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Improving the tolerance of *Escherichia coli* to medium-chain fatty acid production

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ABSTRACT

Microbial fatty acids are an attractive source of precursors for a variety of renewable commodity chemicals such as alkanes, alcohols, and biofuels. Rerouting lipid biosynthesis into free fatty acid production can be toxic, however, due to alterations of membrane lipid composition. Here we find that membrane lipid composition can be altered by the direct incorporation of medium-chain fatty acids into lipids via the Aas pathway in cells expressing the medium-chain thioesterase from *Umbellularia californica* (BTE). We find that deletion of the *aas* gene and sequestering exported fatty acids reduces medium-chain fatty acid toxicity, partially restores normal lipid composition, and improves medium-chain fatty acid yields.

1. Introduction

Microbially derived fatty acids are attractive precursors for a variety of carbon-neutral fossil fuel replacements. Moreover, microbially derived fatty acids do not directly compete with food production, unlike oils and fats from plant and animal sources (Liu and Khosla, 2010; Peralta-Yahya et al., 2012). As a result, engineering microbial fatty acid biosynthesis has been extensively investigated with many successful efforts to overproduce free fatty acids (FFA) in bacteria and yeast (Liu et al., 2010, 2012; Lennen et al., 2010; Steen et al., 2010; Dellomonaco et al., 2011; Tai and Stephanopoulos, 2013; Xu et al., 2013). Moreover, several groups have developed strategies to convert the overproduced fatty acids into biofuels, such as methyl or ethyl esters and medium to long-chain alcohols and alkanes(Steen et al., 2010; Dellomonaco et al., 2011; Kalscheuer et al., 2006; Nawabi et al., 2011; Tan et al., 2011; Choi and Lee, 2013; Schirmer et al., 2010; Zhang et al., 2012). Despite these efforts, yields of FFAs must be improved for economic viability.

Escherichia coli (*E. coli*) is an attractive host organism for production of FFAs as it can grow on a variety of carbon sources, has fast replication rates, and can be genetically manipulated. Moreover, the extensive knowledge of *E. coli* fatty acid biosynthesis facilitates pathway engineering allowing tailoring of the chemical composition of the fatty acids produced (Liu and Khosla, 2010). The *E. coli* fatty acid metabolic pathways relevant to this work are summarized in Fig. 1. Fatty acid biosynthesis starts with acetyl-CoA and proceeds through multiple rounds of elongation and reduction to yield a long-chain fatty acyl group attached to acyl carrier protein (ACP) (Liu et al., 2010). Elongation usually ends with the production of long-chain (16–18 carbon) acyl-ACPs that are used for lipid production (Chan and Vogel, 2010). Generally, fatty acyl intermediates do not exist as free fatty acids in bacteria and are virtually all are esterified to ACP. To prevent build-up of fatty acids, fatty acid biosynthesis is tightly regulated by acyl-ACP feedback inhibition of FA biosynthetic enzymes as well as by the transcription factors FabR and FadR (Chan and Vogel, 2010). FFAs in cells are either degraded by β-oxidation pathway into acetyl-CoA or incorporated into membrane phospholipids (Fujita et al., 2007).

It has been shown that the introduction of acyl-ACP thioesterases in β-oxidation deficient (Δ*fadD*) cells liberates free fatty acids from ACP and redirects lipid biosynthesis into free fatty acid production (Voelker and Davies, 1994). Moreover, elimination of acyl-ACP feedback inhibition allows unregulated fatty acid (FA) production, increasing fatty acid yields (Jiang and Cronan, 1994; Lu et al., 2008). As different thioesterases have distinct fatty acid chain specificities, the length and diversity of fatty acids produced can be tailored by varying the thioesterase. Thioesterases from *Acinetobacter baylyi* and *Umbellularia californica* have been used to make short to medium (C6–C14) chain fatty acids, while *E. coli*, *Cinnamomum camphorum*, *Ricinus communis* and *Jatropha curcus* thioesterases have been used for synthesis of longer chain fatty acids (C14–C18) (Lennen et al., 2010; Steen et al., 2010; Lu et al., 2008; Zheng et al., 2012; Zhang et al., 2011). Although distinct thioesterases have been used to produce a variety of fatty acids in *E. coli*, there is a need to address the cellular toxicity of endogenously-produced free fatty acids, particularly short and medium chain fatty acids, and consequent reductions in cell viability, membrane stability, and fatty acid yields (Lennen and Pfleger, 2012).

