Development of an orthogonal fatty acid biosynthesis system in *E. coli* for oleochemical production

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Abstract

Here we report recombinant expression and activity of several type I fatty acid synthases that can function in parallel with the native *E. coli* fatty acid synthase. *Corynebacterium glutamicum* FAS1A was the most active in *E. coli* and this fatty acid synthase was leveraged to produce oleochemicals including fatty alcohols and methyl ketones. Coexpression of FAS1A with the ACP/CoA-reductase Maqu2220 from *Marinobacter aquaeolei* shifted the chain length distribution of fatty alcohols produced. Coexpression of FAS1A with FadM, FadB, and an acyl-CoA-oxidase from *Micrococcus luteus* resulted in the production of methyl ketones, although at a lower level than cells using the native FAS. This work, to our knowledge, is the first example of *in vivo* function of a heterologous fatty acid synthase in *E. coli*. Using FAS1 enzymes for oleochemical production has several potential advantages, and further optimization of this system could lead to strains with more efficient conversion to desired products. Finally, functional expression of these large enzyme complexes in *E. coli* will enable their study without culturing the native organisms.

Key Words: fatty alcohols, methyl ketones, fatty acid synthase, synthetic biology

1. Introduction

Many valuable chemicals and fuels have been produced in engineered strains of *Escherichia coli* by deregulating this organism's fatty acid synthase. Fatty alcohols (F-OHs) are industrial chemicals that are used in the manufacture of surfactants, detergents and lubricants, with worldwide production exceeding 2 million tons per year (Noweck and Grafahrend 2000). Methyl ketones (MKs) are another class of compounds with promising fuel properties and applications in the flavor and fragrance industries (Goh, Baidoo et al. 2014). While these compounds can be generated from petroleum, plant or animal sources, they have also been produced in *E. coli* by enzymatic conversion of fatty acyl-CoA intermediates, which are derived from the native fatty acid synthase (FAS) (Steen, Kang et al. 2010).

Most bacteria, including *E. coli*, possess type II, or dissociated FASs in which all catalytic domains, and the acyl carrier protein (ACP) that anchors the growing acyl chain, operate as separated discrete enzymes (Figure 1, upper right). While this system can generate oleochemicals at high titers, there are several drawbacks still to overcome. First, fatty acid synthesis is a vital element of primary metabolism and is stringently regulated in bacteria. Engineering this essential pathway requires a means of bypassing this regulation. It has been demonstrated that fatty acyl-ACP, the end product of bacterial type II FAS, allosterically inhibits one or more enzymes involved in fatty acid production including acetyl-CoA carboxylase (ACC) (Jiang and Cronan 1994, Davis 2001). This inhibition can be relieved via the action of the thioesterase 'TsaA, which cleaves the thioester bond linking acyl chains to ACP and

releases free fatty acids (FFAs) (Cho and Cronan 1995) (Figure 1). FFAs cannot be directly converted to F-OH or MK products in the native host; they must be re-activated to acyl-CoA thioesters employing FadD, a CoA-ligase that uses ATP to catalyze the energetically uphill thioesterification (Figure 1). Once formed, acyl-CoAs can be degraded by the β -oxidation pathway, but deletion of the gene encoding the acyl-CoA oxidase FadE prevents loss of acyl-CoAs and leads to improved titers of desired products (Steen, Kang et al. 2010). However, one potential shortcoming of using the coupled action of 'TesA and FadD for generating acyl-CoAs is that 'TesA, which is used to hydrolyze acyl-ACPs, can also hydrolyze acyl-CoAs generated by FadD (Barnes and Wakil 1968). Hydrolysis and resynthesis of acyl-CoAs in this manner creates a futile cycle that consumes one ATP each time a molecule of acyl-CoA is cleaved by 'TesA and then regenerated by FadD.

