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Engineering Escherichia coli for Overproduction of Medium-chain Fatty Acids and One-step In Vivo Conversion to Biodiesel

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Engineering *Escherichia coli* for Overproduction of Medium-chain Fatty Acids and One-step *In Vivo* Conversion to Biodiesel

A dissertation submitted in partial satisfaction of the
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in Biochemistry and Molecular Biology

by

Saken Sherkhanov

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ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Biochemistry and Molecular Biology

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Professor James U. Bowie, Chair

Over the past century, fossil fuels have been an abundant and cheap source for petroleum-derived chemicals and fuels. The production and burning of these carbon-derived fuels have led to adverse global environmental changes such as increase in air pollution, climate change and fluctuations in sea level. To replace dwindling petroleum resources and to curb emissions of CO_2, it is critical to develop alternative and renewable resources for energy and fuels. Genetically engineered microorganisms that can directly produce medium and long chain hydrocarbons have been one of the most promising potential routes to renewable biofuel synthesis.

Microbial fatty acids are an attractive source of precursors for a variety of renewable biofuels such as alkanes, alcohols, and biofuels. Enormous progress has been in engineering microbes to divert endogenous fatty acid synthesis and

overproduce free fatty acids. However, there is an inherent problem of product toxicity that greatly reduces cell viability, increases cell lysis and product titers and there are previous reports suggesting membrane damage as the main mechanism of free fatty acid toxicity. In this work, we metabolically engineered *Escherichia coli (E. coli)* bacteria to overproduce medium chain free fatty acids and identified membrane stress as the leading factor in product toxicity. We found that membrane lipid composition can be altered by the direct incorporation of endogenously produced medium-chain fatty acids into lipids via the Aas pathway. The deletion of the *aas* gene and sequestering exported fatty acids reduces medium-chain fatty acid toxicity, partially restores normal lipid composition, and dramatically improves medium-chain fatty acid yields.

In the second part of this thesis, we genetically engineered *E. coli* to produce fatty acid methyl esters (FAMEs) by direct *in vivo* methylation of free fatty acid in the strains discussed above. Insect *Drosophila melanogaster* Juvenile Hormone Acid *O*-Methyltransferase (*Dm*JHAMT) was identified as a candidate to methylate a variety of endogenous medium chain fatty acids in *E. coli.* By introducing DmJHAMT in *E. coli* engineered to produce medium chain fatty, we obtain medium chain FAMEs at titers of 0.56 g/L, more than two orders of magnitude higher than titers previously achieved. This one-step conversion process was optimized by expression of rat Mat1A gene that increased the *S*-adenosyl-*L*-methionine cofactor pool and providing a physical sink to extract FAMEs from culture.

The work presented here shows the viable method of producing microbial biodiesel by metabolical engineering of *E. coli.* Primary physiological stress associated with production of free fatty acid precursors was identified and higher titers of fatty acid

production were reported. These free fatty acids were then converted to biodiesel in one step by expressing insect enzyme in *E. coli*. Although further work is needed for viable bacterial production of biodiesel, the simplicity of the pathway allows easier optimization and possibility to transport this pathway into photosynthetic microorganisms in the future.

The dissertation of Saken Sherkhanov is approved.

Guillaume Chanfreau

James Liao

James Gober

Benjamin Bonavida

James Bowie, Committee Chair

This work is dedicated to my parents,

Khalilayeva Sholpan and Sherkhanov Rakhym

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Acknowledgements

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CHAPTER 1:

Introduction to Fatty Acids for Next Generation Biofuel Production

1.1. Introduction to Biofuels

With the invention of the internal combustion engine and the development of plastics, petroleum derived from the fossil fuels became one of the most indispensable chemicals for the humankind. For more than 100 years, fossil fuels have made up around 80% of total U.S energy consumption and this trend is likely to continue into the future (1). The burning of these carbon-derived fuels for energy and transportation has led to increase in air pollutants and atmospheric CO_2 concentrations. There is an overwhelming agreement among leading scientists that the increased concentration of carbon dioxide in the atmosphere is the cause of anthropogenic global warming and changes in climate patterns with observable effects on the environment such as sustained heat waves, droughts and rising sea levels (2). In addition, increasingly high demand for fossil fuels will deplete the existing natural stocks that will have wider implications in pharmaceutical and chemical industries. There are other economic and political concerns associated with U.S. fossil fuel consumption such as high energy prices, increasing dependence on petroleum imports and environmental contamination due to extraction and processing of fossil fuels. To address these issues, the development of alternative and renewable resources for energy and fuels is essential for human society. Primary energy sources such as nuclear energy, solar, wind, geothermal and hydropower have already been utilized to produce electricity as a secondary energy source, but thus far have not matched the capacity required by modern living standards. Alternatively, biomass-derived chemicals are recognized as a major alternative to supplement declining fossil fuel resources for transportation and chemical industry.

In a typical petroleum distillation process, the crude oil is fractionated according to the carbon chain length for their appropriate uses in the engines. The alcohols containing short-chain hydrocarbons (C2-C10) can be used as substitutes in gasoline engines or as fuel additives. Longer chain hydrocarbons (C10-C23) are the major components of diesel and jet fuel needed by the heavy transportation sector. Ethanol and biodiesel are two of the most commonly used biofuels produced to date. All biofuel production requires feedstock sugars and extensive treatment and processing as outlined in Figure 1-1 (3). Ethanol has been one of the most successful biochemicals produced on a large, industrial scale. Fuel ethanol is produced by direct fermentation of simple sugars or more complex polysaccharides like lignin and cellulose that can be converted into monomeric sugar molecules. Under anaerobic conditions, the yeast *Saccharomyces cerevisiae* utilizes the well-characterized Embden-Meyerhof glycolytic pathways and alcoholic fermentation of sugars as the sole source of ATP for cellular metabolism and growth. In this manner, hexose sugars are converted into ethanol in yeast at >90% of the theoretical yield (4). More studies have been done to utilize other organisms to produce ethanol. Gram-negative *Zymomonas mobilis* metabolizes hexoses using the distinct Entner-Doudoroff pathway producing less ATP and biomass and metabolic carbon flux is channeled to higher fermentation products (5). As a result, its ethanol productivity is 2.5 fold higher than that of *Saccharomyces* species and *Z. mobilis* has been engineered to convert various simple sugars to ethanol (6). Additionally, thermophilic bacteria *Thermoanaerobacterium saccharolyticum* and *Clostridium thermocellum* have been metabolically engineered to produce ethanol as the only detectable organic product at higher temperatures (7, 8).

Currently, bioethanol is present in 95% of U.S. gasoline to oxygenate the fuel and reduce air pollution (9). While some flexible fuel vehicles can utilize a gasoline with high ethanol blend, there are significant drawbacks of ethanol usage as a fuel. Due to its high water solubility, ethanol requires a significant input of energy to be processed and distilled and currently, most of the first generation ethanol production enterprises require governmental subsidies. In addition, ethanol's hygroscopic nature makes it incompatible with the existing energy infrastructure due to its rusting potential and inability to be delivered by pipeline. Compared to current petroleum-based fuels, ethanol has very low energy content and has far lower mileage per volume of fuel.

Biodiesel is another important renewable biofuel derived from biological sources such as vegetable oil, animal fat and fatty acids produced by yeast, algae or bacteria (10). Due to high energy density and lower water solubility, biodiesel is the most appropriate biofuel for the growing transportation needs. Biodiesel has been already used in existing infrastructure and is widely available in pure form (100% biodiesel known as B100) and in blends with petroleum diesel. Accounting for the biomass-derived carbon, biodiesel reduces net CO_2 emissions by 78.45% compared to petroleum diesel. Using pure biodiesel as a transportation fuel will substantially reduce tailpipe emissions of total particulate matter by 32%, CO by 46% and completely eliminate SO_x emissions (11). Biodiesel is essentially non-toxic, biodegradable and suitable for sensitive environments. Typically, biodiesel consists of monoalkyl esters of fatty acids, mainly fatty acid methyl esters (FAME) produced by the chemical transesterification of plant- and animal-derived triacylglycerides with methanol under alkaline conditions (Figure 1-2). The main characteristics for biodiesel fuels are their

heat of combustion and their cetane number, a measure of ignition quality that is standardized to be 47 in U.S. and 51 in E.U (12). As we can see from Table 1-2, most of the C12-C23 fatty acid derived esters meet or exceed these standards and have been used as a benchmark for producing biodiesel (12). Alkali-catalyzed transesterification process requires more than 3 moles of methanol at higher temperatures (~55-60 ^{o}C) in an anhydrous environment to drive the reaction into FAME production. The majority of the acyl groups in biodiesel are long-chain hydrocarbons (C10-C23) that are suitable for current diesel engines (13). There have been many studies towards engineering higher lipid biosynthesis in plants and fungi and the summary of some of these studies is presented in Table 1-1.

While biodiesel has attracted increasing attention as an alternative to fossil fuels, there are significant drawbacks that will limit the production of biodiesel in the foreseeable future. Direct transesterification method requires pure triacylglycerides as trace amounts of water and free fatty acids lead to saponification and soap formation, lowering the quality of biodiesel. While acid-based catalysis has been developed to convert free fatty acids to biodiesel in a step-wise manner, it required large amount of toxic catalyst and water. Furthermore, the pretreatment of feedstock, esterification and post-methylation processing of FAMEs need large input of energy and none of the methods listed so far are cost effective (3). Most importantly, these first generation biofuels require biomass feedstock and considerable agricultural input that may compete with food supply. In 2005, the U.S. produced 1.48 million liters of ethanol and 256 million liters of biodiesel from corn and soybean, respectively (14). While significant combined, these biofuels accounted for only 1.81% fuel usage in the United States and

reaching beyond these rates is highly unlikely due to contribution of these crops to food supplies.

Based on these fundamental limitations mentioned above, better biofuel alternatives may be directly produced in microorganisms using the tools of genetic engineering and synthetic biology. . Recent progress in metabolic engineering has already allowed scientists to develop microbial hosts that directly produce a wide range of advanced biofuels with similar properties to fossil fuels. In one of the earlier works in biofuel production, *Escherichia coli (E. coli)* was designed to produce 45 g L^{-1} ethanol with almost 90% theoretical maximum yield (15). While native fermentative process in *E. coli* is suboptimal due to other competing by-products, the new strain had genome-integrated *pdc* (pyruvate decarboxylase) and *adhB* (alcohol dehydrogenase) genes from *Zymomonas mobilis* that were shown to produce high level of ethanol in *E. coli* while native *pflB* and *frd* genes responsible for competing formate and succinate biosynthesis were removed (15). In a seminal work, James Liao research group engineered *E. coli* to non-fermentatively produce branched-chain alcohols such as isobutanol and 1-butanol. Introduction of 2 non-native genes, 2-keto-acid decarboxylase from *Lactococcus lactis* with alcohol dehydrogenase 2 from *S. cerevisiae*, diverted endogenous amino acid biosynthesis to alcohol synthesis in *E. coli* (16). This strategy allowed *in vivo* production of longer chain and branched alcohol that have higher energy content and lower hygroscopicity compared to traditional ethanol. Further growth of these strains in bioreactor with *in situ* product removal yielded 50 g/L of isobutanol that is beyond the toxicity limit of this substance on *E. coli.* (17). In a different approach, a heterologous isoprenoid pathway was engineered in *E. coli* to produce isoprenoid-

based C_5 alcohols such as 3-methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol and 3-methyl-1-butanol (18, 19). Other organisms such as *Corynebacterium glutamicum, Clostridium cellulolyticum, Ralstonia eutropha* and *Synechococcuse elongates* have been utilized to produce longer chain alcohols such as isobutanol and 2,3-butanediol from carbon dioxide and various carbon sources including cellulose (18–21). Figure 1-3 summarizes the range of alcohols produced in genetically-engineered *E. coli.*

While alcohols are suitable as gasoline substitutes and fuel additives, longer chain hydrocarbons with higher energy density are required for heavy transportation and many metabolic engineering strategies have been recently focused on studying pathways that produce longer chain carbon molecules. The aliphatic moieties of fatty acids contain high energy density and are readily available, renewable building blocks for biofuel production. Fatty acid biosynthesis is present throughout the Bacteria and Eukarya domains of life and is essential for the production of phospholipids, liposaccharides, secondary messengers in cells and organisms and the energy storage molecules. As outlined in Table 1-1, much has been done to divert and produce free fatty acids in genetically modified organisms. For example, yeast *Yarrowia lipolytica* has been designed to accumulate 61.7% lipid content after overexpression of two enzymes, diacylglycerol acyltransferase and acetyl-CoA carboxylase, that are involved in endogeneous fatty acid biosynthesis (20). Unfortunately, the polar carboxylic acid part of fatty acids makes them incompatible with any model fuels and the last conversion step to biofuel has been challenging and mostly done *in vitro* by chemical esterification or reduction of extracted oils. While yeast can produce very high titers of lipids and ethanol, the latest *in vivo* biodiesel production studies in yeast reported the highest

yields of only 34 mg of fatty acid ethyl ester produced per liter of culture (21). There are clearly many unknown factors and bottlenecks that need to be addressed in plant and fungi-related biofuel research before any industrial scale applications are developed.

While plants, fungi and algae provide ideal platform for the generation of fatty acids from various carbon sources or sunlight and $CO_{2,}$ the main roadblock for designing metabolic pathways in these organisms has been the limited understanding of innate biochemistry and genetics and the lack of genetic manipulation tools. Due to these reasons, Gram-negative *E. coli* was chosen as the model organism for my graduate studies. *E. coli* has well-studied fatty acid biosynthetic pathway that is amenable to genetic manipulations and has a potential to produce fatty acids with different chain lengths and properties. We sought to study the limiting factors such as product toxicity and subsequent growth slowdown due to free fatty acid synthesis and engineer a strain that has both better fitness and higher production rate of fatty acids (Chapter 2). In addition, we developed efficient one step *in vivo* conversion pathway of free fatty acids to biodiesel utilizing endogenous pathways with little or no energy leak in the process (Chapter 3).

The remainder of this chapter will review *E. coli* fatty acid metabolism and regulation, role of fatty acids in membrane biosynthesis and how it changes with fatty acid production, current challenges the field faces and examples of fatty acid derived biofuels in *E. coli* produced to date.

1.2. Fatty Acid Metabolism and Regulation in *E. coli*

1.2.1 Fatty Acid Biosynthesis

E. coli fatty acid synthesis (FAS) is one of the prototypical bacterial Type II pathways found in bacteria. Unlike Type I FAS that utilizes one or two large polypeptide complex to synthesize fatty acids in mammals and other eukaryotes, FAS II is a completely dissociated system where each component is a unique protein catalyzing a single step in a pathway (reviewed in 22–25). Due to being multi-enzyme system, FAS II can modify growing acyl groups at various steps and generate a wide range of fatty acids with different chain length, saturation, branching and chain length modification (22). This diversity is attributed to the fact that all fatty acid intermediates are carried by acyl-carrier protein (ACP) that can be easily diverted into other biosynthetic pathways including phospholipid biosynthesis, acylation of membrane proteins, production of endotoxin lipid A, fatty-acid derived vitamins lipoic acid, biotin and synthesis of quorum sensing molecules (reviewed in 24).

Enzymes in the FAS II act on acyl thioesters that are either coupled to ACP or coenzyme A (CoA). These two cofactors are essential for *E. coli* fatty acid biosynthesis as they are involved in initiation, providing precursors for condensation reactions, carrying the acyl group from one enzyme to another and eventually, transfer to the membrane phospholipids . In *E. coli*, ACP is a small acidic protein (~9 kDa) that is one of the most abundant proteins in the organism (about 0.25% of all soluble proteins) (26). Majority of ACP is maintained in active holo-form that has phosphopantetheine group from CoA attached to Ser-36 of ACP (27, 28). The resulting terminal sulfhydryl of the prosthetic group is used to bind all acyl intermediated of FAS (26, 29).

The general overview of *E. coli* fatty acid metabolism is outlined in Figure 1-4 and the genes, enzymes and their functions are detailed in Table 1-3. The precursors for FAS are derived from acetyl-CoA pool. The first committed step in fatty biosynthesis starts with the conversion of acetyl-CoA to form malonyl-CoA by acetyl-CoA carboxylase complex (AccABCD) (30). Malonyl group is then transferred to ACP by malonyl-CoA-ACP transacylase (FabD) (31). The resulting malonyl-ACP serves as donor of two carbon units for fatty acid elongation reactions. The first step is the condensation of malonyl-ACP with either growing acyl-ACP primer or acetyl-CoA to form β-ketoacyl-ACP. β-ketoacyl-ACP synthase III (FabH) catalyzes the initial acyl formation by condensing acetyl-CoA with malonyl-ACP to form acetoacetyl-ACP, whereas FabB/FabF β-ketoacyl-ACP synthases are part of elongation module and catalyze growing acyl-ACP condensation with malonyl-ACP (32–34). β-ketoacyl-ACP is reduced in a NADPH-dependent manner to β-hydroxyacyl-ACP by β-ketoacyl-ACP reductase (FabG) followed by removal of water molecule to form *trans*-2-enoyl-ACP by either one of two β-hydroxyacyl-ACP dehydrases (FabA or FabZ) (35–37). Finally, acyl-ACP is formed by NADH-dependent reduction of enoyl-ACP by enoyl-ACP reductase (FabI) and the product is cycled back to 2-carbon unit elongation or diverted to phospholipid synthesis (38).

The most important of membrane properties is the ratio of unsaturated to saturated fatty acids in the phospholipids. The key reaction in producing unsaturated fatty acids lies with FabA and FabB (24, 39). FabA, in addition to dehydrating β-hydroxyacyl-ACP , has a unique property of isomerizing the *trans*-2-decenoyl-ACP

product to *cis*-3-decenoyl-ACP (40). FabB uses this *cis*-3-decenoyl-ACP to further elongate the acyl chain (22, 41). The *cis* double bond is retained in the subsequent elongation cycles and each cycle pushes this *cis* bond by two carbon units per cycle from the thioester bond with ACP (reviewed in 22, 24).

1.2.2. Fatty Acid Degradation

E. coli can use exogenously supplied fatty acids as a carbon and energy source through β-oxidative cleavage to acetyl-CoA (Figure 1-4). Long-chain fatty acids are transported through outer membrane protein FadL and inner-membrane-associated acyl-CoA synthase (FadD) esterifies free fatty acids to CoA (42, 43). β-oxidation occurs via repeated cycles of reactions that start with dehydrogenation of acyl-CoA by acyl-CoA dehydrogenase (FadE) (44). Next, two multifunctional homologues FadB and FadJ reduce enoyl-CoA and then dehydrate β-hydroxyl-CoA (45). The resulting β-ketoacyl-CoA is a substrate for β-ketoacyl thiolases FadA and FadI that results in the shortening of original acyl-CoA by two carbon atoms and production of acetyl-CoA (46). The redundancy of enzymes in some reaction steps in β-oxidation (FadB/FadJ and FadA/FadI) and FA biosynthesis (FabB/FabF and FabA/FabZ) is due to different substrate specificities of these enzymes in relation to acyl chain length and saturation (reviewed in 23).

