Fatty Acid Metabolic Engineering: Insights for Bacterial Hydrocarbon Production

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I would not have any of these people to thank if it wasn't for my parents who have extended to me every opportunity in life to succeed, thank you Mom and Dad. Lastly, and most importantly, I thank Brittany for supporting me when I told her I was going back to school and unknowingly agreeing to years of late night time points and "quick stops to the lab".

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Chapter 1

Introduction

Energy use in United States has been dominated by non-renewable fossil-based fuels for a little over a century. Over that same time span, these energy sources were both cheap and abundant, and a vast infrastructure was built around them. Currently, the United States generates approximately eighty-six percent of its energy from non-renewable fossil fuels (Perlack et al., 2005). Forty percent of the energy is derived from petroleum-based fuels, of which two-thirds is used as a liquid transportation fuel (Perlack et al., 2005). Petroleum is a fuel with an increasingly limited supply due to its non-renewable nature and increased global demands. Coupled with awareness of the environmental impacts of using fossil fuels and a desire to achieve energy independence for greater national and economic security, there is increased interest in developing renewable, sustainable, and domestic supplies of fuel that can be integrated into the current infrastructure.

Bioethanol and biodiesel represent current generation alternative fuels that are both renewable and can be utilized in the already established infrastructure. The largest drawback to these fuels is that the biomass feed stocks for fuel production are also human food crops. Sustainability is also an issue, in that if all the corn grown in the United States was used for bioethanol production and all the soybeans for biodiesel production, this would only offset 12% of total gasoline use and 6% of total diesel use (Hill et al., 2006). Additional deficiencies include cost of production and the lack of certain desired fuel quality characteristics such as high energy density and stability. These deficiencies leave the door open for development of additional alternative biofuels. A wave of second generation alternative fuels is on the horizon, but issues must be addressed with the biomass feedstock, biotechnology, scalability, fuel quality, and economic relevance.

Shewanella oneidensis strain MR-1 is an example of a microorganism that synthesizes a second generation fuel-type compound. MR-1 naturally synthesizes a 31 carbon poly-unsaturated compound from a poly-unsaturated fatty acid precursor (Sukovich et al., 2010). These poly-unsaturated hydrocarbons could theoretically be refined using traditional petroleum based techniques to make high quality fuels such as

gasoline or diesel. For this fuel to be a viable alternative to petroleum based fuels, increased production of this or similar hydrocarbons from MR-1 is necessary at a lower cost. This can be achieved by coupling the hydrocarbon producing *Shewanella* to a phototroph to generate energy and carbon precursors that can be assimilated into hydrocarbons by MR-1, resulting in lower production cost, smaller carbon footprint, and elimination of land competition with food crops. MR-1 is an ideal host organism for this engineering because it naturally synthesizes hydrocarbons, it is genetically amenable, and the genome has been sequenced and is available. Additionally, MR-1 and *E. coli* share homologues and general pathway features for both fatty acid synthesis and degradation, providing a framework for engineering an *S. oneidensis* strain capable of producing relevant quantities of hydrocarbons as an alternative fuel.

The aim of this research was to engineer a pathway into *S. oneidensis* that synthesizes high amounts of hydrocarbons. This pathway involves engineering strategies with the end goals of increasing production of fatty acids, deregulating fatty acid synthesis, and disruption of competing pathways for the hydrocarbon precursors. The proposed pathway is outlined in figure 2.1 and lays out the engineering strategies for the following studies. These goals employed several microbial engineering strategies. Chapter two focuses on disruption of a competing pathway by engineering an *S. oneidensis* strain incapable of degrading hydrocarbon precursors. Chapter three focuses on identifying the necessary and optimal gene(s) for substrate formation in *S. oneidensis*. The fourth chapter explores heterologous expression of a protein targeted to deregulate synthesis of precursors, allowing for increased production of fatty acids and overexpression of native genes considered rate-limiting steps in precursor synthesis. Leveraging the broad wealth of knowledge of fatty acid metabolism in *E. coli*, hydrocarbon production in MR-1, and engineering capabilities of MR-1, generating an *S. oneidensis* strain capable of producing relevant quantities of fuel is a possibility.

Chapter 2

Elimination of a competing pathway for acyl-CoA substrates: fatty acid β-oxidation

Introduction

MR-1 is a facultative anaerobe, gram-negative rod, γ-proteobacterium well known for the ability to respire a variety of soluble and insoluble terminal extracellular electron acceptors (Hau and Gralnick, 2007). Hydrocarbons are synthesized in MR-1 by a head-to-head condensation reaction of two polyunsaturated fatty acids by the enzyme OleA (Sukovich et al., 2010). For this reaction to occur, the fatty acids must be in an activated form. Most commonly, activated fatty acids are found in bacteria thioesterified either to acyl carrier protein (ACP) or coenzyme A (CoA) groups. Using OleA from MR-1 as a query subject, homologues are identified in various bacteria. In particular, when one of these OleA homologues from *Stenotrophomonas maltophilia* is heterologously expressed in MR-1, it produces over 17 different long chain fatty acid (LCFA) derived hydrocarbon products (Sukovich et al., 2010). The ability of this recombinant strain to use multiple products from fatty acid synthesis as substrates when OleA from *S. maltophilia* is heterologously expressed provides an opportunity to increase hydrocarbon production. To further explore the fatty acid substrate requirements for hydrocarbon production, experiments were carried out by (Frias et al., 2011) using a purified OleA homologue from another organism, *Xanthomonas campestris,* and results indicated that *in vitro,* this homologue specifically condenses CoA activated long chain fatty acids (LCFA-CoAs) (Frias et al., 2011). Based on the homology between *X. campestris* and *S. maltophilia* OleA, it is likely that *S. maltophilia* OleA also has substrate specificity for LCFA-CoAs, and it was the aim of this research to eliminate competing pathways for LCFA-CoA's in our *S. oneidensis* hydrocarbon overproducing strain.

Fatty acids are energy-rich molecules that can be degraded by the β-oxidation pathway in bacteria to generate energy and carbon for the cell. β-oxidation is an iterative cycle involving the oxidation of the β carbon of the fatty acid with the end result of each cycle being the release of the two-carbon molecule acetyl-CoA along with generation of the reducing equivalents $FADH_2$ and NADH. At this point, the fatty acid is reduced by two carbons and re-enters the β-oxidation cycle to be oxidized again until the fatty acid

chain is reduced to acetyl-CoA. The β-oxidation cycle reviewed in (DiRusso et al., 1999) shows the first committed step in this pathway is the oxidation of the LCFA-CoAs catalyzed an acyl-CoA dehydrogenase (FadE). FadE catalyzes the dehydrogenation of the LCFA-CoA chain and subsequent transfer of two electrons to FAD, resulting in the formation of 2-enoyl-CoA. In the next step, the FadBA multienzyme complex catalyzes a hydration reaction at the double bond, forming a 3-hydroxyacyl-CoA followed by a second dehydrogenation step yielding a 3-ketoacyl-CoA and NADH. In the final step, the FadBA complex functions as a 3-ketoacyl-CoA thiolase that cleaves the β-ketoacyl-CoA from the acyl chain and transfers it to the thiol group of a free CoA (CoASH), resulting in the release of acetyl-CoA. In a strain engineered to overproduce fatty acids for hydrocarbon production, it is imperative to block fatty acid degradation so LCFA-CoA substrates for hydrocarbon synthesis are not immediately degraded. Since FadE is the first committed step in fatty acid degradation and a direct competitor for LCFA-CoAs, disruption of *fadE* in MR-1 should effectively eliminate degradation of the substrate for hydrocarbon synthesis. Disruption of *fadE* has already been characterized in *E. coli*, and this has been shown to effectively eliminate β-oxidation. The phenotypic effect on β-oxidation of the disruption of *fadE* can be assessed by culturing S. *oneidensis* strains in minimal medium with fatty acids as the sole carbon source.

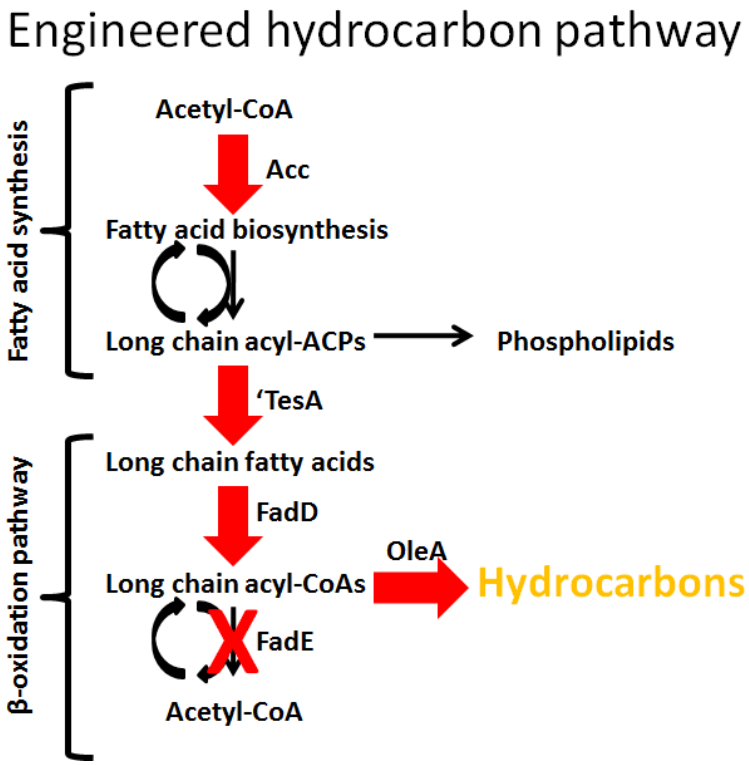

Figure 2.1 Proposed hydrocarbon pathway to be engineered in MR-1. Targeted reaction for overexpression: Acc (acetyl-CoA carboxylase, 'TesA (thioesterase), FadD (acyl-CoA ligase), OleA (3-oxoacyl-ACP synthase III). Targeted reaction for disruption: FadE (acyl-CoA dehydrogenase).

Materials and Methods

Reagents

Restriction enzymes, Antarctic phosphatase, and T4 ligase were from New England Biolabs. GoTaq Green Master Mix was used to amplify DNA and was obtained from Promega. For PCR cleanup and gel extraction, the IBI Gel/PCR DNA fragment extraction kit was used. For plasmid isolation and purification, the Invitrogen PureLink Quick Miniprep Plasmid Kit was used.

FadE-1, FadE-2, and OleA expression vector design

Primers used are listed in Table 2.2. The *fadE-1* (locus tag SO2536) and *fadE-2* (locus tag SO2492) genes were amplified by PCR using an MR-1 colony as template DNA resulting in a 2.5 kb band for *fadE-1* and a 2.2 kb band for *fadE-2*. Primers for *oleA* (locus tag Smlt0205) from Table 2.2 were used to amplify *oleA* from

Stenotrophomonas maltophilia R551-3 genomic DNA and resulted in a 1 kb band. All three genes were digested with BamHI and XhoI restriction enzymes and purified on a 1% w/v agarose gel. The digested genes were excised and purified with the IBI gel extraction kit. The purified genes were then ligated with T4 ligase into pB that had been digested with the same enzymes and purified in the same manner as the genes. The ligation reaction was used to transform UQ950 competent cells and were plated on LB with 50 $\mu g \cdot ml^{-1}$ kanamycin (km) to select for transformed cells. Colonies formed on the transformation plate were screened using colony PCR and primers originating within the pBBRBB backbone that amplified across the multiple cloning site of the vector. Colonies with the appropriate length PCR product were inoculated into 5 mL LB with 50 $\mu g \cdot ml^{-1}$ km and grown overnight. These cultures were pelleted, and the plasmid was isolated and purified. Plasmids were then sent to ACGT Inc. to verify correct nucleotide sequence.

Primer	ID	Sequence
PBBRBB_FADE-1_F	58190016	NNNGGATCCGTGACTACCCTACTTTGGCTCATC
PBBRBB_FADE-1_R	58190017	NNNCTCGAGTTAAGCGGCGCGTTCC
PBBRBB_FADE-2_F	58190018	NNNGGATCCATGTTGACGATTATAATTATTGCCCTGATTG
PBBRBB_FADE-2_R	58190019	NNNCTCGAGTTATAAAGCTTCGAAATCATTCACATTGATGG
PBBRBB_OLEA_F	56337226	NNGGATCCATGCTCTTCAAGAATGTCTCGATCGCCGGC
PBBRBB_OLEA_R	52915779	NNNCTCGAGTTACCAGACCACTTCAGCCATCGAGCAGT
PSMV3_FADE-2_UF	55583305	GGGCCCACCGCGACCCAAATGATG
PSMV3_FADE-2_UR	55583306	GGAATTCATCGTATTGGTTGAAATGAGTTAACTCAG
PSMV3_FADE-2_DF	55583307	GGAATTCAATCAAGACCTTTTATTTAGAAGCCACG
PSMV3_FADE-2_DR	55583308	CGGGATCCGTACTTGTACTAGCAGGTATAGAAGTCTTAG
PSMV3_FADE-1_UF	49846461	GGGCCCAATTCAGCGTAATTTCAAAAGTAAGGCCAAAC
PSMV3_FADE-1_UR	49846462	GAATTCGTATTTCTCCTTAATCATGACCGAGTGCC
PSMV3_FADE-1_DF	49846463	GAATTCTAGCGAAGCTGTACTAGCTAAGCC
PSMV3_FADE-1_DR	49846464	GGATCCGGCCCAAGGTTTGTTAATTTTCGG

Table 2.2 Primers used in this study.

fadE-1 and *fadE-2* deletion construct design

Primers for *fadE-1* and *fadE-2* listed in table 2.2 were designed to amplify approximately 1 kb upstream and 1 kb downstream of the target gene. Primers were designed to disrupt the entire gene, including the start and stop codon. The upstream and

downstream 1 kb regions were digested with ApaI/EcoRI and EcoRI/BamHI, respectively. The digested 1 kb fragments were purified and cleaned, and a three-way T4 DNA ligation with pSMV3 that had been digested with ApaI and BamHI was performed. UQ950 calcium chloride competent cells were then transformed with the ligation reaction containing pSMV3, upstream, and downstream fragments using the heat shock method. After outgrowth of transformed cells, the cells were concentrated and plated on LB agar plates with 50 $\mu g \cdot mL^{-1}$ km freshly spread with 50 µL of 60 $mg \cdot ml^{-1}$ X-gal. The transformation plate was incubated at 37°C overnight. White colonies were selected and screened by colony PCR using M13 forward and reverse primers. PCR products were run on a 1% w/v agarose gel, and 2 kb positive bands were selected. Corresponding colonies were inoculated into 5 mL of LB with 50 $\mu g \cdot mL^{-1}$ km and cultivated at 37°C for 16 hours with shaking at 250 rpm. Plasmids were purified from the cultures, digested with ApaI and BamHI for restriction digest confirmation, and then sent for Sanger sequencing to verify sequence.

