Microbial Cell Factories

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Escherichia coli

Zaigao Tan[1], William Black[1,2], Jong Moon Yoon[1], Jacqueline V. Shanks[1] and Laura R. Jarboe[1*]

Abstract

Background: Construction of microbial biocatalysts for the production of biorenewables at economically viable yields and titers is frequently hampered by product toxicity. Membrane damage is often deemed as the principal mechanism of this toxicity, particularly in regards to decreased membrane integrity. Previous studies have attempted to engineer the membrane with the goal of increasing membrane integrity. However, most of these works focused on engineering of phospholipids and e orts to identify membrane proteins that can be targeted to improve fatty acid production have been unsuccessful.

Results: Here we show that deletion of outer membrane protein *ompF* significantly increased membrane integrity, fatty acid tolerance and fatty acid production, possibly due to prevention of re-entry of short chain fatty acids. In contrast, deletion of *fadL* resulted in significantly decreased membrane integrity and fatty acid production. Consistently, increased expression of *fadL* remarkably increased membrane integrity and fatty acid tolerance while also increasing the final fatty acid titer. This 34% increase in the final fatty acid titer was possibly due to increased membrane lipid biosynthesis. Tuning of *fadL* expression showed that there is a positive relationship between *fadL* abundance and fatty acid production. Combinatorial deletion of *ompF* and increased expression of *fadL* were found to have an additive role in increasing membrane integrity, and was associated with a 53% increase the fatty acid titer, to 2.3 g/L.

Conclusions: These results emphasize the importance of membrane proteins for maintaining membrane integrity and production of biorenewables, such as fatty acids, which expands the targets for membrane engineering.

Keywords: Membrane engineering, Membrane integrity, Outer membrane protein, Tolerance, Fatty acid production

Background

Construction of microbial cell factories for production of biorenewable fuels and chemicals is a promising alternative to current petroleum-driven industries [1, 2]. A variety of microorganisms have been engineered for production of bulk chemicals, biofuels and high-value, fine chemicals [3–7]. However, performance of some biocatalysts can be restricted by various detrimental e ects, including toxicity of the product or components of the feedstock [8, 9]. A variety of adverse e ects could be the cause of this toxicity, e.g. intracellular acidification; DNA, RNA, protein and membrane damage [10]. Among these, membrane damage has been recognized as a common problem [11–15].

Membrane damage can be compared to a reaction vessel that is vulnerable to corrosion by its contents. In this scenario, a typical response would be to change the composition of the reaction vessel in order to increase resistance to corrosion. For microbial biocatalysts, the composition, function and physical properties of the membrane can be altered through targeted, rational genetic manipulation. Such genetic manipulation is consistent with Cameron and Tong's fifth application of cellular and metabolic engineering, "modification of cell properties" [16]. When enzymes, transporters and regulators are involved in this membrane engineering, it is

*Correspondence: ljarboe@iastate.edu
[1] 4134 Biorenewables Research Laboratory, Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011, USA
Full list of author information is available at the end of the article

also consistent with Bailey's 1991 definition of metabolic engineering as “the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory function of the cell with the use of recombinant DNA technology” [17].

is work focuses on membrane engineering to improve production of fatty acids, an attractive class of biorenewable chemicals which can be catalyzed to a variety of products with a large potential market, e.g. alkanes, olefins, esters, fatty aldehydes, and fatty alcohols [18–22]. Unfortunately, these fatty acids have been reported to cause a decrease in membrane integrity of *E. c* during both exogenous challenge and endogenous production [14]. Engineering of membrane phospholipids has proven as a powerful tool in addressing membrane integrity. Decreasing incorporation of medium-chain fatty acids into the membrane increased the average membrane lipid length, decreased the toxicity of fatty acids and increased fatty acid (C12–C14) production in rich medium from 0.60 to 1.36 g/L [23]. Expression of a thioesterase from *G c* sp. Y412MC10 that prevents medium-chain unsaturated acyl-ACPs from being incorporated into the phospholipids was shown to increase membrane integrity during fatty acid production, but there was no increase in fatty acid (C8–C14) production after 24 h in rich medium, with titers of 0.65 g/L observed with and without expression of the secondary thioesterase [24]. Both of these works demonstrate the feasibility of engineering the membrane lipid composition in order to increase membrane integrity and possibly enhance fatty acid tolerance and production [23, 24].

As e orts continue to increase the membrane integrity during production of membrane-damaging compounds, it becomes increasingly important to provide a su cient route of product export. Several studies have shown that increasing the expression of transporters can increase production of inhibitory compounds, such as valine [25] and limonene [8]. With the goal of using this strategy to improve fatty acid production, sixteen possible fatty acid transporters were characterized for their role in fatty acid tolerance and production [26]. is previous study identified several transporters that increased fatty acid tolerance when their expression was increased, but did not identify any such transporters that increased fatty acid production.

e transporters OmpF and FadL were part of the previous study. e OmpF protein exists as a trimer in the outer membrane and participates in the transport of sugars, ions, antibiotics and proteins across the outer membrane [27, 28]. FadL is an outer membrane ligand gated channel that functions in the uptake of exogenous long-chain fatty acids (LCFA), [29, 30], especially palmitic acid (C16:0) and oleic acid (C18:1), yet shows no binding to short-chain fatty acids (SCFA, <C10) [31]. Even though the previous characterization observed that deletion of *F* and *L* had no impact on fatty acid production [26], several other reports related to these two transporters (Table 1) motivated the further exploration of their role in fatty acid tolerance and production described here.