1.2.3 Regulation of Fatty Acid Metabolism

Fatty acid metabolism is largely regulated and coordinated with phospholipid production, the growth and the environmental changes. Rapid responses such as temperature change involve changes in the phospholipid composition that largely do not cause changes in gene expression (phospholipid synthesis will be discussed later

section 1.3) (47). *E. coli* normally shuts down fatty acid and phospholipid synthesis in stationary phase and it was observed that inhibition of phospholipid synthesis resulted in a rapid decrease in the rate of fatty acid synthesis and in the accumulation of acyl-ACPs (48). This concomitant inhibition was fully relieved with the overexpression of *E. coli* thioesterase I (TesA) or other plant thioesterases that cleave acyl-ACPs to generate free fatty acids (48, 49). The accumulated acyl-ACPs are involved in an allosteric regulatory feedback of fatty acid synthesis in a stationary phase and it was subsequently shown that long-chain acyl-ACP products inhibit enzymes FabH, FabI and acetyl-CoA carboxylase complex (AccABCD) (Figure 1-5) (50–52).

Two transcriptional factors, FabR and FadR, have been identified in regulation of fatty acid metabolism. FabR represses expression of the two genes, *fabA* and *fabB,* required for unsaturated fatty acid synthesis (53, 54). On the other hand, FadR serves as an activator of these two genes and it upregulates unsaturated fatty acid biosynthesis in *E. coli* (55, 56). In addition, FadR coordinates the fatty acid degradation by being a global repressor of β-oxidation genes and outer membrane transporter FadL (reviewed in 57, 58). Binding of FadR is specifically inhibited by long chain fatty acyl-CoA compounds that most likely accumulate with basal expression of *fadD* and presence of free fatty acids in the environment (58). The overview of fatty acid metabolism regulation is summarized in Figure 1-5.

1.3. Fatty Acids and Membrane Phospholipid Synthesis

Palmitate (C16:0), palmitoleate (C16:1) and *cis*-vaccenate (C18:1) make up the majority of fatty acids found in *E. coli* membranes (59). Fatty acids biosynthesis

normally ends when the acyl chain is 16 or 18 carbons in length. These long-chain acyl-ACPs are substrates to the acyltransferases that transfer acyl chain into the membrane phospholipids (Figure 1-6). The peripheral membrane protein PlsX cleaves acyl-ACP and transfers acyl group to inorganic phosphate to form reactive acylphosphate (60). This intermediate is then used by integral membrane protein PlsY to acylate glycerol-3-phosphate (G3P) to form 1-acyl-glycerol-3-phosphate or lysophosphatidic acid (LPA) (60). Alternatively, LPA may be generated from either long-chain acyl-ACP or acyl-CoA through PlsB, inner membrane acyltransferase (61). Integral membrane acyltransferase PlcC then adds a second acyl chain to the 2-position of LPA to generate phosphatidic acid (PA) (61). PA is the central intermediate in the formation of three major phospholipid species: phosphatidylethanolamine (PE) that comprises the bulk of the phospholipids (75%), with phosphatidylglycerol (PG) and cardiolipin (CL) forming the remainder (15-20% and 5-10%, respectively) (Figure 1-6) (62). As the only post-synthetic modification mechanism, *E. coli* has cyclopropane fatty acid synthase (Cfa) that forms cyclopropane ring across the *cis* double in unsaturated fatty acids of existing membrane phospholipids (63). This modification affects the membrane fluidity and occurs during the transition from the late log phase to the stationary phase of the cell growth or as a response to an acid stress or temperature change (63, 64).

An unusual and novel free fatty acid incorporation into existing membrane phospholipids has been discovered and characterized in late 80s and early 90s (65–67). Post-translational acylation of lipoproteins uses the acyl group at the 1-position of phosphatidylethanolamine (PE) (68). The resulting 2-acylglycerolphosphoethanolamine (2-acyl-GPE) is membrane disruptive and either destroyed by phospholipases or

recycled by Aas, an inner membrane-associated bifunctional enzyme (66, 67). In *fadD* mutants lacking free fatty acid degradation pathway, Aas, with acyl-ACP synthase and 2-acyl-GPE acyltransferase activities, provides only pathway to incorporate free fatty acids into phospholipids (Figure 1-6) (65). This incorporation is not coupled to fatty acid biosynthesis or degradation and utilizes free fatty acid available to recycle 2-acyl-GPE into plasma membrane (67). While this enzyme is not essential, it affects the cell fitness and growth in *E. coli* in the presence of more toxic fatty acids in the environment. In my graduate study, I showed that Aas can incorporate medium chain free fatty acids directly into plasma membrane and thus, decrease membrane fluidity and exacerbate the toxicity of these fatty acids (Chapter 2).

1.4. Free Fatty Acid Production in *E. coli.*

Due to its high rate of fatty acid biosynthesis and ability to grow on a variety of carbon sources and naturally secrete compounds, *E. coli* has been the focus of recent studies to produce free fatty acids (FFAs) and fatty acid derived biofuels. The first reports of FFA production were reported when periplasmic thioesterase TesA and plant thioesterases expressed cytosolically deregulated fatty acid synthesis (49, 69). Most of these thioesterases cleave acyl-ACPs at different stages of FA elongation and lead to accumulation of FFAs in the culture (reviewed in 25). The diversity of plant thioesterases and their expression in *E. coli* allowed the production of wide range of fatty acids with different chain length and properties. While many strategies were employed to increase fatty acid yields, the combination of three strategies proved to be most effective: upregulation of carbon flux through FA synthesis, either by

overexpression of Acc complex or transcription factor FadR, expression of acyl-ACP thioesterases to cleave acyl-ACP and relieve feedback inhibition and elimination of β-oxidative degradation of fatty acids (refer to Figures 1-4 and 1-5 for FA metabolism) (70–73). Table 1-4 presents some of these genetic modifications and their efficiencies in *E. coli* (74). The amount of work and articles published on FFA production in *E. coli* is very extensive and several excellent reviews on this research field were recently published (25, 74, 75).

While the highest yields of free fatty acids were achieved with the production of less toxic straight long-chain (C16:0-C18:0) fatty acids, their downstream products have higher melting points and are unsuitable in cold temperature applications (Table 1-2) (76, 77). Because of that, production of short and medium-chain fatty acids attracted considerable interest. Pfleger research group studied extensively the production of C12 and C14 fatty acids and achieved titers of 0.8g/L of these FAs (25, 73, 78). While the expression of heterologous thioeterase enzymes and regulation of FA genes can be optimized, the inherent problem with carboxylic acid production is their toxicity (79, 80). Medium chain fatty acids cause membrane damage leading to less fluidity, membrane lysis, interruption of the electron transport chain and possibly affecting nutrient uptake (25, 74, 79, 80). Several approaches such as developing tolerance through evolutionary approach or improving fatty acid export systems have been suggested to improve the cell viability and the results so far have been mixed (79, 81).

In this study, we addressed these toxicity challenges and developed *E. coli* strains that have increased tolerance to and highest yields of medium chain FAs reported (82). Our knock-out genetic screens identified membrane biosynthetic gene

aas as a key factor in causing medium-chain FA toxicity in FFA-producing strains. We showed that *aas* directly incorporated endogenously produced FAs into membrane phospholipids and dramatically decreased cell viability. Deleting this gene partially restored membrane composition, improved cell fitness and subsequently, increased the product yields (Chapter 2).

1.5 Production of Fatty-Acid Derived Biofuels

Endogenously produced free fatty acids as well as intermediates of fatty acid biosynthesis such as acyl-ACPs and β-ketoacyl-ACP have been converted into biofuels and bio-based products *in vivo* (Figure 1-7) (reviewed in 75, 83, 84). Methyl ketones were produced in *E. coli* by overexpressing plant methylketone synthases with yields of 450 mg/L (85). Introduction of plant thioesterase and acyl-CoA reductase from other bacteria in *E. coli* produced fatty aldehydes and fatty alcohols up to 60 mg/L (86). Two cyanobacterial genes expressing acyl-ACP reductase and fatty aldehyde decarbonylase in *E. coli* enabled the production of alkanes and alkenes (87). Other fatty acid derived products synthesized from *E. coli* include α-olefins and long chain alkenes (88, 89).

There are two major reports on the direct production of biodiesel in *E. coli* (18, 90). Keasling lab developed an *E. coli* strain capable of producing fatty acid ethyl esters (FAEE) *in vivo* from sugars (Figure 1-8). Endogenous acyl-CoA pool was enriched by overexpression of both acyl-CoA synthase (*fadD*) and a native *E. coli* thioesterase (*tesA*) in the cytosol and deletion of acyl-CoA dehydrogenase (*fadE*) to remove endogenous β-oxidation (see Figure 1-4 for FA metabolism). The engineered strain also produced ethanol non-fermentatively by heterologous expression of pyruvate

decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) from *Zymomonas mobilis.* The final esterification of acyl-CoA and ethanol to form FAEE was catalyzed by heterologously expressed acyltransferase (wax ester synthase/acyl-CoA:diacylglycerol acyltransferase) encoded by *atfA* from *Acinetobacter baylyi* (86).The highest yield reported in that study was 0.67 g/L of FAEE and subsequent optimization of this strain via a dynamic sensor-regulator system increased titers to 1.5 g/L (86, 91).

The other pathway of *in vivo* production of biodiesel in engineered *E. coli* was constructed by intorducing a fatty acid O-methyltransferase (FAMT) from *Mycobacterium marinum* and catalyzing the reaction with endogenous S-adenosylmethionine (SAM) as a methyl donor. The impacts of this study on biofuel field are limited, as the titers were extremely low (total yields of 16 mg/L) and almost all FAME containing β-hydroxy fatty acids that do not have favorable fuel properties.

In Chapter 3, we report a novel one-step conversion of free fatty acids to biodiesel via heterologously expressed insect methyltransferase in *E. coli* (Figure 1-7). While no enzyme that carboxymethylate long chain fatty acids have been identified so far, we proposed that insect enzyme, *Drosophila melanogaster* Juvenile Hormone III acid methyl transferase (DmJHAMT) could be used to convert free fatty acids to biodiesel in *E. coli* (92, 93). *Dm*JHAMT is an insect enzyme that normally methylates mevalonate pathway-derived hormones and when we heterologously expressed this enzyme in fatty acid producing *E. coli,* we observed the biodiesel production. SAM cofactor, required for the methylation of fatty acids, was enriched in *E. coli* by expressing rat liver SAM synthase. Overall, our titers were 540 mg/L of FAME, the highest yield of FAME produced in *E. coli* and most of the products (C12 and C14

FAME) have better attributes such as lower melting point and comparable cetane number compared to previously published FAME or longer chain FAEE production. This simple system opens a possibility of porting this pathway into the photosynthetic microorganisms for direct production of biodiesel from CO_2 and sunlight in the future.

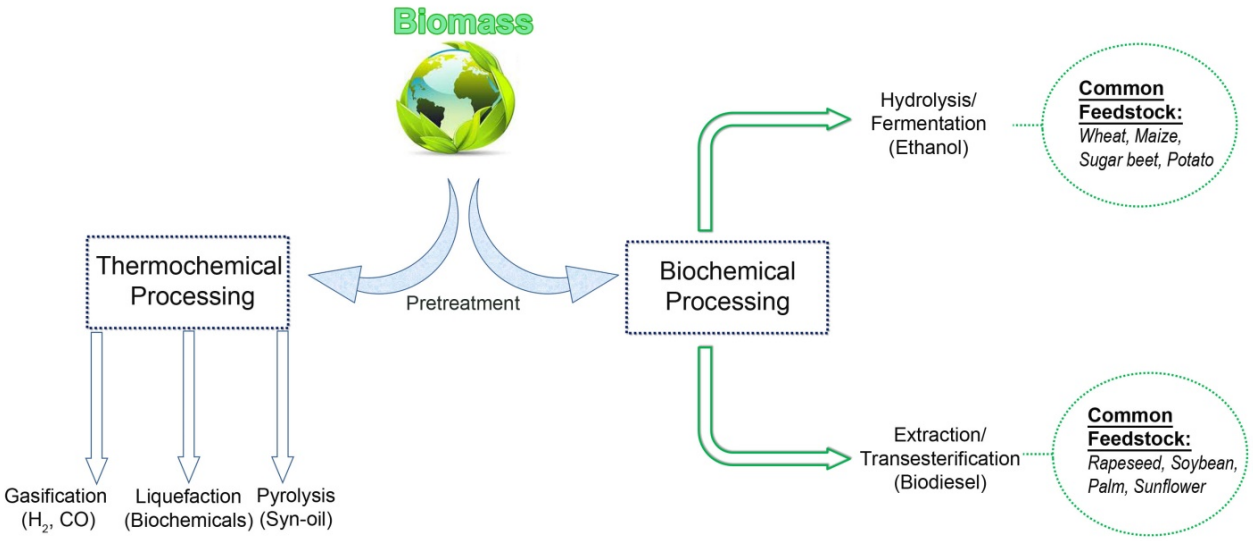

Figure 1-1. Main industrial biomass conversion processes (3). Syn-oil, synthetically produced oil.

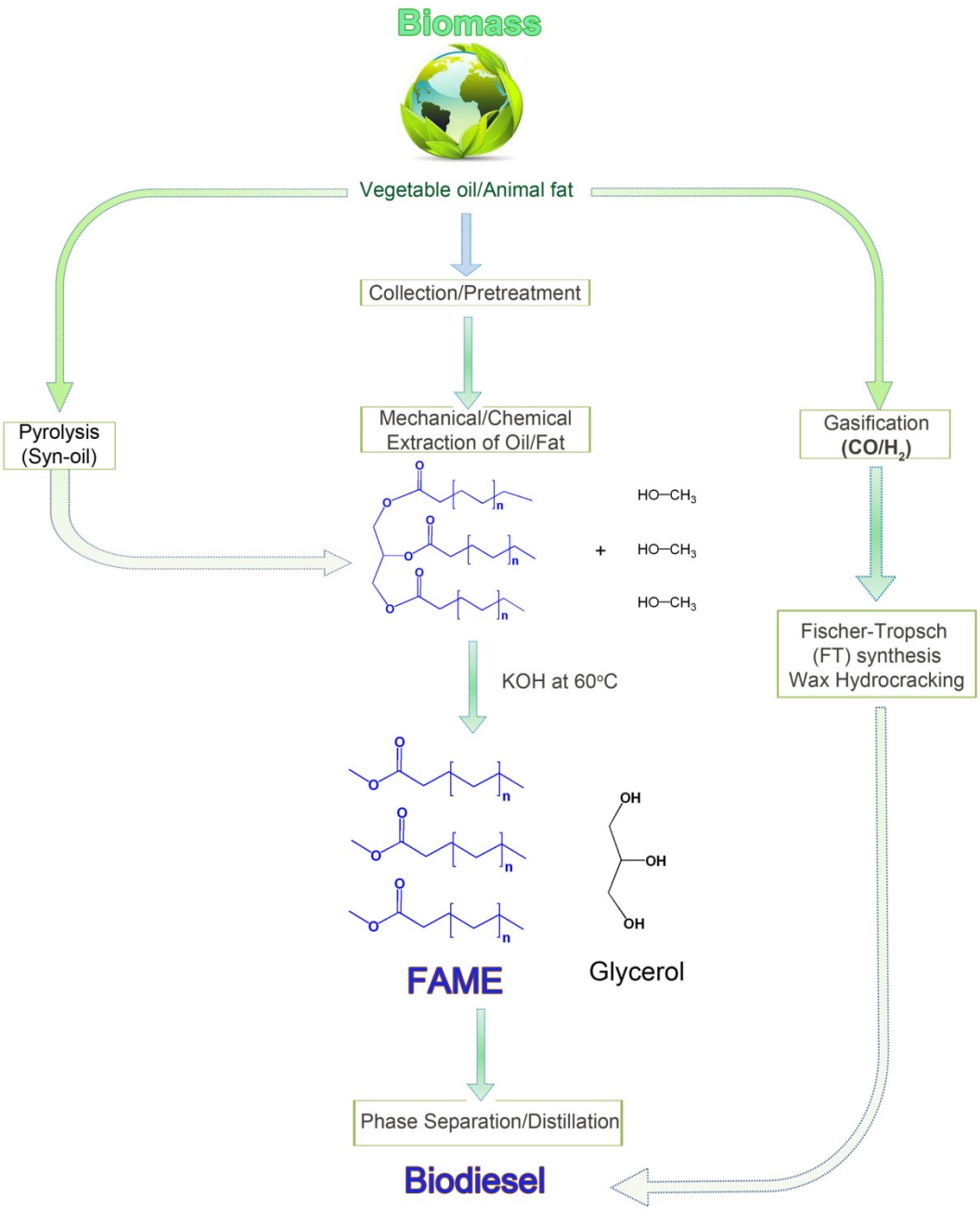

Figure 1-2. Current industrial methods in biodiesel production.

