acidic and the second most abundant (10–25%), CL is also acidic, is the least abundant (< 5%) and exists in the septal/polar regions (Oliver et al., 2014). Fig. 1 shows the biosynthesis pathways for these phospholipids in *E. coli*. CDP-diacylglycerol (CDPG) is the common precursor, and the majority of the CDPG is used by phosphatidylserine synthase (PssA) to produce PE. Phosphatidylglycerophosphate synthase (PgsA) draws CDPG into the PG biosynthesis branch and two molecules of PG can subsequently be used by cardiolipin synthase (ClsA) to produce one molecule of CL. Therefore, PssA, PgsA and ClsA are key enzymes in phospholipid synthesis and altering their abundance is expected to change the relative abundance of these three head groups. To this end, genetic deletion of *pssA*, *pgsA* and *clsA* genes was attempted. The *pssA* gene was successfully deleted, but consistent with previous reports (Baba et al., 2006), *pssA* and *pgsA* were found to be essential in our condition. The Δ *clsA* strain was confirmed to lack detectable CL (Table 1) and, consistent with previous reports (Rowlett et al., 2017),

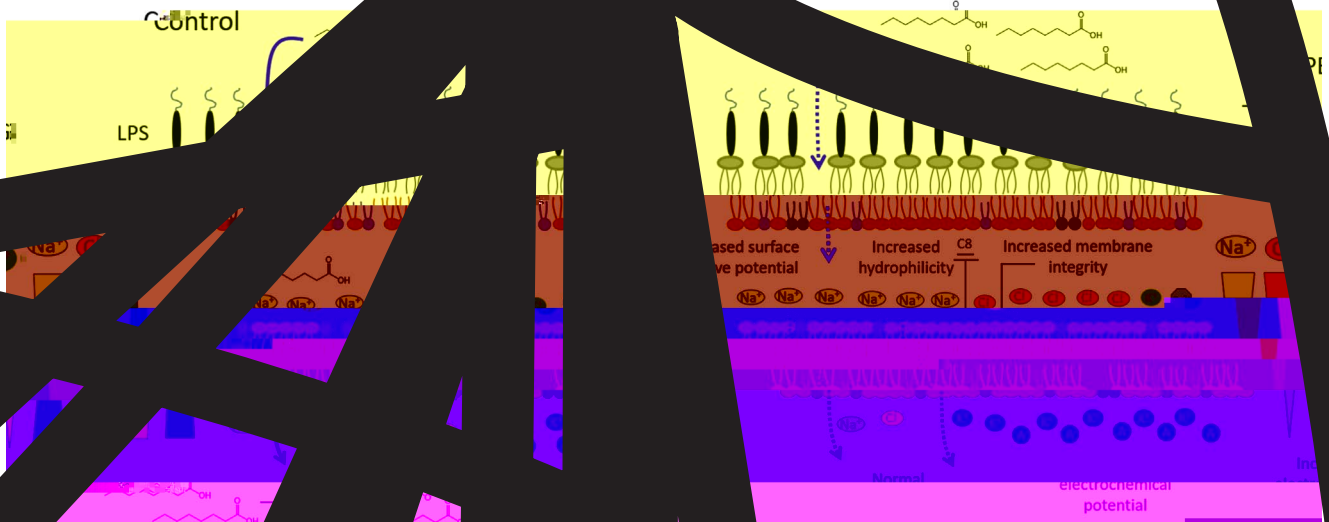
the CL-deficient strain had a decreased (*P* = 0.04) growth rate in defined media (Fig. 1).

Expression of *pssA*, *pgsA* and *clsA* was increased individually by chromosomal insertion of a second copy of each gene, regulated by the strong constitutive promoter M1-93 (Zhu et al., 2014; Tan et al., 2013), to obtain strains + *pssA*, + *pgsA* and + *clsA*. As expected, the relative abundance of PE, PG and CL significantly increased when each of these genes was overexpressed. Specifically, PE increased by 7% (from 88 ± 2 to 93.7 ± 0.4 mol%), PG increased by 38% (from 8.8 ± 0.4% to 12.1 ± 0.3%) and CL increased by 12% (from 3.3 ± 0.1% to 3.7 ± 0.2%) in the respective + *pssA*, + *pgsA* and + *clsA* strains relative to the control (Table 1).

