Modulating Membrane Composition Alters Free Fatty Acid Tolerance in *Escherichia coli*

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Abstract

Microbial synthesis of free fatty acids (FFA) is a promising strategy for converting renewable sugars to advanced biofuels and oleochemicals. Unfortunately, FFA production negatively impacts membrane integrity and cell viability in *Escherichia coli*, the dominant host in which FFA production has been studied. These negative effects provide a selective pressure against FFA production that could lead to genetic instability at industrial scale. In prior work, an engineered *E. coli* strain harboring an expression plasmid for the *Umbellularia californica* acyl-acyl carrier protein (ACP) thioesterase was shown to have highly elevated levels of unsaturated fatty acids in the cell membrane. The change in membrane content was hypothesized to be one underlying cause of the negative physiological effects associated with FFA production. In this work, a connection between the regulator of unsaturated fatty acid biosynthesis in *E. coli*, FabR, thioesterase expression, and unsaturated membrane content was established. A strategy for restoring normal membrane saturation levels and increasing tolerance towards endogenous production of FFAs was implemented by modulating acyl-ACP pools with a second thioesterase (from *Geobacillus* sp. Y412MC10) that primarily targets medium chain length, unsaturated acyl-ACPs. The strategy succeeded in restoring membrane content and improving viability in FFA producing *E. coli* while maintaining FFA titers. However, the restored fitness did not increase FFA productivity, indicating the existence of additional metabolic or regulatory barriers.

Citation: Lennen RM, Pfleger BF (2013) Modulating Membrane Composition Alters Free Fatty Acid Tolerance in *Escherichia coli*. PLoS ONE 8(1): e54031. doi:10.1371/journal.pone.0054031

Editor: Christopher V. Rao, University of Illinois at Urbana-Champaign, United States of America

Received September 28, 2012; Accepted December 7, 2012; Published January 21, 2013

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Funding: This work was funded by the DOE Great Lakes Bioenergy Research Center (DOE BER Office of Sciences DE-FC02-07ER64494). R.M.L. was supported as a trainee in the Chemistry-Biology Interface Training Program (NIH) and by the Department of Chemical and Biological Engineering Dahlke-Hougen Fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

One of the most promising routes for producing renewable substitutes for petrodiesel makes use of intermediates derived from fatty acid biosynthesis in microbes such as *Escherichia coli*. By introducing a cytosolic acyl-acyl carrier protein (ACP) thioesterase, feedback inhibition of enzymes involved in fatty acid biosynthesis by acyl-ACP intermediates is released, increasing flux through fatty acid biosynthesis [1–3]. The released free fatty acids (FFA) can either be separated from the culture medium and catalytically decarboxylated to alkanes [4,5], or they can be directed into heterologous pathways that produce products that include fatty acid ethyl esters, fatty alcohols, alkanes, olefins, methyl ketones, and polyhydroxyalkanoates [6–10].

Past reports have indicated that heterologous expression of the acyl-ACP thioesterase from *Umbellularia californica* (BTE) in *E. coli* results in greatly elevated levels of unsaturated and cyclopropane phospholipids, which are derived from unsaturated acyl-ACPs [11,12]. BTE belongs to the FatB family of plant acyl-ACP thioesterases, and has specificity for predominantly saturated C12 acyl-ACPs (~60–70% of FFAs), while also hydrolyzing unsaturated C12 (~10%), saturated C14 (~10%), and unsaturated C14 (~10%) [12]. The heightened unsaturated membrane lipid content was postulated to result from altered long-chain acyl-ACP pools caused by BTE-mediated depletion of saturated acyl-ACP. Altered membrane content has been observed in cells expressing other thioesterases with the changes directly correlated to thioesterase substrate specificity. For instance, expression of the FatA type thioesterase from *Helianthus annuus* in *E. coli*, which predominantly cleaves unsaturated C16 and C18 acyl-ACPs, increased the saturated phospholipid acyl content by approximately 5% [13]. Overexpression of a cytosolic form of *E. coli* thioesterase I (TesA′), which was reported to generate a FFA distribution of approximately 54% unsaturated and 46% saturated, also resulted in a 7.5% reduction of unsaturated and cyclic phospholipid acyl group content [1].

In prior work, we observed strong decreases in expression of fabA and fabB, genes required for unsaturated fatty acid biosynthesis, in cultures expressing BTE compared to cultures expressing a non-functional thioesterase with a catalytic histidine mutated to an alanine, BTE-H204A [11]. This was concomitant with increased unsaturated C16–C18 fatty acid content associated with membrane phospholipids [11,12], FabA, which catalyzes formation of the cis double bond in elongating acyl chains at the C10 chain length, and FabB, which is essential for condensing cis-3-decenoyl-ACP with malonyl-ACP, are both regulated at the transcriptional level by DNA binding of FabR to their promoter region [14,15] (Figure 1). FabR binds DNA.
and both saturated and unsaturated acyl-ACPs, with a stronger affinity for DNA when bound to unsaturated acyl-ACPs [16,17]. FabR thus plays a key role in modulating unsaturated membrane lipid content [16]. Due to this mechanism of repression, we hypothesized that expression of BTE, which primarily cleaves saturated acyl-ACPs, was enriching the acyl-ACP pool in unsaturated acyl-ACPs (Figure 1). The high proportion of unsaturated acyl-ACPs increased FabR-mediated repression, reducing transcription of fabA and fabB, but this repression was insufficient to prevent a highly elevated unsaturated membrane lipid content. The altered membrane composition would be expected to affect membrane properties, and could be responsible (in full or in part) for the negative phenotypes associated with endogenous FFA production (e.g. increased membrane depolarization and induction of membrane stress responses) [11]. The inability of cells to effectively regulate membrane fatty acid saturation as a result of BTE expression could become a selective pressure against endogenous FFA production and therefore a potential source of strain instability at industrial scale.