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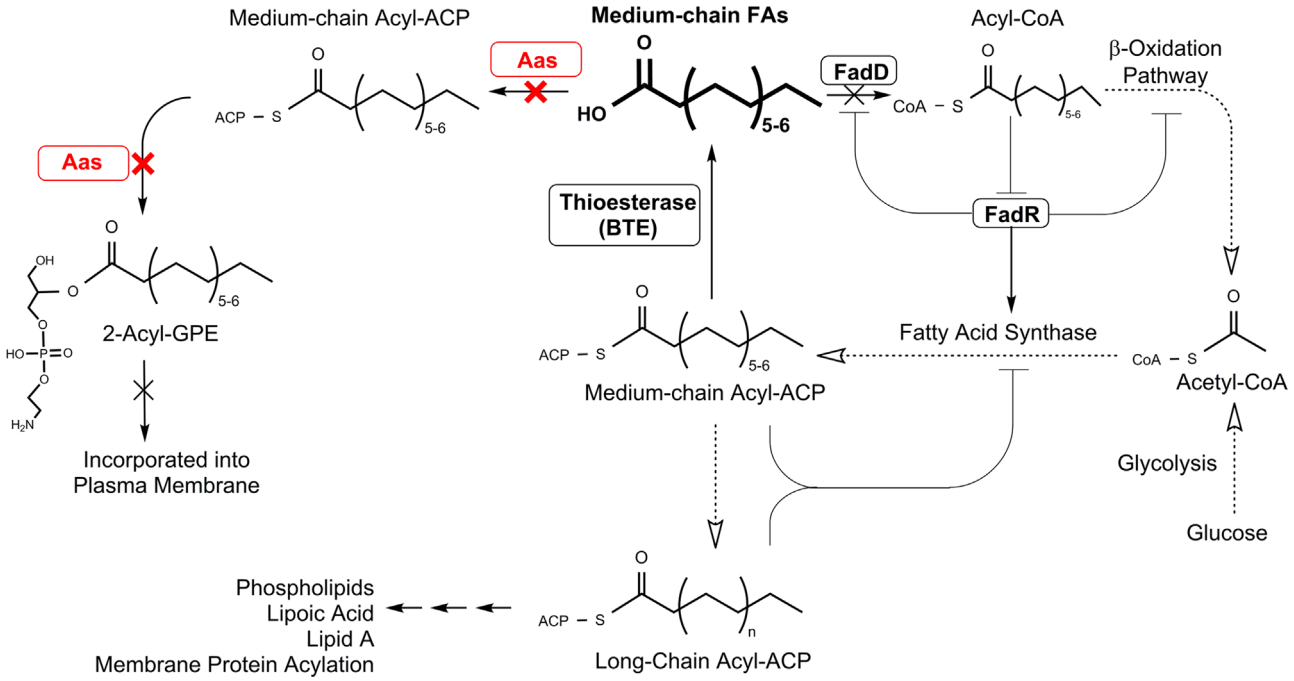