The tolerance of these engineered strains to octanoic acid (C8), a representative membrane-damaging inhibitor, was assessed. Increasing the expression of *pssA* (+ *pssA*) did not result in a significant increase in specific growth rate during challenge with C8 (Fig. 1). An increase in PG content (+ *pgsA*) was found to compromise C8 tolerance: in the presence of 20 mM C8, the specific growth rate of + *pgsA* (0.17 h⁻¹) declined by 50% relative to the control strain (0.34 h⁻¹, *P* < 0.01) (Fig. S1). In contrast, an increase in PE content (+ *pssA*) was found to significantly enhance tolerance to C8. In the presence of 20 mM C8, the specific growth rate of + *pssA* (0.44 h⁻¹) increased by 29% over the control strain (0.34 h⁻¹, *P* < 0.01) (Fig. S1). Since increasing phospholipid PE content resulted in the largest increase in C8 tolerance, we chose the corresponding strain, + *pssA*, for further analysis.

3.2. Membrane leakage and membrane integrity

Membrane leakage is considered a primary mechanism of membrane damage and efforts to improve membrane integrity have been eff



given the M1-... strong activity (Zhu et al., ... relative ... PE content and the ... with ... -based character ... for ... which has lower act ... in previous ... et al., ... demonstrated that increased ... with in- ... to C8, we then test ... engineering production. To this end, ... was con- ... production. The AcrAB-TolC ... has been ... to free fatty acids efflux (Lef ... thus ... overexpressed via chromosomal ... A ... implemented in this strain, result ... JMYEEI82564 plasmid (Royce et al., ... from ... , which primarily ... P to ... C8, was also used, resulting in strain + Another ... manipulations were also performed to the control ... E10. When characterized in MOPS+ 2% (wt/v) ... medium, the + ... - 10 strain produced ... and 280 ± 6 mg/L of total fatty acids (TFA), which ... 46% ... higher than the corresponding control strain ... ± 10 ... , 200 ± 13 mg/L of TFA) respectively (Fig. 3B). The results ... that the membrane engineering strategy of in- ... sing the ... abundance of the PE phospholipid head is effective in ... easing ... toxicity, but also C8 production.

3.4. Membrane thickness and hydrophobic core thickness

The experimental analyses described above show that increasing the relative abundance of the PE phospholipid head group significantly alters a variety of membrane and cell surface properties. Molecular dynamics simulations were used to further our understanding of this membrane engineering strategy. Two membrane models were initially used: one representing the control strain and one representing the + 1.4 Å engineered strain. The relative abundance of the head groups and fatty acid tails in these models (Tables S3 and S4) were based on experimental data (Tables 1, 2). CL was not included in our membrane models, because these lipids are known to segregate to the polar and septal regions to induce curvature (Nishibori et al., 2005) and our approach is to focus on membrane structure outside these minor regions in the bacteria. Snapshots of the end of the simulations are shown in Fig. 4A.

In the absence of inhibitors, the control and + 1.4 Å membrane models both had a predicted surface area of 59 Å²/lipid (Table 3). The electron density profiles (EDPs) had a similar shape for both models (Fig. 4C), but a slight shift toward the center of membrane was seen at the peak point of total electron density profile of the control model. The simulated EDPs and volume probability profiles of the lipids and water were used to calculate the head-to-head group thickness (D_{HH}), bilayer thickness (D_B), and hydrophobic core thickness ($2D_C$) (Table 3). For each of these metrics, the + 1.4 Å membrane model has a higher value than the control model. Among these thicknesses, D_{HH} was statistically larger in the + 1.4 Å model ($P = 0.03$). While our experimental observation of an increase in the average fatty acid tail length in the + 1.4 Å strain (Table 2) suggests an increase in membrane thickness, these simulation results provide a more direct measure of membrane thickness and represent the diffusion barrier across the membrane.