In this study, we investigated the impact of BTE expression on unsaturated membrane lipid content, cell lysis, FFA titers, and fabA and fabB expression levels in the absence of FabR, eliminating the mechanism for feedback repression of unsaturated fatty acid biosynthesis. Higher levels of fabA and fabB expression were observed, correlated with a greatly elevated unsaturated membrane lipid content, a highly exacerbated degree of cell lysis, and depressed FFA titers, underscoring the importance of FabR-mediated control of unsaturated fatty acid biosynthesis toward tolerance of endogenous FFA production. As a demonstration of the ability to modulate membrane lipid composition by acyl-ACP thioesterase selection, a thioesterase from Geobacillus sp. Y112MC10 (GeoTE) which was reported to hydrolyze a high percentage of unsaturated medium chain-length FFAs [18], was expressed by itself and in combination with BTE. Expression of GeoTE both alone and in tandem with BTE reduced the membrane unsaturated fatty acid content, relieved transcriptional repression of fabA and fabB, and decreased the population of lysed cells while maintaining total FFA titers. Our findings emphasize the importance of membrane physiology in biofuel producing strains and illustrate how unintended consequences can arise when native metabolism is manipulated.

**Materials and Methods**

**Chemicals, reagents, enzymes, and oligonucleotide primers**

All chemicals were purchased from Fisher Scientific (Pittsburgh, PA) unless indicated otherwise. Cloning and PCR reagents were purchased from New England Biolabs ( Ipswich, MA), Fermentas (Glen Burnie, MD), Promega (Madison, WI), and Qiagen (Valencia, CA). Oligonucleotides (Table S1) were purchased from Integrated DNA Technologies (Coralville, IA).

**Strain construction**

Bacterial strains and plasmids used in this study are listed in Table 1. The background strain used in this study is RL08ara, a strain derived from RL08 (K-12 MG1655 ΔfadD ΔaraKΔD, [4]) with the additional deletion of araFGH encoding a high-affinity arabinose transporter, and replacement of the native promoter of araE, a low-affinity arabinose transporter, with a constitutive promoter. These modifications were previously shown to generate homogeneous induction from the PBAD promoter across a population of cells, and to enable titration of arabinose to modulate gene expression from a PBAD promoter [19].

Strain RL08ara was constructed by sequential P1 phage transduction using lysates harboring Φ(araEp kan ParaE) and

![Figure 1. Unsaturated fatty acid biosynthesis and regulation.](image_url)

Unsaturated fatty acid biosynthesis begins with the isomerization of trans-2-decenoyl-ACP to cis-3-decenoyl-ACP by FabA. Instead of being reduced, this intermediate is condensed with malonyl-ACP by FabB. The resulting unsaturated β-ketoacyl-ACP is processed analogous to its saturated counterpart until unsaturated C16 and C18 acyl-ACPs are made and incorporated into phospholipids. Unsaturated fatty acid biosynthesis is feedback-inhibited at the transcriptional level by FabR, which exhibits increased repression of transcription of fabA and fabB when bound to enoyl-ACP species [16,17]. Expression of a thioesterase cleaves acyl-ACPs to generate FFA. BTE expression preferentially cleaves saturated C14-acyl-ACPs (solid blue arrow) and minorly cleaves unsaturated C12 (dashed blue arrow) and saturated and unsaturated C18 acyl-ACPs, thereby depleting saturated, long chain acyl-ACPs, the key regulatory signal for controlling fatty acid biosynthesis. As a result, flux through the saturated (prior to C12) and unsaturated pathway increases. Inset: the four arrows represent the elongation (FabB/FabF) (dashed arrow) in which the acyl chain represented by R grows by 2 carbons, keto reduction (FabG), dehydoration (FabZ), and enoyl reduction (FabI) reactions that comprise one round of fatty acid elongation and reduction.
**Table 1.** Strains and plasmids used in this study.

| Strain/plasmid | Relevant genotype/property* | Source/Reference |
|----------------|----------------------------|------------------|
| **Strains**    |                            |                  |
| DH10B          | F− mcrA Δ(mec-hsdRMS-mcrBC) pB8lacZAM15 ΔlacX74 recA1 endA1 araD139 Δ ara, leu7697 galK Δ貂 L rpsL napG | Invitrogen       |
| DHSα           | fhuA2 Δ(parg-facZU1669 phaA glnIV44 F80 ΔlacZ15 M15 gyrA96 recA1 relA1 endA1 thi1 hsdR17 | Invitrogen       |
| BW25113       | lacI88 mB3 F− Δ(araD-araB)567 ΔlacZ174784ΔmB8-3 Δ貂 L rph-1 Δ(hsdR-hsdB)568 hsdR514 | [21]             |
| BW27269       | BW25113 araFGH::kan903      | [19]             |
| BW27270       | BW25113 P araEp kan PC19 araE | [19]             |
| RL08          | K-12 MG1655 Δ todD Δ araBAD | [4]              |
| RL14          | RL08 araFGH::kan           | This work        |
| RL15          | RL08 Δ araFGH              | This work        |
| RL16          | RL08 Δ araBAD              | This work        |
| RL08ara        | RL08 araFGH (P araEp kan PC19 araE) | This work |
| JW3935-4      | BW25113 Δ fabR751::kan     | [21]             |
| RL17          | RL08ara fabR::kan          | This work        |
| RL18          | RL08ara Δ fabR             | This work        |
| **Plasmids**  |                            |                  |
| pCP20         | carries yeast FLP recombinase under constitutive promoter, pSC101 origin, Δ貂 L cI857+, Δ貂 L Rept, AmpR, CmR | [20]             |
| pBAD33        | P Δadd promoter, pACYC origin, CmR | [32]             |
| pBAD18        | P Δadd promoter, pBR322 origin, AmpR | [32]             |
| pBAD33-BTE    | pBAD33 carrying BTE under P Δadd control, CmR | [4]              |
| pBAD33-BTE-H204A | pBAD33 carrying BTE-H204A under P Δadd control, CmR | [4]              |
| pTrc99A       | P Δinc promoter, pBR322 origin, AmpR | [33]             |
| pTrc99A-BTE   | pTrc99A carrying BTE under P Δinc control, AmpR | [11]             |
| pTrc99A-BTE-H204A | pTrc99A carrying BTE-H204A under P Δinc control, AmpR | [11]             |
| pBAD33*       | pBAD33 with araC-C280* mutation | This work |
| pBAD18-GeoTE  | pBAD18 carrying Geobacillus sp. TE under P Δadd control, AmpR | This work |
| pBAD18-GeoTE-H173A | pBAD18 carrying Geobacillus sp. TE with H173A mutation under P Δadd control, AmpR | This work |
| pBAD18-ClosTE | pBAD18 carrying Clostridium thermocellum TE under P Δadd control, AmpR | This work |
| pBAD18-ClosTE-H171A | pBAD18 carrying Clostridium thermocellum TE with H171A mutation under P Δadd control, AmpR | This work |
| pBAD33*-fabR  | pBAD33* carrying fabR under P Δadd Control, CmR | This work |