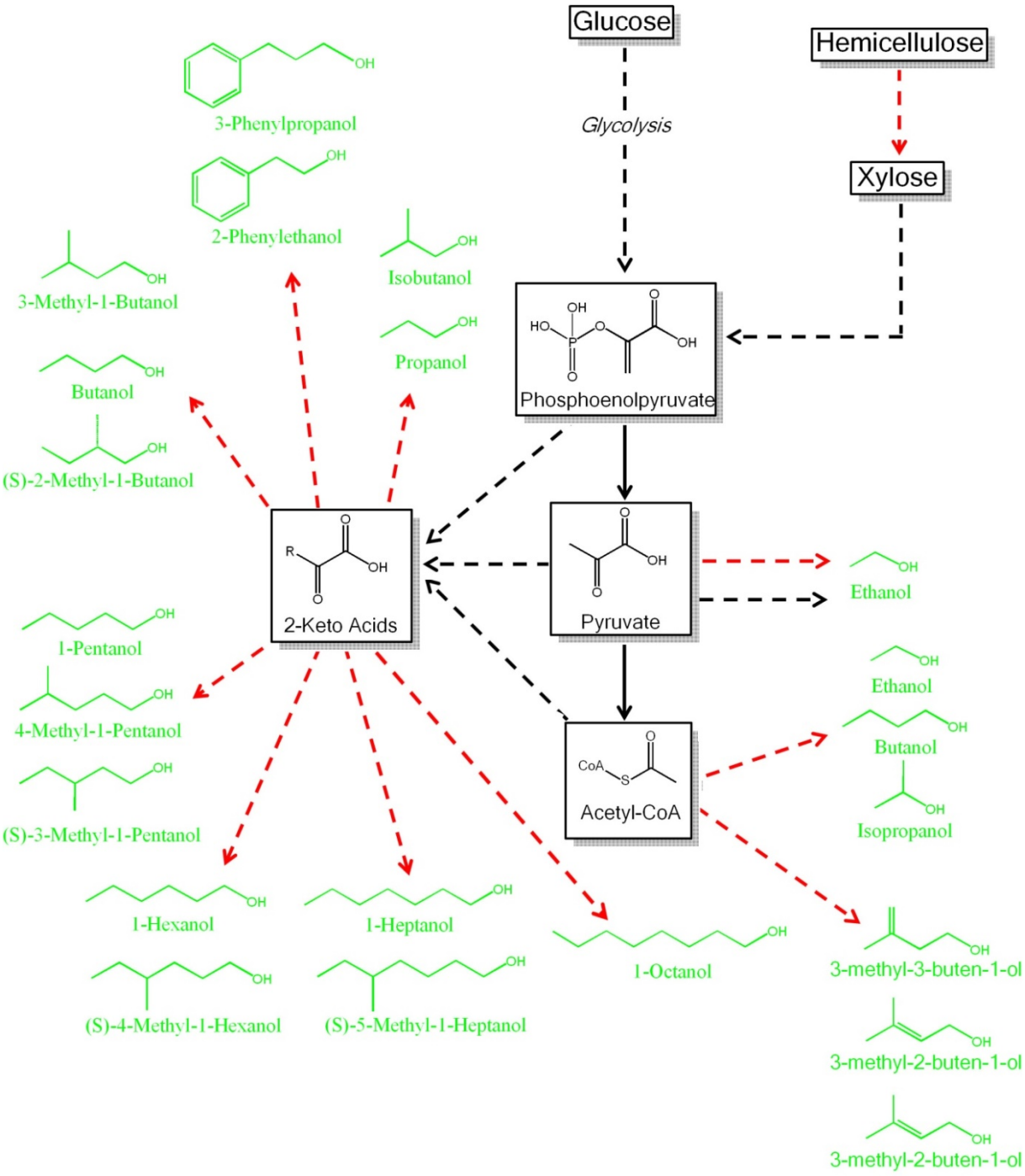

Figure 1-3. The range of alcohols being produced in genetically engineered *E. coli.* The dashed arrows represent multi-enzyme reactions and solid arrows represent single-enzyme catalysis. Black arrows denote native *E. coli* pathways whereas red arrows are heterologous pathways introduced into *E. coli.* Adapted from (13, 18, 75, 94, 95)

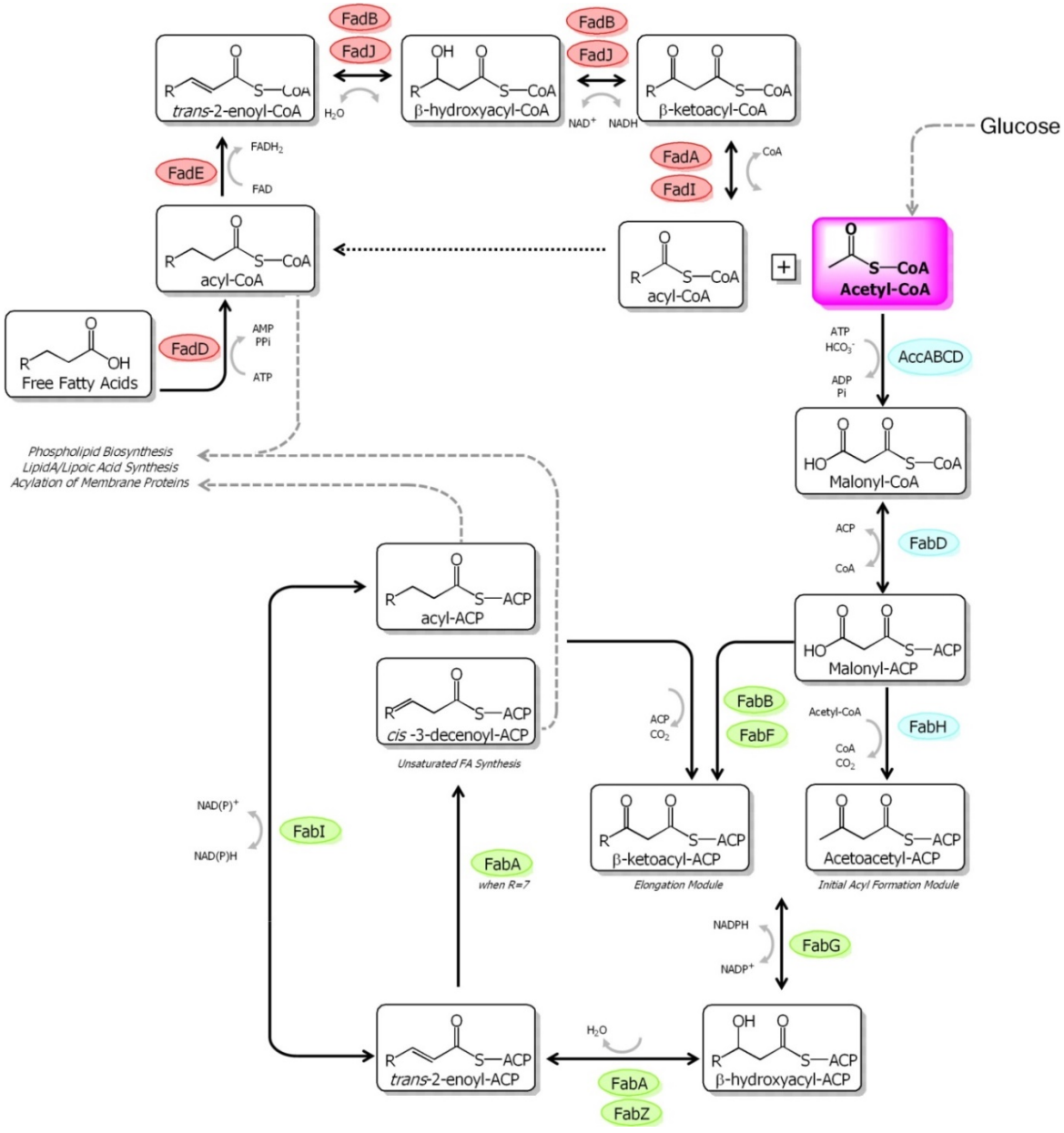

Figure 1-4. Fatty acid (FA) biosynthetic and degradation pathways in *E. coli*. Enzymes in blue represent FA initiation pathway, enzymes in green catalyze FA elongation module and enzymes in red are part of β-oxidation/FA degradation pathway. The list of enzymes and their functions are given in Table 1-3.

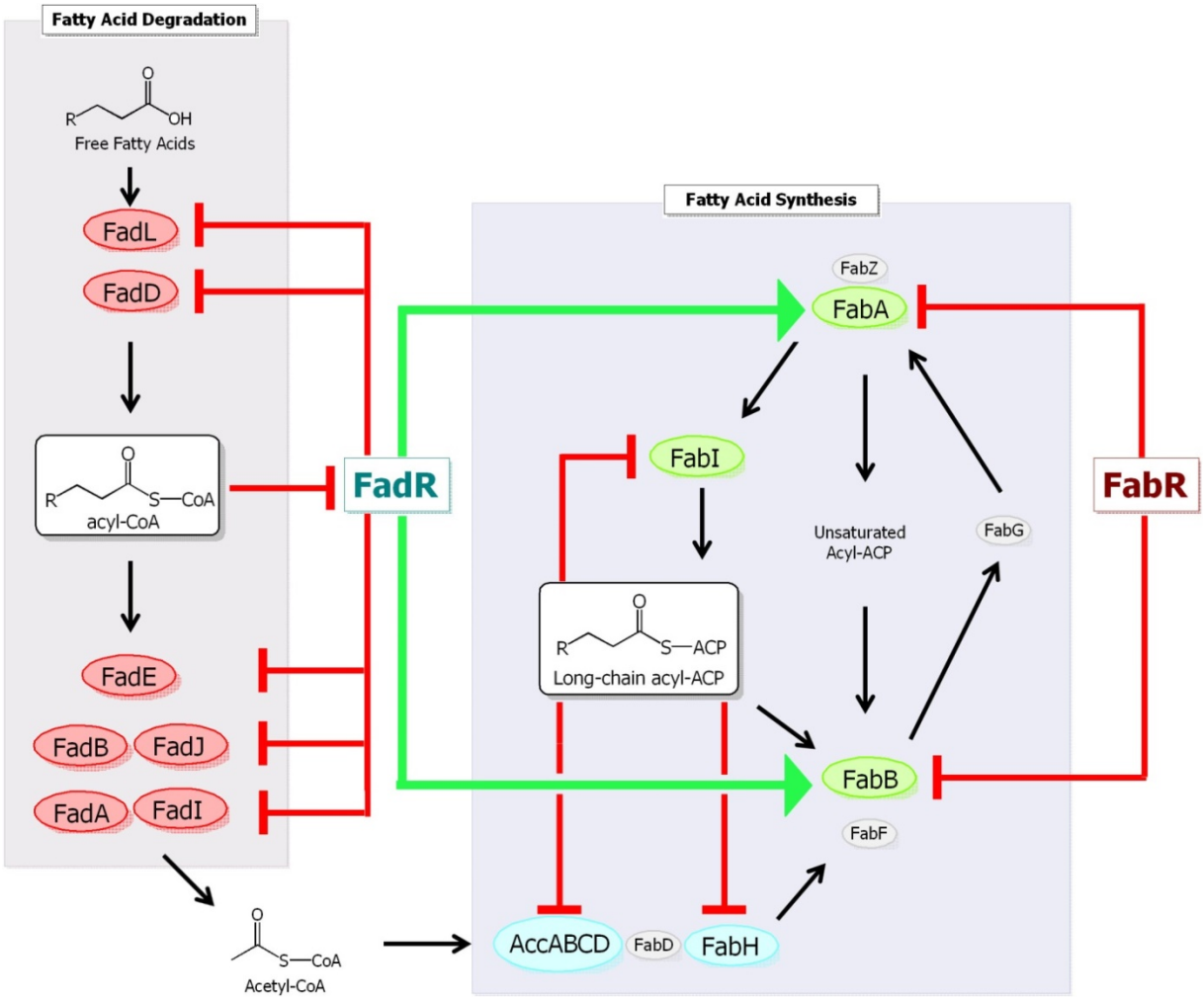

Figure 1-5. Regulation of fatty acid metabolism in *E. coli.* Accumulation of long-chain acyl ACP leads to feedback inhibition of FA biosynthetic enzymes AccABCD, FabH and FabI. Transcriptional factor FabR represses the expression of FabA and FabB. Major transcriptional factor FadR activates FabA and FabB and represses all fatty acid degradation enzymes. FadR is antagonized by long-chain acyl-CoA. For detailed description of enzymes' activities, see Figure 1-4 and Table 1-3.

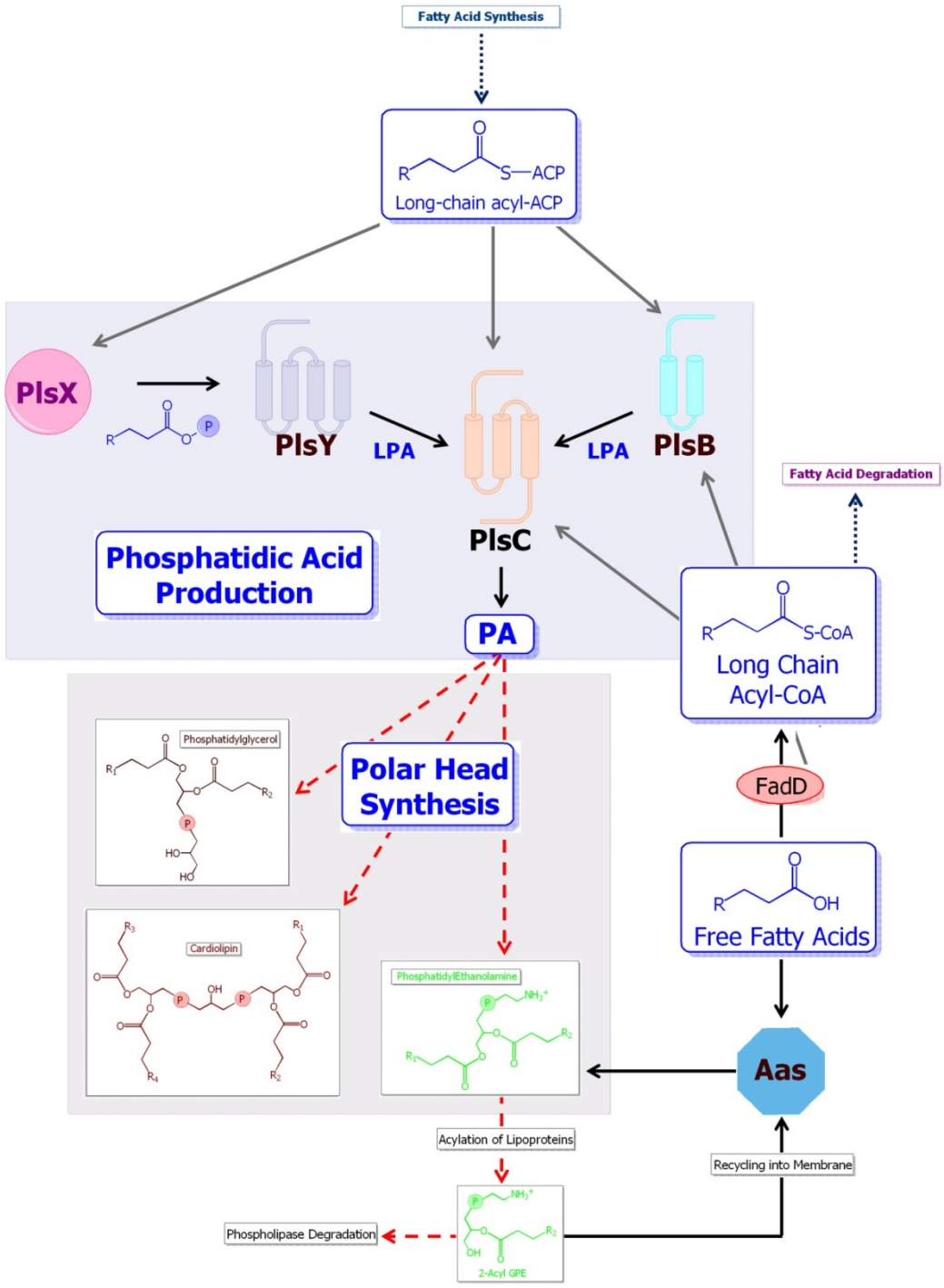

Figure 1-6. Phospholipid biosynthesis in *E. coli.* Membrane phospholipids are formed via two sequential pathways: glycerol-3-phosphate (G3P) acylation (phospatidic acid production) and modification of polar head group (polar head synthesis). Aas uses alternative route to acylate membrane 2-acyl-GPE directly using free fatty acids. The list of enzymes and their functions are given in Table 1-3.

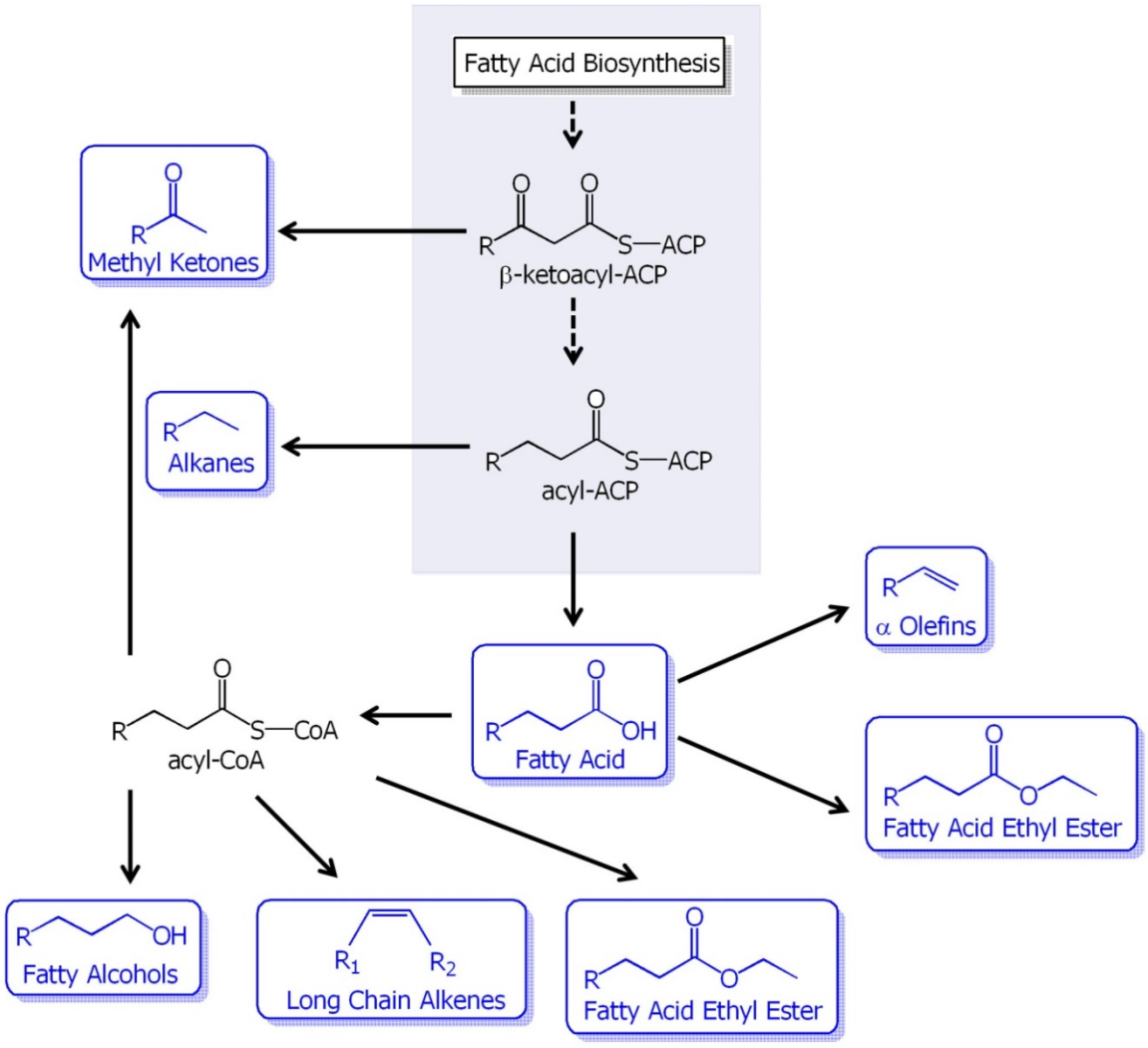

Figure 1-7. Overview of fatty-acid derived fuels and bio-products derived from *E. coli* fatty acid biosynthesis