To compare the fluidity of the two membrane models in the absence of inhibitors, the order parameters of fatty acid chains (S_{CD}) were calculated as the average of C4 to C6 of -1 for all lipids (Table S5). These were the same when comparing the two models in the absence of any inhibitors, which is in agreement with the equivalent lipid average surface areas described above. The area compressibility modulus (K_A) is the measure of rigidity of the membrane and also was not statistically different between the two models ($P = 0.16$). The fluidity experimentally measured by membrane polarization (Table S2) is similar between the strains, which might suggest that this is in agreement with our K_A findings. However, membrane polarization is more a measure of

lipid diffusion whereas K_A is an elastic measure of force required to laterally stretch the membrane. Thus, while the membrane thickness metrics suggest that the + 1.4 Å strain has a thicker membrane than the control, the lateral rigidity of these two membranes is predicted to be similar.

In addition to analyzing the control and + 1.4 Å membrane in the unperturbed condition, we also simulated these membranes during challenge with approximately 4.6 mol% ethanol. Ethanol was selected as a representative membrane-damaging compound due to it being a simple alcohol and because the + 1.4 Å engineered strain showed an approximately 10% increase in specific growth rate during challenge with 2% (v/v) (approximately 0.6 mol%) ethanol (Fig. 5). Ethanol penetration into the bilayers of both membrane types is evident in the snapshots in Fig. 4B. Consequently, the surface area per lipid increased in the presence of ethanol from approximately 59 to 67 Å²/lipid for both models (Table 3). Also, for both models, the total electron density profile peaks were shifted towards the center of the bilayer in the presence of ethanol (Fig. 4C; Fig. S3). For the model of the wild-type membrane, all measures of membrane thickness were substantially and significantly decreased in the presence of ethanol (Table 3). Similar decreases were observed in the + 1.4 Å model for D_{HH} and D_B . However, the impact of ethanol on the hydrophobic core thickness was substantially dampened relative to the wild-type strain (Table 3; Fig. S2). Specifically, the hydrophobic core thickness of the control membrane decreased by 1.4 Å ($P = 0.03$) during ethanol challenge, but only a 0.14 Å ($P = 0.6$) decrease was observed for the + 1.4 Å model. These results indicate that during exposure to ethanol, the + 1.4 Å membrane has a thicker bilayer and hydrophobic core than the control membrane and that its hydrophobic core thickness is more resistant to perturbation by ethanol.

For both models, the upper chain order parameter S_{CD} decreased as the ethanol concentration increased (Table S5), which is consistent with the decreased membrane thickness and increased surface area per lipid. However, the + 1.4 Å model had a significantly higher S_{CD} value for the DOPE phospholipid during challenge with ethanol relative to the control model ($P < 0.001$), which may contribute to the increase in hydrophobic core thickness. The penetration of ethanol into these two membrane types was quantified with component electron density profiles (Fig. S4). For the + 1.4 Å membrane, the electron density peaks for both the phosphate and carbonyl groups decreased as the ethanol concentration increased, due to increases in the surface area per lipid and penetration of ethanol into the head group region. In the presence of 4.6 mol% ethanol, the ethanol penetration into the bilayer is clearly