Plasmid	**Description**	**Reference/ Source**
pB	pBBRBB-*eGFP*	(Vick et al., 2011)
pSMV3	9.5 kb mobilizable suicide vector, oriR6K, mobRP4, *sacB*, Km^rAp^r	(Saltikov and Newman, 2003)
pSMV3-*fadE-1*	*S. oneidensis fadE-1* deletion construct	This study
pSMV3-*fadE-2*	*S. oneidensis fadE-2* deletion construct	This study
pB-*fE-1*	pBBRBB-*fadE-1*	This study
pB-*fE-2*	pBBRBB-*fadE-2*	This study
pB-*o*	pBBRBB-oleA (*S. maltophilia*)	This study
Strain	**Description**	**Reference/ Source**
***S. oneidensis* strain MR-1**	Isolated from Lake Oneida, NY	(Venkateswaran et al., 1999)
***E. coli* strain UQ950**	*E. coli* DH5α, λ (*pir*) for cloning	(Saltikov and Newman, 2003)
***E. coli* strain WM3064**	DAP auxotroph, donor strain for *Shewanella* conjugation	(Saltikov and Newman, 2003)
JG1611	*S. oneidensis*, pB	This study
JG1156	*S. oneidensis,* Δ*fadE-1*	This study
JG1884	*S. oneidensis,* Δ*fadE-2*	This study

JG1885	*S. oneidensis*, Δ*fadE-1,2*	This study
JG1991	*S. oneidensis*, Δ*fadE-1,2* pB-*fE-2*	This study
JG1994	*S. oneidensis*, Δ*fadE-1,2* pB-*fE-1*	This study
JG1883	*S. oneidensis*, pB-*o*	This study
JG1892	*S. oneidensis*, Δ*fadE-1,2* pB-*o*	This study

Table 2.3 Plasmids and strains used in this study.

Conjugal transfer of plasmids

S. oneidensis strains selected for gene disruption were streaked onto an LB agar plate spread with 50 µL of 60 mM diaminopimelic acid (DAP). The *E. coli* donor strain WM3064 carrying the desired plasmid for transformation was streaked over the *S. oneidensis* strain and incubated at 30°C for 5-12 hours for conjugal transfer of plasmid to strain. *S. oneidensis* and WM3064 cells were collected from the plate and streaked for isolation onto an LB plate with 50 ug·ml^{-1} km for selection of the plasmid followed by incubation at 30°C for 16 hours.

Targeted gene disruption in *S. oneidensis*

The technique above was used to transfer suicide deletion constructs into the strain designated for gene disruption. In the last step, selection on an LB plate with 50 ug·ml^{-1} km was used to select for integration of the suicide vector construct into the *S. oneidensis* strain. Colonies that formed on the plate were then restreaked onto an LB plate with 50 ug·ml^{-1} km and incubated at 30°C for 16 hours to ensure plasmid integration. Colonies from this plate were then streaked onto an LB and 5% w/v sucrose plate and incubated at room temperature for two days or until colonies were visible. Counter-selection on the sucrose plate resulted in the second recombination step, resulting in reversion back to wild type or deletion of the targeted gene. Colonies on the sucrose plate were screened with colony PCR using an upstream fragment forward primer and downstream fragment reverse primer to identify colonies with the desired deletion. Colonies with the desired disruption resulted in a 2 kb fragment. Colonies that were positive for the deletion were streaked onto an LB plate with 50 ug·ml^{-1} km to ensure km sensitivity.

Culturing of strains in minimal medium with fatty acid carbon sources

Single colonies from an LB plate (supplemented with 50 $\mu g \cdot ml^{-1}$ km when strains carried plasmids) were used to inoculate LB liquid medium (supplemented with 50 $\mu g \cdot ml^{-1}$ km when strains carried plasmids) and shaken at 250 rpm for 16 hours at 30°C. Overnight cultures were back-diluted to an OD_{600nm} of 0.05 into *Shewanella* basal medium (SBM) as defined previously defined (Hau et al., 2008) with 20 mM sodium lactate (Sigma) (supplemented with 10 $\mu g \cdot ml^{-1}$ km when strains carried plasmids) and shaken at 250 rpm for 16 hours at 30°C. Overnight SBM lactate cultures were back-diluted to an OD_{600nm} of 0.05 into SBM medium with 1.5% w/v Brij 58 (Sigma) containing 5 mM hexanoic acid, 5 mM sodium decanoate, 4 mM sodium dodecanoate, 3 mM sodium tetradecanoate, 1.5 mM sodium hexadecanoate, and 1 mM sodium octadecanoate (Sigma).

Chloroform/methanol extraction of nonpolar molecules

Single colonies were inoculated into 2 mL LB (supplemented with 50 $\mu g \cdot mL^{-1}$ km when strains carried plasmids). Cultures were incubated at 30°C with shaking at 250 rpm overnight. Cultures were back-diluted to an OD_{600nm} of approximately 0.05 in 3 mL LB medium and incubated at 30°C with shaking at 250 rpm for 48 hours. After 48 hours, 50 µL of culture was collected for BCA protein assay normalization. Chloroform/methanol extraction of hydrocarbons was adapted from the method by Bligh and Dyer (BLIGH and DYER, 1959). An internal standard consisting of 25 µL of 0.45 mM 12-tricosanone (C23) in toluene standard was added to the remaining culture tube. 3.75 mL of Chromosolv plus high purity chloroform (Sigma) was added to the culture and vortexed briefly to mix. The mixture was then poured into a glass extraction tube (18x2 cm). 7.5 mL of spectrophotometric grade methanol (Sigma) was added to the extraction tube and vortexed for 15 seconds. An additional 3.75 mL of chloroform was added to the extraction tube and vortexed for 15 seconds. 3 mL of deionized H_2O was added to the extraction tube and vortexed for 15 seconds. Phase separation of the organic layers was allowed for 20 minutes. The lowest layer, containing chloroform was removed with a glass pipette and dispensed into a twice chloroform rinsed vial. Once chloroform had evaporated, the extract was resuspended in 100-200 µL of toluene and dispensed into a GC vial. Hydrocarbons were analyzed by GC-FID-MS.

Direct esterification of fatty Acids

The methyl esterification procedure was adapted from (Griffiths et al., 2010). Fatty acid extracts were resuspended in 500 µL toluene and dispensed in fluorinated ethylene propylene (FEP) tubes. Either 15 µL of 0.5 mM methyl decanoate in toluene or 25 µL of 28 mM heptadecane in toluene was added to resuspended extract as an internal standard. 1 mL of 0.5 N sodium methoxide (Sigma) was then added to the FEP tube and vortexed briefly to mix. The FEP tube was then placed in a stirring 80°C H_2O bath for 20 minutes. FAME samples were then removed from the H_2O bath and cooled for 5 minutes at room temperature. 1 mL of boron trifluoride in 14% methanol (Sigma) was then added to the FAME sample, and the heating and cooling steps were repeated. 400 µL of deionized H_2O and 400 µL hexanes were added to the culture, vortexed on high to mix, then on low for 1 minute. The reaction was centrifuged at 2,000 X g for 5 minutes to facilitate phase separation. The upper hexane layer was removed with a glass pipette and dispensed into GC vial. FAMEs were analyzed by using by GC-FID-MS.

Analysis of hydrocarbons and FAME products by GC-FID-MS

Hydrocarbons and FAME products were analyzed using a gas chromatograph (GC) with a flame ionization detector (FID) HP 7890A (Hewlett Packard, Palo Alto, CA) and mass spectrometer (MS) HP 5975C. Operating conditions for the GC were as follows: helium gas, 1.75ml/min; HP-1ms column (100% dimethylsiloxane capillary; 30 m 250m 0.25 m); temperature ramp, 100-320°C for 5 min., 250°C injection port, and split at the outlet between MS and FID. MS parameters were: electron impact at 70eV and 35A. The FID was set at 250°C with hydrogen flow set at 30 ml/min., air set at 400 ml/min., and helium make up gas set at 25 ml/min.

Results

To eliminate a competing pathway for the fatty acyl-CoA substrate used by OleA, FadE was selected to disrupt oxidation of fatty acyl-CoA chains in the β-oxidation pathway. *E. coli* strain K12 substrain MG1655 FadE (locus tag b0221) was used as a query subject for NCBI protein blast to identify the FadE homologue in MR-1. This blast search identified two potential homologues: FadE-1 (locus tag SO2536, 66% amino acid identity) and FadE-2 (locus tag SO2492, 52% amino acid identity). In order to determine

if either or both homologues are necessary for β-oxidation in MR-1, deletion constructs were designed, and in-frame chromosomal disruptions of both *fadE-1* and *fadE-2* individually and together were created.

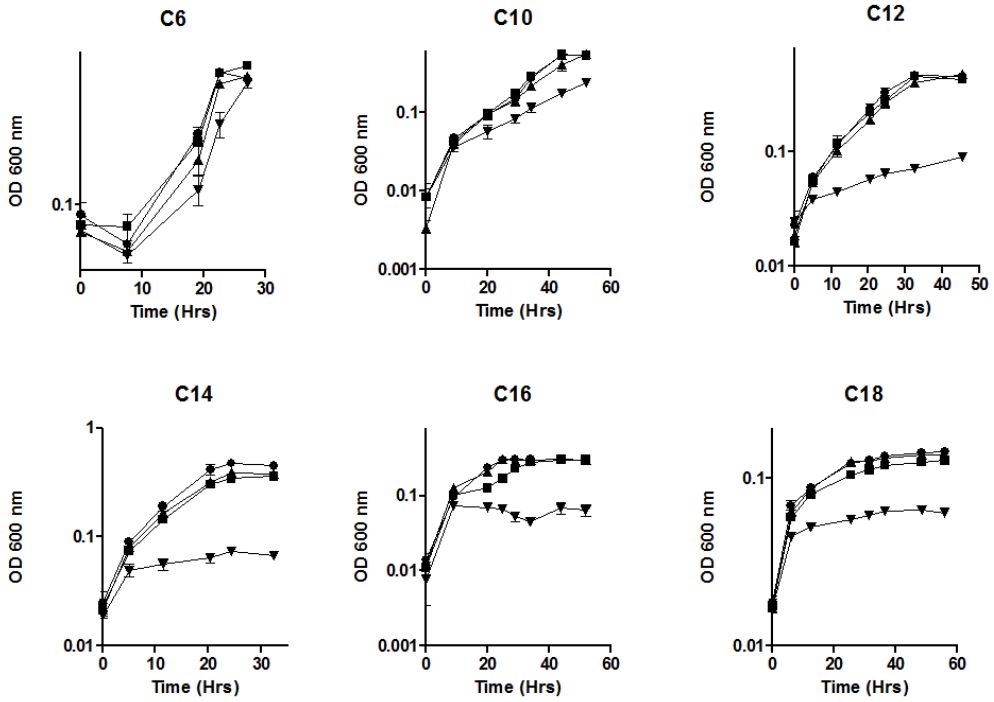

Figure 2.2. Growth analysis of *S. oneidensis fadE* mutants. Growth on saturated fatty acids: C6, C10, C12, C14, C16, C18. (●) *S. oneidensis*, (■) *S. oneidensis* Δ*fadE-1*, (▲) *S. oneidensis* Δ*fadE-2*, (▼) *S. oneidensis* Δ*fadE-1,2*.

Phenotypes for *fadE* mutants in β-oxidation were determined by aerobic culturing of *fadE* mutants in SBM supplemented with varying chain lengths of saturated fatty acids (C6, C10, C12, C14, C16, C18) and a fatty acid solubilizing non-ionic detergent, Brij 58 (Kang et al., 2010). Using C8 (octanoic acid) as a carbon source is not possible for MR-1 since it is toxic to the organism (Dan Coursolle, personal communication). Disruption of *fadE-1* and *fadE-2* individually had marginal effects on growth on all fatty acids tested (figure 2.2). When both homologues are disrupted in the same strain, growth on all fatty acids is inhibited except for C6 and marginally on C10 (figure 2.2). Complementation of the *fadE* double knockout with either *fadE* homologue restored growth to wild type levels except for C10 where *fadE-2* was unable to fully restore growth (figure 2.3). With

growth on long chain fatty acids inhibited, the fatty acid profile of the *fadE* double knockout was analyzed using the FAME protocol to esterify the fatty acids extracted from cultures and quantified by GC-FID-MS. This procedure will identify both free and phospholipids derived fatty acids in the culture. In figure 2.4a, the FAME profile of both MR-1 and the *fadE* double knockout shows no changes in fatty acid profile.