Two 2015 publications directly implicated OmpF in tolerance of exogenously supplied inhibitors, though in one case OmpF played a protective role and in the other it played a damaging role. Most relevant to our goal of improving fatty acid production is the demonstration that deletion of *F* dampened octanoic acid toxicity, with evidence that this deletion of *F* reduced SCFA entry into cells [32] (Table 1). is reduced entry of SCFA into cells was assessed by measuring the decrease in intracellular pH during challenge with exogenously supplied octanoic acid. Contrastingly, OmpF was found to be directly related to tolerance of three exogenously provided phenylpropanoids: rutin, naringenin and resveratrol [33]. Specifically, strains with increased expression of OmpF showed increased tolerance to these compounds and strains with decreased expression of OmpF showed decreased tolerance, leading to the proposition that OmpF participates in the removal of phenylpropanoids from the cell interior. us, OmpF showed a negative role in SCFA tolerance and a positive role in phenylpropanoid tolerance.

ere are also reports of FadL being involved in fatty acid production and tolerance to some inhibitors (Table 1). Increased expression of *L* resulted in increased conversion of exogenously supplied palmitic acid to ω-hydroxy palmitic acid [34]. is improved organism performance was attributed to increased uptake of palmitic acid, as data indicated that FadL was not involved in export of the hydroxylated product. Similarly, FadL seemed to play a crucial role in the import of octane for production of octanol, octanal and octanoic acid [35]. Specifically, production of these compounds from exogenously supplied octane was abolished when *L* was deleted. However, it was noted that this deletion of *L* increased survival during challenge with hexane, with the conclusion that FadL was the main route of hexane entry into the cell [35]. e phenylpropanoid studies described above also noted that FadL abundance was directly related to tolerance of exogenously supplied rutin, naringenin and resveratrol, the same trend was observed for OmpF, with the interpretation that FadL was involved in repairing membrane damage caused by these compounds [33]. However, even though phenol toxicity is often attributed to membrane damage [36], deletion of *L* had no impact on survival during phenol challenge [37]. us, FadL appears to be important to

Table 1 Previous reports of the role of OmpF and FadL in tolerance of membrane-damaging compounds

Compound	Condition	Result	Reference
OmpF, outer membrane porin F			
C_8–C_{14} fatty acids	Production of ~1 g/L fatty acids during growth in LB with glycerol, 37 °C	Deletion of *ompF* from a derivative of MG1655 had no impact on cell viability or membrane integrity	[26]
Octanoic acid (C8)	Challenge with up to 20 mM C8 in minimal media with glucose, tryptone and yeast extract at pH 7.0 and 37 °C	Deletion of *ompF* from BW25113 decreased sensitivity to C8, and increased expression of *ompF* increased sensitivity to C8. Sensitivity was assessed via the maximum OD. Deletion of *ompF* decreased the magnitude of intracellular acidification	[32]
Phenylpropanoids	Challenge with 1 g/L rutin, naringenin or resveratrol in M9 medium with casamino acids and glucose at 30 °C	Increased expression of *ompF* in BL21 increased the maximum specific growth rate during challenge. Decreased growth rate during challenge was observed when *ompF* expression was decreased	[33]
FadL, long-chain fatty acid outer membrane porin			
C_8–C_{14} fatty acids	Production of ~1 g/L fatty acids during growth in LB with glycerol at 37 °C	Deletion of *fadL* from a derivative of MG1655 had no impact on cell viability or membrane integrity	[26]
Palmitic and ω-hydroxy palmitic acids	Addition of 1 mM palmitic acid in potassium phosphate buffer with glucose or glycerol, 30 °C	Increased expression of *fadL* increased conversion of palmitic acid to ω-hydroxy palmitic acid. The increase was smaller in the presence of glycerol than glucose	[34]
Phenol	Challenge with phenol at 50–75% of the MIC in LB at 37 °C	Deletion of *fadL* from BW25113 had no impact on survival	[37]
Octane	Addition of ~20 vol% octane in LB at 37 °C	Deletion of *fadL* from a BW25113 derivative abolished conversion of octane to octanol, octanal and octanoic acid	[35]
Hexane	Challenge with 10 vol% hexane in LB at 37 °C	Deletion of *fadL* from BW25113 increased survival, as assessed by OD	[35]
Phenylpropanoids	Challenge with 1 g/L rutin, naringenin or resveratrol in minimal medium with casamino acids and glucose at 30 °C	Increased expression of *fadL* in BL21 increased the maximum specific growth rate during challenge. Decreased growth rate during challenge was observed when *fadL* expression was decreased	[33]

dextrose and 10 mM octanoic acid (1.44 g/L) in 250 mL ba ed flasks at 220 rpm and initial pH at 7.0, 30 °C. MOPS media contains the following: 8.37 g/L 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.72 g/L tricine, 2.92 g/L NaCl, 0.51 g/L NH_4Cl, 1.6 g/L KOH, 50 mg/L $MgCl_2$, 48 mg/L K_2SO_4, 348 mg/L K_2HPO_4, 0.215 mg/L Na_2SeO_3, 0.303 mg/L $Na_2MoO_4{\cdot}2H_2O$, 0.17 mg/L $ZnCl_2$, 2.5 µg/L $FeCl_2{\cdot}4H_2O$, 0.092 µg/L $CaCl_2{\cdot}2H_2O$, 0.031 µg/L H_3BO_3, 0.020 µg/L $MnCl_2{\cdot}4H_2O$, 0.0090 µg/L $CoCl_2{\cdot}4H_2O$, and 0.0020 µg/L $CuCl_2{\cdot}4H_2O$ [43, 44]. Specific growth rate µ (h^{-1}) was calculated by fitting the equation $OD = OD_0e^{\mu t}$ 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 [45]. Dry cell weight (DCW) was calculated from the optical density at 550 nm (1 $OD_{550} = 0.333$ g DCW/L).

Membrane integrity characterization

Cells were centrifuged, washed twice, and then resuspended in PBS bu er (pH 7.0) at a final OD_{550} of ~1. One hundred microliter (100 µL) of this suspension was mixed with 900 µL of PBS bu er and SYTOX Green (Invitrogen) was added to a final concentration of 5.0 µM. After resting at room temperature for 15 min, cells were analyzed by a BD Biosciences FACSCanto II flow cytometer equipped with standard factory-installed 488 nm excitation laser, signal collection optics, and fluorescence emission filter configuration. Instrument sheath fluid was filtered (0.22 µm) PBS bu er. Green fluorescence from stained cells was collected in the FL1 channel (525/50 nm). Forward scatter (FSC), side scatter (SSC), and FL1 (Green) parameters were collected as logarithmic signals. All data collections were performed at low flow rate setting (~12 µL/min) and cell concentrations were such that the event rate was below 5000 events/s. All samples were analyzed immediately after staining. Background noise and small debris was eliminated from data collection via a side scatter signal threshold that was established by examining samples containing only SYTOX Green staining bu er. Bacteria in SYTOX Green-stained samples were readily identified on the basis of FSC and SSC signals and an appropriate "Cell" gate was drawn to limit FL1 analysis to bacteria and exclude non-cell events. A minimum of 20,000 cell-gated events were collected for each sample. Green fluorescence data for these "cell" events were plotted as histograms showing the signal distribution of bacteria in the sample [14]. Flow cytometry data for this work is available via Flow Repository (https://flowrepository.org) FR-FCM-ZY2B.