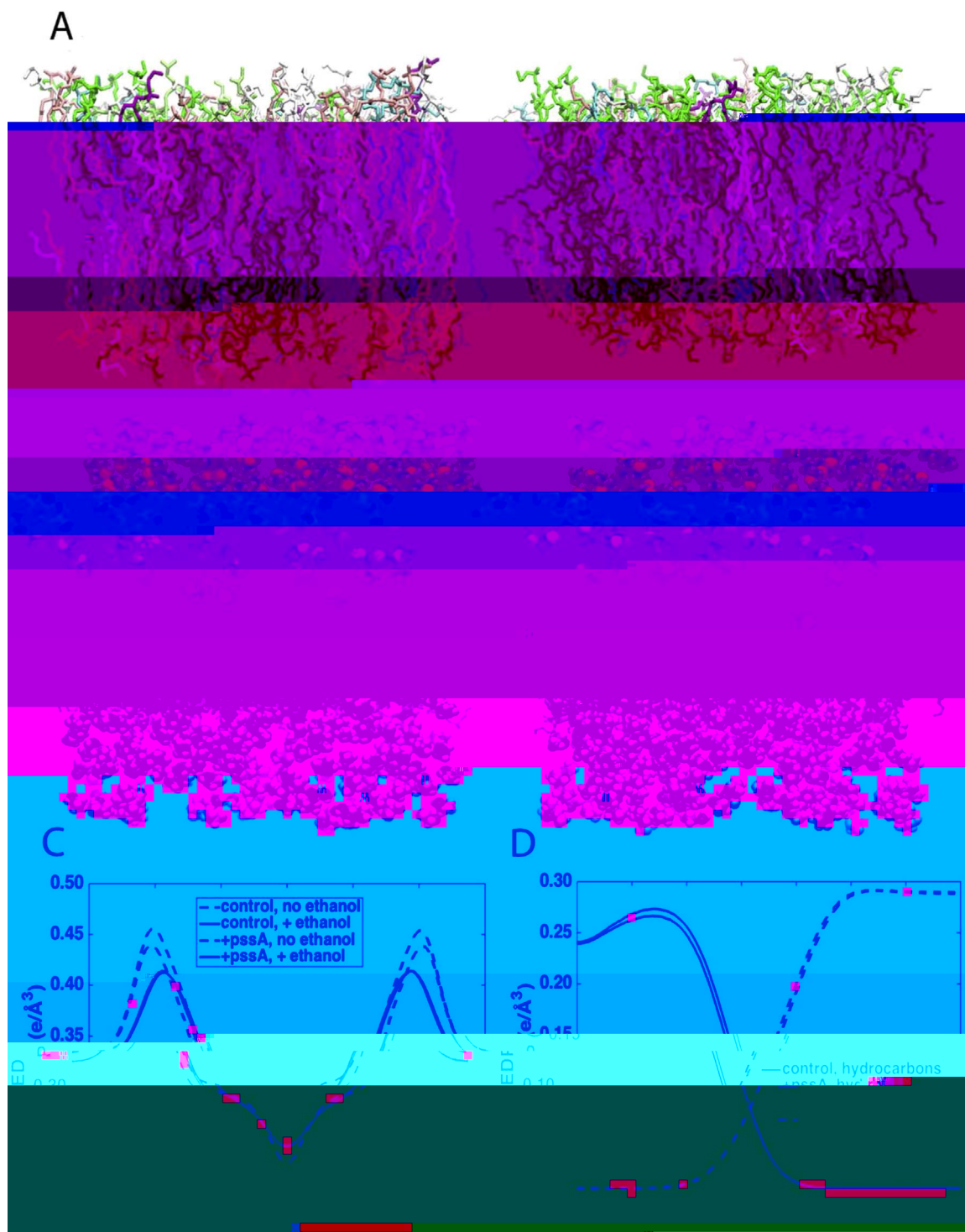


Fig. 4. (A) Left: Snapshot of simulation for the control membrane model. DOPE is shown in light green, DPPE in grey, DSPE in violet, DYPE in cyan, PMPE in pink, and PMPG in dark green. (A) Right: Last snapshot of simulation for membrane in +pssA model. Same color code was used. (B) Left: Simulation snapshot of the control membrane model with 4.64 mol% ethanol. Right: Simulation snapshot of the +pssA membrane model with 4.56 mol% ethanol. Lipids are shown in grey. Hydrogen atoms are not shown for clarity. Ethanol molecules are shown in van der Waals spheres. (C) Total electron density profiles of +pssA and control models with 0 and 4.6 mol% ethanol. (D) Component electron density profiles of water and hydrocarbons ($\text{CH}_3 + \text{CH}_2 + \text{CH}$) in +pssA and control models with 4.6 mol% ethanol.

reduced (Fig. S4C) for the +pssA model compared to control.

Overall, structural changes in the +pssA membrane led to increased ethanol resistance. The stress tolerant strain ultimately results in a thicker bilayer for the hydrophobic core and reduced ethanol penetration. Ultimately, this may decrease permeation of representative membrane-damaging compounds, which is consistent with our experimental C8 results (Figs. 1, 2).

3.2. Membrane thickness and ethanol resistance of the +pssA membrane

As described above, experimental characterization showed not only an altered distribution of the phospholipid head groups in the +pssA strain, but also an altered distribution of the fatty acid tails (Table 2). In order to distinguish the impact of the head group change from the

impact of the fatty acid tail change, we built two hybrid membrane models (Table S3). The + ... Δ control (head group/tail) hybrid model has the same head group composition as the + ... Δ model, while maintaining the fatty acid chains composition of control model. The second control/+ ... Δ hybrid model has the same head group composition as the control strain and the fatty acid chains from the + ... Δ model. Both of these hybrid models were assessed at ethanol concentrations of roughly 4.6 mol%.