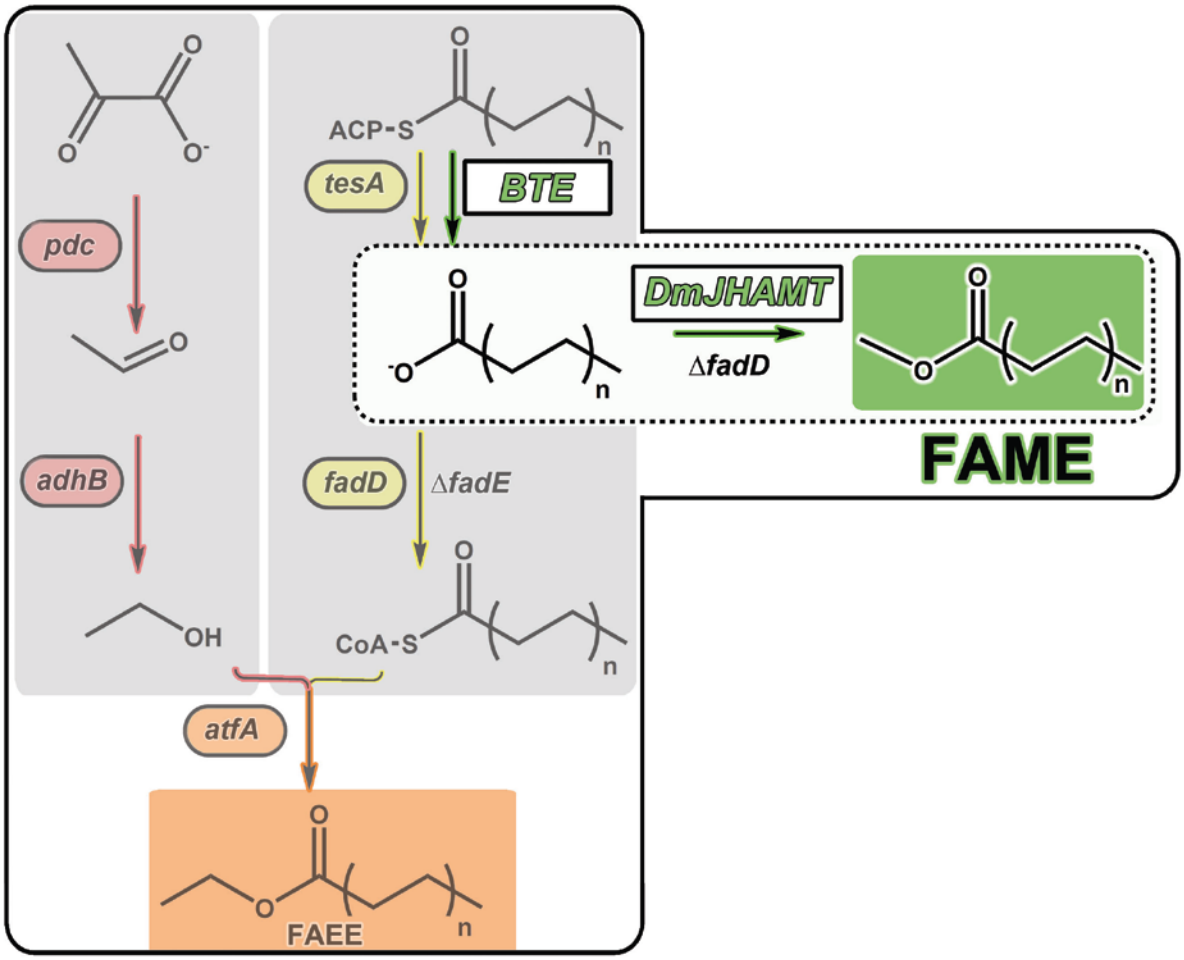

Figure 1-8. Biodiesel metabolic engineering pathways in *E. coli.* Fatty acid ethyl esters (FAEE) are produced by enriching acyl-CoA and ethanol production. Fatty acid methyl ester (FAME) production in our lab is performed in one-step conversion utilizing insect enzyme DmJHAMT.

Genetic Modification	Host Organism	Remarks	Ref.
PLANTS			
Carthamus tinctorius G3P acyltransferase (GPAT) expression	*Arabidopsis thaliana*	10–21 % increase in seed oil content	(96)
Expression of *Thunbergia alata* Δ6 ACP desaturase	*Arabidopsis thaliana*	<10 % of total fatty acid became palmitoleic acid	(97)
Expression of *Umbellularia californica* lauryl-ACP thioesterase	*Arabidopsis thaliana*	24 % of total fatty acid converted to laurate	(98)
Expression of a cytosolic variant of endogenous acetyl-CoA carboxylase (ACCase)	*Brassica napus*	5 % increase in seed oil content	(99)
Expression of ketoacyl ACP synthase III (KASIII) from *Spinacia oleracea*	*Brassica napus*	Increased palmitic acid proportion, decreased total fatty acids 5–10 %	(100)
Saccharomyces cerevisiae G3p dehydrogenase (gpd1)expression	*Brassica napus*	40 % increase in seed oil content	(101)
Saccharomyces cerevisiae sn-2 acyltransferase (SLC1-1) expression	*Brassica napus*	53–121 % increase in erucic acid content	(102)
Arabidopsis thaliana diacylglycerol acyltransferase (DGAT1) expression	*Brassica napus*	Increase in oil content and seed weight	(103)
Expression of *Umbellularia californica* lauryl-ACP thioesterase	*Brassica napus*	58 % of total fatty acid converted to laurate	(104)
Expression of *Cuphea hookeriana* FatB1 thioesterase	*Brassica napus*	Fatty acid content changed to 11 % caprylate and 27 % caprate	(105)
Co-expression of *Cuphea hookeriana* FatB1 thioesterase and ketoacyl ACP synthase (KAS)	*Brassica napus*	30–40 % increase in short-chain fatty acid content over FatB1 expression only	(106)
Co-expression of *Cuphea hookeriana* FatB1 thioesterase and lyso-phosphatidic acid acyltransferase (LPAAT) from *Cocos nucifera*	*Brassica napus*	67 % of total fatty acid content converted to laurate	(107)
Arabidopsis Acc1 (cytosolic ACC) expression	*Brassica napus*	1–2× plastid ACC + 6 % fatty acid content	(108)
Down regulation of FAD2 desaturase and FatB hydrolase	*Glycine max*	85 % increase in oleic acid levels	Revie-wed in (109)
Expression of *Coriandrum sativum* Δ4palmitoyl acyl carrier protein desaturase (ACP desaturase)	*Nicotiana tabacum*	<10 % of total fatty acid became palmitoleic acid	(110)

Continued next page

Genetic Modification	Host Organism	Remarks	Ref.
Arabidopsis thaliana acyl-CoA:diacylglycerol acyltransferase 2 (DGAT2)	Nicotiana benthamiana	20 fold increase in **triacylglyceride** (TAG) content in leaf	(111)
Arabidopsis Acc1 (cytosolic ACC) expression	*Solanum tuberosum*	Fivefold increase in TAG content	(112)
Rat ACL expression	Tobacco	16 % increase in lipid content	(113)
FUNGI			
Malic enzyme isoforms expression from *Mortierella alpina* and *Mucor circinelloides*	*Mucor circinelloides*	2.5-Fold increase in lipid accumulation	(114)
Deletion of the glycerol-3-phosphate dehydrogenase gene (GUT2)	*Y. lipolytica*	Threefold increase in lipid accumulation	(115)
Overexpression of the G3P dehydrogenase GPD1, deletions of the acyl-CoA oxidase (POX) genes	*Y. lipolytica*	Accumulation of more than 80 % of its mass as lipids	(116)
Expression of *Arabidopsis* DGAT	Yeast	Threefold increase in TAG accumulation	(117)
Overexpression of DGA1 and ACC1	*Y. lipolytica*	~11.5 fold improvement in TAG conversion yield	(118)

Table 1-1. Examples of genetic modifications used for enhanced lipid synthesis in plants and fungi (adapted from original tables published in (119) and (120) with corrected and updated references)

Ester	Cetane number	Melting point (°C)	Heat of combustion (kJ/mol)
Methyl octanoate (8:0)	39.75	−37.3	5523.76
Methyl decanoate (10:0)	51.63	−13.1	6832.24
Methyl laurate (12:0)	66.70	4.6	8138.42
Methyl myristoleate (14:1)	—	−52.2	9238.27
Methyl palmitate (16:0)	85.9	30	10 669.20
Methyl palmitoleate (16:1)	56.59	−33.9	10 547.86
Methyl stearate (18:0)	101	39	11 962.06
Methyl oleate (18:1)	56.55	−19.5	11 887.13
Methyl linoleate (18:2)	38.2	−35	11 690.10
Methyl linolenate (18:3)	22.7	−52	11 506.00
Ethyl octanoate	42.19	−44.5	6129.56
Ethyl decanoate	54.55	−19.8	7447.52

Table 1-2. Properties of common fatty esters used as a biodiesel related to ignition quality, combustion and melting point (12, 121).

Gene	Protein Function(s)	Notes
Acyl Carrier Protein Synthesis		
acpP	Acyl carrier protein (ACP)	Growing acyl chain is coupled to ACP
acpS	Acyl carrier protein synthase	
Fatty Acid Biosynthesis		
accA	Acetyl-CoA carboxyltransferase subunit	Acc complex (AccABCD) is downregulated by long chain acyl-ACP
accB	Biotin carboxy carrier protein	
accC	Biotin carboxylase	
accD	Acetyl-CoA carboxyltransferase subunit	
fabA	β-hydroxydecanoyl-ACP dehydratase	Involved in unsaturated fatty acid biosynthesis
fabB	β-ketoacyl-ACP synthase I	Involved in unsaturated fatty acid biosynthesis
fabD	Malonyl-CoA-ACP transacylase	
fabF	β-ketoacyl-ACP synthase II	
fabG	β-ketoacyl-ACP reductase	
fabH	β-ketoacyl-ACP synthase III	
fabI	Enoyl-ACP reductase I	Downregulated by long chain acyl-ACP
fabK	Enoyl-ACP reductase II	
fabZ	β-hydroxyacyl-ACP dehydrases	
Fatty Acid Degradation		
fadA	β-ketoacyl-CoA thiolases	
fadB	Enoyl-CoA hydratase I/β -hydroxyacyl-CoA dehydrogenase I and epimerase/*cis*-β-*trans*-2-enoyl-CoA isomerase	
fadD	Acyl-CoA synthase	- Couples free FAs to CoA - First step in fatty acid degradation pathway
fadE	Acyl-CoA dehydrogenase	
fadF	Enoyl-CoA hydratase II/ β -hydroxyacyl-CoA dehydrogenase II	
fadL	Long-chain fatty acid transporter	Transports C16 and longer chain fatty acids
Transcriptional Regulation		
fabR	Transcriptional regulator	Represses fabA/fabB
fadR	Transcriptional regulator	- Upregulates fabA/fabB - downregulates fatty acid degradation pathway - repressed by long chain acyl-CoA

Continued next page

Gene	Protein Function(s)	Notes
Phospholipid Biosynthesis		
aas	Acyl-ACP synthase/2-acyl-GPE acyltransferase	- Directly incorporates free fatty acids into membrane phospholipids
cfa	Cyclopropane fatty acid synthase	- Modifies acyl chains in existing membrane phospholipids
plsB	Glycerol-3-phosphate acyltransferase I	
plcC	1-acyl-n-glycerol-3-phosphate acyltransferase	
plsX	Phosphate acyltransferase	
plsY	Glycerol-3-phosphate O-acyltransferase II	
Other		
tesA	Acyl-CoA/acyl-ACP thioesterase I	- Periplasmic enzyme - Uncouples fatty acids from acyl-ACP/acyl-CoA when expressed in the cytosol

Table 1-3. Genes of Lipid Metabolism in *E. coli*

Variable	Background	Improvement of the total FFA yield (x-fold)	Ref.
tesA'/BTE/ AcTesA' overexpression	Wild-type	12-fold to 35-fold (1)	(73, 86, 122)
Δ*fadD*	Wild-type	3-fold to 10-fold (1)	(72, 73, 123)
Δ*fadE*	Wild-type	5-fold (1)	(124)
tesA'/BTE	Δ*fadD*	1.5-fold to 11.5-fold (2)	(72, 73, 86)
tesA'	Δ*fadE*	4-fold (2)	(124)
Δ*fadD*	*tesA'*	2-fold (2)	(86)
Δ*fadE*	*tesA'*	3-fold (2)	(86)
accABCD	Δ*fadD* or Δ*fadD* + *tesA' or BTE*	1.1-fold to 1.33-fold (2)	(72, 73)
fabF	*tesA'* + Δ*fadE*	15-fold diminished or 3-fold enhanced (2)	(124, 125)
fabZ	*tesA'* + Δ*fadD* or Δ*fadE*	3-fold enhanced or no change (2)	(124, 126)
fabG; *fabZ*; *fabI*	*tesA'* + Δ*fadE*	1.5-fold (2)	(124)
fabA	*tesA'* + Δ*fadE*	1.1-fold (2)	(125)
fabB	*tesA'* + Δ*fadE*	2.3-fold (2)	(125)
fabBA	*tesA'* + Δ*fadE*	1.7-fold (2)	(125)
fadR	*tesA'* + Δ*fadE*	7.4-fold (2)	(125)

Table 1-4. Genetic modifications in *E. coli* in free fatty acid production. The original table is published by Janßen and Steinbüchel in reference (74).

(1) Wild-type = 0.02 g/L (72). (2) Compared with the reference strain of the same study. For calculation of the yield improvement, the final fatty acid concentration of the background strain was compared with the same strain plus deletion or overexpression of the respective gene. Thioesterases from different organisms have been tested, but were always expressed as a cytosolic enzyme. *tesA', E. coli* Acyl-CoA/acyl-ACP thioesterase I; *BTE, Umbellularia californica* acyl–ACP thioesterase; *AcTesA', Acinetobacter baylyi* acyl–ACP thioesterase.

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Chapter 2

Improving the Tolerance of *Escherichia Coli* to Medium-Chain Fatty Acid Production

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2.1 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.

2.2 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 (1, 2). 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 (3–9). 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(5, 6, 10–15). 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 (1). The *E. coli* fatty acid metabolic pathways relevant to this work are summarized in Figure 2-1. 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 toacyl carrier protein (ACP) (3). Elongation usually ends with the production of long-chain (16-18 carbon) acyl-ACPs that are used for lipid production (16). 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 (16). FFAs

in cells are either degraded by β-oxidation pathway into acetyl-CoA or incorporated into membrane phospholipids (17).

It has been shown that the introduction of acyl-ACP thioesterases in β-oxidation deficient (*ΔfadD*) cells liberates free fatty acids from ACP and redirects lipid biosynthesis into free fatty acid production (18). Moreover, elimination of acyl-ACP feedback inhibition allows unregulated fatty acid (FA) production, increasing fatty acid yields (19, 20). 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) (4, 5, 20–22). 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 (23).

The cytosolic acyl-ACP thioesterase from *U. californica* (BTE) has been used extensively in *E. coli ΔfadD* strains for the overproduction of medium-chain FFAs (4, 18). 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 (4, 18). 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 (24). Toxicity can be reduced by including a second thioesterase that

prefers unsaturated chains, although an increase in fatty acid titers was not observed (24). Efforts to overexpress efflux transporters have shown moderate success (25, 26), but these approaches do not directly addresses the potential toxic effects of incorporation of free fatty acids into membrane phospholipids (24)

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 acitivity of 2-acyl-glycerophosphoethanolamine (2-acyl-GPE) acyltransferase/acyl-ACP synthetase (Aas)(27, 28). Here, we demonstrate that medium-chain FFAs produced as a result of thioesterase activity can be directly introduced into membrane lipids via Aas activityas shown in Fig 2-1 (27, 28). 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.

2.3 Results and Discussion

2.3.1 *Δaas* alleviates medium-chain FFA toxicity

To test whether deletion of the *aas* gene could reduce the toxicity of medium-chain FFAs, we tested the effects on growth when exogenous FFAs were added to the growth medium. *E. coli* strains SS0 (*ΔfadD*) and SS19 (*Δaas ΔfadD*) were grown to log phase and then plated on LB agar plates containing 1 mg/ml of different medium-chain fatty acids. Cell viability was assessed by counting colony forming units (CFU) after one day of incubation. As shown in Fig. 2-2A, the SS0 (*ΔfadD*) strain showed more than 50% reduction of CFU counts in the presence of exogenous medium-chain fatty acids. Saturated medium-chain fatty acids were significantly less toxic to the SS19 (*Δaas ΔfadD*) strain, with 70-72% of cells forming colonies in the presence of exogenously added C12:0 and C14:0 medium-chain fatty acids (Fig. 2-2A). The deletion of the *aas* gene had no notable effect on the toxicity of the unsaturated C14:1 FFA, however, indicating the toxicity of this FFA occurs by an independent mechanism.

We next tested whether deletion of *aas* could protect cells lacking a competent fatty acid degradation pathway (*ΔfadD*) from the toxicity of medium-chain FFAs produced endogenously. Cells expressing BTE thioesterase from a plasmid were grown and cell viability was assessed by measuring the CFUs over time. As shown in Fig. 2-2B, strain SS22 (Δ*aas* Δ*fadD* BTE) grew faster and reached a higher level of saturation than strain SS20 (Δ*fadD* BTE) alone. Moreover, after several days of growth, strain SS22 (Δ*aas* Δ*fadD* BTE) remained viable, whereas strain SS20 (Δ*fadD* BTE)

began to die off. Thus, deletion of *aas* in a Δ*fadD E. coli* strain led to better cell fitness and higher cell counts in the presence of both exogeneously added and endogenously produced medium-chain FFAs.

2.3.2 *Δaas* reduces incorporation of FFAs into membrane phospholipids

We hypothesized that the *aas* deletion reduces toxicity by eliminating incorporation of medium-chain FFAs into membrane lipids. To test this hypothesis, we analyzed the membrane lipid composition of each BTE-expressing strain. Fig. 2-3A shows the membrane lipid composition in SS20 (Δ*fadD* BTE) and SS22 (Δ*aas* Δ*fadD* BTE) strains after 2 days of growth. We saw a decrease in saturated FA content, especially of palmitic (C16:0) acid and an increase in unsaturated fatty acids, including C12:1, C14:1 and C18:1 FAs in membranes of strains expressing BTE, as previously reported (24). We also found, however, that the ratio of medium-chain fatty acids (C12 and C14) to C16 fatty acids increased dramatically in SS20 (Δ*fadD* BTE) membranes (Fig. 2-3B). The altered composition is consistent with previous reports that BTE-expressing cell lines have higher membrane stress (24, 34). Deletion of *aas* in strain SS22 (Δ*aas* Δ*fadD* BTE) partially restored normal membrane phospholipid composition by decreasing the amounts of medium-chain fatty acids relative to C16 fatty acids in the phospholipid membrane (Fig. 2-3B).