Membrane lipid composition characterization

e membrane lipids were extracted by using the Bligh and Dyer method with minor modifications [14, 46]. Cells were centrifuged, washed twice with cold double-distilled water (ddH_2O), resuspended in 1.4 mL methanol and transferred to a new glass tube. Ten µicroliter of 1 µg/µL pentadecanoic acid (C15:0) dissolved in ethanol was added as internal standard. en, samples were sonicated, incubated at 70 °C for 15 min and centrifuged at 5000× for 5 min. e supernatant was transferred to a new glass tube and the cell pellet was resuspended in 0.75 mL of chloroform, shaken at 37 °C, 150 rpm for 5 min. Transferred supernatant and pellet suspension were combined, vortexed for 1 min and centrifuged at 5000×

Sequences of L primers for qRT-PCR are CTGAAATGTGGGAAGTGTC/GAAGGTCCAGTTATCATCGT, Primers for A are TGGCTCAGATTGAACGC/ATCCGATGGCAAGAGGC. e qRT-PCR was performed with the StepOnePlus™ Real-Time PCR System (ermo Fisher Scientific). e PCR mixture was held at 95 °C for 10 min and then subjected to 40 cycles of incubation at 95 °C for 15 s, then 60 °C for 1 min.

Fermentation for fatty acid production

Individual colonies were selected from Luria Broth (LB) plates with ampicillin and inoculated into 3 mL of LB liquid medium with ampicillin for 4 h. en, 0.5 mL of culture was added to 20 mL LB with ampicillin at 30 °C, 220 rpm overnight for seed culture preparation. Seed culture was collected, resuspended in MOPS 2.0% (wt/v) dextrose medium, and transferred into 50 mL MOPS 2.0% (wt/v) dextrose containing ampicillin and 1 mM of isopropyl-β- -thiogalactopyranoside (IPTG) in 250 mL ba ed flasks. e target initial cell density was OD_{550} ~0.1. Cultures were grown in 250 mL ba ed flasks with initial pH 7.0 at 30 °C, 220 rpm for 72 h.

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–MS) after carboxylic acid extraction. Briefly, 100 µL of whole liquid media sample was taken and 10 µL of 1 µg/µL C7:0/C11:0/C17:0 was added as internal standards. Two milliliter of ethanol: sulfuric acid (98:2 v/v) mixture was added, mixed and incubated at 65 °C for 30 min. en, 2 mL of 0.9% (wt/v) NaCl solution and 1 mL of hexane were added, vortexed and centrifuged at 2000× for 2 min. e top hexane layer was then analyzed by GC–MS, as described in "Strain tolerance characterization" section.

Statistical analysis

e two-tailed t test method was employed to analyze the statistical significance of all data in this study and P value <0.05 is deemed statistically significant.

Results

E□ects of *ompF* or *fadL* deletion on tolerance and production of fatty acids

It was previously reported that OmpF facilitates transport of SCFA, such as octanoic acid (C8), into *E. c* , and that deletion of F in *E. c* BW25113 decreased the impact of C8 on biomass production [32]. To evaluate the e ect of OmpF on C8 tolerance in MG1655, we also constructed an F deletion strain (Δ F) and confirmed that this engineering strategy improved tolerance to C8. In the absence of C8, the specific growth rates (µ) of both strains were approximately 0.39 h^{-1}. During C8 challenge, the specific growth rate of the Δ F mutant was 0.33 h^{-1}, which is 7% higher than that of MG1655 (0.31 h^{-1}) (Fig. 1a), which is consistent with the previous report [32].

Decreased membrane integrity has been previously described as a primary cause of C8 toxicity, where decreased membrane integrity is evidenced by leakage of metabolites and ions, such as Mg^{2+}, out of the cell or the entry of membrane-impermeable molecules, such as SYTOX, into the cell [14, 24, 48]. We next characterized the membrane integrity changes after disruption of F. Consistent with the growth results, deletion of F dampened the impact of C8 on membrane integrity. Specifically, the percentage of cells with intact membranes, i.e. SYTOX impermeable, during challenge with exogenously provided 10 mM C8, increased by 18% compared with the wild-type control strain ($P < 0.05$) (Fig. 1b).

Given that increased tolerance might lead to increased production of bio-products, we next applied the F deletion strategy to fatty acid production. e plasmid pXZ18Z (TE) harboring the heterologous thioesterase from . c [42], which primarily releases tetradecanoic acid (C14:0), palmitoleic acid (C16:1) and hexadecanoic acid (C16:0), was transformed into the Δ F strain and the corresponding control for fatty acid production in minimal MOPS 2.0% (wt/v) dextrose medium. We observed that deletion of F increased fatty acid production (Fig. 1c): in the Δ F + TE mutant, the titer of C14:0 was increased by 10% ($P = 0.03$) to 875 mg/L, C16:1 was increased by 17% ($P = 0.24$) to 71 mg/L and C16:0 was increased by 11% ($P = 0.01$) to 711 mg/L. All of these increases led to a 10% improvement of total fatty acids produced by the Δ F + TE mutant compared to MG1655 + TE strain, with titers of 1500 ± 20 and 1660 ± 40 mg/L, respectively ($P = 0.005$). It should be noted that previous studies concluded that deletion of F from *E. c* strain TY05 did not significantly increase fatty acid (C8–C14) production [26]. e di erence from this previous report and the findings presented here may be due to the use of di erent thioesterases (from . c c vs. from . c), growth media (nutrient-rich LB + 0.4% (v/v) glycerol vs. minimal MOPS + 2% (wt/v) glucose) and temperature (37 vs. 30 °C).