A significant increase in head-to-head and bilayer thickness was predicted for the hybrid + ... Δ control model relative to the control (Table 3). Contrastingly, the control/+ ... Δ model did not show a significant difference in D_{HH} or D_B relative to the control. Therefore, the increase in bilayer thickness D_B observed for the + ... Δ model during ethanol challenge can be attributed to the change in the head group distribution.

However, it appears that the increase in hydrophobic core thickness in the + ... Δ strain relative to the control cannot be attributed only to the change in head group distribution or the change in fatty acid tail distribution. A significant increase in D_C was observed only in the + Table rg 3.55255 (inThid)-2ascd

compound. Given that an increase in PE content is a general membrane change, we hypothesized that it should not only confer with increased robustness to C8, but may also be effective in other conditions that possibly cause membrane damage. To this end, three general classes of inhibitory stresses were considered: (A) inhibitors found in hydrolysate of lignocellulose, (B) industrial fermentation products, and (C) adverse industrial conditions (Fig. 5). Each of the investigated bio-products is an attractive biorenewable chemical and each of the processing conditions can impact the process cost.

There are three main classes of inhibitors in dilute acid-treated lignocellulose feedstock: furans, weak carboxylic acids and phenolic monomers (Mills et al., 2009). Furans can cause DNA damage and inhibit metabolic processes (Mills et al., 2009; Miller et al., 2009). Weak carboxylic acids, such as acetate, can disrupt transmembrane potential and decrease intracellular pH (Mills et al., 2009; Trcek et al., 2015). Some phenolic monomers have been shown to cause membrane leakage and repress respiration (Mills et al., 2009; Fitzgerald et al., 2004). For cost-effective utilization of lignocellulosic biomass, construction of inhibitor-resistant host strains is desirable. We observed that increasing membrane PE content improved tolerance to most of these inhibitors (Fig. 5). Most dramatically, the + PE strain had a 41% increase in its specific growth rate (0.36 h^{-1}) relative to the control strain (0.25 h^{-1}) in the presence of 1.0 g/L furfural ($P < 0.001$). Further analysis showed that the + PE strain has a 2.7-fold increase in the concentration of furfural required for 50% inhibition (IC_{50}), from 0.34 g/L to 0.94 g/L (Fig. 5). Increased robustness was also observed to another furan derivative, hydroxymethylfurfural (HMF). In the presence of 2 g/L of HMF, the specific growth rate of + PE was 12% higher ($P < 0.001$) than the control strain. For weak carboxylic acids, the + PE strain had a 21% higher specific growth rate ($P < 0.001$) during challenge with 1.8 g/L of acetate, and it also had significantly increased tolerance to levulinic acid (Fig. 5). For phenolic monomers, the + PE strain showed a roughly 10% increase in specific growth rate relative to the control strain when challenged with vanillin, vanillic acid, ferulic acid and 4-hydroxybenzoate ($P < 0.05$) (Fig. 5).

Considering that the + PE strain showed improved tolerance to individual inhibitors known to be present in lignocellulose-derived feedstocks, and it also increased the production of bio-octanoic acid (Fig. 3B), we then tested them in combination. Three representative inhibitors, furfural, acetate and vanillic acid (FAV) were added to MOPS+ 2% (wt/v) glucose minimal salts medium for bio-octanoic acid production at final concentrations of 0.8, 1.2 and 0.5 g/L, respectively. The engineered + PE strain, + PE-TE10, produced $155 \pm 5 \text{ mg/L}$ of C8 and $216 \pm 8 \text{ mg/L}$ of TFA, which is 66% and 42%

more than the Control-TE10 ($93 \pm 11 \text{ mg/L}$, $153 \pm 14 \text{ mg/L}$), respectively (Fig. 6). This result confirms the usefulness of this engineering strategy during the production of a membrane-damaging bio-product in the presence of biomass-derived inhibitors. However, when we tested the effect of the + PE engineering strategy on the utilization of corn stover acid hydrolysate, a decrease in both C8 and TFA titers was observed relative to the control strain (Fig. S5). These results suggest that there may some inhibitor present in the hydrolysate whose toxicity was increased by this membrane engineering strategy.