Fig. 1. Pathways employed for medium-chain free fatty acid (FFA) overproduction in *E. coli*. Expression of *U. californica* acyl-ACP thioesterase BTE in β-oxidation- and phospholipid synthesis-deficient (Δ*fadD* Δ*aas*) *E. coli* strain leads to increased accumulation of medium-chain FFAs in cells and culture. FA, fatty acid; ACP, acyl-carrier protein; GPE, glycerophosphoethanolamine; FadD, acyl-CoA synthetase; Aas, 2-Acyl-GPE acyltransferase/acyl-ACP synthase; BTE, *U. californica* acyl-ACP thioesterase. The Aas enzyme targeted in this work is highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The cytosolic acyl-ACP thioesterase from *U. californica* (BTE) has been used extensively in *E. coli* Δ*fadD* strains for the overproduction of medium-chain FFAs (Lennen et al., 2010; Voelker and Davies, 1994). It has been shown that BTE has a preference for saturated fatty acids, yielding a pool of FFAs with a relatively high degree of saturation (Lennen et al., 2010; Voelker and Davies, 1994). However, one consequence of BTE expression in *E. coli* is the depletion of saturated chains in the lipid biosynthesis pathway, leading to a toxic replacement by unsaturated lipids in the membrane (Lennen and Pfleger, 2013). Toxicity can be reduced by including a second thioesterase that prefers unsaturated chains, although an increase in fatty acid titers was not observed (Lennen and Pfleger, 2013). Efforts to overexpress efflux transporters have shown moderate success (Doshi et al., 2013; Lennen et al., 2013), but these approaches do not directly addresses the potential toxic effects of incorporation of free fatty acids into membrane phospholipids (Lennen and Pfleger, 2013)

Here we explore an alternative mechanism for increasing medium chain FFAs by focusing on the alteration of bilayer composition and resulting toxicity caused by the production of medium-chain FFAs. It has been shown previously that exogenous free fatty acids can be directly incorporated into membrane phospholipids via the activity of 2-acyl-glycerophosphoethanolamine (2-acyl-GPE) acyltransferase/acyl-ACP synthetase (Aas) (Hsu et al., 1991; Jackowski et al., 1994). Here, we demonstrate that medium-chain FFAs produced as a result of thioesterase activity can be directly introduced into membrane lipids via Aas activity as shown in Fig. 1 (Hsu et al., 1991; Jackowski et al., 1994). We find that deletion of the *aas* gene reduces the levels of medium-chain fatty acids incorporated into the membrane, lowers medium-chain fatty acid toxicity and increases FFA yields.

2. Materials and methods

2.1. Materials

T4 DNA ligase and restriction endonucleases were obtained from New England Biolabs. DNA Polymerase Mastermix was from Denville Scientific. QIAprep Miniprep kits and QIAquick gel extraction kits were purchased from Qiagen. The λDE3 Lysogenization Kit was from EMD Chemicals. All reagents were from Sigma Aldrich except for LB agar and Terrific Broth which were obtained from Fisher Scientific. Oligonucleotide primers were synthesized by Valuegene. Gene sequencing and gene synthesis were performed by Genewiz.

2.2. Vector construction

The 897-bp portion of *U. californica* BTE gene lacking the thylakoid targeting sequence was prepared synthetically by Genewiz and the BTE gene was amplified with primers XhoI-pBAD/p15A-BTE and NsiI-pBAD/p15A-BTE. The PCR product was purified, digested with XhoI and NsiI and ligated into XhoI and PstI digested plasmid pBAD/HisA/p15A (Massey-Gendel et al., 2009) to produce BTE-pBAD/p15A. The BTE-pBAD/p15A plasmid was digested with XhoI and SfuI and the gel-purified BTE gene was sub-cloned into XhoI/SfuI-digested pBAD/HisA plasmid (Invitrogen) to yield BTE-pBAD/HisA. The FadR expression construct (FadR-AG1) was obtained from the ASKA (−) collection (Kitagawa et al., 2005).

2.3. E. coli strains

E. coli strains JW 1794-1 (Δ*fadD*) and JW2804-1 (Δ*aas::kan*) were used as the starting point for strain construction (Baba et al., 2006). A λDE3 prophage was integrated into these two cell lines to yield strain SS0 (Δ*fadD*) and SS18 (Δ*aas*) strains. Removal of the Kan cassette from SS18 and subsequent knock-out of the *fadD* gene was performed according to the protocol from Datsenko and Wanner (2000). The PCR products employed to knock-out the *fadD* gene were generated by using primers FadD-P1-pKD4-Primer1 and FadD-P2 pKD4-Primer1 using pKD4 plasmid as template. The gel-purified PCR product was further extended using FadD-P1-pKD4-Primer2 and FadD-P2-pKD4-Primer2 and the final PCR product was used to knock-out *fadD* as described previously, yielding the double knock-out strain SS19 (Δ*aas* Δ*fadD*)