Fatty acyl-CoAs can be reduced to F-OHs by CoA-reductases (Figure 1, right). Youngquist and coworkers compared fatty alcohol production in *E. coli* using CoA-reductases from a variety of organisms, and found that a reductase from the marine bacteria *Marinobacter aquaeolei* gave the highest F-OH titers (Youngquist 2013). Maqu2220, a robust reductase from *Marinobacter aquaeolei*, was reported to reduce fatty aldehydes using NADPH (Wahlen, Oswald et al. 2009), and was later found to efficiently reduce a number of acyl-CoA and acyl-ACP thioesters to their corresponding F-OH (Hofvander, Doan et al. 2011). Recently it was shown that expression of Maqu2220 coupled with previously reported methods of acyl-CoA enhancement (described above) improved F-OH production (Liu, Tan et al. 2013).

Methyl ketones are also derived from acyl-CoA intermediates; oxidation catalyzed by Mlut_11700 and FadB yields β -keto-acyl-CoAs. FadM catalyzes hydrolysis of these intermediates to β -keto-fatty acids which spontaneously decarboxylate to form methyl ketones (Goh, Baidoo et al. 2014). Because acyl-CoAs are important intermediates in both of these pathways, we hypothesized that direct production of acyl-CoAs, without free fatty acid intermediates, could result in more efficient production strains. To this end, we investigated the use of heterologous type I FASs (FAS1s) in *E. coli*.

Type I FAS enzymes, in which the ACP and all catalytic domains are housed on a single polypeptide (Figure 1, lower left), are common in mammals and fungi and are present in some bacterial hosts. In these systems, after the acyl chain reaches its mature length, it is transferred to CoASH and released as fatty acyl-CoA. Using FAS1 for F-OH and MK production offers a number of potential advantages. When producing F-OHs with FAS1, there is no need for a thioesterase that catalyzes counterproductive acyl-CoA hydrolysis. Direct production of acyl-CoAs removes the requirement of a CoA-ligase that consumes ATP. Furthermore, the heterologous FAS1 enzymes are not subject to downregulation through physiological feedback mechanisms as is the case with the native *E. coli* FAS. Thus, we hypothesized that this orthogonal FAS could decouple oleochemical production from native fatty acid synthesis and minimize perturbation of the cells' primary metabolism.

2. Methods

2.1. Plasmid construction

Codon-optimized versions of *C. glutamicum fas-IA* (YP_226736.1, 8988 bp), *C. glutamicum fas-IB* (YP_225128.1, 8907 bp), and *Norcardia farcinica fasI*, were synthesized and assembled at the Joint Genome Institute. Codon optimization was performed using an empirically derived codon-usage table for high expression in *E. coli* (Quan, Saaem et al. 2011). Full sequences of codon-optimized genes are provided in the supplemental information. Genes were initially partitioned into 1-Kb blocks using GeneDesign (Richardson, Nunley et al. 2010) with 25 bp overlaps. Full-length genes were generated through two rounds of Gibson assembly and sequence verified using a PACBIO RSII system (Pacific Biosciences). The *Bifidobacterium adolescentis fasI* (BAD_RS01360) gene was amplified from genomic DNA. *Mycobacterium tuberculosis fasI* (NP_217040.1) was synthesized by Genscript. All FAS1 genes were integrated into biobrick vector pBbE5C. The gene encoding Maqu2220 was codon optimized and synthesized by IDT as two gblocks. The gene was then PCR amplified and integrated into pBbB5K using Gibson cloning (Gibson, Young et al. 2009), and we refer to this plasmid as pB5K.Maqu2220. Codon optimized sequences are listed in the supplemental information. The genes encoding 'Tesa and FadD were PCR amplified from previously reported plasmids (Zhang, Carothers et al. 2012), and integrated into vector pBbE5C using Gibson cloning (Gibson, Young et al. 2009). The genes encoding FadM, FadB, and Mlut_11700 were amplified from previously reported plasmids and integrated into

pBbB5K. We refer to this plasmid henceforth as pB5K.MK. Gene expression was driven by P_{lacUV5} .