*Abbreviations: Amp, ampicillin; Cm, chloramphenicol; R, resistance; ts, temperature sensitive.

doi:10.1371/journal.pone.0054031.t001

**araFGH::kan** loci from strains BW27271 and BW27269, respectively [19]. Antibiotic resistance genes were removed after each transduction using pCP20 [20]. Plasmid pCP20 was removed by repeated elevated temperature cures at 43°C, and the presence of all desired FRT-site containing loci were confirmed by colony PCR using primers 1–8 (Table S1). Promoter replacement of araE was verified sequentially following both transduction and elevated temperature cure of pCP20.

Strain RL08ara Δ fabR was constructed by P1 phage transduction of the fabR::kan cassette from strain JW3935-4 [21]. The kanamycin resistance gene was removed using pCP20 as described above, and all FRT-site containing loci were confirmed by colony PCR using primers 9–10.

**Gene synthesis and plasmid construction**

Codon-optimized genes encoding *Geobacillus* sp. Y412MC10 (recently renamed *Paenibacillus* sp. Y412MC10, GenBank genome accession number CP001793.1) acyl-ACP thioesterase (GeoTE), and *Clostridium thermocellum* acyl-ACP thioesterase (ClosTE, GenBankABN54268) were synthesized by GeneArt (Life Technologies, Regensburg, Germany). Ribosome binding sites (RBS) predicted to lead to a high rate of translation (~25000 au) were designed using the Ribosome Binding Site Calculator [22]. The RBS and flanking restriction sites at the 3' (XmaI) and 5' (HindIII) termini were added to the final sequence to be synthesized. The ordered gene sequences are shown in Figure S1. A site-directed mutant was also ordered from GeneArt, following alignment of GeoTE and ClosTE with BTE (Figure S2), which identified a conserved catalytic histidine at positions 175 and 171, respectively. The full
synthesized sequences were amplified by PCR using primers 13–14 for GeoTE, and 15–16 for CloST (3’ hexahistidine tags were added), digested with XmaI and HindIII, and ligated into plasmid pBAD18 to generate plasmids pBAD18-GeoTE, pBAD18-CloST-H173A, pBAD18-CloST, and pBAD18-CloST-H171A. The fabH gene was amplified by PCR from MG1653 genomic DNA with flanking 5’ (XmaI) and 3’ (HindIII) restriction sites and an artificial RBS generated from the RBS Calculator forward design tool, using primers 17–18. This vector was generated by PCR using primers 11–12 with template pBAD33, which introduced the C280* mutation and an XhoI restriction site at the 5’ and 3’ ends. The PCR product was then digested with XhoI and ligated to form plasmid pBAD33*. The aqueous upper layer and interfacial cell debris was removed, and the bottom organic layer was evaporated to dryness under a nitrogen stream. To the dried residue, 0.5 ml of 0.5 M sodium methoxide in methanol (Sigma) was added, and the reactions were allowed to proceed at 50°C for 10 minutes [25]. To quench the reaction, 0.1 ml of glacial acetic acid was added, followed by 5 ml of deionized water. Fatty acid methyl esters (FAME) were extracted twice into 0.5 ml of hexane, and the collected hexane layers were quantified for total fatty acids. Gas chromatography/mass spectrometry (GC/MS) analysis and peak identification and quantification was performed on a model 7890 Agilent GC with a model 5975 mass spectrometer as described previously [4]. Average fatty acid concentrations from biological triplicate cultures were determined by normalizing to recovered FFA internal standards (pentadecanoic and heptadecanoic acid) from acid-catalyzed methylations, and from the recovered fatty acids derived from the phospholipid internal standard in base-catalyzed methylations (heptadecanoic acid was added for verification that FFA were not being methylated).

**Cell cultivation**

Cell cultures used in viability, RNA quantification, and fatty acid production experiments were grown at 37°C with shaking (250 rpm) in 250 ml shake flasks with a 4 x headspace in Difco LB medium [BD, Franklin Lakes, NJ] supplemented with 0.4% v/v glycerol. Chloramphenicol was added to a concentration of 34 μg/ml in strains harboring pBAD33* derived plasmids. Ampicillin was added to a concentration of 50 μg/ml in strains harboring pBAD18 derived plasmids, and 100 μg/ml in strains harboring pTrc99A derived plasmids. Cultures were induced with IPTG at 50°C for expression of genes on pBAD33*, pBAD33*, and pBAD33*-fabR in strains RL08ara and RL08ara (C280*) that enables co-induction of IPTG-inducible and arabinose-inducible promoter systems [23]. This vector was generated by PCR using primers 13–14 with template pBAD33*, which introduced the C280* mutation and an XhoI restriction site at the 5’ and 3’ ends. The PCR product was then digested with XhoI and ligated to form plasmid pBAD33*.