2.3.3 *aas* deletion increases fatty acid production

We speculated that the reduction in toxicity due to medium-chain FFA production might allow for increased production of FFA in *E. coli*. Growth of SS20 (Δ*fadD* BTE) in rich media yielded 0.60±0.05 grams of FFA per liter of culture, primarily consisting of

lauric (C12:0), cis-5-dodecanoic (C12:1), and cis-7-tetradecanoic (C14:1) acids (Fig. 2-4). Indeed, in strain SS22 (Δ*aas* Δ*fadD* BTE), FFA production increased about 20% to 0.69±0.12 gr L^{-1}.

2.3.4 Expression of FadR in Δ*aas* background boosts production of medium-chain FFA

FadR is a positive transcriptional regulator controlling genes of fatty acid synthesis and overexpression of FadR is known to increase FFA production in the presence of thioesterases, albeit those specific for long chain (≥16C) acyl-ACPs (35). When we attempted to express both BTE and FadR in a *ΔfadD* strain, growth essentially stopped after induction. However, deletion of the *aas* gene in strain SS23 (*ΔfadD Δaas* BTE/FadR), restored normal growth rates, reduced medium-chain FFA incorporation into membrane phospholipids and increased FFA production to 0.90±0.14 gr L^{-1} (Fig. 2-2B, Fig. 2-3 and Fig.2- 4). These results suggest that the *aas* deletion was able to alleviate the increased toxicity caused by enhanced production of medium-chain FFAs induced by FadR expression.

2.3.5 Extraction of FFAs from culture medium further increases production

The addition of a dodecane layer on top of the culture medium has been shown to increase the yield of fatty acid derivatives such as biodiesel (5). We wondered whether it might also be possible to trap fatty acids in a dodecane layer, thereby removing FFAs from the culture as well as mitigating their toxic effects. Preliminary tests indicated that a majority of fatty acids produced are trapped in the dodecane layer providing a physical sink for FFA accumulation. As shown in Fig. 2-4A, the dodecane

layer significantly enhances FFA production in all strains tested. Ultimately we could raise the total medium-chain FFA production to 1.36±0.04 gr L^{-1}.

A more detailed analysis of the free fatty acids in the dodecane layer from each strain is shown in Figure 2-4B. As expected, a majority of the free fatty acids present in each strain are C12 saturated fatty acids since BTE is specific for C12 acyl-ACP (4, 18). Notably, the introduction of FadR into a BTE–expressing double mutant (*Δaas ΔfadD*) significantly increases the production of C14:1 fatty acids. Because FadR is a transcriptional activator of FabAB expression, it is expected that C14:1 free fatty acid production would increase. The FadR-induced increase in unsaturated C14:1 fatty acid content in the SS23 (*Δaas ΔfadD* BTE/FadR) strain did not increase the incorporation of C14 fatty acids in the membrane, however (Fig. 2-3A). Thus, the *aas* deletion helps to protect membranes from changes caused by the incorporation of FFAs.

2.4 Conclusion

Overall, we achieved a 126% increase in medium-chain FFA yields (1.36±0.04 gr L^{-1} versus 0.60±0.05 gr L^{-1}) by knocking out the *aas* gene, co-expressing FadR and BTE in a *ΔfadD* background, and introducing a physical sink for FFAs at the stationary phase compared to BTE-expressing *ΔfadD* control strain. The final yield of FFAs is higher than any reported yield for medium-chain FFAs to our knowledge. The challenges facing fatty acid derived biofuel production are still considerable, but our results add another tool for improving metabolic efficiency of free fatty acid producing *E. coli* strains.

2.5 Materials and Methods

2.5.1 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.

2.5.2 Vector construction

The 897-bp portion of *U. californica BTE (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 (29) 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 (30).

2.5.3. *E. coli* strains

E. coli strains JW 1794-1 (*Δfad::kan*) and JW2804-1 (*Δaas::kan*) were used as the starting point for strain construction (31). A λDE3 prophage was integrated into these two cell lines to yield strain SS0 (*Δfad*) and SS18 (*Δaas*) 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 (32). 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 (*Δaas ΔfadD)* (32). The BTE-pBAD/HisA plasmid was transformed into SS0 and SS19 to yield SS20 (*ΔfadD* BTE) and SS22 (*Δaas ΔfadD* BTE). SS21 (*ΔfadD* BTE/FadR) and SS23 (*Δaas ΔfadD* BTE/FadR) strains were made by transforming FadR-AG1 plasmid into SS20 and SS22 strains respectively.

2.5.4. Cell growth

Terrific broth with 1.5 % glycerol (TB) was used for cell growth, supplemented with ampicillin (50 μg ml^{-1}), chloramphenicol (34 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) as appropriate. Single colonies of each strain were inoculated into 5 mL of TB and cultured overnight at 37 °C. The seed cultures were then used to inoculate 30 mL TB medium with appropriate antibiotics in 150 mL culture tube and cultivated at 25°C in a rotary shaker (210 rpm). BTE and FadR expression was induced at OD_{600} of 0.1 with 50 μM Isopropyl-β-D-thio-galactoside and/or 0.002 % L-arabinose. For samples with

dodecane overlay, 6 ml of dodecane were added after 24 hours of growth. Cultures were grown for 2 days prior to FA analysis as described below.

2.5.5. Cell viability measurements

SS0 and SS19 cell lines were grown from overnight culture to an OD_{600} of 0.4, serially diluted in TB medium and plated on LB agar plates containing 1 mg/ml of various FAs. For time-course measurements of cell viability, samples from various time points were collected, serially diluted and plated on LB agar plates containing appropriate antibiotics. Individual colonies were counted after overnight incubation at 37°C. Every measurement was done either in duplicate or triplicate.

2.5.6. Membrane lipid composition

Membrane-lipid acyl composition was analyzed as previously described (33) with a few modifications. 10 mL of cell cultures were collected after 48 hours of growth and were initially incubated at room temperature for 10 minutes in 4 ml of 10 mM Tris-HCl pH 7.5 and 5 mL of 100 mg/ml BSA solution to remove any FFAs in the solution. Cells were lysed by sonication and collected by centrifugation at 3800 x g for 5 min. Membrane bound acyl groups were methylated to produce fatty acid methyl esters (FAMEs), extracted and analyzed as described below (33). The optical density of the cultures was assessed at 600 nm (Abs_{600}) on a SpectraMax M5 plate reader and the membrane lipid composition was normalized to optical density measurements. For lipid ratio calculations, C12 and C14-derived fatty acids were added and divided by C16-derived fatty acids present in membrane phospholipids.

2.5.7. Metabolite extraction and identification

FFAs and FAMEs were extracted by addition of 6 mL of a 2:1 chloroform/methanol mixture to 5 ml of culture or 1 mL of dodecane layer spiked with 0.15 mg/L methyl heptadecanoate as an internal control. Quantification of FFAs was conducted by GC-FID in HP 5890 Series II gas chromatograph equipped with HP-Innowax Column (0.32mm x 30 m x 0.25µm, Agilent). All samples were analyzed using the following parameters: inject: 1 µl; inlet at 250°C with split ratio 1:1; carrier gas: helium; flow: 5 ml/min; oven temperature: initial temperature of 160°C, hold 3 min; 255°C at 5°C/min; hold 3 min; inlet temp: 270°C, detector temp: 330°C. The amount of FFA was determined by comparison to a standard curve of various FAs and methyl heptadecanoate concentrations. The identity of metabolites was confirmed by GC/MS using an Agilent 6890-5975 equipped with HP-Innowax Column (0.32mm x 30 m x 0.25µm, Agilent).

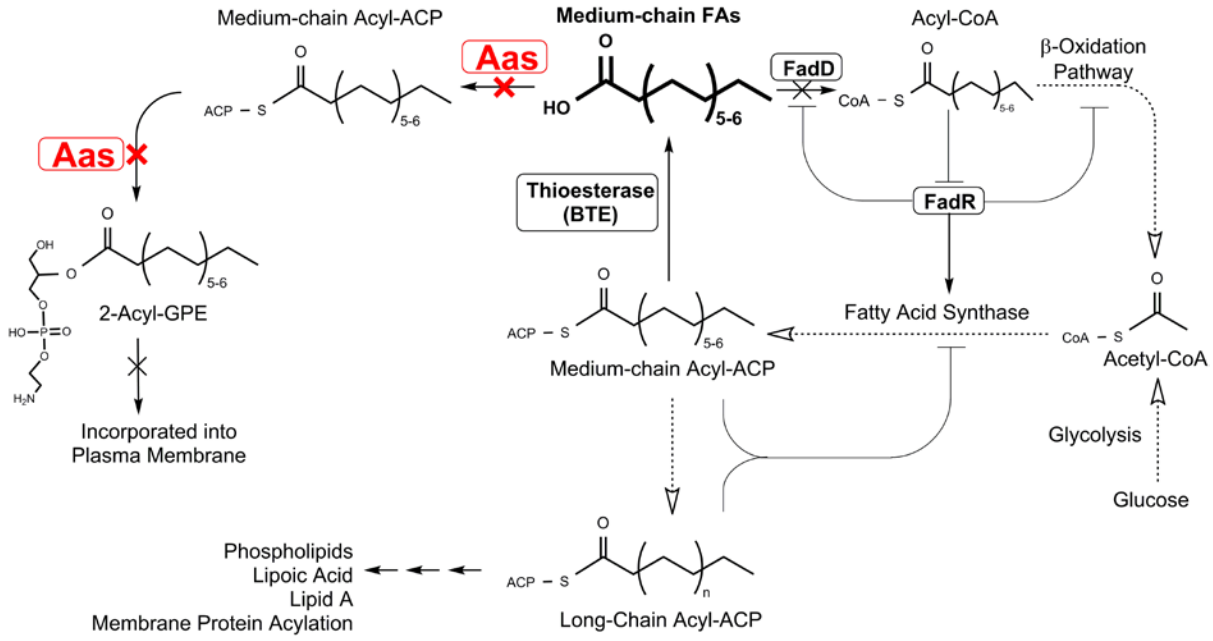

Figure 2-1. Pathways employed for medium-chain free fatty acid (FFA) overproduction in *E. coli*. Expression of *U. californica* acyl-ACP thioesterase BTE in β-oxidation- and phospholipid synthesis-deficient (*ΔfadD Δaas*) *E. coli* strain leads to increased accumulation of medium-chain FFAs in cells and culture. FA, fatty acid; ACP, acyl-carrying protein; GPE, glycerophosphoethanolamine; FadD, acyl-CoA synthetase; Aas, 2-Acyl-GPE acyltransferase/acyl-ACP synthase; BTE, *U. californica* acyl-ACP thioesterase.

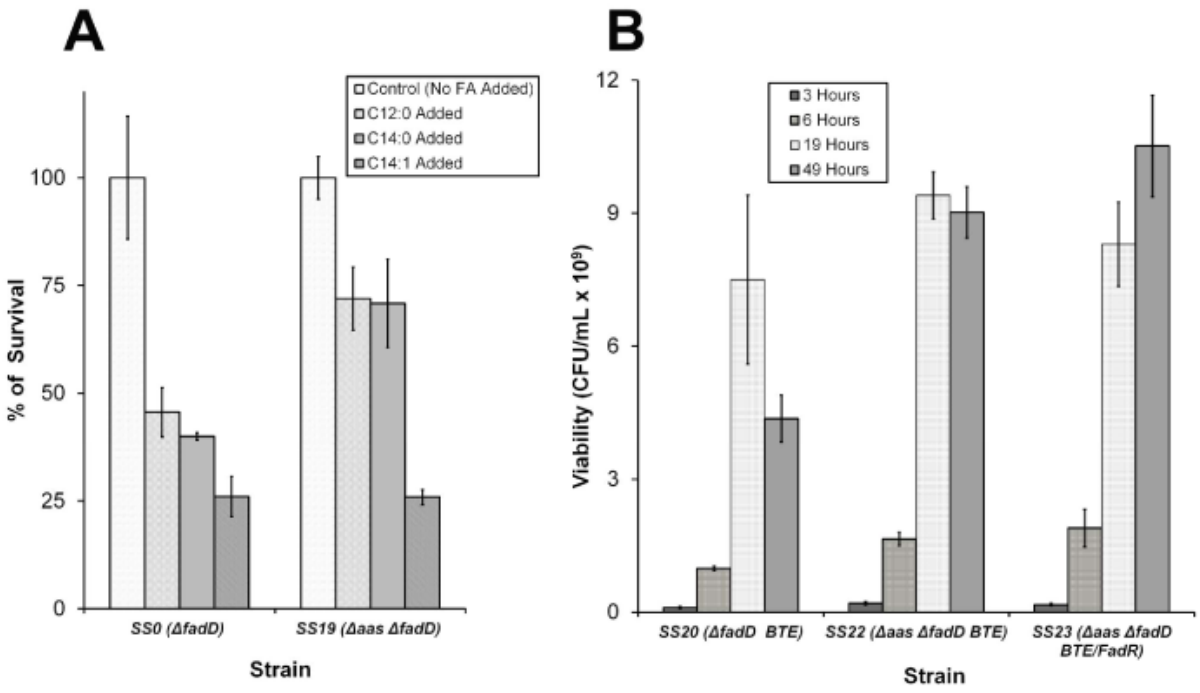

Figure 2-2. *aas* gene deletion decreases medium-chain FFA toxicity. (A) Log phase SS0 (*ΔfadD*) and SS19 (*Δaas ΔfadD*) strains were plated on LB plates containing 1mg/mL of medium chain FAs and the CFU counts were measured after 1 day of growth. (B) Viable cell counts of BTE-expressing *E. coli* strains cultured over a 2-day period. The OD_{600} of strains after 2 days of growth were as follows: SS0 11.4±0.6, SS19 11.6±0.4, SS20 12.1±0.3, SS22 13.1±0.4, SS23 11.7±0.4

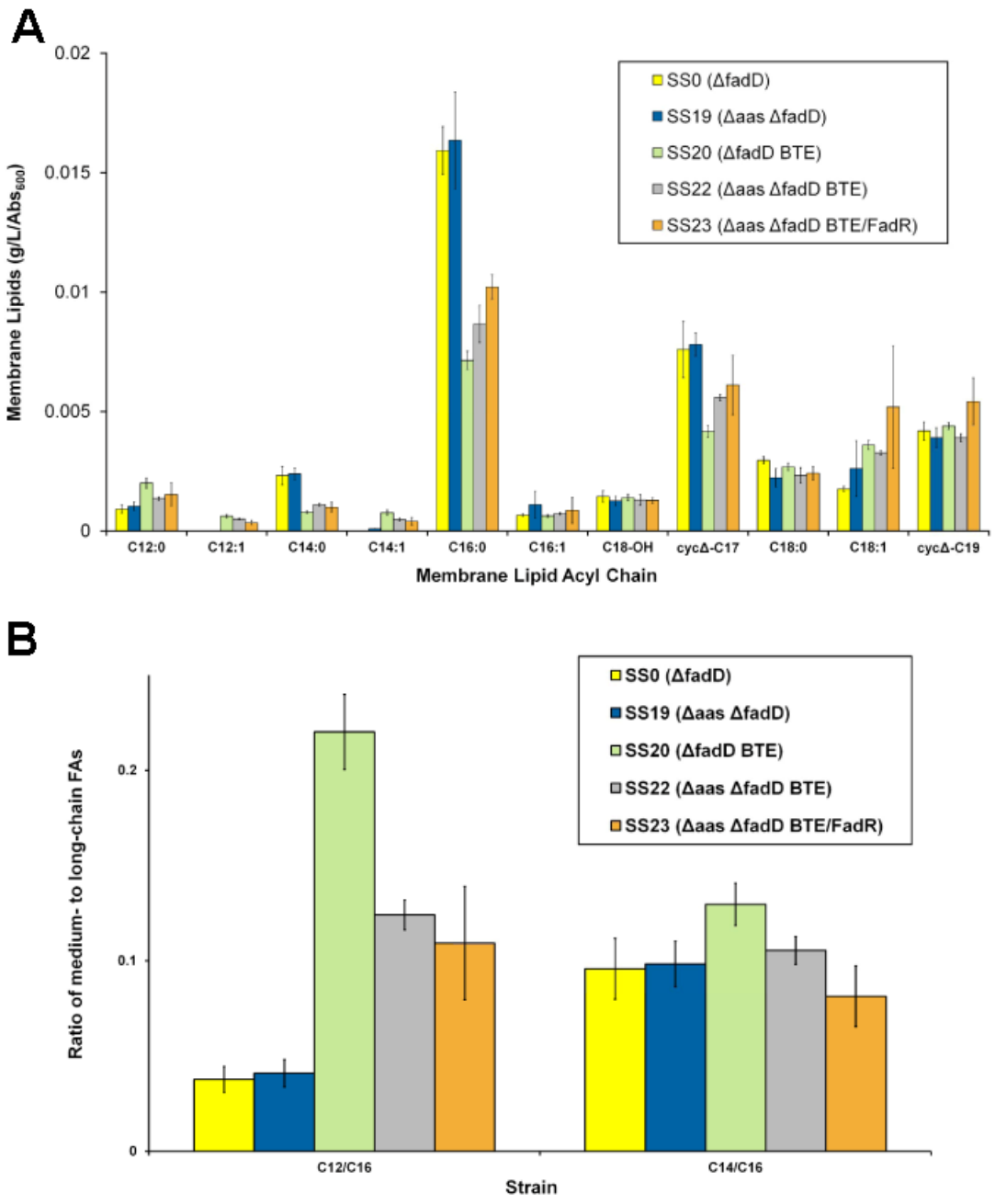

Figure 2-3. *aas* gene deletion prevents incorporation of medium-chain FAs into membrane phospholipids. (A) Normalized membrane lipid composition of *E. coli* strains. (B) Ratio of membrane-bound medium-chain fatty acids to C16-derived fatty acids, the major components of *E. coli* membrane lipids, in strains used in this study.