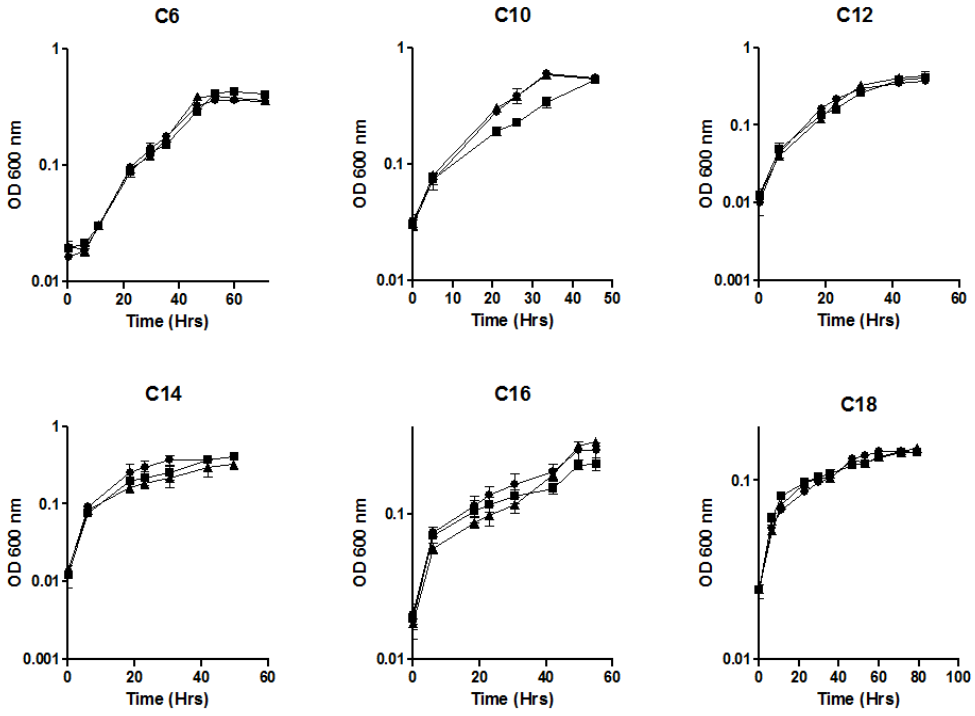

Figure 2.3. Growth analysis of *S. oneidensis fadE* mutants complemented with *fadE* homologues. Growth on saturated fatty acids: C6, C10, C12, C14, C16, C18. (●) *S. oneidensis* E.V., (■) *S. oneidensis* Δ*fadE-1,2* pB-*fE-1* (▲)*S. oneidensis* Δ*fadE-1,2* pB-*fE-2*

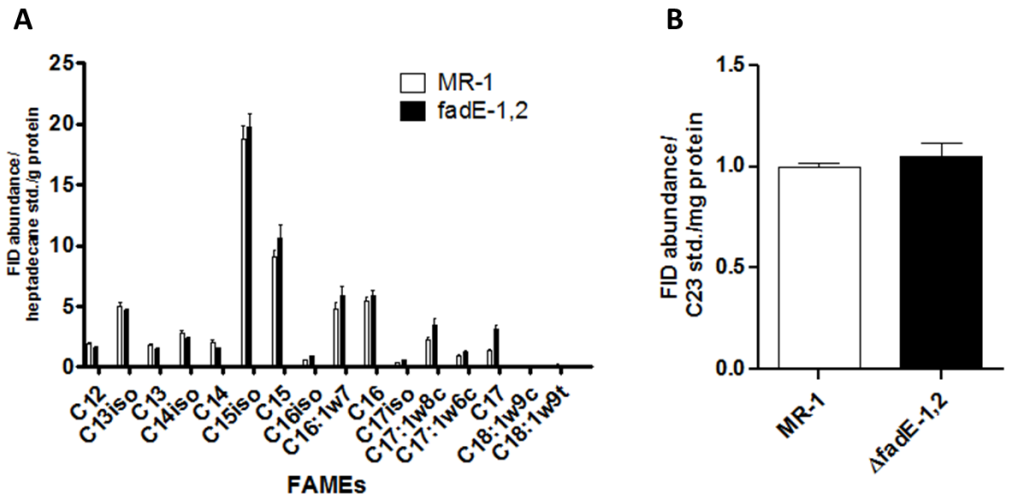

Figure 2.4 FAME profile and hydrocarbon production of MR-1 and *S. oneidensis fadE* double knockout. FAME results are reported as FID abundance normalized to an internal standard of heptadecane normalized per gram of protein in the culture. Hydrocarbon production is reported as FID abundance normalized to 12-tricosanone (C23) standard normalized per mg protein in culture and amounts are displayed relative to MR-1.

Discussion

Initial experiments to eliminate fatty acid degradation targeted FadE-1, the homologue with the highest shared amino acid identity (66%) to *E. coli* FadE. The *fadE-1* mutant was tested for its ability to grow on fatty acids (C6, C10, C12, C14, C16, C18), and results showed that strains grew equally well as MR-1 on all fatty acids tested. This result suggested that additional FadE homologues were present in MR-1. The blast search revealed a second homologue, FadE-2, with 52% amino acid identity to *E. coli* FadE. When *fadE-2* was disrupted in combination with *fadE-1*, the strain was unable to grow on all fatty acids tested except C6 and marginally on C10. The growth observed on C6 and C10 with the *fadE* double knockout is most likely associated with a medium chain acyl-CoA dehydrogenase yet to be identified. Blast searches do not suggest an additional homologue based on the 30% shared amino acid identity estimate for homologues. The next likely candidate is annotated as an isovaleryl-CoA dehydrogenase (SO1897), an enzyme involved in branched chain amino acid metabolism with 23% amino acid identity and 40% gene coverage to FadE in *E. coli*. Blast searches reveal

several other putative acyl-CoA dehydrogenases with low amino acid identity present in MR-1 that may be responsible for β-oxidation of the medium chain length fatty acids C6 and C10.

The ability of each FadE homologue to essentially maintain wild type MR-1 β-oxidation levels (assumed by correlation to growth) in the absence of the other homologue suggests a redundant specificity for the enzymes under the experimental conditions tested. It is possible that each enzyme exhibits different specificities for fatty acid substrates based on chain length but the acyl-CoA dehydrogenase reaction catalyzed by FadE may not be a rate limiting step in the MR-1 β-oxidation pathway. It is also possible that naturally, expression of each homologue is differentially regulated by factors yet to be identified. Interestingly, the gene immediately upstream of *fadE-2* in MR-1 is annotated as *psrA*. The product of this gene is a known transcriptional regulator of genes involved in the transition to stationary phase and recently has been shown to repress β-oxidation in *Pseudomonas aeruginosa* (Kang et al., 2008). PsrA has been shown to bind LCFAs as opposed to LCFA-CoAs, as done by the *E. coli* transcriptional regulator FadR. In *P. aeruginosa,* binding of LCFAs to PsrA relieves transcriptional repression of *fadBA*, thereby inducing β-oxidation (Kang et al., 2008). This represents a novel transcriptional regulation of β-oxidation yet to be thoroughly explored. Discovery of the second and potentially third acyl-CoA dehydrogenase represents a difference in β-oxidation when compared to *E. coli*. The reason for expression of two or potentially three FadE homologues remains unknown and could be the subject of additional experiments to determine under what conditions these genes are naturally transcribed and expressed and substrate specificities for each. The ability of MR-1 to degrade and grow on fatty acids that are 6 and 10 carbons long does not occur in *E. coli*. In *E. coli*, β-oxidation is negatively regulated by the transcriptional regulator FadR, and inhibition is released only when LCFA-CoAs 12 carbons or longer bind FadR, allowing the transcription of genes involved in β-oxidation. This leads to the possibility that FadR in MR-1 is capable of binding shorter chain fatty acyl-CoAs or that additional or different transcriptional regulators exist in the MR-1 genome for regulating β-oxidation.

In order to determine if the double deletion of *fadE-1* and *fadE-2* causes changes in fatty acid abundance or profile, cultures of MR-1 and the β-oxidation deficient strain were subjected to FAME analysis. By esterifying the fatty acids with methanol the fatty acids present in a culture can be easily identified. The FAME profiles and corresponding abundance of fatty acids for both strains were compared and determined that there were no significant changes (figure 2.4a). This was not an unexpected result since fatty acid synthesis is a tightly regulated pathway controlled by long chain acyl-ACP feedback inhibition and fatty acids are effectively recycled for use in phospholipid membrane homeostasis (Zhang and Rock, 2008). Hydrocarbon production was also determined for the *fadE-1,2* mutant expressing *S. maltophilia* OleA on a multicopy plasmid. As expected, hydrocarbon production for the MR-1 control strain and the β-oxidation mutant showed no significant differences as well, since fatty acid precursors remained unchanged (figure 2.4b).

In this study, the disruption of the β-oxidation pathway in MR-1was successful for long chain fatty acids (C12, C14, C16 and C18) by disrupting the two *fadE* homologues. This effectively eliminates degradation of the longer chain fatty acids (C12-C18) used as substrates by OleA. No increase in fatty acid or hydrocarbon production was detected, but as the strain is further engineered to over-produce fatty acids, the β-oxidation deficiency should be essential.

Chapter 3

Identification of optimal *fadD* gene(s) necessary for substrate formation

Introduction

In order for OleA to condense LCFAs into hydrocarbons, the fatty acid substrates need to be activated with CoA as discussed in chapter 2. This activation is accomplished by FadD, an acyl-CoA ligase that ligates CoA onto free fatty acid chains. The FadD polypeptide has two signature domains: an ATP/AMP binding domain and a LCFA binding domain. The ATP/AMP binding domain is essential for catalyzing the ligation of CoA onto the fatty acid chain using the energy from the cleavage of pyrophosphate from ATP. FadD is an essential gene for bacterial growth on fatty acids. Fatty acid transport into the cell and activation is reviewed in (Kunau et al., 1995) and briefly described below. In *E. coli*, exogenous fatty acids shorter than 12 carbons diffuse through the outer membrane, but those larger than 12 carbons are transported across the outer membrane by a fatty acid transport protein FadL. Once fatty acids enter the periplasm, they can flip and diffuse across the inner membrane into the cytoplasm. The transport of fatty acids across these membranes is thought to be facilitated by vectorial thioesterification in which CoA is thioesterified to fatty acids near the cytoplasmic side of the inner membrane by FadD providing unidirectional transport of fatty acids by trapping LCFA-CoAs in the cytoplasm. Following activation with CoA, fatty acids can then enter β-oxidation for degradation or be assimilated into phospholipids.

By overexpressing the acyl-CoA ligase FadD in MR-1, it should be possible to convert free fatty acid chains being overproduced from fatty acid synthesis to the corresponding CoA derivatives that can then be condensed by OleA to form hydrocarbon products. Two methods were employed to determine which *fadD* gene(s) should be overexpressed in the hydrocarbon producing strain. First, growth of *S. oneidensis fadD* mutant strains in minimal medium with fatty acids as the sole carbon source should indicate which *fadD* gene(s) are essential for β-oxidation of exogenous fatty acids. Secondly, by taking those same *fadD* mutants and expressing OleA on a multicopy plasmid, and quantifying hydrocarbon production, it should be apparent which *fadD*

gene(s) are essential for hydrocarbon production. This study explored the *fadD* genes in MR-1 and the role played in β-oxidation as well as hydrocarbon production.

Materials and Methods

Reagents

Previously described in Chapter 2.

FadD-1 and FadD-2 expression vector design

Primers used are listed in Table 3.1. The *fadD-1* (locus tag SO2581) and *fadD-2* (locus tag SO3664) genes were amplified by PCR using an MR-1 colony as template DNA resulting in a 1.6 kb band for both fadD-*1* and *fadD-2*. Both genes were digested with BamHI and XhoI restriction enzymes, and the cloning protocol was followed as previously described in chapter 2.

fadD-1 and *fadD-2* deletion construct design

Primers for *fadD-1* and *fadD-2* deletion constructs are found in table 3.1. Deletion construct design was completed as previously described in chapter 2.

Primer	ID	Sequence
PBBRBB_FADD-1_F	57237172	CGGGATCCGTGGATCAGCCTTGGATTAGACATTTACCAAAAGATG
PBBRBB_FADD-1_R	52457104	CCGCTCGAGTTACGCACGCTTAACTTCGTCTCTAAGTTCTCG
PBBRBB_FADD-2_F	57237173	CGGGATCCATGGCATACGATCAAGAGTCACAACTCGAAC
PBBRBB_FADD-2_R	52457106	CCGCTCGAGCTAGTTTTTAAGCTCTCTGCGCAAAATTTTACCCACG
PBBRBB_OLEA_F	56337226	NNGGATCCATGCTCTTCAAGAATGTCTCGATCGCCGGC
PBBRBB_OLEA_R	52915779	NNNCTCGAGTTACCAGACCACTTCAGCCATCGAGCAGT
PSMV3_FADD-1_UF	49604679	GGGCCCGTTTATCCCAACGCCATTGTAAATACTC
PSMV3_FADD-1_UR	49604680	GAATTCGATTGAAGCCGGCAATAAGCC
PSMV3_FADD-1_DF	49604681	GAATTCTACTTTCTCCTAAATCCCCACAACTG
PSMV3_FADD-1_DR	49604682	GGATCCGGTCGATTATGGGGGACGAAAG
PSMV3_FADD-2_UF	52457107	NNNGGGCCCCGGGGATTGTGTCGATTGC
PSMV3_FADD-2_UR	52457108	NGAATTCGCCTAACTACCTCAAATAGGTTTAGTCG
PSMV3_FADD-2_DF	52457109	NGAATTCGTTTAATAAACCAGACAAACCCCAGAATC
PSMV3_FADD-2_DR	52457110	CGGGATCCGGGCGGCGAACTCTC
K12 PKD13 FADD_F	78153348	TGCGATGACGACGAACACGCATTTTAGAGGTGAAGAAGTGTAGGCTGGAGCTGCTTC
K12 PKD13 FADD_R	78153349	CGCCGGATTAACCGGCGTCTGACGACTGACTTAACGCATTCCG

GGGATCCGTCGACC

Table 3.1 Primers used in this study.