While OmpF has been previously characterized in terms of SCFA transport, FadL predominantly functions in the uptake of LCFA [29, 30]. To investigate the e ect of FadL on fatty acid tolerance and production, a L deletion mutant (Δ L) was constructed. Interestingly, the Δ L mutant showed decreased tolerance to C8.

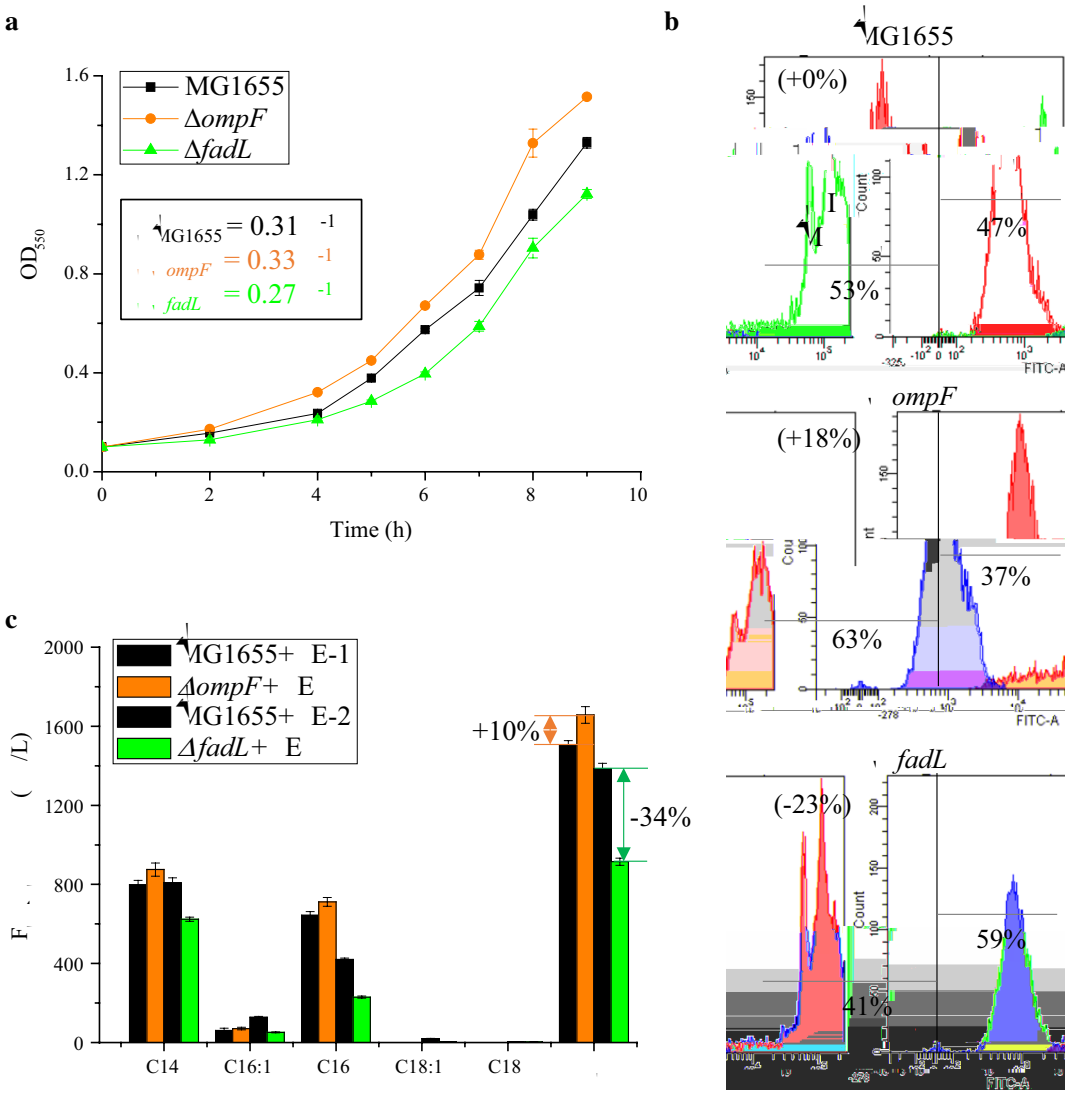
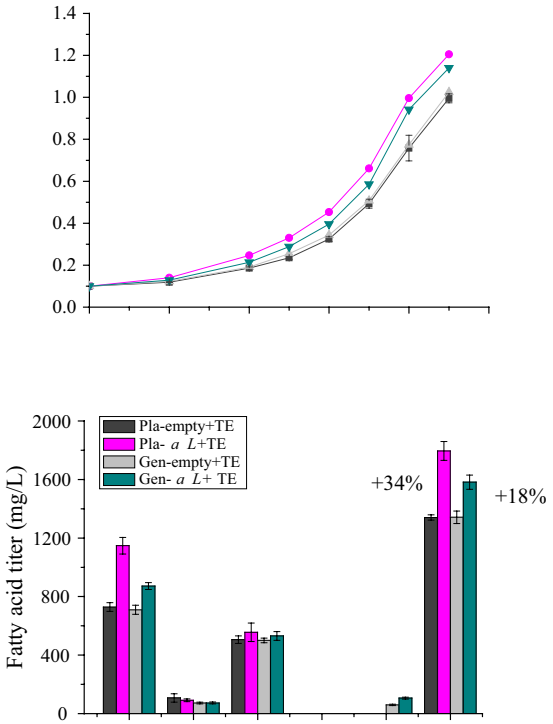