Next, four different groups of bio-products were considered: short-chain fatty acids, organic alcohols, organic acids and other aromatic compounds distinct from those selected as representative of lignocellulose-derived feedstocks. Similar to the results observed for C8,)GYUIBPUSSE-aaub%ds9NOLP .f9f- h.66450C8S3;Gna50 > A* D2E*EK9D00

biorenewable fuels and chemicals at economically viable titers and yields on a large scale, toxicity of the bio-product and/or the feedstock is often problematic and frequently attributed, at least in part, to membrane damage (Lennen et al., 2011; Liu et al., 2013; Royce et al., 2013; Zaldivar and Ingram, 1999; Huffer et al., 2011). Given the magnitude of this problem, modification of the membrane composition in order to increase its robustness is an attractive approach. Currently, most membrane engineering efforts have focused on changing the distribution of the phospholipid fatty acids tails, particularly in terms of fatty acid length and abundance and conformation of double bonds and cyclic groups (Lennen and Pfleger, 2013; Luo et al., 2009; Tan et al., 2016; Besada-Lombana et al., 2017; Sherkhanov et al., 2014).

Previous simulations of lipid membranes demonstrated the variability of the interaction of different phospholipid head groups with membrane-penetrating molecules, such as ethanol (Konas et al., 2015), suggesting that altering the distribution of these head groups could change the sensitivity of the membrane to such compounds. However, reports of experimental characterization of *E. coli* strains deficient in the PE, CL or PG/CL head groups concluded that such changes had a largely negative impact on cell physiology and the response to environmental stresses (Rowlett et al., 2017). Here, we report that increasing the expression of a gene specific to synthesis of the PE head group, without eliminating PG or CL production, increased tolerance of a variety of chemical inhibitors, such as short-chain fatty acids, furans, acetate, ethanol, and toluene (Figs. 1, 3 and 5), and industrially-relevant harsh conditions, such as high temperature, low pH, and high osmotic pressure (Fig. 5). Our strategy of increasing PE content also seems to be novel in the context of microbial response mechanisms (Weber and de Bont, 1996; Ramos et al., 2002). Specifically, in the presence of toluene, *E. coli* strains decreased the relative abundance of PE while increasing PG and CL (Ramos et al., 2002), while here we observed that increasing PE content while decreasing PG and CL increased the specific growth rate in the presence of toluene by 30% relative to the corresponding control strain (Fig. 5).

Molecular dynamics simulations of the + *PE* membrane showed an increased bilayer (D_B) and hydrophobic core thickness during ethanol challenge relative to the control membrane (Table 3). This increased resistance of the + *PE* model membrane core to penetration by ethanol is consistent with the increased membrane integrity (Fig. 2A) and with the increased resistance of the + *PE* strain to intracellular acidification by exogenous octanoic acid (Fig. 2E). The increase in abundance of the electron carrier lipid ubiquinone-8 (Q8) during osmotic stress has also been suggested as a strategy of increasing the membrane hydrophobic thickness (Sevin and Sauer, 2014), though the branched-chain Q8 could also possibly increase the membrane rigidity by increasing the lateral compressibility modulus (Lim and Klauda, 2011).

Given previous reports that altering the phospholipid head group distribution has little impact on the fatty acid tail distribution (Rowlett et al., 2017), it was surprising that nearly all fatty acid tails had a significantly altered relative abundance in the + *PE* strain compared to the control strain (Table 2). The fact that the + *PE* strain has substantial changes in both the phospholipid head groups and in the fatty acid tails makes it challenging to distinguish the contribution of these two molecule types to the physical properties of the engineered membrane. The molecular dynamics simulations played a vital role in assessing the relative effects of these changes by allowing the generation and assessment of two hybrid models which would have been very difficult to develop in an experimental system. Besides being useful in addressing solvent tolerance, this simulation strategy can probably also be applied to other membrane-damaging conditions, such as the role of Q8 in osmotic stress tolerance (Sevin and Sauer, 2014).