Conjugal transfer of plasmids

Previously described in chapter 2.

Targeted gene disruption in *S. oneidensis*

Previously described in chapter 2.

Targeted gene disruption in *E. coli*

Primers from table 3.1 were designed with 35 nucleotide base pair homology extensions flanking the targeted gene for deletion. Primers also had twenty nucleotide base pair homology regions to the template plasmid pKD13 to amplify the km resistance marker (Datsenko and Wanner, 2000). Primers were used to amplify pKD13 km cassette, the product was separated on a 1% agarose gel using electrophoresis, excised, and cleaned. *E. coli* strain K12 substrain MG1655 was transformed with pKD46 and plated on LB with 100 $\mu g \cdot mL^{-1}$ amp and grown at the permissible temperature of 30°C for 16 hours. A single colony was inoculated into 2 mL LB with 100 $\mu g \cdot mL^{-1}$ amp and grown overnight at 30°C. The following day, the culture was back-diluted to an OD_{600nm} of 0.05 in 10 mL of LB with 100 $\mu g \cdot mL^{-1}$ amp, induced with 0.5% w/v L-arabinose, and grown at 30°C to a mid-log OD_{600nm}. Cells were centrifuged at 4,000 X g and washed three times in 1 mL of 10% w/v ice cold glycerol. Cells were resuspended in 100 µL 10% w/v glycerol, and 100-500 ng of cleaned PCR pKD13 product was added to the tube. The transformation mixture was added to a 1 mm gap electroporation cuvette and electroporated. 1 mL of LB was added to the cuvette to resuspend the electroporated cells. The cells were incubated at 37°C for 1 hour then plated on LB with 50 $\mu g \cdot ml^{-1}$ km. The plate was incubated at 37°C overnight to cure pKD46 and select for cassette integration and targeted gene disruption with km. Colonies that formed on the plate were screened for targeted disruption using primers originating outside of the homology regions used for gene disruption. A positive colony for km cassette insertion was inoculated into 2 mL LB with 50 $\mu g \cdot ml^{-1}$ km and shaken at 250 rpm at 37°C. The following morning, the culture was back-diluted to an OD_{600nm} of 0.05 in 5 mL LB with 50 $\mu g \cdot ml^{-1}$ km. The culture was grown to an OD_{600nm} of 0.6-0.8, harvested by

centrifuging at 4,000 X g for 10 minutes, and the pellet was resuspended in 100 µL of transformation and storage solution (TSS) (Chung et al., 1989). Normal heat shock procedure was used to transform the K12 *fadE* deletion strain with the Flp recombinase plasmid pCP20 (Cherepanov and Wackernagel, 1995). The strain was plated on LB with 100 µg·mL^{-1} amp to select for pCP20 and incubated at the permissive temperature 30°C. The following day, amp resistant colonies were streaked onto an LB plate and incubated at 42°C to cure pCP20. Colonies present the following day were streaked onto an LB plate with 50 µg·ml^{-1} km and an LB plate with 100 µg·mL^{-1} amp to ensure loss of the pKD13 cassette from the chromosome and pCP20 respectively. A final colony PCR step was used to verify the pKD13 cassette was removed leaving a clean deletion of *fadD*.

Plasmid	**Description**	**Reference/Source**
pB	pBBRBB-*eGFP*	(Vick et al., 2011)
pSMV3	9.5 kb mobilizable suicide vector, oriR6K, mobRP4, *sacB*, Km^{r}Ap^r	(Saltikov and Newman, 2003)
pSMV3-*fadD-1*	*S. oneidensis fadD-1* deletion construct	This study
pSMV3-*fadD-2*	*S. oneidensis fadD-2* deletion construct	This study
pB-*fD-1*	pBBRBB-*fadD-1*	This study
pB-*fD-2*	pBBRBB-*fadD-2*	This study
pB-*o*	pBBRBB-*oleA* (*Stentrophomonas maltophilia*)	This study
pKD46	curable arabinose inducible lambda red recombinase plasmid	(Datsenko and Wanner, 2000)
pKD13	template plasmid for K12 homologous recombination	(Datsenko and Wanner, 2000)
pCP20	curable yeast Flp recombinase plasmid	(Cherepanov and Wackernagel, 1995)
Strains	**Description**	**Reference/Source**
***S. oneidensis* strain MR-1**	Isolated from Lake Oneida, NY	(Venkateswaran et al., 1999)
***E. coli* strain K12 substrain MG1655**	Wild type	Michael Sadowsky at the University of Minnesota
***E. coli* strain UQ950**	*E. coli* DH5α, λ (*pir*) for cloning	(Saltikov and Newman, 2003)
***E. coli* strain WM3064**	DAP auxotroph donor strain for *Shewanella* conjugation	(Saltikov and Newman, 2003)
JG1611	*S. oneidensis*, pB	This study
JG2159	*S. oneidensis,* Δ*fadD-1,2* pB-*fD-1*	This study
JG2161	*S. oneidensis,* Δ*fadD-1,2* pB-*fD-2*	This study
JG1161	*S. oneidensis,* Δ*fadD-1*	This study

JG1850	*S. oneidensis*, Δ*fadD-2*	This study
JG1851	*S. oneidensis*, Δ*fadD-1,2*	This study
JG2258	*E. coli* K12 Δ*fadD*	This study
JG2297	*E. coli* K12 Δ*fadD* pBBRBB-*fadD-1* (MR-1)	This study
JG2296	*E. coli* K12 Δ*fadD* pBBRBB-*fadD-2* (MR-1)	This study
JG2304	*E. coli* K12 pB	This study
JG1883	*S. oneidensis*, pB-*o*	This study
JG1851	*S. oneidensis*, Δ*fadD-1,2* pB-*o*	This study

Table 3.2 Plasmids and strains used in this study.

Growth of strains in minimal medium with fatty acid carbon sources

Previously described in Chapter 2.

Chloroform and methanol extraction of nonpolar molecules

Previously described in Chapter 2.

Direct esterification of fatty Acids

Previously described in Chapter 2.

Analysis of hydrocarbons and FAME products by GC-FID-MS

Previously described in Chapter 2.

Results

Utilizing FadD (locus tag b1805) from *E. coli* K12 MG1655 as the query subject, 2 homologues were identified in *S. oneidensis* MR-1: FadD-1 (locus tag SO2581, 69% amino acid identity) and FadD-2 (locus tag SO3664, 48% amino acid identity). Deletion constructs were designed to disrupt both of the *fadD* homologues in MR-1. Each *fadD* mutant was grown aerobically in minimal media containing saturated fatty acids (C6, C10, C12, C14, C16, C18) to identify substrate specificity *in vivo,* followed by expression of OleA in *fadD* mutants to assess hydrocarbon production. Strains with *fadD* disruptions that make less or no hydrocarbons would be a strong indication that it is essential for hydrocarbon production.

Analysis of *fadD* mutants by growth in minimal media supplemented with different length saturated fatty acid chains (C6, C10, C12, C14, C16, C18) indicated that FadD-1 and FadD-2 have different fatty acid chain length specificity (figure 3.1).

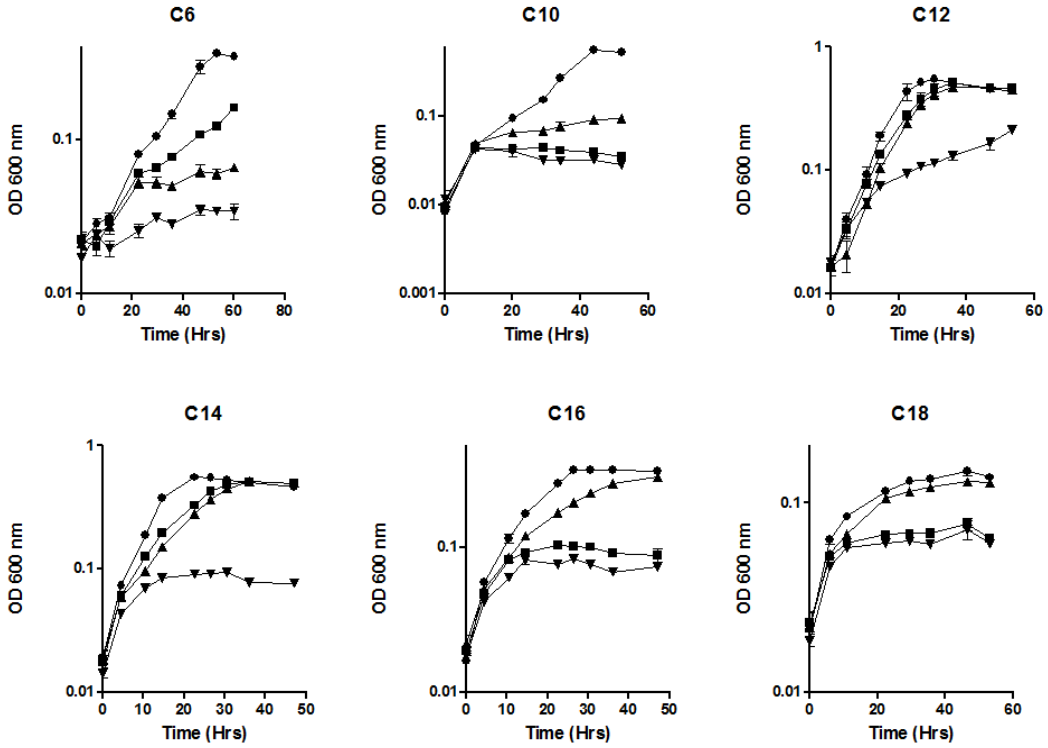

Figure 3.1. Growth analysis of *S. oneidensis fadD* mutants. Growth on saturated fatty acids: C6, C10, C12, C14, C16, C18. (●) *S. oneidensis*, (■) *S. oneidensis* Δ*fadD-1*, (▲) *S. oneidensis* Δ*fadD-2*, (▼) *S. oneidensis* Δ*fadD-1,2*.

When *fadD-1* is disrupted, growth on C6 is possible but at a reduced rate compared to MR-1, growth on C10 is greatly reduced, C12 and C14 growth is minimally effected, and C16 and C18 growth is severely reduced (figure 3.1). When *fadD-2* is disrupted, growth on C6 and C10 is greatly reduced compared to MR-1 and growth on C12, C14, C16, and C18 had near wild type growth rates. When both *fadD-1* and *fadD-2* are disrupted in the same strain, growth is abolished, when compared to no carbon controls, on all fatty acids tested (figure 3.1). When the *fadD* double mutant is complemented with FadD-1 on a multicopy plasmid, there is no growth on C6, near wild type growth on C10-C16, and faster growth on C18 (figure 3.2).

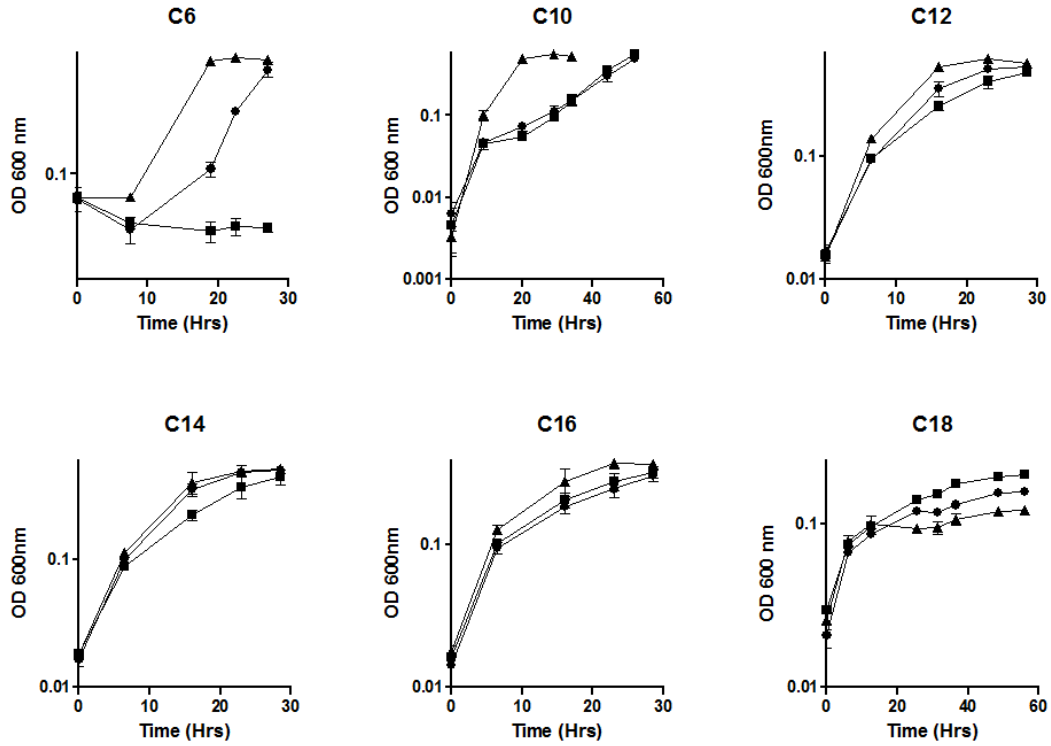

Figure 3.2. Growth analysis of *S. oneidensis fadD* mutants complemented with *fadD* homologues. Growth on saturated fatty acids: C6, C10, C12, C14, C16, C18. (●) *S. oneidensis* E.V., (■) *S. oneidensis* Δ*fadD-1,2* pB-*fD-1* (▲)*S. oneidensis* Δ*fadD-1,2* pB-*fD-2*

When the *fadD* double mutant is complemented with FadD-2 on a multicopy plasmid, the growth rate is much higher than MR-1 on C6 and C10, and marginally higher than MR-1 on C12-C16, and lower than MR-1 on C18 (figure 3.2).