**SYTOX flow cytometry assays**

To assess cell permeability, cell pellets were collected and stained by addition of 1 μL of 3 mM SYTOX Green in DMSO (Invitrogen) and measured by flow cytometry as described in [11,24]. Two distinct populations were evident from the green fluorescence histograms, allowing a logarthmic-scale green fluorescence intensity of 420 to serve as the cut-off between cells counted as intact (less than or equal to 420) and non-intact (greater than 420).

**Cell viability measurements from plate counts**

Volumes of cell culture were serially diluted in phosphate buffered saline (PBS) (137 mM NaCl, 27 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) and spread onto LB agar plates (containing no antibiotics) at indicated times. Individual colonies were counted after overnight incubation at 37°C and additional overnight incubation as specified.

**Fatty acid extraction and analysis**

Total fatty acids were extracted from cell cultures and methylated by acid catalysis as previously described [4]. In separate analyses, bound fatty acids were extracted and methylated by base catalysis. Samples were extracted from 2.5 mL of cell culture spiked with 5 μl of 10 mg/mL heptadecanoic acid (Fluka) in ethanol and 10 μL of 2.5 mg/mL 1,2-dipentadecanoyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids, Alabaster, AL) in chloroform with 5 mL of 1:1 chloroform:methanol, vortexed thoroughly, and centrifuged at 1000g for 10 min. The SYTOX flow cytometry assays are performed on a model 7890 Agilent GC with a model 5975 mass spectrometer as described previously [4]. Average fatty acid concentrations from biological triplicate cultures were determined by normalizing to recovered FFA internal standards (pentadecanoic and heptadecanoic acid) from acid-catalyzed methylations, and from the recovered fatty acids derived from the phospholipid internal standard in base-catalyzed methylations (heptadecanoic acid was added for verification that FFA were not being methylated).

**RNA extraction and qPCR**

From shake flask cultures expressing combinations of pBAD33-BTE-H204A, pBAD33-BTE, pBAD18-GeoTE, and pBAD18-GeoTE-H173A, cells were collected in mid-log phase (OD ~0.8, 3.25 hours post-inoculation) and in early stationary phase (OD varied between 0.6 to 4.6, 5.25 hours post-inoculation) by spinning down 0.8 ml and 0.333 ml of culture, respectively, centrifuging at 16,000g for 1 min, aspirating off the supernatant, and flash freezing the cell pellet in a dry ice/ethanol bath. Cell pellets were stored at −80°C. To extract RNA, cell pellets were resuspended in 100 μl of TE buffer, pH 8.0, containing 400 μg/ml lysozyme and incubated for 5 minutes. From this point forward, the Qiagen RNeasy Plus Kit was used according to the manufacturer’s instructions. For shake flask cultures expressing combinations of pTrc99A-BTE, pTrc99A-BTE-H204A, pBAD33*, and pBAD33*-fabR in strains RL08ara and RL08ara ΔfabR, approximately 0.7 OD600/ml of cells (i.e. 0.7 ml of OD600 1.0) were collected in early stationary phase (4.6 h post-inoculation) and centrifuged at 16,000g for 1 min. The supernatant was removed by aspiration and cell pellets were flash frozen in a dry ice/ethanol bath prior to storage at −80°C. RNA was extracted and purified as described above, with the exception of using the Qiagen RNeasy Kit. Following RNA clean-up, contaminating DNA was removed using the RNA-free™ Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer’s instructions. The absence of DNA was confirmed by using 0.5 μl of each RNA sample as template in a PCR using primers 25–26 (Table S1). First-strand cDNA synthesis was performed for the first set of shake flask cultures using the Promega GoScript™ Reverse Transcription System according to the manufacturer’s instructions, with 0.5 μg of RNA, random primers, 2.5 mM MgCl2, and a 1 hour extension time at 42°C. First-strand cDNA synthesis for the second set of shake flask cultures was performed using the Bio-Rad iScript™ Reverse Transcription Supermix (Hercules, CA) for RT-qPCR with 1 μg RNA template. The presence of cDNA was confirmed in each reaction using 0.5 μl of each sample as template as described above for confirming the lack of DNA contamination in RNA samples. To quantify the relative expression of fabA, fabB, and fabR transcript in each sample, qPCR reactions were set up using Fermentas Maxima SYBR Green master mix was used according to the manufacturer’s directions, with 2.0 μl of 5-fold diluted cDNA in water was used as template, and primers 19–24 (Table S1). SYBR green fluorescence was monitored with a Bio-Rad
CFX96 optical reaction module. Cycle quantification (Ct) values were calculated by Bio-Rad CFX Manager software, and relative expression values to the double negative controls were calculated as $2^{-\Delta\Delta Ct}$.

**Results**

**Effect of fabR deletion on unsaturated membrane-bound fatty acids**

To verify that the elevated levels of unsaturated C16 and C18 fatty acids found in BTE expressing *E. coli* cultures [11,12] were located in membrane lipids, we compared fatty acid methyl ester (FAME) profiles prepared by acidic and base catalysis. Acid catalysis methylates both FFA and bound fatty acids in phospholipids and other species, whereas base catalysis methylates only bound fatty acids. FAME samples were prepared from replicate cultures of *E. coli* RL08ara harboring pBAD33-BTE, and pBAD18-GeoTE-H173A. The difference between the total and bound fatty acid profiles provided the FFA profile, which demonstrated that BTE does not significantly cleave C16 to C18 chain length fatty acids (Figure S3).