Fig. 1 E□ects of *ompF* or *fadL* deletion on membrane integrity during short-chain fatty acid challenge, short-chain fatty acid tolerance and production of C12 and C14 fatty acids. **a** Deletion of *ompF* or *fadL* impact the specific growth rate relative to the wild type MG1655 during challenge with 10 mM C8. *Inset values* are the specific growth rate, h^{-1}. **b** Deletion of *ompF* or *fadL* alters the percentage of cells with intact membranes (membrane integrity), assessed using SYTOX Green, during challenge with 10 mM C8. **c** Deletion of *ompF* increased fatty acid production and deletion of *fadL* decreased fatty acid production. MG1655 + TE-1 and MG1655 + TE-2 indicates experiments performed with the same strain, but on di□erent days. For **a** and **b**, experiments were performed in MOPS + 2% (wt/v) dextrose shake flasks at 220 rpm 30 °C with an initial pH of 7.0, 10 mM octanoic acid (C8). For **c**, strains carry the pXZ18Z plasmid (TE) for LCFA (C14–C16) production. Fermentations were performed in MOPS + 2% (wt/v) dextrose shake flasks at 220 rpm 30 °C with an initial pH of 7.0, 1.0 mM IPTG. Values are the average of at least three biological replicates with *error bars* indicating one standard deviation. Percent increase values are shown only for di□erences that were deemed statistically significant (P < 0.05)

For example, the specific growth rate of the Δ , *L* strain was 12% lower than that of MG1655 (0.27 vs. 0.31 h^{-1}) (P < 0.05) (Fig. 1a). Further membrane characterization showed that the percentage of cells with intact membranes was 23% lower for the Δ , *L* strain than MG1655 (P < 0.05) (Fig. 1b). When this , *L* deletion strategy was applied to fatty acid production (+TE), titers of C14:0 decreased by 23% to 623 mg/L, C16:1 decreased by 60% to 51 mg/L and C16:0 decreased by 45% to 230 mg/L. Each of these changes had a P value less than 0.05. Together, these changes led to a 34% reduction of total fatty acids in the Δ , *L* + TE mutant compared with MG1655 + TE strain (from 1390 ± 30 to 920 ± 20 mg/L) (P < 0.05) (Fig. 1c). It should be noted that the fatty acid titer of MG1655 + TE here (1390 ± 30 mg/L) is slightly lower than the 1500 ± 20 mg/L described above for the

F results, due to di erences between batches, similar to the results described elsewhere [26]. As with deletion of *F*, our results di er from previous reports of the e ect of *L* deletion on fatty acid production. is previous characterization employed *E. c* strain TY05 in rich medium with glycerol and found no significant change in production of C8–C14 fatty acids upon deletion of *L* [26]. However, our observation that deletion of *L* can increase sensitivity to membrane-damaging



of C14:0, 90 mg/L of C16:1, 930 mg/L of C16:0 and 2330 mg/L of total fatty acids after 72 h fermentation. ese titers are 47, 25, 29 and 38% higher than the strain in which only the F deletion was implemented (Δ F + Pla-empty + TE, 885 mg/L of C14:0, 72 mg/L of C16:1, 722 mg/L of C16:0 and 1680 mg/L of total fatty acid) and 25, 10, 18 and 20% higher than the strain in which only the L overexpression was implemented (Pla- L + TE, 1040 mg/L of C14:0, 83 mg/L of C16:1, 786 mg/L of C16:0 and 1930 mg/L of total fatty acid). Note that all of these comparisons have $P < 0.05$, except for C16:1. e combined strain has an approximately 50% improvement in fatty acid titers relative to the corresponding un-engineered control, Pla-empty + TE, which produced 801 mg/L of C14:0, 65 mg/L of C16:1, 653 mg/L of C16:0 and 1520 mg/L of total fatty acid (Fig. 3c). ese results again demonstrate the e ectiveness of concurrent utilization of F deletion and increased expression of L for increasing fatty acid production.

Functional mechanism of OmpF and FadL on increased membrane integrity

In this study, engineering the abundance of the membrane proteins OmpF and FadL increased membrane integrity, fatty acid tolerance and fatty acid production.