The role of each FadD homologue was further explored by complementing *E. coli* strains that had a *fadD* chromosomal disruption. The *E. coli fadD* mutant strain was assayed for the ability to degrade fatty acids C10, C12, C14, and C16. Disruption of *fadD* resulted in no growth on all four fatty acid chains tested (figure 3.3), and longer fatty acids were metabolized at a faster rate than shorter chain fatty acids. The *E. coli fadD* mutant was then complemented with either FadD-1 or FadD-2 from MR-1 on a multicopy plasmid and assayed for growth on C10, C12, C14, and C16. Growth analysis indicated that FadD-1 complementation was able to restore growth to wild type *E. coli* levels, with the growth rate exceeding wild type on C10 and slightly below wild type for

C12, C14, and C16 (figure 3.4). FadD-2 complementation had a significant lag phase before growth and there was high variation between replicates for C10, C12, C16 and never reached cell densities achieved in wild type *E. coli* or the FadD-1 complemented strain (figure 3.4). This long lag in growth could be contributed to mutations related to the *fadD*-2 gene on the multicopy plasmid since in figure 3.2 the *fadD* disruption does not display a similar growth phenotype which would be indicative of a suppressor mutation.

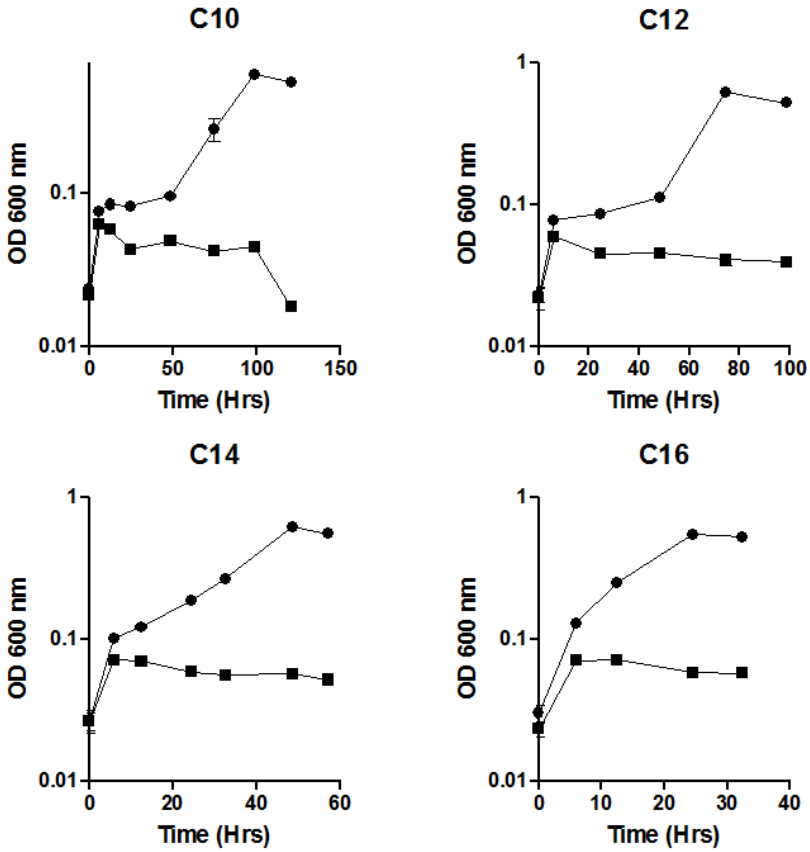

Figure 3.3. Growth analysis of *E. coli fadD* mutant. Growth on saturated fatty acids: C10, C12, C14, C16. (●) *E. coli* WT, (■) *E. coli* Δ*fadD*.

To measure the effect of inhibiting β-oxidation on fatty acid profile in the mutant strain, fatty acids were extracted from *S. oneidensis fadD* double knockout and wild type MR-1and methyl esterified. The FAMEs were analyzed by GC-FID-MS, and a fatty acid profile was generated (figure 3.5a). The profile revealed small increases in fatty acids

C13iso, C15iso, C15, in the *fadD* double knockout when compared to wild type MR-1. Next, hydrocarbon production was assayed in the *fadD* double knockout strain

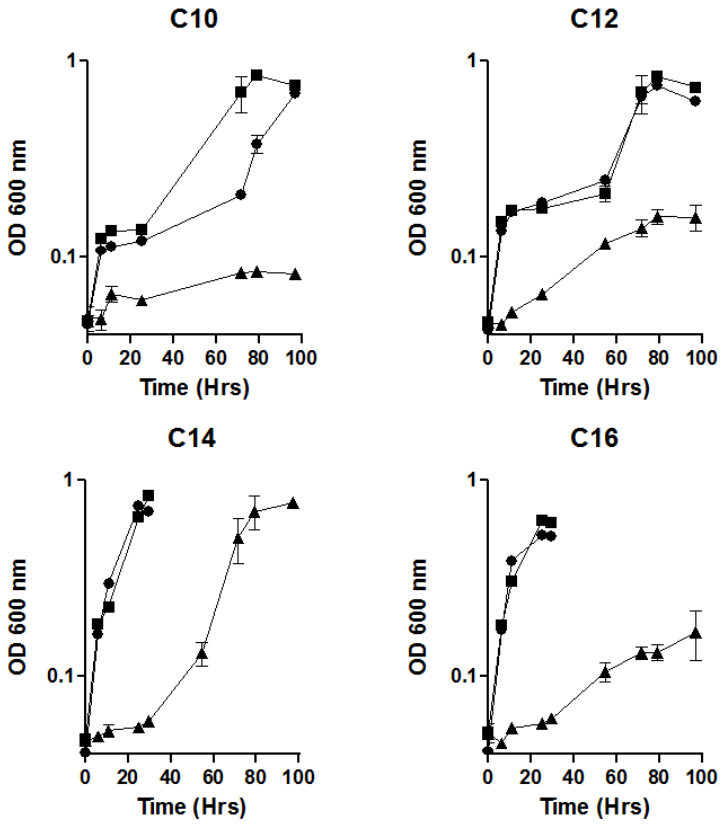

Figure 3.4 Growth analysis of *E. coli fadD* mutant complemented with MR-1 FadD homologues. Growth on saturated fatty acids: C10, C12, C14, C16. (●) *E. coli* E.V., (■) *E. coli* Δ*fadD* pB-*fD-1* (▲) *E. coli* Δ*fadD* pB-*fD-2.*

transformed with OleA on a multicopy plasmid. This strain was grown in rich medium, 5 mL LB, and in minimal medium, 50 mL SBM with 40 mM Lactate as the carbon and energy source. MR-1 with OleA in multicopy plasmid was also expressed in both media for comparison. Minimal medium was tested to verify that the substrates were not being provided from the yeast extract or other component in the rich medium. The minimal medium study was scaled up to 50 mL due to lower hydrocarbon yields and the study was completed in uniplicate of each strain. Hydrocarbon production in rich medium in the *fadD* double knock out background was 10% higher than wild type background and in

minimal medium the *fadD* double knockout background was 5% lower than wild type (figure 3.5).

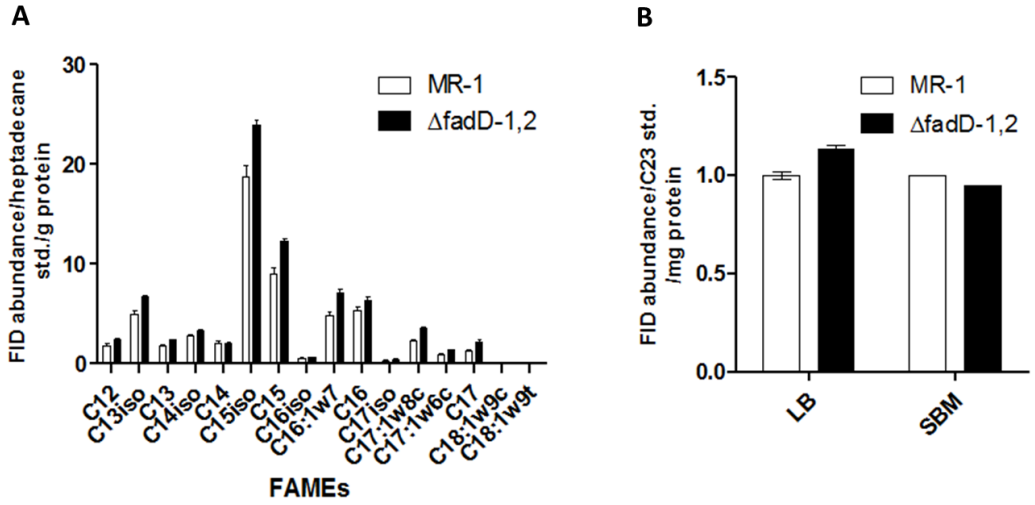

Figure 3.5. FAME profile and hydrocarbon production of *fadD* double knock out and MR-1. A) FAME profile is determined as FID abundance as a percent of the heptadecane standard normalized to gram of protein in culture. B) Hydrocarbon production in MR-1 and *fadD* double knockout strains expressing OleA on a multicopy plasmid. Hydrocarbon quantities reported as FID abundance as a percent of 12-tricosanone (C23) standard and normalized to total mg protein in the culture relative to MR-1.

Discussion

Disruption of each *fadD* homologue and culturing the strains in minimal media with fatty acids as the sole carbon and energy source provides valuable information as to the substrate specificity of f*adD* homologues *in vivo*. If long chain acyl-CoA ligase activity is successfully disrupted, this should result in a *S. oneidensis* strain incapable of utilizing fatty acids as a carbon and energy source, and this strain should also be unable to produce hydrocarbons since the LCFA-CoAs are not synthesized. Growth analysis of *S. oneidensis fadD* mutants in SBM with fatty acids indicates that *in vivo*, each homologue has different but overlapping substrate specificity for varying fatty acid chain lengths. Disruption of both homologues results in inability to utilize C6, C10, C12, C14, C16, and C18 fatty acids. When *fadD-1* is disrupted, the growth rate on C6 is slightly reduced compared to wild type and greatly reduced when *fadD-2* is disrupted. This

suggests that FadD-2 is better able to utilize C6 as a substrate *in vivo*. Growth on C10 is greatly impaired for both homologues, but some reduced growth still occurs when *fadD-2* is disrupted compared to wild type and no growth is present when *fadD-1* is disrupted. If either *fadD-1* or *fadD-2* is disrupted individually when grown on C12 or C14 fatty acids, either homologue is capable of maintaining near wild type growth rates suggesting similar substrate specificities. Growth on C16 and C18 is severely limited when *fadD-1* is disrupted and near wild type when *fadD-2* is disrupted. Results from these experiments show that FadD-1 is responsible for CoA thioesterification of longer chain fatty acids (C16 and C18). It appears that FadD-2 is responsible for CoA thioesterification of short chain fatty acids (C6).

To further explore the role of the FadD homologues in β-oxidation, the *fadD* double knockout strain was complemented with either FadD homologue and assayed for the ability to degrade fatty acids as done previously. These experiments should provide more insight into substrate specificity for each homologue since they are being constitutively expressed from the same promoter from the same plasmid. FadD-1 was able to restore growth on all fatty acids tested except for C6, in which it did not grow at all, agreeing with the results obtained from growth of the *fadD-1* disruption strain on C6. Surprisingly, FadD-2 complementation increased the growth rate above wild type levels for the shorter chain fatty acids C6 and C10 and restored to wild type levels for C12, C14, and C16 but was unable to fully complement growth on C18. The high growth rate of the FadD-2 complementation on C6 and C10 suggests that this step is rate limiting in the β-oxidation pathway and overexpressing FadD-2 eliminates the rate limiting step, allowing for faster growth rates. Under normal conditions, it is possible the *fadD-2* gene may be regulated by transcriptional repressor that binds shorter chain fatty acids less efficiently and when *fadD-2* is expressed from a constitutive promoter on a multicopy plasmid, this is alleviated. For comparison, it is well established in *E. coli* that β-oxidation is negatively regulated by FadR, and the longer the acyl-CoA chain, the stronger the binding to FadR (DiRusso et al., 1992).

To further explore the role of FadD-1 and FadD-2, the multicopy plasmids with each gene were used to transform the *E. coli fadD* mutant strain that is deficient in β-

oxidation. This phenotype was confirmed by culturing the strain in minimal media with fatty acids as the sole carbon and energy source as previously described. Complementation of FadD-1 and FadD-2 was assayed by culturing the strains as previously described using C10, C12, C14, and C16 fatty acids. Results from growth of each *fadD* homologue showed strikingly different results when expressed in *E. coli fadD* knockout versus expression in *S. oneidensis fadD* double knockout. Expression of FadD-1 resulted in near wild type growth rates on C12, C14, and C16 and on C10 grew faster than wild type. Expression of FadD-2 was unable to complement the *E. coli fadD* disruption like it had in the *S. oneidensis fadD* double knock out.

By disrupting both *fadD* homologues in *S. oneidensis*, a strain is generated that is deficient in β-oxidation on medium and long chain fatty acids that are 6-18 carbons long. This strain is unable to activate the fatty acids with CoA and cannot be targeted for β-oxidation. Based on these results, it was expected that this strain would also be unable to produce hydrocarbons when OleA from *S. maltophilia* was heterologously expressed in the *fadD* double mutant, since long chain acyl-CoAs have been shown to be substrates for an OleA homologue from *X. campestris* using *in vitro* assays. Surprisingly, expression of OleA in the *fadD* double mutant in rich and minimal media resulted in essentially no change in hydrocarbon production. One conclusion that can be drawn from this result is that the acyl chains present in the hydrocarbon products are from the condensation of a different substrate than acyl-CoAs. The most likely alternative substrates are activated acyl chains in a thioester linkage to ACP, the product of fatty acid synthesis. Fatty acids can be found esterified to other compounds in the cell, especially in membrane lipids, but these are not considered activated forms. Time courses of hydrocarbon production in MR-1 show that hydrocarbon production is maximal in late stationary phase (Sukovich and Wackett, personal communication). One possibility is that cultures that are in late stationary phase are undergoing lipid remodeling, and OleA condenses fatty acid derivatives involved in that remodeling. Stationary phase lipid remodeling is not a novel mechanism; it has been shown in *E. coli* that once cultures enter stationary phase, the cells modify lipids by converting unsaturated fatty acid lipid tails to cyclopropane groups

(Chang and Cronan, 1999). The reason for this modification is not fully understood, but strains that are deficient in this activity have low acid tolerance.