If our hypothesis (FabR mediated repression of fabA/fabB is insufficient to overcome the BTE-mediated alteration of acyl-ACP pools) is correct, deletion of fabR should result in both increased levels of fabA and fabB, as well as increased unsaturated membrane content (a phenotype that should be further amplified by expression of BTE). To test this hypothesis, the levels of fabA and fabB transcript in early stationary phase (approximately 4.5 hours after inoculation) were quantified by qPCR (Figure 2a). Sampled cultures included RL08ara or RL08ara fabR harboring combinatorial plasmids (pTrc99A-BTE-H204A or pTrc99A-BTE, and pBAD33* or pBAD33*-fabR) that were induced at an OD600 of 0.05 and 0.12. Strain RL08ara/pTrc99A-BTE exhibited statistically significant (P < 0.05) decreased levels of fabA and fabB relative to strain RL08ara/pTrc99A-BTE-H204A, consistent with previous results comparing BTE- and BTE-H204A-expressing strains under different growth conditions [11]. Strains with deletions in fabR exhibited heightened levels of fabA and fabB expression in both BTE-H204A (fabA, P = 0.069; fabB, P = 0.033) and BTE-expressing strains (fabA, P = 0.034; fabB, P = 0.031) relative to RL08ara/pTrc99A-BTE-H204A. The fold-change in fabA and fabB expression in response to fabR deletion was larger in the BTE-expressing strains than in the BTE-H204A-expressing strains, consistent with the hypothesis of an increased degree of FabR-mediated repression that is present when BTE is expressed.

Fatty acid titers were analyzed following extraction 8 h and 24 h post-inoculation. As predicted by the increased levels of fabA and fabB, strain RL08ara ΔfabR/pTrc99A-BTE-H204A also exhibited a higher percentage of unsaturated C16-C18 fatty acids at 8 h (58.8 ± 0.9%) than RL08ara/pTrc99A-BTE-H204A (49.3 ± 2.5%), and strain RL08ara ΔfabR/pTrc99A-BTE exhibited an even larger increase (82.7 ± 0.3%) than RL08ara/pTrc99A-BTE, (67.5 ± 1.5%) (Figure 2b). Fatty acid compositions followed the same trend when extracted 24 h post-inoculation. These percentages include C17 cyclopropane fatty acids, which are derived from methylation across the double bond of 16:1 fatty acids in phospholipids by cyclopropane fatty acid synthase (Cfa).

Given the impact of FabR on regulating fabA and fabB expression in the presence of BTE, we hypothesized that overexpression of FabR could increase repression and restore the unsaturated fatty acid membrane content towards original levels. Measurement of relative fabR transcript levels by qPCR indicated a 106.6 (P < 0.001) and 82.5 (P < 0.001) fold increase in strain RL08ara ΔfabR/pTrc99A-BTE-H204A and RL08ara ΔfabR/pTrc99A-BTE overexpressing fabR on pBAD33*-fabR, respectively, over the native levels of fabR expression in strain RL08ara harboring pBAD33*. Despite growth defects in strains carrying pBAD33*-fabR, overexpression of fabR restored decreased levels of fabB in RL08ara ΔfabR (Figure 2a), and also restored unsaturated C16-C18 fatty acid levels in RL08ara ΔfabR expressing either BTE-H204A or BTE to similar or lower levels as those present in RL08ara (Figure 2b). While not further considered in this study, fabR overexpression at lower levels may be a viable strategy for reverting the increased unsaturated fatty acid membrane composition resulting from expression of acyl-ACP thioesterases with predominantly saturated acyl-ACP substrate specificity.

**Effect of fabR deletion on cell membrane integrity**

Cells from the same cultures described in 3.1 were collected 8 h post-inoculation and stained with SYTOX Green. The green fluorescence of individual cells in the population was measured by flow cytometry. SYTOX Green is ordinarily impermeable to intact inner cell membranes, but results in a bright green fluorescence upon nucleic acid binding in cells with non-intact inner membranes [26]. RL08ara/pTrc99A-BTE-H204A was 98.4 ± 0.2 percent intact while RL08ara ΔfabR/pTrc99A-BTE-H204A exhibited only a slight reduction to 94.0 ± 0.6 percent intact (Figure 2c). However, RL08ara/pTrc99A-BTE was 75.5 ± 4.0 percent intact while RL08ara ΔfabR/pTrc99A-BTE had a dramatic reduction to 14.2 ± 5.2 percent intact (Figure 2c). Therefore deletion of fabR has a specifically deleterious effect on cell membrane integrity under conditions of fatty acid overproduction.

**Effect of fabR deletion on FFA production**

The negative physiological consequences of fabR deletion manifested in FFA production of BTE-expressing strains. RL08ara/pTrc99A-BTE exhibited higher titers of C8–C14 fatty acids at both 8 h and 24 h post-inoculation compared to RL08ara ΔfabR/pTrc99A-BTE (Figure 2d).

**Selection and expression of a predominant enoyl-ACP thioesterase**

BTE is in the FatB family of plant acyl-ACP thioesterases, all of which exhibit predominantly saturated acyl-ACP substrate specificities [27]. Conversely, the FatA family of plant acyl-ACP thioesterases primarily hydrolyze cis-18:1A9-ACP [27], and no FatA thioesterase has been characterized in the literature that can hydrolyze medium-chain-length unsaturated species as a significant portion of its product profile. Recently, a number of bacterial acyl-ACP thioesterases were characterized by heterologous expression in a *fadD* deficient strain of *E. coli* [18]. Thioesterases from *Clostridium thermocellum* and *Geobacillus sp*. Y412MC10 produced greater quantities of unsaturated than saturated C12–C14 species and lower levels of octanoic acid than other tested thioesterases. Therefore codon-optimized genes encoding these acyl-ACP thioesterases (hereafter referred to as ClosTE and GeoTE) were chemically synthesized. Catalytic histidines were identified that aligned with His-204 and shared a NXHVNN motif previously identified by alignment of plant FatA and FatB thioesterases [28], with the exception of GeoTE having a leucine residue in place of valine (Figure S1). Non-functional mutations of ClosTE (ClosTE-H171A) and GeoTE (GeoTE-H173A) were generated in order to provide negative controls that account for protein production effects. ClosTE, GeoTE, ClosTE-H171A, and
GeoTE-H173A were cloned into pBAD18 and tested in strain RL08ara. Both non-functional bacterial thioesterases produced fatty acid profiles similar to BTE-H204A-expressing cells (Figure S4). CloTTE expressing cells did not increase C15-C18 fatty acid titers significantly (36±1 mg/L from 12±0 mg/L) and was therefore not employed in subsequent studies.