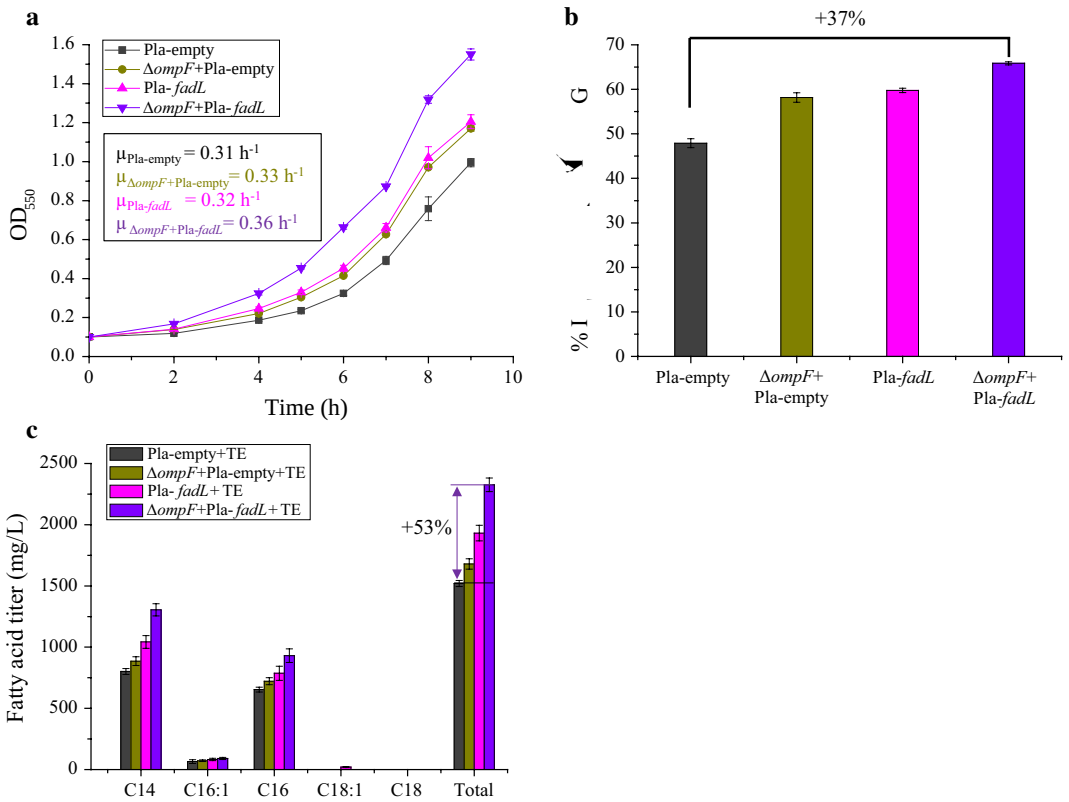


Fig. 3 Deletion of *ompF* and increased expression of *fadL* have an additive effect on increasing membrane integrity, fatty acid tolerance and production. **a** Combinatorial deletion of *ompF* (Δ*ompF*) and increased expression of *fadL* (Pla-*fadL*) increases the specific growth rate during challenge with 10 mM C8 relative to the starting strain (Pla-empty), individual *ompF* deletion strain (Δ*ompF* + Pla-empty), and individual overexpression of *fadL* (Pla-*fadL*). *Inset values* are the specific growth rate, h^{-1} **b** Percentage of cells with intact membrane (membrane integrity), assessed using SYTOX Green. Combinatorial deletion of *ompF* and increased expression of *fadL* improves membrane integrity during challenge with 10 mM C8 relative to Pla-empty, Δ*ompF* + Pla-empty and Pla-*fadL* strains. **c** The combined implementation of *ompF* deletion and increased expression of *fadL* supports increased fatty acid titers relative to each engineering strategy implemented individually. For **a** and **b**, experiments were performed in MOPS + 2% (wt/v) dextrose shake flasks at 220 rpm 30 °C with an initial pH of 7.0, 10 mM octanoic acid (C8). For **c**, all strains carry the pXZ18Z plasmid (TE, *fabZ*) for LCFA (C14–C16) production. Fermentations were performed in MOPS + 2% (wt/v) dextrose shake flasks at 220 rpm 30 °C with an initial pH of 7.0, 1.0 mM IPTG. Values are the average of at least three biological replicates with *error bars* indicating one standard deviation. Percent increase values are shown only for differences that were deemed statistically significant ($P < 0.05$). Pla-empty: MG1655 + pACYC184-Kan; Δ*ompF* + Pla-empty: MG1655, Δ*ompF* + pACYC184-Kan; Pla-*fadL*: MG1655 + pACYC184-Kan-*fadL*; Δ*ompF* + Pla-*fadL*: MG1655, Δ*ompF* + pACYC184-Kan-*fadL*

Prior studies showed that increasing the average length or the saturated:unsaturated (S/U) ratio of *E. c* membrane lipids can alleviate the decreased membrane integrity caused by fatty acids [23, 24]. In order to determine whether the increased membrane integrity here could be attributed to such changes in the phospholipid tail distribution, we measured the membrane lipid composition in the wild-type MG1655, Δ *F*, Δ *L* and Pla- *L* strains (Table 3). However, no significant changes in membrane composition were observed. Similarly, the average lipid length in wild-type MG1655 was 16.4 ± 0.2, which is comparable to the value observed for the Δ *F*, Δ *L* and Pla- *L* strains (Table 3). Additionally, the membrane lipid S/U ratio in the wild-type MG1655 was 1.06 ± 0.02, which is similar to the ratios for the Δ *F*, Δ *L* and Pla- *L* strains (Table 3). ese results indicate that the previously-described membrane engineering mechanisms of increasing the membrane lipid and S/U ratio are not the underlying reason for increased membrane integrity here.

Since the membrane consists of lipids and proteins, altering the abundance of FadL and OmpF might a ect the total membrane lipid content. e Δ *F* strain had a comparable membrane lipid content to MG1655 (Table 3), which indicates that *F* deletion did not significantly impact membrane lipid production. However, unlike *F*, altering the abundance of *L* remarkably a ected membrane lipid content. For example, the

Table 3 Membrane lipid content and composition changes in the wild type MG1655, Δ*ompF*, Δ*fadL*, Pla-*fadL* strains

DCW aS/U ratio

membrane lipid content of Δ L is only 62 ± 3 mg/g DCW, which is an 11% decrease compared to MG1655 ($P < 0.05$). Consistently, Pla- L had a 13% increase in membrane lipid content relative to MG1655 ($P < 0.05$) (Table 3). is result indicates that, unlike OmpF, FadL might be involved in membrane lipid synthesis, and therefore altering the abundance of L a ects the membrane lipid content and thus membrane integrity. It should be noted that the relative distribution of the lipid tails is not changed in the Pla- L strain (Table 3).