From this study, we better understand the role of the FadD homologues and the role played in β-oxidation, but questions have been raised about the substrate for OleA necessary for hydrocarbon production.

Chapter 4

Increasing the production of precursors and hydrocarbons

Introduction

Fatty acid synthesis can be carried out by two methods: type I is generally used by eukaryotes, and a single polypeptide codes for all enzymatic steps; type II is generally used by bacteria and individual polypeptides catalyze each step. MR-1 utilizes type II fatty acid synthesis for fatty acid production. Type II fatty acid synthesis in *E. coli* is reviewed in (White et al., 2005) and briefly described here. Fatty acid synthesis is initiated with the formation of malonyl-CoA from acetyl-CoA and CO_2, which is catalyzed by the enzyme acetyl-CoA carboxylase (Acc). This ATP dependent reaction has been shown to be a key rate-limiting step (Davis et al., 2000) and is the first committed step in fatty acid synthesis and is required for all chain elongation steps. FabD, a malonyl-CoA:ACP transacylase, converts malonyl-CoA to malonyl-ACP. Fatty acid synthesis is initated by β-ketoacyl-ACP synthase III (FabH), which condenses malonyl-ACP and acetyl-CoA to form acetoacetyl-ACP. Malonyl-ACP is then used to elongate the chain for each subsequent fatty acid cycle. Either FabB or FabF, which are 3-ketoacyl-ACP synthases, condense malonyl-ACP with the acyl-ACP chain, thereby elongating the fatty acid chain. After the acyl-ACP chain is condensed with malonyl-ACP, a series of reduction reactions occur. FabG, a 3-ketoacyl-ACP reductase, reduces the 3-ketoacyl-ACP chain while oxidizing NADPH. FabZ, a 3-hydroxyacyl-ACP hydratase, then dehydrates the 3-hydroxyacyl-ACP to enoyl-ACP. FabI, an enoylacyl-ACP reductase, reduces the enoyl-ACP while oxidizing NADH to form acyl-ACP, which can then be used again for the elongation cycle. Once the acyl-ACP chain reaches an appropriate length, it is then transferred to glycerol-3-phosphate by an acyl-transferase for membrane phospholipid synthesis.

Fatty acid synthesis is an energy intensive pathway, and for that reason, it is also tightly regulated. Long chain acyl-ACPs (LCFA-ACPs) (C12-C16), the product of fatty acid synthesis, have an inhibitory effect on several steps in fatty acid synthesis. LCFA-ACPs exhibit allosteric inhibition on Acc (Davis and Cronan, 2001), FabH (Heath and Rock, 1996b), and FabI (Heath and Rock, 1996a) effectively blocking precursor

formation, fatty acid chain initiation, and chain elongation, respectively. Inhibition of these 3 enzymes effectively regulates fatty acid synthesis.

A periplasmic thioesterase isolated from *Escherichia coli* strain K12 substrain MG1655 identified as thioesterase I (TesA) is an enzyme that cleaves the activated ACP and CoA groups esterified to LCFA (Cho and Cronan, 1993). A modified TesA, 'TesA, with the N-terminal periplasmic leader sequence removed, results in cytoplasmic localization. Overexpression of 'TesA effectively catalyzes the removal of ACP from LCFA-ACPs yielding free fatty acids and eliminating feedback inhibition in *E. coli* (Cho and Cronan, 1995).

In order to effectively increase fatty acid synthesis, two engineering strategies will be employed. First, due to the stringent regulation of fatty acid synthesis, expression of a thioesterase, 'TesA that cleaves long chain acyl-ACPs will be expressed on a multicopy plasmid deregulating key fatty acid synthesis reactions. Expression of 'TesA will successfully deregulate fatty acid synthesis, allowing overproduction of free fatty acids. Deregulation of fatty acid synthesis using 'TesA has been demonstrated in *E. coli* (Cho and Cronan, 1995) and in the cyanobacteria *Synechocystis* sp. PCC6803 (Liu et al., 2011). Second, to increase the flux of acetyl-CoA from central metabolism, the rate limiting step of malonyl-CoA formation catalyzed by Acc will be overexpressed on a multi-copy plasmid. Prior work in *E. coli* has shown that increasing expression of Acc increases fatty acid production in *E. coli* (Davis et al., 2000). Since expression of 'TesA and Acc results in the accumulation of free fatty acids, it is necessary to express an acyl-CoA ligase (FadD) to reactivate the fatty acids, followed by condensation by OleA to make hydrocarbons. This study explores first the deregulation of fatty acid synthesis by 'TesA in *S. oneidensis* but also as a proof of concept, in *E. coli*. Secondly, hydrocarbon production is explored in both *S. oneidensis* and *E. coli* strains engineered to overproduce them.

Materials and Methods

Reagents

Previously described in Chapter 2.

BioBrick™ expression system

For construction of the hydrocarbon synthesis pathway in MR-1, a plasmid that can express multiple genes, each under control of individual constitutive promoters, is desired. For this reason, the pBBRBB-eGFP BioBrick™ plasmid was selected. Salient features of this plasmid are a mutated operator region of the lac promoter allowing constitutive expression of genes, BioBrick™ ability to easily add promoter and gene "bricks" to constructs, proper mobilization genes and sequence elements to allow for conjugal transfer of the plasmid to host, and an optimized high affinity ribosomal binding site for each gene (Vick et al., 2011).

Bacterial culturing and growth

Strains and plasmids used in this study are listed below in Table 4.1. Single colonies of *S. oneidensis* were used to inoculate liquid LB medium and cultivated overnight at 30°C with shaking at 250 rpm. The following day, cultures were back-diluted to an OD_{600nm} 0.05 and cultivated for the required amount of time depending on the experiment at 30°C with shaking at 250 rpm. *E. coli* was cultured as described above except at a temperature of 37°C.

Primer	ID	Sequence
PBBRBB_TESA_F	55781876	NNNAGATCTATGGCGGACACGTTATTGATTCTGGG
PBBRBB_TESA_R	55781877	TTTTTCCTTTTGCGGCCGCTTATGAGTCATGATTTACTAAAGGCTGCAACT
PBBRBB_ACCADC_F	51150622	CGGGATCCTTTTCAGAGGCTAAAGCAGAGGATCTTTTGATGACCAGC
PBBRBB_ACCADC_R	51150623	TTTTCCTTTTGCGGCCGCTTAATCTTCAACTGGCGATACGACGAAGAGTAAGTCACCTTG
PBBRBB_ACCB_F	57237175	CGGGATCCATGGCCGTTGACCTGCGGAAAATTAAAAAACTG
PBBRBB_ACCB_R	51150625	CATGCCATGGTTAAGAAATCTCGATTAACGTAAATAGGGGTTGGTCAAAGGCG
K12 PKD13 FADE_F	78153350	AGTGGTCAGACCTCCTACAAGTAAGGGGCTTTTCGTTGTGTAGGCTGGAGCTGCTTC
K12 PKD13 FADE_R	78153351	GGATAAAGAAACGGAGCCTTTCGGCTCCGTTATTCATATTCCGGGGATCCGTCGACC

Table 4.1 Primers used in this study.

Plasmid	Description	Reference/Source
pB-*t*	pBBRBB-*'tesA* (cloned from K12)	This study

pB-*t,a*	pBBRBB-*'tesA, accADC, accB*	This study
pB-*t,o,a*	pBBRBB-*'tesA, oleA, accADC, accB*	This study
pB-*t,o,a,fD-2*	pBBRBB-*'tesA, oleA, accADC, accB, fadD-2*	This study
pKD46	curable arabinose inducible lambda red recombinase plasmid	(Datsenko and Wanner, 2000)
pKD13	template plasmid for K12 homologous recombination	(Datsenko and Wanner, 2000)
pCP20	curable yeast Flp recombinase plasmid	(Cherepanov and Wackernagel, 1995)
Strains	**Description**	**Reference/Source**
***S. oneidensis* strain MR-1**	Isolated from Lake Oneida, NY	(Venkateswaran et al., 1999)
***E. coli* strain K12 substrain MG1655**	Wild type	Michael Sadowsky at the University of Minnesota
***E. coli* strain UQ950**	*E. coli* DH5α, λ (*pir*) for cloning	(Saltikov and Newman, 2003)
***E. coli* strain WM3064**	DAP auxotroph donor strain for *Shewanella* conjugation	(Saltikov and Newman, 2003)
JG1611	*S. oneidensis*, pB	This study
JG1946	*S. oneidensis*, Δ*fadE-1,2* pB	This study
JG1892	*S. oneidensis*, Δ*fadE-1,2* pB-*o*	This study
JG1950	*S. oneidensis*, Δ*fadE-1,2* pB-*t*	This study
JG1988	*S. oneidensis*, Δ*fadE-1,2* pB-*t,o*	This study
JG2082	*S. oneidensis*, Δ*fadE-1,2* pB-*t,o,a*	This study
JG2332	*S. oneidensis*, Δ*fadE-1,2* pB-*t,o,a,fD-2*	This study
JG2304	*E. coli* K12 pB	This study
JG2306	*E. coli* K12 Δ*fadE*	This study
JG2307	*E. coli* K12 Δ*fadE* pB-*o*	This study
JG2308	*E. coli* K12 Δ*fadE*pB-*t,o*	This study
JG2314	*E. coli* K12 Δ*fadE* pB-*t*	This study
JG2328	*E. coli* K12 Δ*fadE* pB-*t,a*	This study
JG2344	*E. coli* K12 pB-*t*	This study
JG2310	*E. coli* K12 Δ*fadE* pB-*t,o,a*	This study
JG2327	*E. coli* K12 Δ*fadE* pB-*t,o,a, fD-2*	This study

Table 4.2 Plasmids and strains used in this study.

Acc expression vector design

Primers used are listed in Table 4.2. Acc subunits AccA (locus tag b0185), AccB (locus tag b3255), AccC (locus tag b3256), AccD (locus tag b2316) from *E. coli* strain K12 substrain MG1655 were used as a query subjects for NCBI protein blast. The Acc homologue in the MR-1 genome was identified as two gene products, one being a 4.5 kb

gene representing subunits AccA, AccD, and AccC (locus tag SO0840), and the second being a 0.5 kb gene representing subunit AccB (locus tag SO0511). An MR-1 colony was used as the template for PCR amplification of *accADC* and *accB.* BamHI and NotI were used to digest the *accADC* PCR product, and BamHI and NcoI were used to digest the *accB* PCR product. Techniques for cloning were described previously in chapter 2.

'TesA expression vector design

An *E. coli* strain K12 substrain MG1655 colony was used as a PCR template to amplify *'tesA* (locus tag b0494). Primers were designed to amplify the *'tesA* gene excluding the nucleotides coding for the N-terminal 26 amino acid leader sequence for localization to the periplasm. The forward primer contained a BglII restriction site, followed by atg to introduce a new start codon and the homologous nucleotide sequence for priming starting at amino acid 27. The *'tesA* PCR product was digested with BglII and NotI restriction enzymes and cloned as described previously in chapter 2.

Conjugal transfer of plasmids

Previously described in chapter 2.

T**argeted gene disruption in *E. coli***

Primers for disrupting *fadE* in *E. coli* are listed in 4.1. Targeted disruption in *E. coli* was described previously in chapter 3.

'TesA activity assay

Single colonies of each strain were inoculated into 2 mL LB supplemented with 50 μg·mL^{-1} km. Cultures were incubated at 30°C with shaking at 250 rpm overnight. Cultures were back-diluted to an OD_{600nm} of approximately 0.05 in 5 mL of LB and 50 μg·mL^{-1} km. Cultures were incubated at 30°C with shaking at 250 rpm until late log phase (OD_{600nm} 0.6 to 0.9). 50 μL of culture was collected for BCA total protein analysis. The remaining culture was pelleted at 4,000 X g for 15 minutes at 4°C. The supernatant was decanted, and the pellet was resuspended in 1 mL of ice cold lysis buffer (20 mM phosphate buffer pH 7.4, 0.5 M NaCl, EDTA-free protease inhibitor from Roche Applied Science). Cells were lysed by sonication using the Branson Digital Sonifier, 20 cycles of 1 second pulse and 3 seconds rest at 30% amplitude. Cultures were centrifuged at 14,000 X g for 10 minutes at 4°C, and the soluble fraction was transferred to a clean

1.5-mL microcentrifuge tube. Per reaction volume of 100 µL in a 96 well plate: 1 mM DTNB, 20 mM phosphate buffer pH 7.4, 40 µM acyl-CoA substrate, and 5 µl of soluble cell extract. The above reagents were added to the 96-well plate, and the absorbance at 412 nm was measured every 20 seconds to determine the V_0 of thioesterases on different acyl-CoA substrates (C10, C12, C14, C16).

Growth of strains in minimal medium with fatty acid carbon sources

Previously described in Chapter 2.

Chloroform/methanol extraction of nonpolar molecules

Previously described in Chapter 2.