2.2. Strain construction

Strain JBEI-3111, MG1655 with *fadE* deleted, was reported previously (Bokinsky, Peralta-Yahya et al. 2011). The gene encoding the *C. glutamicum* phosphopantetheinyl transferase, *acpS* (YP_226732.1), was codon optimized and ordered from IDT as a gblock, and then was integrated into the chromosome of strain JBEI-3111 at the *araD* site using a two step scarless homologous recombination method in which *araD* is deleted and then restored to its original location with an extra “passenger” gene, generating strain JBEI-5029 (Groff D 2012)

JBEI-5029 was transformed with pE5C.cgFAS1A and pB5K.Maqu2220 to give strain JBEI-8085. We generated strain JBEI-9018, containing plasmids pE0C.RFP and pB5K.Maqu2220, as a negative control strain lacking any means of acyl-CoA overproduction. We also generated a F-OH production strain akin to strain AL322 (Liu, Tan et al. 2013), where we transformed JBEI-5029 with pE5C.'tesA.fadD, and pB5K.Maqu2220, which we will refer to henceforth as strain JBEI-9017.

We produced three methyl ketone production strains in a similar manner: JBEI-9019 contains plasmids pE0C.RFP and pB5K.MK and was used as a negative control, JBEI-9020 contains pE5C.cgFAS1A and pB5K.MK, and strain JBEI-9021 contains pE5C.'tesA.fadD and pB5K.MK.

2.3. Cell culture

Production strains were grown in LB medium containing the appropriate antibiotics overnight, and then adapted into M9 minimal medium containing 3% glucose. F-OH production cultures additionally contained 0.1% tritonX-100. 50 mL cultures were inoculated and grown at 37°C for 8 hours, at which point gene expression was induced by addition of 1 mM IPTG. Cultures were incubated with shaking at 30C (F-OH strains) or 37C (MK strains) for an additional 71-87 hours and the fatty alcohol or methyl ketone contents were measured periodically.

2.4. Total lipid, fatty alcohol and methyl ketone analyses

In order to measure total lipid content of strains carrying each FAS1 enzyme, cells were subjected to base-catalyzed hydrolysis and the resulting lipids were converted to the corresponding methyl ester for GC-MS analysis. 100 µL of culture was mixed with 5 µL of 40% tetrabutylammonium hydroxide and incubated at 50°C for one hour, and then extracted with 100 µL dichloromethane containing 1.25% iodomethane and 100 mg/L methyl tricosanoate as an internal standard (Haushalter, Kim et al.). Fatty alcohol content was measured using methods reported previously (Liu, Tan et al. 2013). We mixed 100 µL of cell culture with 100 µL of 2:1 chloroform/methanol containing 1 g/L pentadecanol (C15:F-OH) as an internal standard, and the mixture was vortexed for 30 minutes. After extraction, the organic layer was removed and transferred to a GC vial where it was evaporated to dryness. The sample was finally resuspended in hexanes and analyzed by GC-MS.

Fatty alcohol titers were quantified by integration of total ions and comparison with the 1 g/L pentadecanol standard.

In methyl ketone production experiments, we added 5 mL of decane containing 0.5 mg/L tetradecan-3-one to each culture when inducing expression with IPTG. 50-100 μ L of decane overlay was removed at periodic time points, diluted 20-fold with hexanes, and analyzed by GC-MS using the tetradecan-3-one as an internal standard.

3. Results

3.1. Expression of bacterial FAS1 enzymes and screening for increased lipid production.

All acyl carrier proteins, whether in a type I or II FAS system, must be post-translationally modified with a phosphopantetheine (PPT) prosthetic group that bears the terminal thiol group to which acyl chains are appended as thioesters. In bacteria that possess FAS1s, the phosphopantetheinyl transferase (PPTase) AcpS is expressed as a separate enzyme, unlike the FAS1 from *S. cerevisiae*, in which the PPTase is part of the FAS1 enzyme complex (Stuible, Meier et al. 1997). We therefore used *E. coli* strain JBEI-5029, which harbors *C. glutamicum acpS* integrated adjacent to *araD*, to construct our F-OH and MK production strains.

First, we set out to assay our panel of FAS1 enzymes for soluble expression in *E. coli*. Strain JBEI-5029 was transformed with a pBbE5C harboring FAS1A or FAS1B from *C. glutamicum*, *B. adolescentis* FAS, *N. farcinica* FAS, or *M. tuberculosis* FAS and the cell

lysates were analyzed by SDS-PAGE. We confirmed that all of our FAS1 constructs expressed solubly in *E. coli* (Figure 2, left).