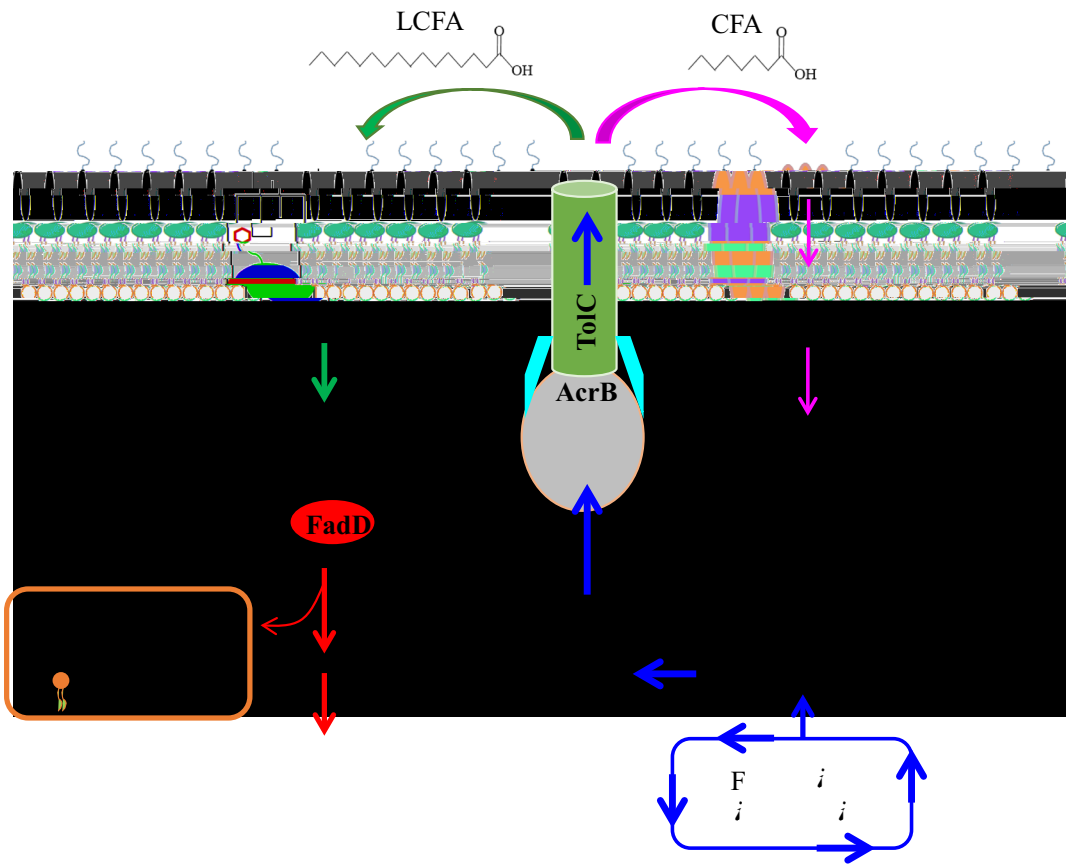
Discussion

Product toxicity is often an obstacle for cost-e ective production of biofuels and chemicals [9, 10]. erefore, construction of robust production organisms tolerant to these biorenewables is critical for industrial applications and has attracted increasing attention in recent years [12, 45, 49, 50]. Given its importance to overall cell function, membrane integrity has become an attractive engineering target for enhancing robustness [13, 24]. In the case of fatty acids, a variety of engineering e orts have been applied to increasing membrane integrity, with mixed results. Most of these engineering strategies focused on altering the distribution of the membrane lipids of *E. c* , such as by altering the average lipids length or degree of saturation [23, 24], though there have also been e orts to identify an e ux system that can improve fatty acid production [26].

Here we focused on two membrane proteins, OmpF and FadL, and found that they have distinct e ects on maintaining membrane integrity during fatty acid challenge and production. OmpF has been reported to function as the general di usion porin of *E. c* , through which a variety of inhibitory molecules, e.g. antibiotics, colicin and SCFA, can enter the cell [32, 51, 52]. Rodriguez-Moya et al. showed that OmpF facilitates transport of C8 into *E. c* , disrupting intracellular pH and oxidative balance [32]. It has also been suggested that OmpF is involved in the removal of phenylpropanoids from the cell interior [33]. In this study, we further characterized the role of OmpF in maintaining membrane integrity and used the *F* deletion strategy to increase fatty acid production. Although we employed the thioesterase specific for release of LCFA (C14–C16), some SCFAs were produced (e.g. C8 and C10) (Additional file 1: Figure S1). ese endogenously produced SCFAs can be exported, i.e. by AcrAB-TolC [26], to the extracellular environment. Conversely, they can also re-enter across the outer membrane through *E. c* porins (e.g. OmpF) (Fig. 4), which can cause severe membrane damage to *E. c* even at low concentrations [14].

One possible explanation for our observations is that after the endogenously produced fatty acids exit the cell, presumably via AcrAB-TolC [26], some of the SCFA re-enter the cell via OmpF. Deletion of *F* blocks this re-entry and thereby increases membrane integrity, which in turn reduces the leakage of important cellular molecules such as Mg^{2+} [14, 53], thereby elevating fatty acid tolerance and production (Fig. 4). e unexpected driving force for such transport may be due to the nature of the AcrAB-TolC transporter. Specifically, this transporter spans the periplasmic space [54–56] and thus the periplasm should be relatively depleted in fatty acids.

Our results demonstrate that, in addition to membrane engineering strategies that alter the distribution of the membrane lipid tails, altering the abundance of membrane protein OmpF can also a ect membrane integrity and production of fatty acids, which provides another strategy for future membrane engineering. Increasing the expression of an e ux pump has been shown to improve the production of inhibitory products, such as valine [25] and limonene [8] and these e ux pumps are also an important part of antibiotic resistance [57]. To the best of our knowledge, this is the first demonstration that deletion of a transporter is associated with increased production of a membrane-damaging compound.



In contrast to the *F* deletion strategy, deletion of *L* was found to decrease membrane integrity, tolerance and production of fatty acid. FadL is the only known outer membrane protein capable of importing exogenous hydrophobic LCFA compounds in *E. c* [32, 34, 58, 59]. Imported LCFA can be degraded through the β-oxidation pathway as sources of carbon and energy, or serve as precursors for membrane phospholipid biosynthesis [30, 59–61]. Since there was still residual glucose at the end of our experiments (data not shown), it is not likely that the decreased fatty acid tolerance and decreased fatty acid production of the Δ *L* mutant was caused by carbon or energy limitations. Membrane lipid biosynthesis in *E. c* requires acyl chains (C16:0, C16:1 and C18:1), of which there are two sources: (1) endogenous long chain acyl-ACP produced by the fatty acid biosynthesis pathway; and (2) long chain acyl-CoA derived from exogenous LCFA [62, 63]. Upon inactivation of FadL, uptake of exogenous LCFA will be decreased and thus membrane lipid biosynthesis will be impaired (Fig. 4). Our experimental results verify this hypothesis, as membrane lipid content was decreased in the Δ *L* strain and increased in the Pla- *L* strain. Since lipids are the primary structural component of the membrane, changing the membrane lipid content is likely to alter the membrane integrity.

is altered membrane lipid content by Δ *L* or Pla- *L* does not change the distribution of the di erent membrane lipid types (Table 3), which suggests that FadL is only responsible for supplying LCFA precursors instead of directly participating in the biosynthesis of phospholipids.