Extraction of fatty acids from culture medium

This method is adapted from (Lalman and Bagley, 2004). After 48 hours of growth, 50 µL of culture was removed for protein quantification for normalization. 3 mL of culture was poured into an FEP tube. 6 mL of 1:1 v/v 95% hexane and MTBE was added to the empty culture tube and vortexed, and then poured into the FEP tube. 510 µL of 5 M NaCl was added to the tube, followed by 240 µL of 50% w/v H_2SO_4. The tube was vigorously mixed by hand for two minutes and centrifuged for 2 minutes at 2,000 rpm to facilitate phase separation. The upper hexane layer was then removed with a glass pipette and dispensed into a twice chloroform rinsed vial. The direct FAME protocol then could be carried out to methyl esterify fatty acids.

Direct esterification of fatty acids

Previously described in Chapter 2.

Analysis of hydrocarbons and FAME products by GC-FID-MS

Previously described in Chapter 2.

Results

Attempts to clone a mutation-free *'tesA* gene into expression vectors were not successful. Multiple clones were isolated and sequenced, revealing deletion and substitution mutations in different regions of the sequence in every construct assembled (table 4.3).

tesA mutants		
Mutant ID	**Description of Mutation**	**Amino Acid Change**
1	287 bp T-->C, 349 bp C-->T	96 V-->A, 117 R-->C
2	226 bp G-->A, 497 bp T-->A	76 G-->S, 166 F-->Y
3	278 bp T deletion	NA
4	209 bp A-->G	70 E-->G
5	180 bp A-->G, 224 bp A-->G, 233 bp G-->A	75 D-->G, 78 R-->H
6	500 bp T-->A	167 I-->N

Table 4.3. Various *'tesA* mutations identified from cloning in *E. coli* UQ50. Both the nucleotide base mutation is identified and the corresponding amino acid change.

The persistence of mutations at random locations within the *'tesA* sequence suggests that expression of 'TesA is toxic to the cell and strong selective pressures resulted in cloning of mutated, less toxic forms of *'tesA*. An unmutated *'tesA* gene was successfully cloned into an expressionless vector. Efforts to restriction digest this unmutated *'tesA* out of the expressionless vector and clone it into a vector with expression tightly regulated by the araBAD promoter resulted in no colonies with the *'tesA* insert most likely due to toxicity of the gene product. Surprisingly, previously published research accomplished this by expressed 'TesA from a multicopy plasmid utilizing an arabinose inducible system to reduce potential toxicity in cultures in lag and early growth phase (Cho and Cronan, 1995). Upon *'tesA* induction, they showed that these cultures exhibited no adverse growth defects. The collected *'tesA* mutants were tested to identify the most ideal *'tesA* mutant for overproduction of fatty acids.

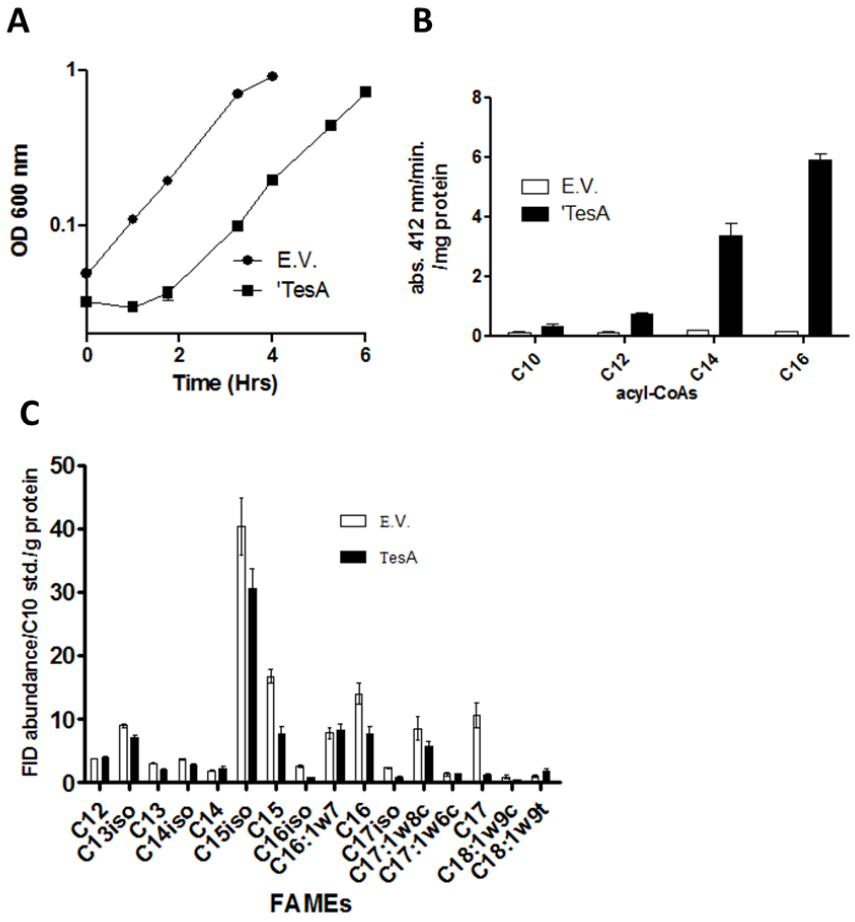

Figure 4.1 Analysis of 'TesA expression in *S. oneidensis fadE* double knockout. A) Growth of strain expressing 'TesA. B) Cell lysate thioesterase activity assay using 40 µM C10, C12, C14, C16 CoA substrates normalized to mg of protein. C) FAME profile of strain expressing 'TesA reported as FID abundance as a percent of the methyl decanoate (C10) standard per gram of protein.

These mutants were assayed for growth defects, which would suggest phospholipid biosynthesis competition and acyl-CoA thioesterase activity. Cellular stress is an indication that 'TesA expression causes substrate competition for acyl-ACPs which are required for phospholipid synthesis. Based on growth experiments in rich medium, TesA 1 had the most extreme phenotype, highlighted by flocculating cells and a long lag before entering growth phase relative to the other *'tesA* mutants and for all the following experiments this mutant was used (figure 4.1a). To assay the activity of 'TesA, a simple colorimetric thioesterase activity assay using DTNB was employed. This assay takes

advantage of the release of CoA from acyl-chains, resulting in free CoASH in the reaction. The sulfur group of the free CoASH readily reacts with and cleaves the disulfide bond of DTNB, forming CoA-NTB product and a NTB^{2-} product that absorbs at 412 nm. As more CoA is released, the increase in absorbance at 412 nm can be measured to determine the initial velocity and specificity of thioesterases for different length acyl-CoA substrates. Ideally, the 'TesA thioesterase activity of interest is on acyl-ACP substrates to deregulate fatty acid synthesis. Using the DTNB assay for acyl-ACPs would require purification of native MR-1 ACP and conjugation to acyl-chains before assaying, making the use of acyl-ACP as substrates expensive and time consuming. Acyl-CoAs are instead used as an approximation to acyl-ACP thioesterase activity. 'TesA was assayed for activity against decanoyl (C10), dodecanoyl (C12), tetradecanoyl (C14), and hexadecanoyl-CoA (C16).

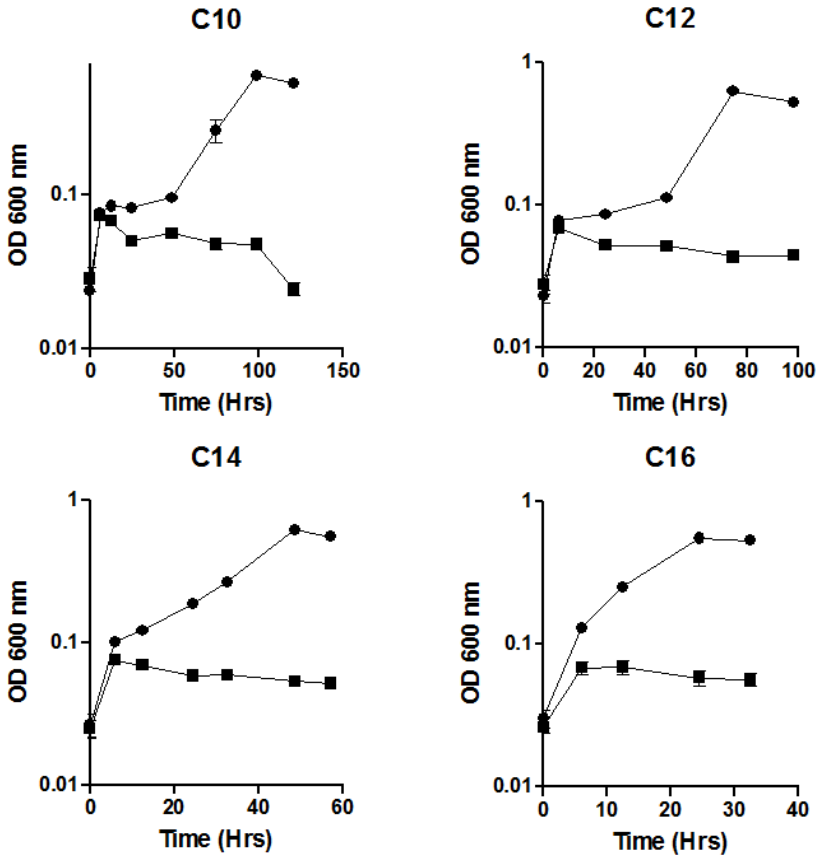

Figure 4.2. Growth analysis of *E. coli fadE* mutant. Growth on saturated fatty acids: C10, C12, C14, C16. (●) *E. coli* WT, (■) *E. coli* Δ*fadE*.

Previously published literature indicates that ‘TesA has the highest specificity for hexadecanoyl-CoA (Cho and Cronan, 1995). DTNB-based thioesterase activity assays indicate that ‘TesA has the highest specificity for hexadecanoyl-CoA, followed by tetradecanoyl-CoA, dodecanoyl-CoA, and decanoyl-CoA (figure 4.1b). From these criteria, *‘tesA* mutant 1 was selected due to the distinct growth phenotype (figure 4.1a) and high acyl-CoA thioesterase activity (figure 4.1b) compared to the other *‘tesA* mutants. This strain was then assayed for fatty acid production by extraction from cultures and subsequent fatty acid methyl esterification of the extract (figure 4.1c). The resulting fatty acid profile provided evidence that *‘tesA*, when expressed in the *S. oneidensis fadE* double knockout, produced fewer fatty acids overall. *E. coli* was transformed with the ‘TesA expression vector to determine if the mutant *‘tesA* still can deregulate fatty acid synthesis or if it has lost this activity as a result of the mutations. In order to carry out this experiment, an *E. coli* strain deficient in β-oxidation was generated by disrupting *fadE* and confirmed by culturing the *E. coli fadE* mutant in SBM with saturated fatty acids C10, C12, C14, C16 (figure 4.2).

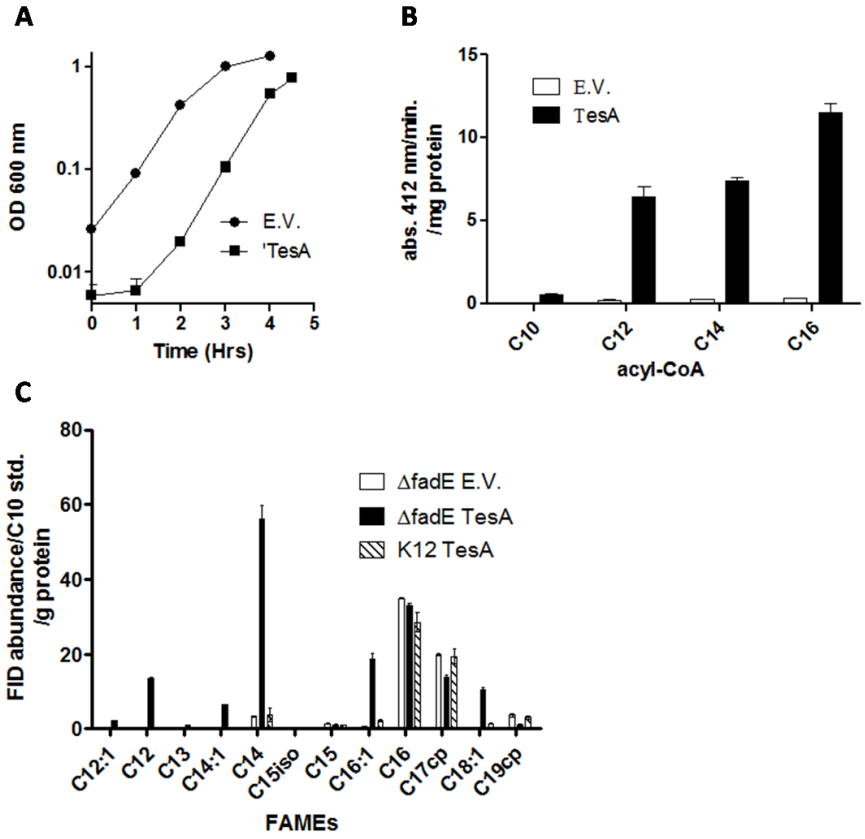

Figure 4.3 Analysis of 'TesA expression in *E. coli fadE* knockout. A) Growth of strain expressing 'TesA in rich medium. B) Cell lysate thioesterase activity assay using 80 µM C10, C12, C14, C16 CoA substrates normalized to mg of protein. C) FAME profile of strain expressing 'TesA reported as FID abundance as a percent of the methyl decanoate (C10) standard per gram of protein.