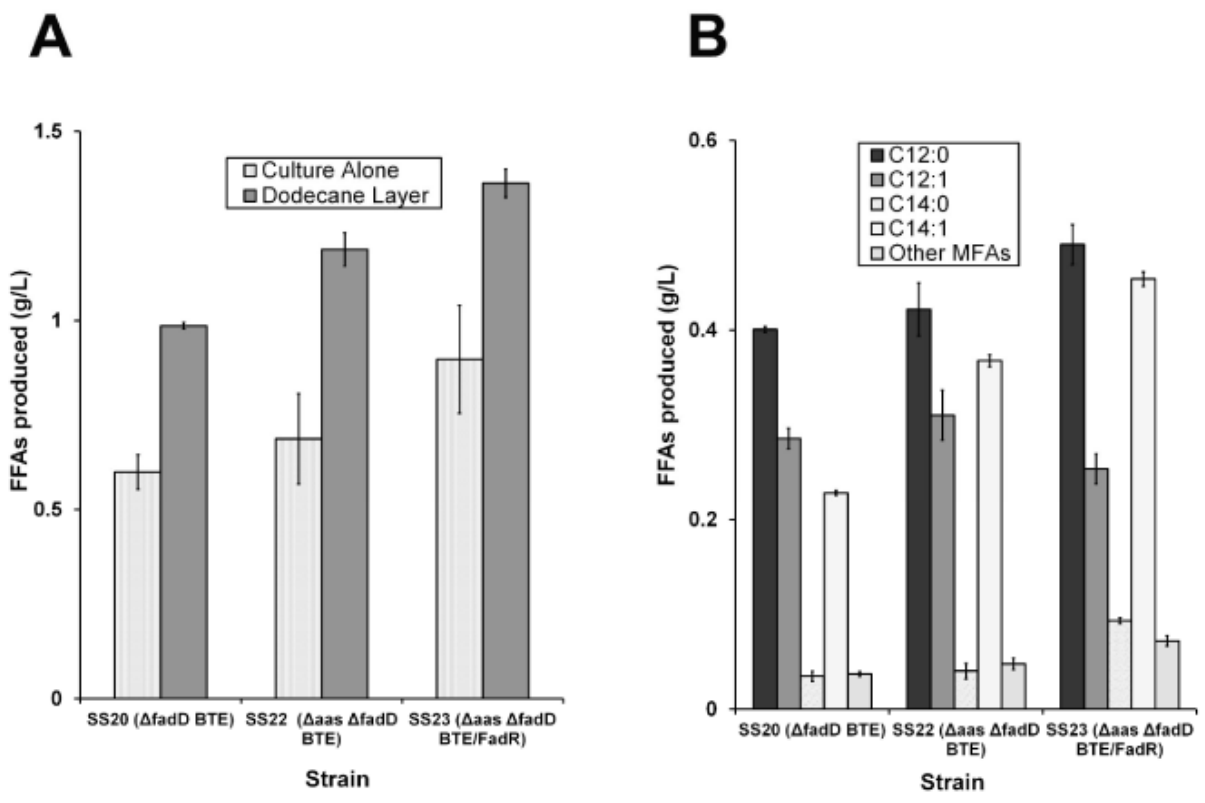

Figure 2-4. Medium-chain free fatty acid production by recombinant *E. coli* strains. (A) Total FAs yields of engineered strains after 2 days of incubation with and without dodecane overlay. Total FA recovered: SS20, 0.60±0.05 gr L^{-1} culture alone, 0.99±0.01 gr L^{-1} with dodecane overlay; SS22, 0.69±0.12 gr L^{-1} culture alone, 1.19±0.04 gr L^{-1} with dodecane overlay; SS23, 0.90±0.14 gr L^{-1} culture alone, 1.36±0.04 gr L^{-1} with dodecane overlay. (B) The distribution of medium-chain FAs in the dodecane layer.

Strains	Genotype	Ref.
BW 25113	*F-, λ,- Δ(araD-araB)567, Δ(rhaD-rhaB)568, hsdR514, ΔlacZ4787(::rrnB-3), rph-1*	(31)
JW 2804-1	BW25113 *Δaas761::Kan*	(31)
JW 1794-1	BW25113 *ΔfadD730::Kan*	(31)
SS0	JW 1794-1 λ(DE3)	This study
SS19	JW 1794-1 λ(DE3) *Δaas761*	This study
SS20	SS0 BTE-pBAD/HisA/p15A	This study
SS22	SS19 BTE-pBAD/HisA/p15A	This study
SS23	SS22 fadR-pCA24N	This study
Plasmids	**Description**	**Ref**
pBAD/HisA	*ara*BAD promoter, pBR322 origin, Amp^R	Invitrogen
pBAD/HisA/p15A	*ara*BAD promoter, p15A origin, Cm^R	(29)
BTE-pBAD/p15A	BTE cloned into pBAD/HisA/p15A	This study
BTE-pBAD/HisA	BTE cloned into pBAD/HisA	This study
fadR-AG1	fadR cloned into pCA24N (-gfp) from ASKA (-) collection	(30)
Primers	**Sequence**	
Xho1-pBAD/p15A-BTE 5'Forward	5'- GGGTTTTCTCGAGGAGTGGAAGCCGAAGCCGAA-3'	
NsiI-pBAD/p15A-BTE 3' Stop	5'- GGGTTTTATGCATTTACACCCTCGGTTCTGCGGGTA-3'	
FadD-P1-pKD4-Primer1	5'- GACGACGAACACGCATTTTAGAGGTGAAGAAGTGTAGGCTGGAGCTGCTTC-3'	
FadD-P2 pKD4-Primer1	5'-GATTAACCGGCGTCTGACGACTGACTTAACGCATGGGAATTAGCCATGGTCC-3'	
FadD-P1-pKD4-Primer2	5'-TATCATTTGGGGTTGCGATGACGACGAACACGCATTTTAG-3'	
FadD-P2-pKD4-Primer2	5'-GCGTCAAAAAAA ACGCCGGATTAACCGGCGTCTGACGACTG-3'	

Table 2-1. Bacterial strains, plasmids and primers used in Chapter 2.

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Chapter 3:
Production of FAME Biodiesel in *E. coli* by Direct Methylation with an Insect Enzyme

*This chapter has been submitted for review/publication

3.1 Abstract

Most biodiesel currently in use consists of fatty acid methyl esters (FAMEs) produced by transesterification of plant oils with methanol. To reduce competition with food supplies, it would be desirable to directly produce biodiesel in microorganisms. Medium chain FAMEs also have potential alternative uses as fragrances and biodegradable pesticides. To date, the most effective pathway for the production of biodiesel in bacteria yields fatty acid ethyl esters (FAEEs) at up to ~1.5 g/L. A much simpler route to biodiesel produces FAMEs by direct *S*-adenosyl-*L*-methionine (SAM) dependent methylation of free fatty acids, but FAME production by this route has been limited to only ~16 mg/L. We hypothesized that the low production of FAMEs was due to the lack of a suitable, broad spectrum methyltransferase (MT). We searched for possible candidate MTs and found a potential enzyme, *Drosophila melanogaster* Juvenile Hormone Acid *O*-Methyltransferase (*Dm*JHAMT) that is capable of methylating a variety of medium chain fatty acids in addition to its endogenous substrate. By introducing DmJHAMT in *E. coli* engineered to produce medium chain fatty acids and overproduce SAM, we obtain medium chain FAMEs at titers of 0.56 g/L, a 35-fold increase over titers previously achieved. Although considerable improvements will be needed for viable bacterial production of FAMEs and FAEEs for biofuels, it may be easier to optimize and transport the FAME production pathway to other microorganisms because it involves fewer enzymes.

3.2 Introduction

Increasing energy consumption and the detrimental environmental impact of fossil fuels has led to increased interest in developing sustainable and renewable sources of energy. The utilization of engineered microorganisms to produce chemicals from renewable biomass is a promising alternative to petroleum-derived fuels and chemicals. Fatty acid derived compounds are particularly promising because fatty acid derivatives are highly reduced, aliphatic compounds with high energy density that are not miscible with water (1). Notably, their similarity to diesel fuels makes them compatible with existing infrastructure. As a result, many strategies have been developed to overproduce microbial fatty acids and then further convert the fatty acids into biofuels such as alkanes, fatty alcohols, and fatty acid methyl or ethyl esters (2–8).

Microbial production of fatty acid methyl or ethyl esters (FAME, FAEE respectively) is of particular interest because FAME and FAEE are the main component of biodiesel currently in use. Typically, biodiesel is made by transesterification of triacylglyceride oils extracted from renewable biomass with short chain alcohols (e.g methanol or ethanol) using an alkaline catalyst (9). However, the use of feedstock oils needed for biodiesel production is a major obstacle for the broader use of biodiesel due to lack of arable land and competition with the food supply. Therefore, a possible alternative to plant and animal oil-based biodiesel is the direct biosynthetic production of biodiesel in metabolically engineered microorganisms (reviewed in 10).

Steinbuchel and co-workers were the first to develop a pathway for the production of FAEE biodiesel in *E. coli,* and their approach was further developed by the

Keasling group for increased yields of FAEE and fatty alcohols (2, 4, 7). To produce FAEEs, two orthogonal pathways were introduced that simultaneously generated ethanol and fatty acyl-CoA. In the last step, ethanol and fatty acyl-CoA were then condensed to the FAEE using a wax ester synthase (Fig. 3-1) (2, 7). After optimization, titers as high as 1.5 g of long chain FAEEs per liter of culture were obtained.

As a more straightforward approach to produce biodiesel in microorganisms, The Lykidis group attempted to produce FAMEs in *E. coli* through direct methylation of fatty acids by the action of an *S*-adenosyl-*L*-methionine (SAM) dependent bacterial methyltransferase from *M. marinum* (3). The Lykidis pathway has the advantage of being much simpler than the FAEE production pathway by using endogenous compounds (SAM and fatty acids) produced in *E. coli.* Nevertheless, the FAME titers obtained were nearly two orders of magnitude lower than the FAEE titers (16 mg/L). The low level of FAME production is likely due to the high specificity of the methyltransferase employed, which prefers rare fatty acids containing a 3-hydroxy group (3).

We hypothesized that if we could find a broad range fatty acid methyl transferase, perhaps we could improve upon the Lykidis approach for FAME production. Here we show that *Drosophila melanogaster* Juvenile Hormone Acid *O*-Methyltransferase (*Dm*JHAMT) has broad specificity for medium chain free fatty acids and can be used to produce FAMEs in *E. coli*. By introducing *Dm*JHAMT to engineered *E. coli* strains tolerant to high levels of endogenously produced medium chain fatty acids, we observed *in vivo* FAME production (11). Enriching the endogenous SAM pool

further increased FAME production with final titers showing a 35-fold increase from titers previously reported (3).

3.3 Results and Discussion

3.3.1 DmJHAMT is robustly expressed and broadly active on medium chain fatty acids.

Several SAM-dependent juvenile hormone acid methyltransferases have been previously found to methylate insect sesquiterpenoid hormones that play central roles in the development and growth of these organisms (12–14). *D. melanogaster* Juvenile hormone acid *O*-methyltransferases (DmJHAMT) appeared to be a promising enzyme for FAME production because it showed some activity with unbranched saturated medium and long-chain fatty acids such as lauric and palmitic acids, and could be expressed in *E. coli* (13).

We expressed the *Dm*JHAMT protein recombinantly in *E. coli* to investigate its substrate specificity. *Dm*JHAMT expression was robust in *E. coli* (up to 200 mg of protein per liter of culture) with no apparent effect on cell growth. As shown in Fig. 3-2A, *Dm*JHAMT is active on fatty acids ranging in size from C12 to C16. We saw no activity with shorter chain, C8:0 and C10:0, fatty acids, however. *Dm*JHAMT is most active on medium chain fatty acids, showing the highest activity with lauric acid (C12:0) among the substrates tested. The kinetic parameters with lauric acid were K_m =59 μM and k_{cat} = 0.15 min^{-1} (Fig. 3-2B). Although the low k_{cat} indicates that the enzyme is not very efficient with these non-natural substrates, the high expression and broad

specificity of *Dm*JHAMT suggested that it might be effective at producing FAMEs, particularly medium chain FAMEs, in *E. coli*.

3.3.2 *Dm*JHAMT produces FAME biodiesel in *E. coli.*

E. coli has been utilized as a host for over-production of free fatty acids (FFAs) of various lengths and properties (2, 5, 15–19). Introduction of bacterial and plant acyl-ACP thioesterases in a *ΔfadD* mutant *E. coli* strain defective in fatty acid degradation allows overproduction of free fatty acids by liberating fatty acids attached to acyl-carrying proteins (ACPs), while simultaneously removing acyl-ACP mediated regulation of the fatty acid biosynthesis pathway, effectively redirecting lipid biosynthesis into free fatty acid production (20). Since *Dm*JHAMT is most active with medium chain FFAs, we opted to utilize the acyl-ACP thioesterase from *Umbellularia californica* (BTE), which has a preference for medium chain fatty acids and leads to accumulation of lauric acid when expressed in an *E. coli* Δ*fadD* strain (5, 21).

We first prepared strain SS3B (Δ*fadD* DmJHAMT/BTE) bearing a Δ*fadD* mutation and expressing *Dm*JHAMT and BTE from plasmids (Table 3-1). In strain SS3B we observed relatively high production of medium-chain fatty acid methyl esters (Fig 3-3). The initial titer of FAMEs was 240 ± 15 mg/L of culture, already a dramatic improvement over prior results (3). Since medium-chain FAMEs are somewhat volatile, we added dodecane as an organic overlay at the stationary phase to trap the FAMEs, which further increased the titer of FAMEs to 312 mg/L of culture (2). The majority of FAMEs

contained 12-carbon acyl chains (73 %), mostly unsaturated C12 methyl laurate (Fig. 3-3).

While a high level of FAMEs were produced, we were surprised to find that strain SS3B (Δ*fadD Dm*JHAMT/BTE) still produced a considerable amount of free fatty acids (FFA) that were not methylated (860 ± 20 mg/L of culture). Indeed a majority of the FFAs generated in strain SS3B were not converted into FAMEs. We therefore sought to increase the conversion of the excess FFAs to FAMEs.

3.3.3 Increasing SAM levels

We hypothesized that SAM levels may be a limiting factor in the conversion of FFAs to FAMEs. To test the possibility that low SAM levels during stationary phase contributed to low FAME production, we lysed strain SS3B after two days of growth and supplemented the lysate with exogenous SAM. We observed increases in all FAME species indicating that the *Dm*JHAMT remained active but the SAM levels may be limiting (Fig. 3-4A).

To increase SAM production, we introduced the methionine synthase protein from rat liver, Mat1A, into *E. coli* strain SS3B (22, 23). Mat1A was shown to dramatically increase the intracellular SAM pool in *E. coli* cells (23). Mat1A expression from a plasmid in strain SS4B (Δ*fadD* DmJHAMT/BTE/Mat1A) increased SAM levels 8.5-fold (from 73.3 to 636.8 nmoles per gram of cells) compared to the control strain SS3B (Δ*fadD* DmJHAMT/BTE) after two days of growth. Nevertheless, we found that Mat1A overexpression actually decreased both FFA and FAME titers. Mat1A overexpression may have unexpected deleterious effect on FAME production such as

toxicity, competition for expression with other proteins, high metabolic ATP demand for SAM production, or the complication of harboring three different plasmids, among other possibilities (24).

To simplify the system and reduce the expression of Mat1A, we incorporated a single copy of the Mat1A gene into the *E. coli* genome under the control of a T7 promoter. When Mat1A was incorporated into the genome, we observed ~3-fold increase of SAM levels in strain SS33 (ΔfadD::Mat1A BTE/DmJHMAT) compared to SS3B (ΔfadD BTE/DmJHMAT) after two days of growth (192 ± 3 nmoles SAM per gram of cells compared to 71 ± 19 nmoles per gram of cells in control the strain, Fig. 3-4B). More importantly, we saw a 19% increase in FAME production, from 312 mg/L to 370 mg/L in cells carrying Mat1A in the genome. In addition, this strain had a higher ratio of SAM to *S*-adenosylhomocysteine (SAH), a by-product SAM-dependent methylation and a potent inhibitor of methyltransferases (Fig. 3-4C). While the levels of SAH were similar in these strains, the levels of SAM showed considerable increases in Mat1A-carrying strains after 48 hours of growth. (25). Overall, Mat1A expression improved the production of FAMEs.

3.3.4 *Δaas* further increases the FAMEs yields in *E. coli*

Short and medium chain FFAs are toxic to *E. coli* cells, most likely due to membrane stress (26–29). It is possible that the production of excess FFAs in our strains is deleterious to FAME production. We recently reported that the deletion of the

aas gene can alleviate medium chain FFA toxicity (11). The Aas protein acts in a FFA salvage pathway that can incorporate exogenous medium chain FFAs directly into the lipid bilayer with deleterious consequences. We therefore attempted to reduce the toxicity of the medium chain fatty acids by deleting the *aas* gene in strain SS3B to produce strain SS34 (Δaas ΔfadD::Mat1A BTE/DmJHMAT). Indeed strain SS34 showed an almost 50% increase in the FAME production (559 mg/L of culture) compared to the same strain with a wild type *aas* gene SS33 (ΔfadD::Mat1A BTE/DmJHMAT). Overall, strain SS34 (Δaas ΔfadD::Mat1A BTE/DmJHMAT) overlaid with a dodecane layer showed 137% increase from the starting strain SS3B (ΔfadD BTE/DmJHMAT) (Fig. 3-5A).

3.3.5 Spectrum of FAMEs produced

The best FAME producing stain, SS34, generated a broad spectrum of medium chain FAMEs. While the saturated aliphatic FAME methyl laurate (C12:0) was the most abundant, we also observed 3-hydroxy C12 (C12-OH), cyclopropanedodecanoic (cyclo-C13) acid, unsaturated straight chain (C12:1, C12:2 and C14:1) and saturated C14 (C14:0) fatty acid methyl esters (Fig. 3-5B). While saturated (C12:0, C14:0), unsaturated (C12:1, C14:1) and hydroxylated C12 fatty acids have been previously observed in BTE-expressing *E. coli* strains, the cyclopropanedodecanoic (cyclo-C13) and unsaturated C12:2 fatty acids are unusual products of bacterial fatty acid biosynthesis (5, 11, 21). Bacterial phospholipid acyl chains are regularly modified as a response to temperature and increasing organic solvent concentrations and these fatty

acids may be a by-product of phospholipase turn-over activity on membrane-disruptive 2-acyl glycerophosphoethanolamine (2-acyl-GPE), especially in *Δaas ΔfadD* strains that lack both 2-acyl-GPE acyltransferase and fatty acid degradation pathways (30–32). The broad specificity of *Dm*JHAMT and availability of SAM in the SS34 strain allows the conversion of these fatty acids to their methyl ester derivatives.

3.4 Conclusion

We have engineered a strain of *E. coli* that produces FAMEs at levels comparable to the best FAEE production strain and at levels that are more than an order of magnitude greater than FAME titers previously attained (2, 3). Essential developments were the identification of a FFA methyltransferase that has broad specificity for fatty acids and could be overproduced in *E. coli* and deletion of the *aas* gene to reduce incorporation of toxic medium chain-length FFAs into the bilayer. The fact that more than half of the FFAs generated (1.45 g of FFAs vs 559 g FAME) are not methylated in the highest producing strain (SS34) suggests that there is still considerable room for improvement. We do not know why FFAs are not fully converted to FAMEs, but presumably some portion of the FFAs is sequestered from *Dm*JHMAT (e.g in the membrane) because there is still sufficient SAM (211 nmoles per g of cells) and active enzyme present after several days, yet FFAs remain. It is also possible that the FFAs that escape from the cell are not reabsorbed efficiently due to the Δ*fadD* mutation, the normal route for uptake of long-chain free fatty acids. Poor re-uptake may be particularly problematic for medium chain FFAs even with *fadD* intact (33), so

perhaps better results will be obtained with strains that can produce longer chain (C16 and C18) FFAs on which *Dm*JHAMT is active. Screening of other methyltransferases or the engineering or methyltranferases for broader specificity should allow for still further improvements and diversification of the FAME products. While heat of combustion and cetane number, a measure of diesel ignition quality, are similar in these molecules, increasing the proportion of unsaturated acyl groups in this biofuel mix adds beneficial properties such as lower cloud point and lower freezing temperature (34, 35). Current studies are underway to increase branched and unsaturated fatty acid yields in *E. coli* that could potentially be used in our one-step biodiesel production method (36–38).