As with OmpF, a driving force for fatty acid uptake via FadL is not expected to exist during fatty acid production. Here, we again refer to the nature of the AcrAB-TolC e ux pump as a possible reason for the existence of this driving force. Since the AcrAB-TolC system spans the periplasmic space [54–56], the periplasm may be depleted of fatty acids relative to the extracellular medium. is direct relationship between L expression and tolerance of membrane-damaging compounds has been noted elsewhere, specifically in regards to phenylpropanoids [33]. is protective e ect of FadL against rutin, naringenin and resveratrol was attributed to FadL's role in repairing membrane damage, though there is no apparent exogenous source of the fatty acids used for this membrane repair [33].

Current membrane engineering strategies focus on altering membrane lipids composition, such as with the goal of increasing membrane lipid length or S/U ratio, to increase membrane integrity. Our results show that increasing the whole membrane lipid content possibly also contributes to increased membrane integrity, tolerance and production of fatty acids, which may serve as a novel strategy for membrane engineering in the future.

Our qRT-PCR results showed that there is a positive relationship between L mRNA abundance and fatty acid titer, and they also show that the native L gene is maintained at a high expression level, which indicates the importance of FadL in maintaining normal phospholipids biosynthesis. Concurrent deletion of F and increased expression of L synergistically increased fatty acid tolerance and production, accompanied by increased membrane integrity, possibly due to an increase in membrane lipid content and prevention of re-entry of the SCFA.

Bae et al. [34] found that deletion of D and overexpression of L in *E. c* increased hydroxy long-chain fatty acid production. In that study, it was concluded that overexpression of L contributes to the improvement in the production of ω-hydroxy palmitic acid, primarily due to increased ability to transport exogenously fed palmitic acid (C16). e present work mainly focuses on the e ect of L overexpression on the import of exogenous LCFA for membrane lipid synthesis and thus maintaining membrane integrity during the production of or challenge with membrane-damaging fatty acids. Prior research showed that deletion of F or L in *E. c* did not a ect fatty acid production [26], which is di erent from our results. ere are two possible reasons for this di erence: (A) the use of di erent thioesterases; and (B) the use of di erent growth conditions. e previous studies used a C8–C14-producing thioesterase enzyme from . c c , while here we used a C14–C16-producing thioesterase from . c . is previous study also used nutrient-rich LB with 0.4% (v/v) glycerol at 37 °C, while we used the nutrient-poor minimal MOPS with 2% (wt/v) dextrose at 30 °C. It is interesting to note that the studies that identified a positive relationship between OmpF abundance, FadL abundance and phenylpropanoid tolerance were also performed at 30 °C [33].

e use of glycerol in the previous fatty acid production studies may also be a complicating factor. e increase in hydroxy-palmitic acid production upon overexpression of FadL was smaller in the presence of glycerol relative to glucose [34] and the presence of glycerol has previously been reported to alter the phospholipid composition of microbial cell membranes [64–66]. Under di erent growth conditions, the membrane composition and associated amount of membrane damage caused by the fatty acids is expected to vary, and therefore the roles of OmpF and FadL may di er.

is engineering method appears to increase fatty acid production as a direct function of increased abundance of the microbial biocatalyst. us, it di ers from a previously described membrane engineering method that increased fatty acid titers by 50% without impacting the final culture OD [23] and evolutionary strain development that improved fatty acid production fivefold while only increasing growth during fatty acid production threefold [50]. e strategy described here also di ers from provision of valine-producing *E. c* with a valine exporter, which increased valine titers by 50% without changing the final OD [25]. us, additional strain engineering would be needed in order for this strategy to be e ective in improving fatty acid production in fed-batch or continuous culture systems. However, this work clearly demonstrates that these two membrane proteins are two viable engineering targets for improving fatty acid production.

Conclusions

Membrane damage of the microbial biocatalyst is a widespread problem in the problem of biorenewable fuels and chemicals. Here we have demonstrated two strategies for dealing with membrane damage in our condition. e first is to increase the abundance of FadL, which we propose increases the ability of the organism to repair the membrane damage incurred by fatty acids. e second method is to delete OmpF, which we propose prevents re-entry of the inhibitory product.

Additional le

Additional le 1: Figure S1. Fatty acids profile of *E. coli* MG1655 harboring pXZ18Z plasmid which carries thioesterase gene from *Ricinus communis* and *fabZ* gene from *E. coli*. Some short chain fatty acids (e.g. butanedioic acid, octanoic acid and decanoic acid) were found in the fermentation broth.

Abbreviations
OmpF: outer membrane porin F; FadL: long-chain fatty acid outer membrane porin; SCFA: short-chain fatty acids; LCFA: long chain fatty acids; C7: heptanoic acid; C8: octanoic acid; C11: undecanoic acid; C14: tetradecanoic acid; C15: pentadecanoic acid; C16:1: palmitoleic acid; C16:0: hexadecanoic acid; C17: heptadecanoic acid; TE: pXZ18Z; DCW: dry cell weight; IPTG: isopropyl-β-D-thiogalactopyranoside; ORF: open reading frame; *ldhA*: lactate dehydrogenase gene.

Authors' contributions
ZT and LRJ designed research. ZT, WB, and JMY performed research. ZT, JVS and LRJ analyzed data, and LRJ wrote the paper. All authors read and approved the final manuscript.

Author details
[1] 4134 Biorenewables Research Laboratory, Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011, USA. [2] Present Address: Department of Chemical Engineering and Materials Sciences, University of California, 916 Engineering Tower Irvine, Irvine, CA 92697-2575, USA.

Acknowledgements
We thank ISU Flow Cytometry Facility for help with SYTOX Green cells analysis, ISU DNA Facility for help with real-time quantitative PCR analysis and ISU W.M. Keck Metabolomics Research Laboratory for help with membrane fluidity analysis and GC–MS analysis. We would also like to thank Edward Yu and Thomas Mansell for helpful discussion of these results.

Competing interests
This work will be included in patent applications by Iowa State University.

Availability of data and materials
The dataset supporting the conclusions of this article is included within the article.

Funding
This work was supported by the NSF Engineering Research Center for Biorenewable Chemicals (CBiRC), NSF Award number EEC-0813570. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Published online: 28 February 2017

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