Once we confirmed soluble expression of the FAS1 enzymes in *E. coli*, we analyzed them for *in vivo* activity. We first wanted to see if FAS1 expression results in an increase in total lipid content. To this end, we hydrolyzed the total lipids in our FAS1 expressing strains and derivatized the lipids to the corresponding methyl esters. As a negative control, we used a strain containing plasmid pE5C.RFP. We found that *C. glutamicum* FAS1A (cgFAS1A) expression caused the largest increase in lipid production (data not shown).

3.2. Fatty alcohol production using the ACP/CoA-reductase Maqu2220

Our next goal was to use cgFAS1A to produce industrially relevant compounds in *E. coli*, such as fatty alcohols. The enzyme Maqu2220 has been shown to reduce acyl-CoAs to the corresponding fatty alcohol. We compared strain JBEI-8085, harboring pE5C.cgFAS1A and pB5K.Maqu2220, with strain JBEI-9018, which harbors pE0C.RFP and pB5K.Maqu2220. pE0C.RFP lacks a promoter and does not encode any enzymes that catalyze acyl-CoA overproduction. For comparison with the native FAS, we additionally assayed strain JBEI-9017, which harbors plasmids pE5C.'tesA.fadD and pB5K.Maqu2220. All three strains contained Maqu2220 and differed only in the upstream acyl-CoA production pathway.

Surprisingly, we found that all three strains produced similar amounts of fatty alcohols (Figure 3, left). Even more surprisingly, we found that all three strains produced more than 3.5 g/L fatty alcohols, representing a more than 8-fold increase from previously reported strains expressing '*tesA*, *fadD*, and *maqu2220* (Liu, Tan et al. 2013). JBEI-8085, expressing cgFAS1A, produced 3.78 ± 0.090 g/L F-OH with a yield of 0.126 g F-OH/g glucose while JBEI-9017 produced 3.82 ± 0.050 g/L F-OH with a yield of 0.127 g F-OH /g glucose. These results are quite significant considering these are, to our knowledge, the highest fatty alcohol titers reported in shake flask cultures to date. However, our goal here is to evaluate cgFAS1A for oleochemical production, and the results of these experiments with regards to cgFAS1A activity are convoluted. Robust F-OH production in strain JBEI-9018, lacking any means of acyl-CoA overproduction, suggests that Maqu2220 must efficiently reduce acyl-ACP substrates *in vivo*. It appears that NADPH-dependent thioester reduction of acyl-ACPs results in deregulation of the native FAS similar to that seen with 'TesA. Indeed, Maqu2220 has been confirmed to reduce acyl-ACP substrates (Hofvander, Doan et al. 2011). Thus, it is not possible to decouple fatty alcohol production via the native FAS from that of cgFAS1A using this reductase.

When we compared the chain length distribution of fatty alcohols in the three strains, we found a considerably higher proportion of hexadecanol (C16:0-OH) in the FAS1A expressing strain than in the other two strains (Figure 3, right). This is consistent with previous reports that this enzyme produces primarily C16 saturated lipids (Radmacher 2005), and suggests that at least some of the carbon flux is being

routed through the heterologous cgFAS1A. However, with nearly equal fatty alcohol production in all three strains, we cannot unambiguously determine the extent to which cgFAS1A is operating in the cell. We therefore turned our attention to a methyl ketone production pathway. Because this pathway is completely acyl-CoA dependent, we hypothesized that we could relieve the ambiguities of the F-OH production experiments using this pathway.

3.3. Methyl ketone production with cgFAS1A and comparison with 'Tesa and FadD

Methyl ketones are produced via decarboxylation of β -keto-fatty acids *in vivo*, which are derived from β -keto-acyl-CoAs produced through a modified β -oxidation pathway (Goh, Baidoo et al. 2014). Because the downstream pathway cannot operate on acyl-ACP intermediates, and since *E. coli* does not have a natural means of producing high levels of acyl-CoAs *in vivo*, we anticipated very low MK production in strains lacking FAS1A or 'Tesa and FadD. We consolidated the genes necessary to convert acyl-CoAs to methyl ketones (*fadM*, *fadB*, and *mlut_11700*) into vector pBbB5K in two operons, and combined this plasmid, pB5K.MK, with pE0C.RFP, pE5C.cgFAS1A, or pE5C.'tesA.fadD.