Many membrane physical characteristics were found to be altered in the + *PE* engineered strain (Fig. 2). We observed a substantial increase in membrane integrity during octanoic acid challenge in the + *PE* strain (Fig. 2A), similar to previous reports of an association

between membrane integrity and octanoic acid tolerance (Lennen and Pfleger, 2013). These prior efforts to increase membrane integrity have included an increase in the average length of the phospholipid tails (Sherkhanov et al., 2014), with an increase in average length also observed in the + *PE* strain (Table 2), and decreasing the abundance of unsaturated lipid tails (Lennen and Pfleger, 2013), where the + *PE* strain actually had an increase in unsaturated fatty acid abundance (Table 2). It is also possible that the change in head group distribution contributes to the altered membrane integrity. Specifically, the PE head group is zwitterionic, and thus increased PE abundance could result in fewer instances of charge repulsion by the negatively-charged PG and CL head groups (Fig. 2F). However, the models predict similar surface area per lipid values for both strains (Table 3). The increased membrane integrity could instead be attributed to the thicker hydrophobic core of the + *PE* membrane and its increased resistance to chemical penetration, which is due to both the change in head group distribution and the change in lipid tail distribution.

Changes in the membrane surface negative potential and the membrane electrochemical potential were also observed (Fig. 2). Our observed association of increased membrane electrochemical potential with increased tolerance is consistent with the recent report that increasing the opposing potassium (K^+) and proton (H^+) electrochemical membrane gradients in *E. coli* increased resistance to multiple alcohols (Lam et al., 2014). Moreover, the membrane-associated electron transport chain generates the proton motive force ($\Delta\mu$), which is usually directed inward, driving H^+ flux into the cytoplasm and ATP formation. The proton motive force is a function of the membrane electrochemical potential $\Delta\psi$ and the hydrogen ion chemical gradient (ΔpH) (Fischer et al., 2000) and thus it is possible that the engineered + *PE* strain may have increased ATP production, though this was not measured. Note that previous characterization of strains lacking PE or CL showed decreased ATP content, but no change in membrane potential relative to the corresponding wild-type control (Rowlett et al., 2017).

The + *PE* strain was found to have a substantial decrease in cell surface hydrophobicity (Fig. 2D), consistent with reports that tuning cell surface hydrophobicity can impact tolerance of biomass hydrolysate (Westman et al., 2014) and solvents, such as nonane (Perpina et al., 2015). This decrease in hydrophobicity for the + *PE* strain can possibly be attributed to the fact that the PE head group ($-CH_2-CH_2-NH_3^+$) is more polar ($-CH_2-CHOH-CH_2-OH$) than the PG and CL groups ($-CH_2-CHOH-CH_2-O-PG$). *E. coli* strains lacking PE or CL have been reported to have altered ability to form biofilms and adhere to surfaces (Rowlett et al., 2017), though hydrophobicity of these strains was not reported. The octanol/water partition coefficient K_{OW} is widely recognized as being directly related to a molecule's toxicity (Inoue and Horikoshi, 1989) and similar to other membrane engineering efforts (Tan et al., 2016), we observed a dual relationship between the K_{OW} value for the organic acids tested here and the resulting increase in specific growth rate of the + *PE* strain (Fig. S6). Specifically, the + *PE* strategy was most helpful for increasing tolerance of organic acids with a log K_{OW} value between -0.5 and 1.0 .

Although product toxicity is often regarded as a primary limiter of strain performance, increased resistance does not always lead to increased production. Here, our rational membrane design of enabling PE increase not only improved tolerance to exogenously added C8 but also remarkably increased the C8 titer. The robust + *PE* membrane was expected to block the re-entry of C8 from the broth and thus efficiently alleviate the toxicity. Increased C8 production was also observed even under lignocellulose-derived FAV inhibitors conditions. These examples not only demonstrate the effectiveness of the + *PE* engineering strategy for increasing biocatalyst performance, but also suggest its potential for application to utilization of toxic lignocellulose feedstock for biorenewables production, which can achieve the goal of using cheap feedstocks with inhibitory components to produce bio-products at higher titers. Although previous studies of furan toxicity in *E. coli*

have not reported observation of membrane damage. (Mills et al., 2009; Zaldivar et al., 1999), we found that the + ... strain had drastically improved tolerance to furfural.

Author contributions

L.R.J., Z.T. and J.B.K. designed research; Z.T., Y.C., J.L., and J.M.Y. performed experimental research, and P.K. performed theoretical research; Z.T., J.V.S., L.R.J., P.K. and J.B.K. analyzed data; and Z.T., P.K., L.R.J. and J.B.K. wrote the paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ymben.2017.08.006>.

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