3.5 Materials and Methods

3.5.1 Materials

T4 DNA ligase and restriction endonucleases were obtained from New England Biolabs. DNA Polymerase Mastermix was from Denville Scientific. Ni-NTA Superflow, 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 and IDT. Gene sequencing and gene synthesis were performed by Genewiz. Assembly master mix (AMM) used for cloning was prepared as outlined in (39). All DNA and protein concentrations were measured with Thermo Fisher Scientific Nanodrop 1000 Spectrophotometer.

Colorimetric enzyme-coupled assays were performed in 96-well plates and measured with Molecular Devices SpectraMax M5 microplate reader.

3.5.2 Plasmid construction

An 897-bp portion of *U. californica BTE (BTE)* gene lacking the thylakoid targeting sequence was prepared synthetically by Genewiz and the synthetic BTE gene was amplified by polymerase chain reaction (PCR) using primers XhoI-pBAD/p15A-BTE and NciI-pBAD/p15A-BTE (See Table I for primer sequences). The PCR product was digested with XhoI and NciI and ligated into XhoI/PstI
digested plasmid pBAD/HisA/p15A (40) to produce BTE-pBAD/p15A. A plasmid containing the mature DmJHAMT gene was obtained from the Drosophila Genomics Resource Center at Indiana University. The DmJHAMT gene was amplified (primers DmJHAMT NdeI Forward and DmJHAMT XhoI End) and cloned into pET-28a(+) (Novagen). The resulting plasmid was then digested with NcoI and XhoI to excise the DmJHAMT gene and cloned into pET-15b (Novagen) to swap the his-tag to the C-terminus. The 5'-methylthioadenosine/S-adenosylhomocysteinenucleosidase (MTAN) gene from *E. coli* was amplified (primers 5' E. coli SAH NdeI, 3' E. coli SAH SacI) and cloned into NdeI/SacI digested pET-28a(+). The S-ribosylhomocysteinase (LuxS) gene from *Bacillus subtilis* was amplified (primers 5' B. sub LuxS NheI and 3' B. sub LuxS EagI) and cloned into NheI/EagI digested pET-28a(+). The resulting plasmid was used to clone the LuxS gene into pET-22b(+) with NdeI/Bpu1102I digestion and ligation. Rat liver S-adenosyl-L-methionine synthetase (Mat1A) was amplified with 5' KpnI Mat1A Forward and 3' XhoI Mat1A End primers from rat liver cDNA and cloned into pCDF-1b plasmid using XhoI and KpnI restriction sties

To knock-in genes into the *E. coli* genome, we generated a plasmid, called pCDF-Cat, that contains a chloramphenicol resistance gene (*cat*) flanked by the FLP recognition target (FRT) sites (41). To do that, the *cat* gene cassette containing FRT sites was amplified from the pKD3 plasmid using pKD3-Cat-pCDF-Forw and pKD3-Cat-pCDF-Rev primers and the pCDF-1B plasmid was amplified using pCDF 385-Rev and pCDF 425-Forw primers. The resulting PCR fragments were ligated together using the AMM kit so that the *cat* gene was inserted into the 385-425-base pair region of the pCDF-1B plasmid (39). The Mat1A gene was then cloned into pCDF-Cat the same way as Mat1A was inserted in pCDF-1B vector and the resulting Mat1A-pCDF-Cat plasmid was used as template to amplify the Mat1A-FRT-cat-FRT fragment that was inserted into the *E. coli* genome (see below). The primers used for the cloning are listed in Table 2. All cloned genes were verified by sequencing.

3.5.3 *E. coli* strains construction

E. coli strains K-12 MG1655, JW 1794-1 (*Δfad::kan*) and JW2804-1 (*Δaas::kan*) were used as the starting point for strain construction (42). The SS19 strain carrying a double *Δfad Δaas* deletion was generated as previously described (11). A Mat1A knock-in PCR fragment was generated by using the primers FadD KO – pCDF1 P1-1 and FadD KO – CAT P2-1 for amplification on the Mat1A-pCDF-Cat plasmid and further extended in a second round of PCR using the primers FadD-P1-pKD4-Primer2 and FadD-P2-pKD4-Primer2. This PCR fragment was employed to insert Mat1A into the fadD gene region of the K-12 MG1655 and JW2804-1 strains.

Subsequent *cat* gene removal was performed according to protocol from Datsenko and Wanner (41). A λDE3 prophage was integrated and BTE-pBAD/p15A and DmJHAMT-pET15b plasmids were transformed into each strain. The list of strains and their genotypes are in Table 3-2.

3.5.4 Protein Expression and Purification

DmJHAMT was expressed from DmJHAMT-pET-28a(+) plasmid in a BL21(DE3) strain and purified using Ni-NTA affinity chromatography. 2 mL of an overnight starter culture was transferred to 2 L of LB media containing 50 µg/ml kanamycin and incubated at 37°C. When the OD_{600} of the culture reached 0.6, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM and incubated at 18°C with shaking for 24 h. The bacterial cells were harvested by centrifugation, resuspended in 100 mL of 50 mM Tris-HCl pH 7.5, 0.3 M KCl, 5% glycerol and 1 mg/ml lysozyme, incubated at 4°C for 30 min with gentle shaking and stored at –80°C. The frozen bacterial pellet was thawed, lysed by sonication and centrifuged (15,000 rpm, Sorvall SS34 Rotor 40 min, 4°C). The lysate supernatant was incubated with 10 mL of Ni-NTA superflow resin at 4°C for 30 minutes. The beads were washed 4 times with 10 mL of 50 mM Tris-HCl pH 7.5, 0.3 M KCl, 5% glycerol, 5 mM imidazole and the protein was eluted with 50 mM Tris-HCl pH 7.5, 0.3 M KCl, 5% glycerol, 250 mM imidazole. MTAN and LuxS were purified as described previously (43, 44). All proteins were dialyzed into 50 mM Tris-HCl pH 8.0, 20% glycerol, 0.2 M KCl solution and stored at –80°C.

3.5.5 Enzyme Assays

DmJHAMT activity was measured using an enzyme-coupled colorimetric assay for SAM-dependent methyltransferases (45). Enzyme assay solutions contained 20 µM LuxS, 10 µM MTAN, 500 µM SAM and various concentrations of fatty acid substrates in degassed 50 mM potassium phosphate [pH 8.0] at a final volume of 500 µL. 3 µM DmJHAMT was used for the k_{cat}/K_m calculations of lauric acid (Fig. 3-2B) and 10 µM DmJHAMT was employed for reaction rate calculation with other fatty acids (Fig. 3-2A). Fatty acids were added from stock solutions prepared at 1 mg/mL in 100% ethanol. C16 palmitic acid was insoluble at concentrations >50 µM so comparison of the reaction rates for different fatty acid substrates was performed at 40 µM fatty acid. 5 - 200 µM range of lauric acid was used to obtain k_{cat}/K_m values for this specific substrate (46). All components of the assay except DmJHAMT were combined and mixed and the reaction was initiated by addition of 10 µM DmJHAMT at 30°C. 60 µL of the reaction mixture was taken out at various time points and quenched by adding 180 µL of 260 µM DTNB, 0.5 mM EDTA, 6 M GuHCl (room temperature) and the absorbance at 412 nm read after a 20 min incubation. A standard curve for SAH consumption by MTAN/LuxS was developed and used to quantify FAME production in the enzyme-coupled assays. All experiments were done in duplicate or triplicate and standard deviation from the mean value was used for error bars.

3.5.6 SAM/SAH Assay

The SAM/SAH measurement protocol from cultures was modified from (47). *E. coli* cells were pelleted by centrifugation (6000 rpm, Eppendorf F45-30-11 rotor, 5 min,

4°C) and the wet cell weight was measured for each sample. The cells were resuspended and lysed by vortexing in 5% trifluoroacetic acid at 4°C for 2 min (4 ml/g of wet cell weight). The cell lysate was clarified by centrifugation (13000 rpm, Eppendorf F45-30-11 rotor, 5 min, 4°C) and 120 µl of supernatant was analyzed by high performance liquid chromatography (HPLC) as described in (47). The concentrations were calculated using SAM and SAH standards of known concentrations. All measurements were performed in triplicate.

3.5.7 Cell growth

Most of the strains did not reach saturation point in minimal media supplemented with either glycerol or glucose and terrific broth (TB) with 1.5 % glycerol was used for cell growth and subsequent analysis. The media was supplemented with ampicillin (50 µg ml^{-1}), chloramphenicol (34 µg ml^{-1}) or kanamycin (50 µg ml^{-1}) as appropriate. 5 mL of TB-glycerol were inoculated from a single colony and cultured overnight at 37 °C. The seed cultures were then used to inoculate 30 mL TB-glycerol medium with appropriate antibiotics in 150 mL culture tubes and cultivated at 25°C in a rotary shaker (210 rpm). BTE and DmJHAMT expression was induced at an OD_{600} of 0.1 with 50 µM Isopropyl-β-D-thio-galactoside and/or 0.002 % L-arabinose. For samples with a dodecane overlay, 6 ml of dodecane were added after 24 hours of growth. Cultures were grown for an additional 1 day prior to FA/FAME analysis as described below.

3.5.8 Metabolite extraction and identification

FFAs and FAMEs were extracted by addition of 6 mL of a 2:1 chloroform/methanol mixture (spiked with 0.15 mg/L of either methyl tridecanoate or

methyl heptadecanoate as an internal control) to 5 ml of culture. For consistency in data analysis, 1 mL of dodecane layer was similarly treated with 6 mL of a 2:1 chloroform/methanol mixture before gas chromatography (GC) analysis. Quantification of FAs/FAMEs was conducted by GC-FID using an HP 5890 Series II gas chromatograph equipped with an HP-Innowax Column (0.32mm x 30 m x 0.25µm, Agilent). All samples were analyzed using the following parameters: inject: 1 µl; inlet temperature 250°C with split ratio 1:1; carrier gas: helium; flow: 5 ml/min; oven temperature: initial temperature of 160°C, hold 3 min; gradient to 255°C at 5°C/min; hold 3 min; inlet temp: 270°C, detector temp: 330°C. The amount of FAs/FAMEs was determined by comparison to a standard curve of various FAs and FAMEs and methyl tridecanoate or methyl heptadecanoate concentrations. To identify all FA/FAME products, GC/mass spectrometry analysis was additionally performed using an Agilent 6890-5975 equipped with HP-Innowax Column (0.32mm x 30 m x 0.25µm, Agilent). The identity of metabolites was confirmed by comparing with known standards (C12:0, C12:1, C14:0 and C14:1 FAs and FAMEs) and to the NIST Mass Spectral Database (cyclo-13, hydroxylated-C12 and C12:2 FAMEs).

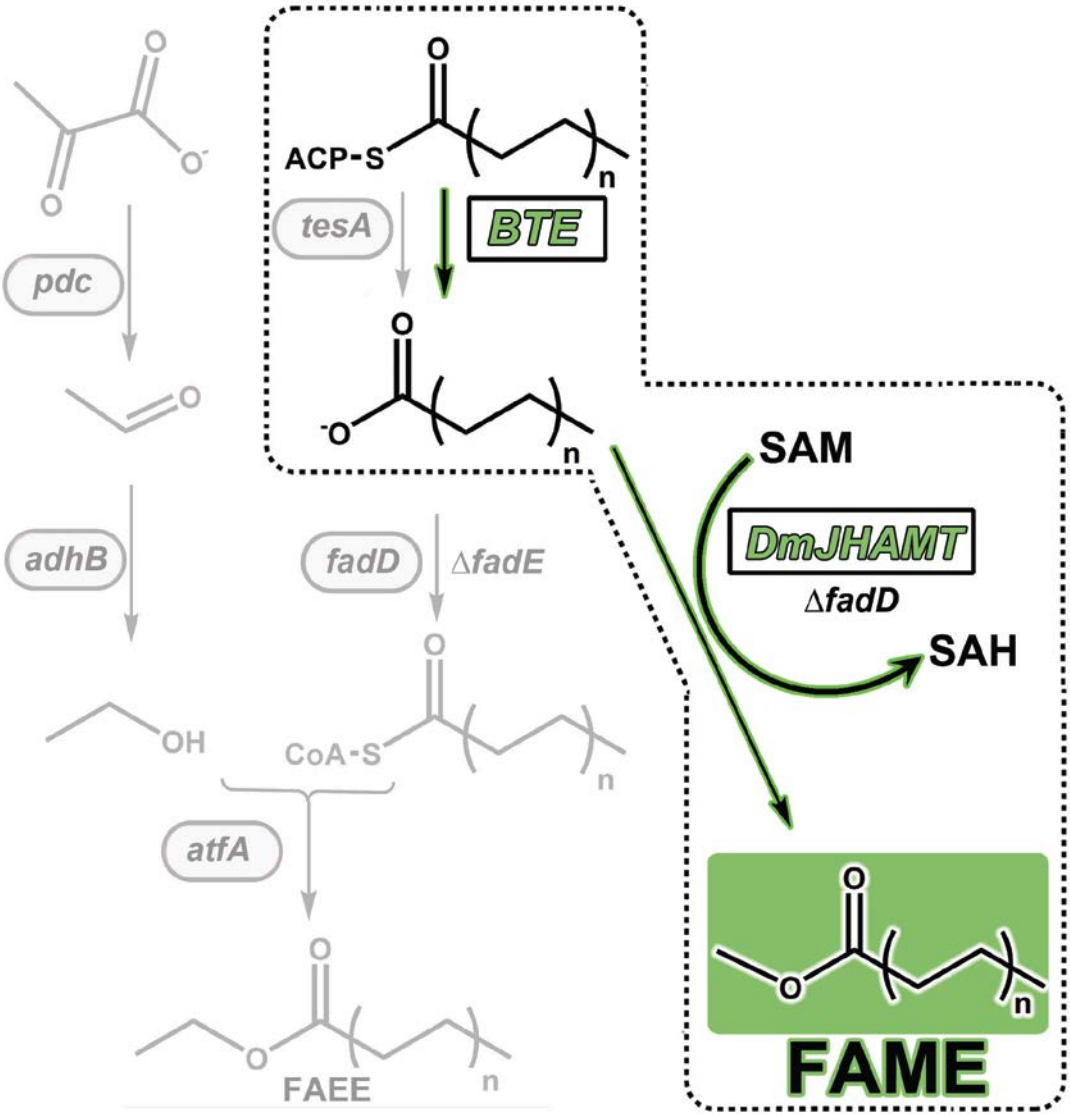

Figure 3-1. Two-step FAME pathway. The FAEE microdiesel pathway previously implemented in *E. coli* is outlined in the left panel in gray (3, 7). In the FAEE pathway, ethanol is produced by the introduction of pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) from *Zymomonas mobilus*. Acyl-Coenzyme A (CoA) thioesters are simultaneously produced by diverting fatty acid biosynthesis with the expression of various thioesterases (TES) and a yeast acyl-CoA ligase (ACL). Wax ester synthase

(atfA) condenses the ethanol and acyl-CoA to make FAEE. In this study (dashed box, black/green), biodiesel is produced by the introduction of *Dm*JHAMT into a fatty acid producing strain. The medium-chain free fatty acid (FFA) pool is enriched in by expressing *U.californica* acyl-ACP thioesterase (BTE) in a β-oxidation-and phospholipid synthesis-deficient *E. coli* strain (*ΔfadD Δaas*). Medium-chain FFAs are then methylated in *S*-adenosyl-*L*-methionine (SAM)-dependent manner to fatty acid methyl esters (FAMEs) by DmJHAMT. Internal SAM levels are upregulated by introducing *S*-adenosylmethionine synthetase gene Mat1A from rat liver into *E. coli* genome. FA, fatty acid; ACP, acyl-carrier protein; GPE, glycerophosphoethanolamine; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosylhomocysteine; FadD, acyl-CoA synthetase; Aas,2-Acyl-GPE acyltransferase/acyl-ACPsynthase; BTE, *U.californica* acyl-ACP thioesterase; Mat1A, rat *S*-adenosylmethionine synthetase; *Dm*JHAMT, *D. melanogaster* Juvenile hormone acid *O*-methyltransferase.

Fig. 3-2A

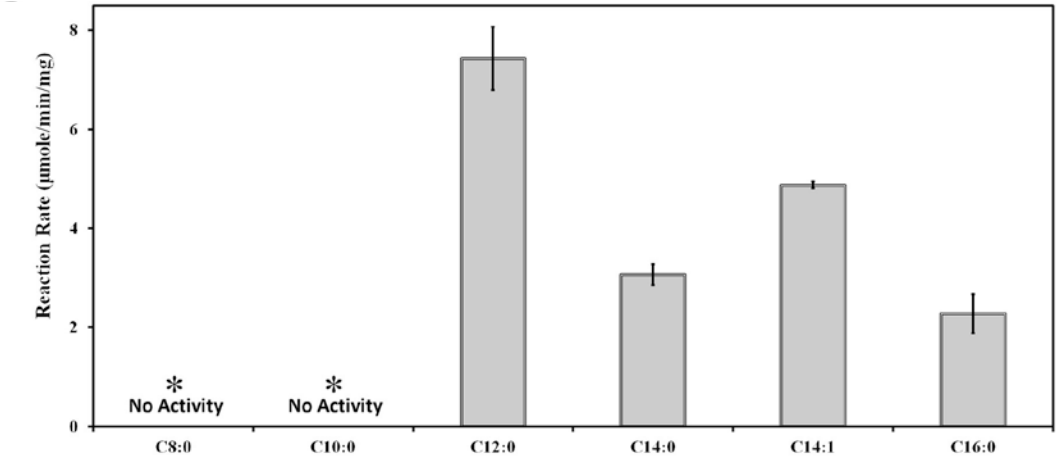

Fig. 3-2B

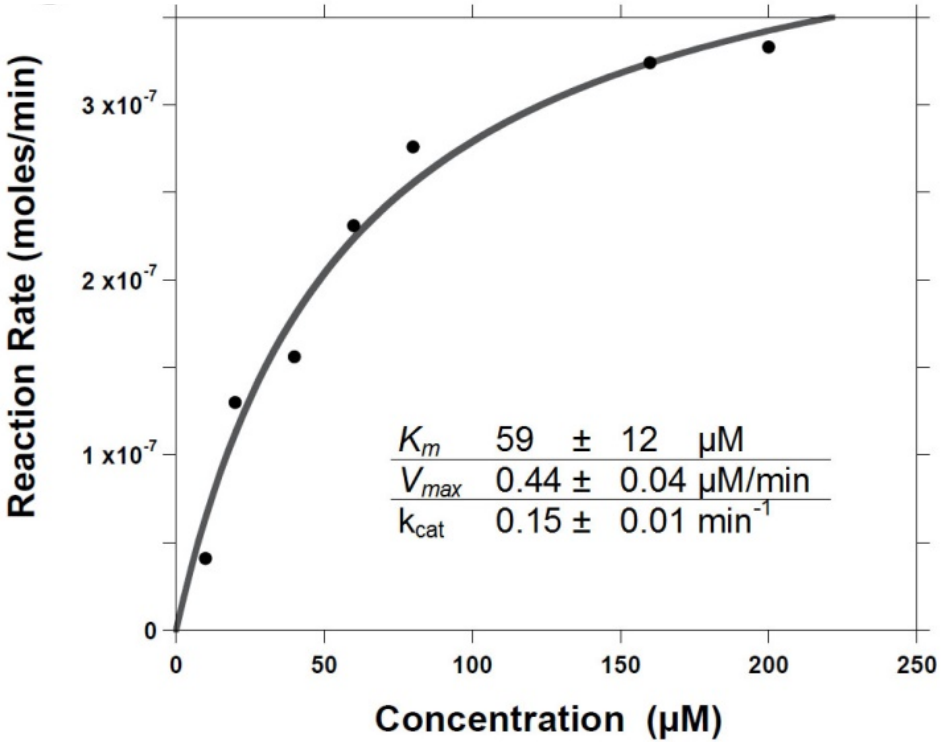

Figure 3-2. *Dm*JHAMT methylates a broad spectrum of fatty acids. (A) *Dm*JHAMT methylates straight and branched medium-chain fatty acids *in vitro*. The reaction rates observed using 40 μM of each fatty acid are shown. (B) Kinetic analysis of recombinant DmJHAMT activity with lauric acid.