Expression of GeoTE produced highly elevated levels of 9:0, 10:1, 10:0, 11:1, 12:0, 14:0, and 14:0 fatty acids and detectable levels of odd-chain 9:0, 11:0, and 13:0 fatty acids (Figure S4). β-hydroxylated octanoic, decanoic, dodecanoic, tetradecanoic, and hexadecanoic species were also detected according to their elution time patterns, molecular ions, and primary m/z ion of 103 (data not shown). Smaller tridecanoic and pentadecanoic peaks were also identified from their mass spectra (data not shown). These compounds were likely generated by spontaneous decarboxylation following hydrolysis of 3-oxotetradecanoyl-ACP and 3-oxohexadecanoyl-ACP, similar to observations by Goh and coworkers [8] by overexpression of E. coli FadM. Elevated levels of 16:1 but decreased levels of 16:0, 18:1, and 18:0 were observed in cells expressing GeoTE compared with cells expressing GeoTE-H173A. Tiers of C15-C18 fatty acids were 248±55 mg/L compared with 12±9 mg/L in GeoTE-H173A-expressing cultures, and the proportion of C15-C18 species that were unsaturated was 55 percent, compared with a typical value in BTE-expressing cultures of 23 percent. As a result of this high level of medium-chain length fatty acid production with a higher percentage of unsaturated species than BTE, GeoTE was selected for further characterization of its impact on membrane fatty acid content.

Unsaturated long-chain fatty acid biosynthesis in BTE and GeoTE expressing cultures

The effect of GeoTE expression and GeoTE/BTE co-expression on membrane lipid composition was compared to that of BTE expression. Analysis of bound fatty acids by base-catalyzed methylation revealed that cultures co-expressing GeoTE and BTE-H204A, as well as cultures co-expressing GeoTE and BTE,
maintained a lower percentage of C16–C18 unsaturated fatty acids than cultures co-expressing BTE and GeoTE-H173A (with P<0.05 for all values) and a similar percent unsaturation to negative control cultures co-expressing BTE-H204A and GeoTE-H173A (Figure 3a). These trends were consistent with the anticipated highest depletion of saturated acyl-ACPs by BTE, followed by BTE and GeoTE co-expression, followed by GeoTE alone. Interestingly, GeoTE and BTE co-expressing cultures exhibited the highest percentage of bound fatty acids as cyclic C17.

If expression of GeoTE, or co-expression of GeoTE and BTE resulted in a shift toward a larger ratio of saturated to unsaturated acyl-ACPs, it would be expected that fabA and fabB levels would be restored to higher levels than in cultures expressing only BTE. The levels of fabA and fabB transcript were quantified by qPCR from biological triplicate cultures of each strain (Figure 3b). Indeed, in both the GeoTE/BTE-H204A and GeoTE/BTE co-expressing cultures, levels of fabA and fabB were restored to levels present in the control culture (co-expressing BTE-H204A and GeoTE-H173A), and were higher than levels present in the BTE/GeoTE-H173A cultures. Expression of fabB was higher with statistical significance (P<0.05) in the GeoTE/BTE-H204A expressing cultures than in the GeoTE-H173A/BTE-H204A expressing cultures, indicating that expression of a thioesterase with this degree of unsaturated acyl-ACP substrate specificity resulted in a lower amount of FabR repression than is present in wild-type cells. The fact that expression of fabA was not higher with statistical significance was also consistent within this framework, as fabA is repressed less strongly, and by analogy also de-repressed less strongly, by FabR than fabB due to weaker binding of FabR within the promoter region upstream of fabA relative to the promoter region upstream of fabB [17].

Figure 3. Modulating E. coli membrane content via co-expression of saturated and unsaturated acyl-ACP targeting thioesterases. a) The percentage of unsaturated C16–C18 and cyclopropane (C17) fatty acids were calculated from FAMEs made by base-catalyzed methylation of fatty acids extracted from cultures expressing combinations of BTE and GeoTE at 8 h and 24 h post-inoculation. GeoTE-expressing cells (GeoTE + BTE) and GeoTE/BTE co-expressing cells (GeoTE+ BTE+) have a reduced unsaturated content relative to BTE-expressing cells (BTE+ GeoTE–). Error bars represent standard deviations about the mean of biological triplicate samples. * = P-value<0.05, ** = P-value<0.01 compared to BTE+ GeoTE– cultures at the same sampling time.