As expected, strain JBEI-9019, containing pE0C.RFP and pB5K.MK, and lacking any means of acyl-CoA overproduction, produced negligible amounts ($< 5\text{mg/L}$) of methyl ketone products (Figure 4, left). On the other hand, strain JBEI-9020 expressing cgFAS1A and the downstream MK pathway resulted in $27.8 \pm 1.4\text{ mg/L}$

methyl ketones, confirming that FAS1A is capable of supplying acyl-CoA precursors *in vivo*. Furthermore, a vast majority of the MK products were C15:0 MKs (69.1%), the decarboxylated products of the expected cgFAS1A C16:0 acyl-CoA products. When we compared JBEI-9020 with strain JBEI-9021, in which cgFAS1A is replaced with ‘TesA and FadD, we observed a higher MK titer of roughly 174.0 ± 11.6 mg/L. This result indicates that in this plasmid-based, P_{LacUV5} -driven system, cgFAS1A does not match or exceed the flux seen with the native FAS.

4. Conclusions

We have demonstrated recombinant expression of several type I fatty acid synthase enzymes and used cgFAS1A as a novel means of producing oleochemicals in *E. coli*. Recombinant expression of these large enzyme complexes is significant in that these enzymes can now be produced in *E. coli* for biochemical and structural characterization without culturing the native organisms, some of which are human pathogens. Current methods for microbial production of F-OHs, MKs, and other oleochemicals are based on enzymatic conversion of activated acyl-CoA intermediates. However, all these reported systems depend on the tightly regulated native FAS, and include a counterproductive cycle of free fatty acid release and reactivation. We have introduced an alternative system to produce acyl-CoAs in *E. coli* that lacks this counterproductive cycle of thioester hydrolysis and regeneration. Our MK production experiments conclusively showed that cgFAS1A produces acyl-CoA precursors *in vivo*. Despite direct production of acyl-CoAs, cgFAS1A expression results in lower MK production than the native FAS. A possible explanation for this

observation is that, without a thioesterase, acyl-ACP from the native FAS accumulates and inhibits the acetyl CoA carboxylase (ACCase) complex, thus limiting malonyl-CoA supply to cgFAS1A (Davis 2001).

A comparison of strain JBEI-9017 with strain AL322 (Liu, Tan et al. 2013) illustrates the drastic impact gene expression strength, number of plasmids, plasmid copy number, and codon optimization can have on F-OH production. While both JBEI-9017 and AL322 express '*tesA*, *fadD*, and *maqu2220*, the strain background, promoters, number of plasmids, and codon usage in *maqu2220* varied in the two strains (Liu, Tan et al. 2013). These variations resulted in a roughly 8-fold increase in F-OH production. These results highlight the potential for further optimization of our FAS1 based system. We are currently exploring potential avenues for improvement of the system. F-OH production could be improved further by using engineered *E. coli* strains that have been selected for fatty alcohol tolerance (Liu, Zhu et al., 2014) Expressing FAS1 enzymes from additional organisms, variation of codon usage, chromosomal integration, and dynamic regulation of FAS1 expression rather than IPTG-induction are other potential strategies we are exploring. Finally, this orthogonal FAS system will require sustained malonyl-CoA production in the presence of acyl-ACP to achieve high oleochemical titers, possibly from a heterologous ACCase.

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Table 1. Plasmids and strains used in this study