Fig. 3-3

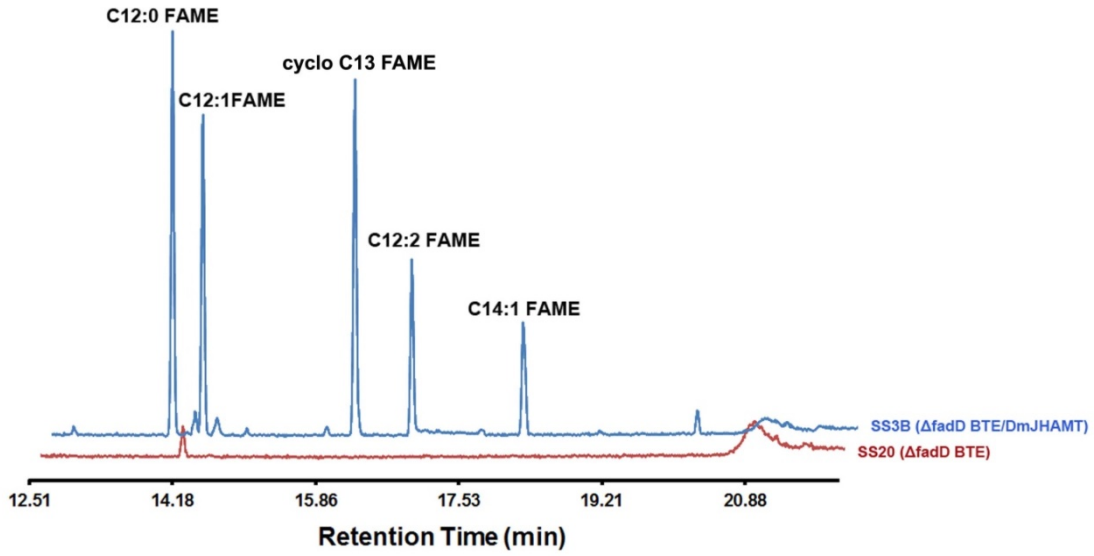

Figure 3-3. Production of FAMEs in *E. coli.* GC-MS analysis of FAMEs produced in strain SS3B (Δ*fadD* BTE/*Dm*JHAMT; blue), compared to the identical strain, SS20 (Δ*fadD* BTE; red) that does not produce *Dm*JHAMT. Most of the FAMEs produced in SS3B contain 12-carbon acyl groups with majority being methyl laurate. C12:0, methyl laurate; C12:1, cis-5-dodecenoic acid methyl ester; cyclo C12, cyclopropanedodecanoic acid methyl ester; C12:2, 3,6-dodecadienoic acid methyl ester; C14:1, methyl myristoleate.

Fig. 3-4A

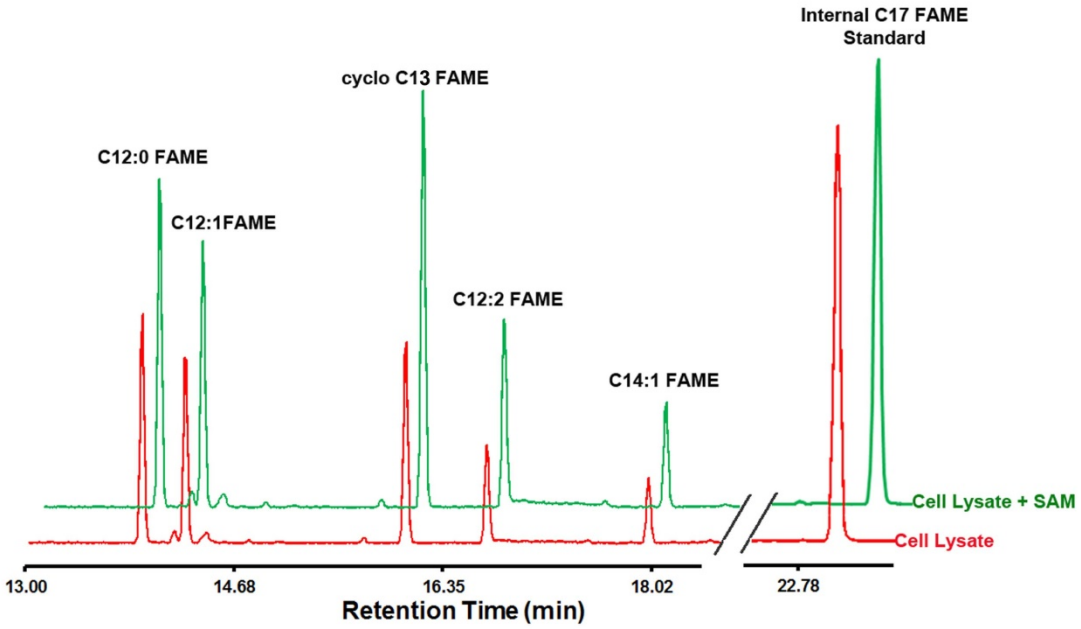

Fig. 3-4B

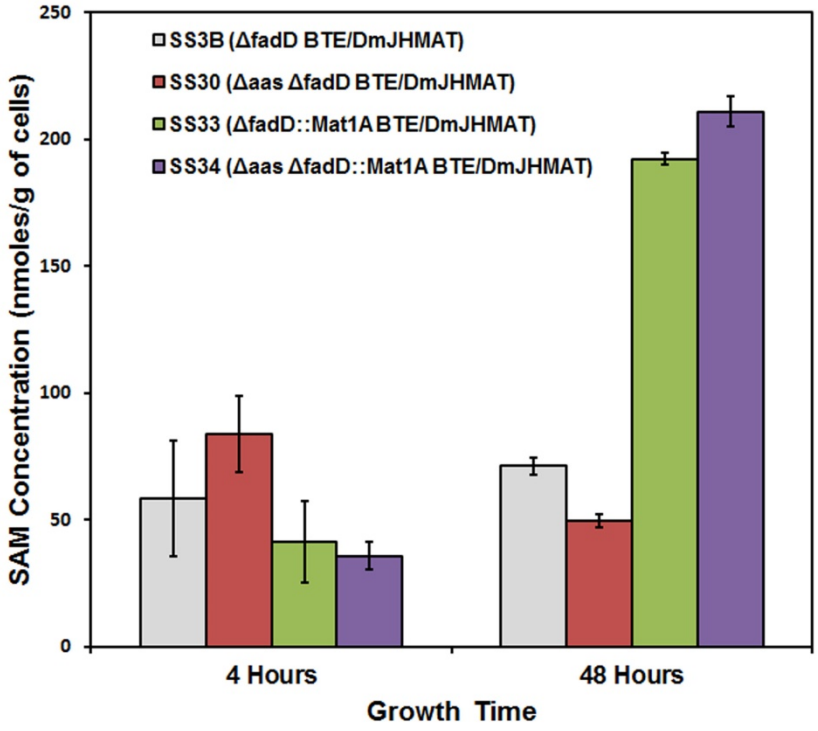

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Fig. 3-4C

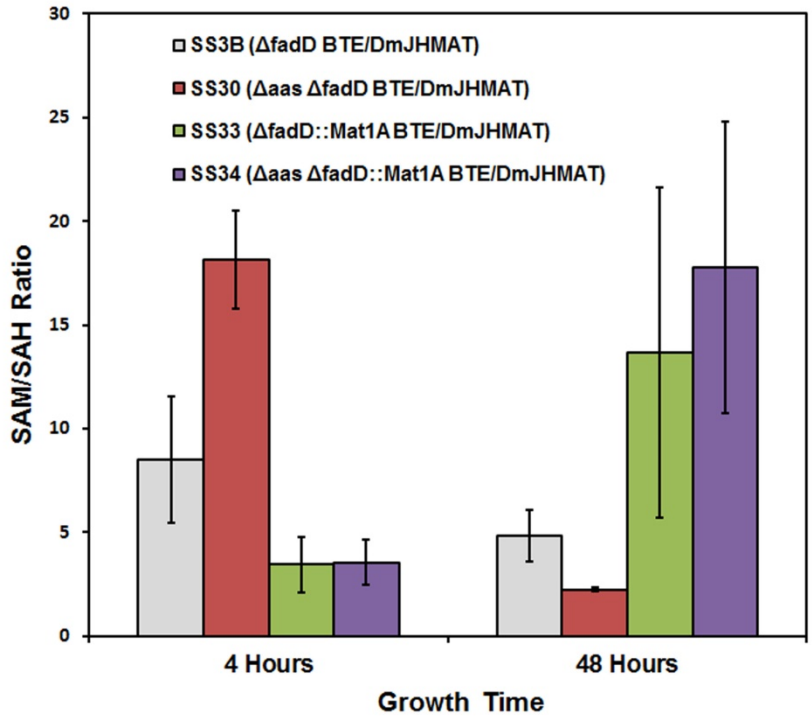

Figure 3-4. SAM is a limiting factor for *in vivo* production of FAMEs. (A) FAME-producing strain, SS3B (ΔfadD BTE/*Dm*JHAMT; red), was lysed and exogenous 500 μM SAM was added to the lysate and incubated for 40 min at 25 ^{o}C (green). An internal standard (methyl heptadecanoate) was added to cell culture prior to lysis. (B) SAM levels in strains producing FAMEs. (C) SAM/SAH ratios in the FAME-producing cell line. While the SAM/SAH ratios are normally higher in the exponential phase of cell growth, this ratio persists in the cells that have Mat1A gene incorporated into their genome.

Fig. 3-5A

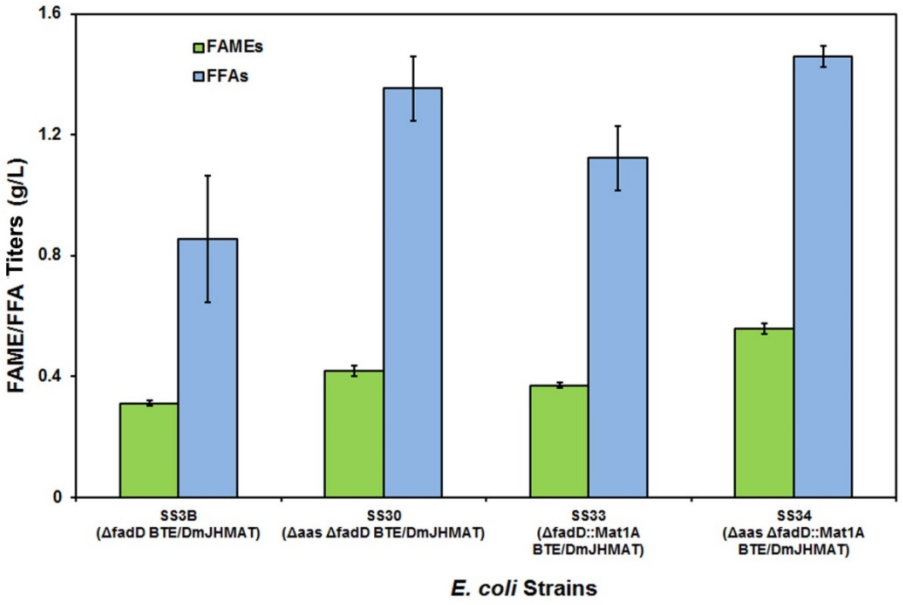

Fig. 3-5B

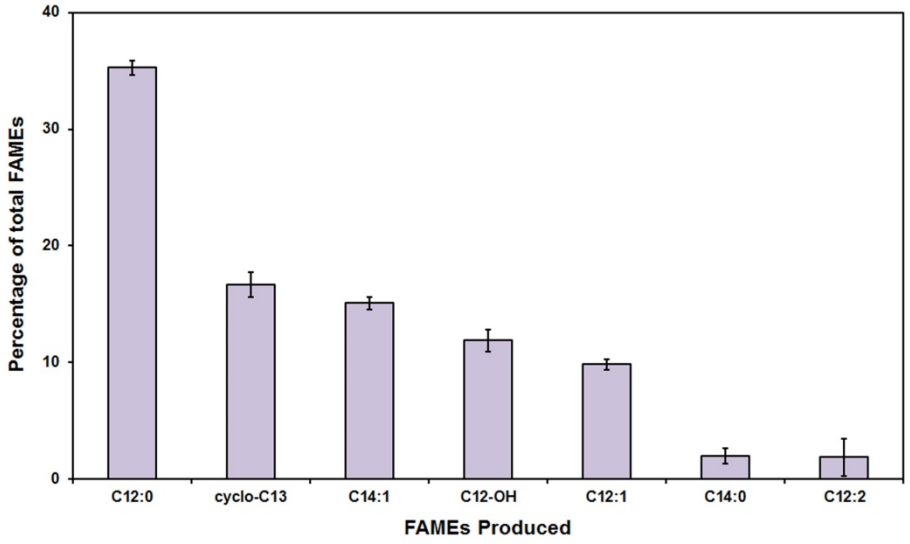

Figure 3-5. *Δaas* and Mat1A increase FAME production in medium chain FA-producing *E. coli* cells. (A) Total FAME and FFA yields in engineered *E. coli* strains overlayed with organic layer. (B) The distribution of different FAMEs produced in SS34 (Δaas ΔfadD::Mat1A BTE/DmJHMAT) strain.

Strain	Description/Genotype	Source
K-12 MG1655	F-, *λ,ˉ Δ(araD-araB)567, Δ(rhaD-rhaB)568, hsdR514, ΔlacZ4787(::rrnB-3), rph-1,*	CGSC, New Haven, CT
JW 1794-1	K-12 MG1655 *ΔfadD730::kan*	CGSC, New Haven, CT
JW2804-1	K-12 MG1655 *Δaas-761::kan*	CGSC, New Haven, CT
SS00	JW 1794-1 λ(DE3)	(11)
SS3B	SS00 pDmJHAMT-pET15b, pUcBTE-pBAD/p15A (*ΔfadD* BTE/DmJHAMT)	This study
SS4B	SS3B pMat1A-pCDF1B (*ΔfadD* BTE/DmJHAMT/Mat1A)	This study
SS19	JW2804-1 *ΔfadD730::kan (Δaas ΔfadD)* λ(DE3)	(11)
SS30	SS19 pDmJHAMT-pET15b, pUcBTE-pBAD/p15A (*Δaas ΔfadD* BTE/DmJHAMT)	This study
SS33	SS3B Δ*fadD*::Mat1A (ΔfadD::Mat1A BTE/DmJHMAT)	This study
SS34	SS30 *ΔfadD730*::Mat1A (Δaas ΔfadD::Mat1A BTE/DmJHMAT)	This study

Table 3-1. *E. coli* strains used in Chapter 3.

Name of Primer	Sequence
XhoI-pBAD/p15A-BTE	5’-GGGTTTTCTCGAGGAGTGGAAGCCGAAGCCGAA-3’
NciI-pBAD/p15A-BTE	5’ GGGTTTTATGCATTTACACCCTCGGTTCTGCGGGTA-3’
DmJHAMT NdeI Forward	5’-GGGAATTCCATATGAATCAGGCCTCTCTATATCAGCAC-3’
DmJHAMT XhoI End	5’-GGCCGCTCGAGTTAATTTATTCCCTTAACCAAGTTTTG-3’
5’ E. coli SAH NdeI	5’-GCATGGGAATTCCATATGAAAATCGGCATCATTGGTGCAATGG AAGAAGAAGTTAC-3’
3’ E. coli SAH SacI	5’-CAAGCTTGTCGACCGAGCTCTCATTAGCCATGTGCAAGTTTCTG CACCAGTGACTC-3’
5’ B. sub LuxS NheI	5’-CAAGCTTGATGGCTGCTAGCCCTTCAGTAGAAAGTTTTGAGCTT GATCATAATGCG-3’
3’ B. sub LuxS EagI	5’- GTGCGGCCGCGCCAAATACTTTTAGCAATTCTTCTTTATCCTGTG AAAAGCC-3’
5’ KpnI Mat1A forward	5’-GCCGGTACCATGAATGGACCTGTGGATGG-3’
3’ XhoI Mat1A end	5’-GCACTCGAGGCTTTACTAAAACACAAGCTTCTTGGG-3’
pKD3-Cat-pCDF-Forw	5’-GGCATTTGAG AAGCACACGG TCACAGTGTAGGCTGGAGCTGCTTC-3’
pKD3-Cat-pCDF-Rev	5’- CAGGGTCGTTAAATAGCCGCTTATG ATGGGAATTAGCCATGGTCC-3’
pCDF 385-Rev	5’-TGTGACCGTGTGCTTCTCAAATGCC-3’
pCDF 425-Forw	5’- CATAA GCGGCTATTTAACGACCCTG-3’
FadD KO – pCDF1 P1-1	5’- GACGACGAACACGCATTTTAGAGGTGAAGAAGGTTTTGCGCCATTCGA TGG-3’
FadD KO – CAT P2-1	5’- GATTAACCGGCGTCTGACGACTGACTTAACGCCAGGGTCGTTAA ATAGCCGC -3’
FadD-P1-pKD4-Primer2	5’- TATCATTTGGGGTTGCGATGACGACGAACACGCATTTTAG-3’
FadD-P2-pKD4-Primer2	5’-GCGTCAAAAAAA ACGCCGGATTAACCGGCGTCTGACGACTG-3’

Table 3-2. Oligonucleotide Primers used in Chapter 3. The restriction sites are underlined.

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