b) Transcript levels of fabA and fabB determined by qPCR on samples harvested 1 hour post-induction were normalized to BTE+ GeoTE– (RL08ara pBAD33-BTE-H204A pBAD18-GeoTE-H173A) samples. Levels of fabA and fabB were decreased in cells expressing only BTE. Conversely, levels of fabA or fabB were statistically the same or higher in cells expressing GeoTE or GeoTE and BTE. Error bars represent propagated standard errors about the mean of biological triplicate samples. * = P-value<0.05 for Cq values compared against fabA or fabB in cultures expressing only non-functional thioesterases (BTE+ GeoTE–). c) The percentage of intact cells were calculated from histograms of cells stained with SYTOX Green 8 h post-inoculation. Cultures expressing only BTE were the least intact. Cultures expressing only GeoTE, and both GeoTE and BTE were over 50% intact. Error bars represent standard errors about the mean of biological triplicate samples. * = P-value<0.05 compared to BTE+ GeoTE–. d) FFA titers from strains expressing combinations of BTE and GeoTE. FFA titers were determined at 8 and 24 h post-inoculation. Cultures expressing only GeoTE exhibited the highest titer at 8 h, with a minor increase observed after 24 h. Nearly equivalent titers were reached after 24 h in cultures expressing only BTE, or co-expressing BTE and GeoTE. Error bars represent standard deviations about the mean of biological triplicate samples. * = P-value<0.05, ** = P-value<0.01 compared to BTE+ GeoTE– cultures at same sampling time.

doi:10.1371/journal.pone.0054031.g003
Cell growth and viability analysis of GeoTE and BTE expressing cultures

SYTOX Green staining and flow cytometry analysis of cell samples collected 8 h post-inoculation revealed differences in the percentage of intact cells between strains expressing the two thioesterases (Figure 3e). BTE-H204A and GeoTE-H173A expressing cells exhibited a typical non-functional thioesterase expressing value of 94.8±1.3 percent intact, while cells expressing only functional BTE were 34.6±2.1 percent intact. Expression of only functional GeoTE, however, resulted in a higher percentage of intact cells (77.8±5.2 percent), while co-expression of functional GeoTE and BTE resulted in 67.7±4.5 percent intact cells. These data were consistent with the intermediate C16 to C18 unsaturated fatty acid content of the dual thioesterase expressing strain.

A percent viable cells analysis obtained by dividing CFU mL⁻¹ by the number of flow cytometry events measured per mL of original cell culture generally followed trends in the SYTOX Green data for percent intact cells (Table 2). While non-FFA overproducing BTE-H204A and GeoTE-H173A expressing cells were 90±14 percent viable, BTE and GeoTE-H173A expressing cells were only 5±1 percent viable, and BTE-H204A and GeoTE expressing cells were 45±12 percent viable. In contrast to the SYTOX Green assay, BTE and GeoTE expressing cells were only 3±2 percent viable. As we have observed previously [11], plated cells from BTE-expressing cultures exhibited a wide variety of different colony sizes after one night incubation at 37°C, and one additional night incubation at room temperature was required for all colonies to become visible. Plated cells from GeoTE-expressing cultures exhibited an even larger distribution of colony sizes, with new colonies appearing after 1 night at 37°C and 4 nights at room temperature. BTE plus GeoTE expressing cultures similarly required a total of 5 days before new colonies stopped appearing. Therefore it appeared that large percentages of strains expressing one or both thioesterases are non-lysed but also non-culturable on LB agar.

Fatty acid production of GeoTE and BTE expressing cultures

After 8 h growth, cultures expressing only functional GeoTE exhibited a higher total fatty acid titer (513±12 mg L⁻¹) than cultures expressing only functional BTE (364±42 mg L⁻¹) (Figure 3d). This higher productivity could be due to a larger percentage of viable, intact cells in these cultures (Table 2). Cultures expressing both functional thioesterases produced 255±117 mg L⁻¹ after 8 hours, reflecting the lower cell counts and reduced growth in these cultures. After 24 hours, titers for GeoTE, BTE, and both GeoTE and BTE expressing cultures reached 569±8, 651±57, and 653±60 mg L⁻¹ respectively (Figure 3d). The fatty acid composition of cultures expressing both thioesterases was intermediate between cultures expressing only functional GeoTE or BTE.

Discussion

In prior work, we observed cell lysis, decreased viability, increased membrane unsaturated fatty acid content, and transcriptional evidence of membrane stresses, cell depolarization, and impaired aerobic respiration when BTE was expressed in E. coli [11]. The present study correlated cell lysis and decreased cell viability with elevated unsaturated fatty acid content in the membrane, suggesting a causative relationship in strains producing FFA. Achieving the maximum theoretical yield of FFAs (as determined by constraint-based modeling) requires aerobic respiration and the use of the membrane-bound transhydrogenase PntAB, and therefore maintenance of inner cell membrane integrity and properties. To develop commercial strains for advanced biofuel applications, it is critical to maintain necessary active metabolic pathways throughout the course of production and to understand the source of disruptions to these pathways. Furthermore, it is important to not induce selective pressures that favor loss of endogenous FFA production. In the last five years, many groups have implemented strategies for producing FFA that are based on expression of acyl-ACP thioesterases in E. coli. In these studies, a wide range of titers (and correspondingly, yields in batch cultures) have been reported when different thioesterases were expressed [3]. In most cases there is no clear rationale to explain the differences in observed titers. We postulate that the degree to which a thioesterase alters the long-chain acyl-ACP pool destined for incorporation in membrane phospholipids is one source of varying titers reported in different engineered FFA-overproducing strains. For example, this hypothesis may explain the results of Lu et al. [29], wherein co-expression of a cytosolic form of E. coli TesA (thioesterase I) and the plant FatB-type thioesterase from Cinnamomum camphoratum (CcTE) produced higher titers than expression of CcTE alone. The substrate specificity for CcTE is most enriched in saturated C14 [30], whereas TesA has a broad C12–C18 specificity that is highest toward unsaturated C16 and C18 fatty acids [1]. High titers were also reported from a strain designed to undergo reversal of b-oxidation and overexpressing an acyl-CoA thioesterase (E. coli FadM) [31], may be due to this pathway being independent from fatty acid biosynthesis and therefore lacking impacts to membrane-bound fatty acids such as those imposed by acyl-ACP thioesterase expression. If true, this hypothesis motivates further work to control membrane content independent of thioesterase specificity.