	Name	Description	Reference
Plasmid	pE5C.cgFAS1A	colE1 ori, Cm ^r , P _{LacUV5} - <i>fas-IA</i> (<i>C. glutamicum</i>)	This study
Plasmid	E5C.cgFAS1B	colE1 ori, Cm ^r , P _{LacUV5} - <i>fas-IB</i> (<i>C. glutamicum</i>)	This study
Plasmid	E5C.nfFAS	colE1 ori, Cm ^r , P _{LacUV5} - <i>fas-I</i> (<i>N. farcinica</i>)	This study
Plasmid	E5C.baFAS	colE1 ori, Cm ^r , P _{LacUV5} - <i>fas-I</i> (<i>B. adolescentis</i>)	This study
Plasmid	E5C.mtbFAS	colE1 ori, Cm ^r , P _{LacUV5} - <i>fas-I</i> (<i>M. tuberculosis</i>)	This study
Plasmid	E5C-'tesA.fadD	colE1 ori, Cm ^r , P _{LacUV5} - <i>'tesA.fadD</i>	This study
Plasmid	pE0C.RFP	colE1 ori, Cm ^r , RFP (no promoter)	This study
Plasmid	pB5K.Maqu2220	BBR1 ori, Kan ^r , P _{LacUV5} - <i>Maqu2220</i>	This study
Plasmid	pB5K.MK	BBR1 ori, Kan ^r , P _{LacUV5} - <i>fadM</i> . P _{LacUV5} - <i>fadB.mlut_11700</i>	This study
Strain	MG1655	F- lambda- <i>ilvG- rfb-50 rph-1</i>	Blattner, et al.
Strain	JBEI-3111	MG1655 Δ <i>fadE</i>	Bokinsky, et al.
Strain	JBEI-5029	MG1655 Δ <i>fadE</i> , <i>araD::acpS</i> (<i>C. glutamicum</i>)	This study
Strain	JBEI-8085	JBEI-5029 bearing E5C-CG.FAS1A and pB5K.Maqu2220	This study
Strain	JBEI-9018	JBEI-5029 bearing pE0C.RFP and pB5K.Maqu2220	This study
Strain	JBEI-9017	JBEI-5029 bearing pE5C.'tesA.fadD and	This study

		pB5K.Maqu2220	
Strain	JBEI-9019	JBEI-5029 bearing pE0C.RFP and pB5K.MK	This study
Strain	JBEI-9020	JBEI-5029 bearing pE5C.cgFAS1A and pB5K.MK	This study
Strain	JBEI-9021	JBEI-5029 bearing pE5C.'tesA.fadD and pB5K.MK	This study

Figures

Figure 1. Alternative acyl-CoA production pathways. The native FAS produces fatty acyl-ACP, which feedback-inhibits the FAS. This inhibition is relieved by 'TesA, which releases free fatty acids. Free fatty acids are esterified to CoASH by the ATP-dependent CoA-ligase FadD. Alternatively, heterologous type I FASs release acyl-CoAs directly, obviating the need for 'TesA, which hydrolyzes acyl-CoA intermediates, and FadD which consumes ATP. The CoA/ACP-reductase Maqu2220 efficiently reduces either acyl-ACP or acyl-CoA intermediates to fatty alcohols. Acyl-CoA intermediates can also be enzymatically converted to methyl ketones using the enzymes Mlit_11700, fadB, and fadM. Abbreviations: ACP, acyl carrier protein; AT, acyltransferase; KS, ketosynthase; KR, ketoreductase; DH, dehydratase; ER, enoylreductase; MPT, malonyl-palmitoyltransferase.

Figure 2. Recombinant expression of type I FAS enzymes and effect on lipid content. (Left) SDS-PAGE analysis of cells expressing FAS1 enzymes from different organisms. All five of the constructs tested expressed solubly. (Right) Overlaid GC-MS traces of extracts from cells expressing cgFAS1A and RFP shows an increase in total lipids when we express cgFAS1A.

Figure 3. Fatty alcohol production in strains expressing alternate acyl-CoA pathways and Maqu2220. (Left) Total fatty alcohol titers were measured in strains JBEI-9018 (red diamonds), JBEI-8085 (blue squares), and JBEI-9017 (green triangles) at three time points over 71 hours. (Right) The chain length distributions of the three strains are indicated. While the total fatty alcohol titers were similar in the three strains, the proportions of different chain lengths varied. Error bars indicate standard deviations from triplicate biological replicates.

Figure 4. Methyl ketone production in strains expressing alternate acyl-CoA pathways. (Left) Methyl ketone titters in strains JBEI-9019 (red diamonds), JBEI-9020 (blue squares), and JBEI-9021 (green triangles) were measured at five time points over 87 hours (Right) Chain length distributions of methyl ketones are indicated. Error bars indicate standard deviations from triplicate biological replicates.