### Table 2. Viability analysis of strains expressing combinations of BTE and GeoTE.

| BTE   | GeoTE | Flow Cytometer (events mL⁻¹) | Plate Counts (CFU mL⁻¹) | Normalized CFU (CFU event⁻¹) |
|-------|-------|-----------------------------|-------------------------|-------------------------------|
| (−)   | (−)   | (5.75±0.43)×10⁹             | (5.2±0.7)×10⁹           | 0.90±0.14                     |
| (−)   | (+)   | (4.93±0.15)×10⁹             | (2.20±0.60)×10⁹         | 0.45±0.12                     |
| (+)   | (−)   | (3.61±0.50)×10⁹             | (1.9±0.4)×10⁹           | 0.05±0.01                     |
| (+)   | (+)   | (1.37±0.76)×10⁹             | (4.0±1.6)×10⁹           | 0.03±0.02                     |

Cultures of R LB8044 harboring combinations of pBAD33-BTE-H204A (BTE⁻¹) or pBAD33-BTE (BTE⁻¹), and pBAD18-GeoTE-H173A (GeoTE⁻¹) or pBAD18-GeoTE (GeoTE⁻¹) grown in LB+0.4% glycerol and antibiotics at 37°C for 8 hours. Reported values are forward scatter triggered flow cytometry events per mL original culture volume of SYTOX Green stained cells, plate counts (CFU mL⁻¹) after 5 days incubation, and plate counts normalized to flow cytometry events (CFU event⁻¹), as another estimate of percentage live cells.

doi:10.1371/journal.pone.0054031.t002
While alteration of thioesterase substrate specificity by expression of GeoTE succeeded in restoring membrane unsaturated fatty acid content and reducing cell lysis, it resulted in a more diverse product profile. This outcome may be acceptable when fuels or other chemical mixtures are targeted, but not when specific oleochemicals are targeted. This study further underscored the criticality of FabR in maintaining membrane integrity in thioesterase-expressing cells, and strongly suggests that the level of FabR repression is insufficient in fabR<sup>−</sup> BTE-expressing cultures, possibly due to cells being unable to down-regulate fabA and fabB to a sufficient degree to counter the effects on the acyl-ACP pool resulting from BTE expression. While overexpression of FabR resulted in severe impacts toward growth, a more promising strategy to pursue in the future entails promoter engineering of P<sub>fabA</sub> and P<sub>fabb</sub> to achieve increased repression by FabR.

**Conclusion**

The unsaturated fatty acid content of the membrane is a key parameter that influences cell lysis under conditions of endogenous FFA production. While FabR is a major controller, its level of repression appears to be insufficient to prevent elevated unsaturated acyl-ACP pools as a result of expression of BTE, which hydrolyzes primarily saturated acyl-ACPs. The current data cements the relationship between acyl-ACP thioesterase expression and membrane unsaturated fatty acid content. It was shown that acyl-ACP pools can be modulated by thioesterase selection. Expression of a thioesterase with primarily unsaturated acyl-ACP substrate specificity (GeoTE) reduced membrane unsaturated fatty acids, increased cell viability, reduced lysis, and increased FFA productivity during the first 8 hours of growth. Further strategies geared toward modulation of membrane lipid content for improved cellular fitness are being pursued that do not result in alteration of the FFA product profile, such as promoter engineering of FabR binding sites upstream of fabA and fabB.

**Supporting Information**

**Figure S1** Synthetic codon-optimized gene sequences for the acyl-ACP thioesterases from *Geobacillus Y412MC10 (GeoTE)* and *Clostridium thermocellum* (ClosTE). The location of the H173A mutation in GeoTE is shown in underlined italics and involved substitution of CAT with GCT. The location of the H171A mutation in ClosTE is shown in underlined italics and involved substitution of CAT with GCC. Restriction sites (for XmaI and HindIII) used for cloning are underlined. The ribosome binding site is shown in blue, and the spacer sequence is shown in red. Start and stop codons are bolded. (DOC)

**Figure S2** Alignment of amino acid sequence of BTE with bacterial acyl-ACP thioesterases from *Geobacillus Y412MC10 (GeoTE)* and *Clostridium thermocellum* (ClosTE). The catalytic histidine at position 204 in BTE and HVNN motif aligns with histidine-173 in GeoTE and histidine-171 in ClosTE. Valine-203 in BTE aligns with a similar leucine residue in GeoTE. (TIF)

**Figure S3** Comparative analysis of acid and base catalyzed FAME preparations from cultures expressing BTE and GeoTE-H173A. Cultures were sampled 8 h and 24 h post-inoculation. Free fatty acids can be calculated by subtracting the base titers from acid titers. BTE hydrolyzes predominantly 12:1, 12:0, 14:1, and 14:0 fatty acids, and does not appreciably hydrolyze C<sub>16</sub>–C<sub>18</sub> fatty acids. GeoTE hydrolyzes a wide range of C<sub>5</sub> to C<sub>18</sub> fatty acids, with the highest activity towards 12:1, 12:0, 14:1, 14:0, 16:1, and 16:0 species. (TIF)

**Figure S4** Total fatty acid analysis of functional and mutagenized GeoTE and ClosTE. Cultures were grown for 24 hours in LB plus 0.4% glycerol at 37°C. Expression of GeoTE resulted in high titers of overexpressed FFAs with a diverse profile and 12:1 as the highest concentration product. Expression of ClosTE produced low FFA titers. (TIF)

**Table S1** Oligonucleotide primers used in this study. (DOC)

**Author Contributions**

Conceived and designed the experiments: RML, BFP. Performed the experiments: RML. Analyzed the data: RML, BFP. Contributed reagents/materials/analysis tools: RML, BFP. Wrote the paper: RML, BFP.

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