The *E. coli* strain was cultured in the same medium, extracted, and esterified using the same procedures as those used for *S. oneidensis*. The same assays were carried with the *E. coli* β-oxidation mutant expressing 'TesA as those done with the *S. oneidensis* characterizing growth of the strain (figure 4.3a), thioesterase activity on acyl-CoAs (figure 4.3b), and the fatty acid profile (figure 4.3c). A similar lag in growth and acyl-CoA activity was observed for the *E. coli* strain when compared to *S. oneidensis.* After 24 hours of growth, a thin oil sheen was present on the surface of the culture that was not observed in the vector control strain, suggesting fatty acid production. That observation was confirmed by the results from the FAME analysis, indicating significant increases of

fatty acids C12, C14, and C16 not observed in the *S. oneidensis* strain expressing ‘TesA. Furthermore, fatty acid production overall was increased approximately 2.5- fold and 15 to70- fold for some individual fatty acids (table 4.4). Additionally, overexpression of ‘TesA in an *E. coli* strain that has a functional β-oxidation pathway results in a similar fatty acid profile as the β-oxidation mutant control strain (figure 4.3c). To verify the lack of fatty acid production observed in *S. oneidensis* was not due to a mutation in *‘tesA*, the ‘TesA expression vectors were isolated from both the *E. coli* and *S. oneidensis* strains, sequenced, and transformed into the corresponding strain and tested. No additional mutations were identified in either ‘TesA expression vector and the *S. oneidensis* strain transformed with the ’TesA expression vector purified from the *E. coli* β-oxidation mutant resulted in the same FAME profile and the *E. coli* strain transformed with the ‘TesA expression vector from *S. oneidensis* still overproduced fatty acids (data not shown).

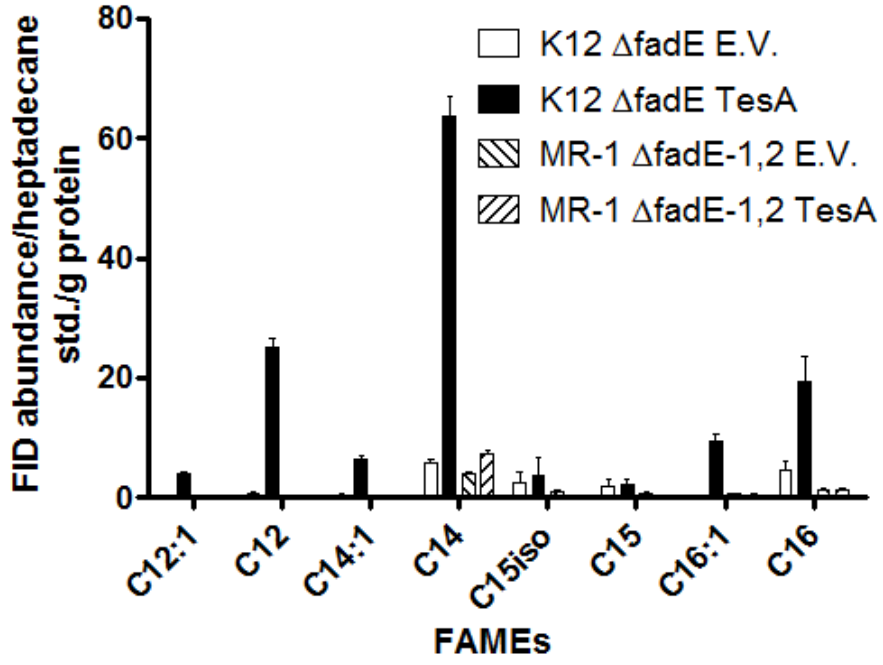

Figure 4.4. Comparison of fatty acids secreted into the medium from *S. oneidensis* and *E. coli* β-oxidation mutants expressing TesA. Results are reported as FID abundance as a percent of the methyl decanoate (C10) standard per gram of protein.

The presence of an oil sheen on the aqueous surface of the culture suggests that fatty acids were being secreted into the medium. In order to verify this observation and to quantify how much and which fatty acids were being secreted, the culture was subjected to a hexane/MTBE nonpolar extraction (Lalman and Bagley, 2004) that leaves cellular membranes intact. Fatty acids from the extract were methyl-esterified as described previously and showed that C12, C14, and C16 fatty acids were being secreted into the medium in the *E. coli* β-oxidation mutant expressing ‘TesA but minimal fatty acids were present in the vector control (figure 4.4). Both the *S. oneidensis* β-oxidation mutant expressing ‘TesA and vector control had minimal fatty acids present in the medium relative to the *E. coli* β-oxidation mutant expressing ‘TesA as expected.

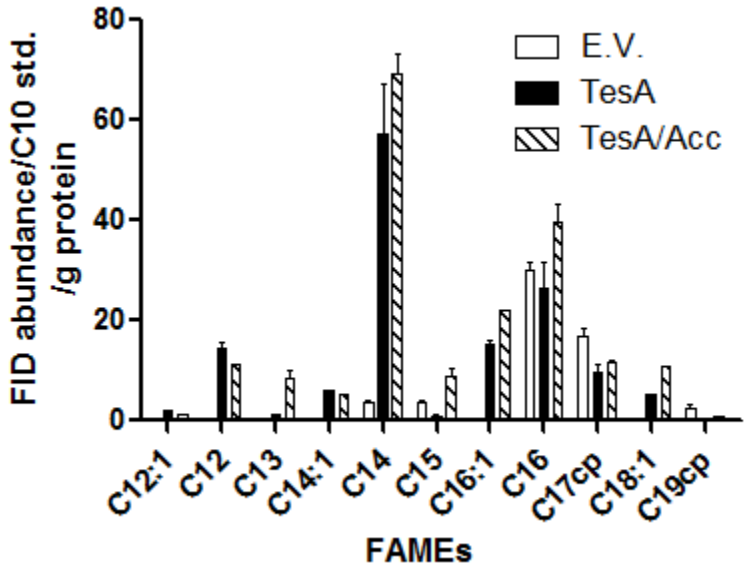

Figure 4.5. Fatty acid production from *E. coli* strains co-expressing TesA and Acc. Results reported as FID abundance as a percent of the methyl decanoate (C10) standard per gram of protein.

Compared to the control strain, the *E. coli* β-oxidation mutant expressing ‘TesA overproduces fatty acids by approximately 2.5-fold. The first committed step in fatty acid synthesis is the formation of malonyl-CoA from acetyl-CoA by Acc. This is a critical step since acetyl-CoA is fed into the fatty acid synthesis pathway from central metabolism and this step has been shown to be a rate limiting in *E. coli*. Therefore, the

acc genes from *S. oneidensis* were cloned and added to the BioBrick™ vector already containing *'tesA* to see if this would increase fatty acid production by funneling more precursors into the fatty acid synthesis pathway. The fatty acid profile of the 'TesA and Acc co-expression vector was determined and compared to the 'TesA expression vector in the *E. coli* β-oxidation mutant background and showed that co-expression of 'TesA with Acc resulted in a 3.3-fold increase in fatty acid production over the control strain (figure 4.5 and table 4.4). Interestingly, the C13 and C15 fatty acids were the most significant increases compared to the 'TesA expression strain.

Hydrocarbon production was next assayed by cloning *oleA* into the BioBrick™ vectors in different combinations with other genes targeted for increasing hydrocarbon production. The following constructs were then used to transform the *S. oneidensis* β-oxidation mutant and were assayed for hydrocarbon production: pB-*o*, pB-*t,o*, pB-*t,o,a*, pB-*t,o,a,fD-2*. These results showed that hydrocarbon production was unchanged by expression of any of the above genes targeted to increase hydrocarbon production (figure 4.6). These same constructs were then used to transform the *E. coli* β-oxidation mutant strain in hopes that since 'TesA deregulated fatty acid synthesis in *E. coli* that these targeted genes would increase hydrocarbons in this strain. Results indicated that they did not, and that expression of additional genes caused a decrease in hydrocarbon production (figure 4.6). The observation was also made that *E. coli* and *S. oneidensis* strains expressing OleA make approximately the same amount of total hydrocarbons, but *E. coli* hydrocarbons consist of fewer hydrocarbon species when compared to *S. oneidensis* (data not shown).

Plasmid	**E.V.**		**TesA**			**TesA, Acc**		
Fatty Acids	Average	std. dev.	Average	std. dev.	fold inc./E.V.	Average	std. dev.	fold inc./E.V.
C12:1	0.00	0.00	2.00	0.02	NC	1.22	0.13	NC
C12	0.21	0.05	14.28	2.21	68.23	11.23	0.26	53.67
C13	0.00	0.00	1.29	0.24	NC	8.55	1.99	NC
C14:1	0.18	0.03	5.88	0.29	33.12	5.26	0.24	29.59
C14	3.79	0.20	57.44	13.70	15.15	69.32	5.41	18.28
C15	3.60	0.38	0.99	0.37	0.28	9.02	2.02	2.50
C16:1	0.25	0.10	15.13	1.61	59.81	22.26	0.13	87.62
C16	30.18	2.26	26.54	7.44	0.88	39.86	4.96	1.32

C17cp	16.89	2.33	9.59	2.29	0.57	11.47	0.94	0.68
C18:1	0.00	0.00	5.10	0.51	NC	10.76	0.37	NC
C19cp	2.37	1.03	0.00	0.00	0.00	0.77	0.09	0.33
Total FA	57.47		138.24			189.62		
Total FA fold inc/E.V.	1.00		2.41			3.30		

Table 4.4. Fatty acid (FA) quantities represented as FID abundance as a percent of the methyl decanoate (C10) standard per gram of protein. Background strain used for all experiments was the *E. coli fadE* mutant. NC=not calculable. Colon and subsequent number indicate the number of double bonds in fatty acid, cp indicates cyclopropane group within the fatty acid.

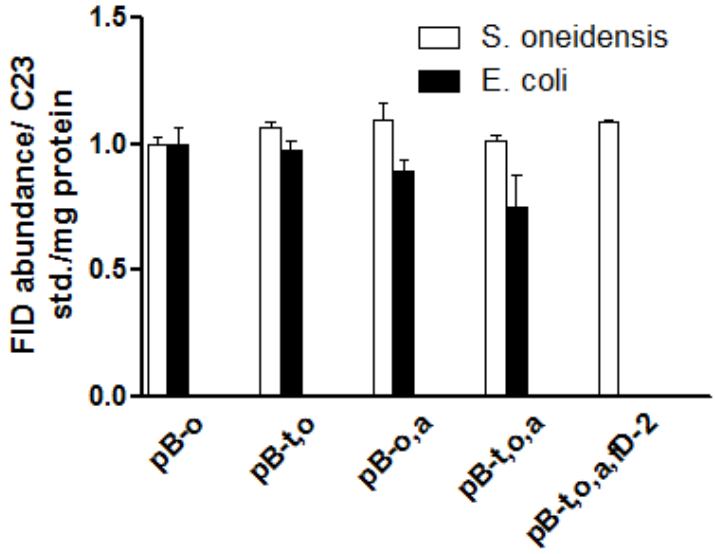

Figure 4.6. Hydrocarbon production in *S. oneidensis* and *E. coli fadE* mutants deficient in β-oxidation of LCFAs. Hydrocarbon production is reported as a percent of the 12-tricosanone (C23) standard per mg of protein with results displayed as values relative to pB-*o* for each strain.

Discussion

Results from the extraction and methyl esterification show that the fatty acid profile has decreased levels of fatty acids in the strain expressing ‘TesA when compared to the vector control in *S. oneidensis*. This result was unexpected when considering the results obtained from expression of ‘TesA in an *E. coli fadE* knockout resulting in a significant increase in fatty acids (2.5-3 fold) (Cho and Cronan, 1995). Cho and Cronan observed no growth defects upon induction of the arabinose inducible pBAD22 vector

expressing *'tesA* and there is no mention of mutations within the gene. In order to determine if the mutation in the gene of *'tesA* rendered it unable to cleave acyl-ACPs and thereby deregulate fatty acid synthesis, 'TesA was used to transform an *E. coli* strain deficient in β-oxidation by deletion of *fadE*. FAME analysis indicated approximately a 2.5-fold increase in the amount of fatty acids being produced relative to the control strain for the mutated *'tesA*. Fatty acid overproduction in the *E. coli* β-oxidation mutant expressing 'TesA confirms that the mutation in *'tesA* is not responsible for the lack of fatty acid accumulation in *S. oneidensis*, and in fact, produces nearly the same amount of fatty acids as compared to published results by Cho and Cronan using the arabinose inducible pBAD22 expressing the *'tesA*. Furthermore, isolation of the plasmid from both the *S. oneidensis* and *E. coli* strains showed the nucleotide sequence for *'tesA* to be identical. Thioesterase assays using the cell lysate of the *S. oneidensis* and *E. coli* strains expressing 'TesA support that 'TesA is active in the cell on acyl-CoA substrates as well. It is possible that 'TesA does effectively cleave acyl-ACP substrates, suggested by the strong growth defect observed from *S. oneidensis* strains expressing 'TesA, but unlike *E. coli*, fatty acid synthesis is not negatively regulated by the presence of acyl-ACPs. Fatty acid synthesis is a highly conserved process across bacteria, but one of the few areas of differentiation is the primers used for fatty acid synthesis. *S. oneidensis* and *E. coli* have very different fatty acid profiles largely due to the expression of FabH homologues that have different specificity for priming molecules for fatty acid synthesis. FabH from *E. coli* almost exclusively uses acetyl-CoA to prime fatty acid synthesis resulting in straight chain, even numbered fatty acids (Choi et al., 2000) whereas FabH from *S. oneidensis* uses short branched chain CoA substrates as primers resulting in the branched chain, odd numbered fatty acids observed in the fatty acid profile. Gene expression analysis of FabH homologues might reveal differences in transcription levels when 'TesA is expressed in *S. oneidensis* and *E. coli* and this may explain why 'TesA induces fatty acid overproduction in *E. coli* but not *S. oneidensis*.

The presence of an oil sheen on cultures of the *E. coli* β-oxidation mutants expressing 'TesA suggests fatty acids are being secreted into the medium and could pose a significant problem in a hydrocarbon overproducing strain. It is possible that the fatty

acids extracted from the medium in the *E. coli* β-oxidation mutant expressing ‘TesA are the result of lysis, although evidence supports secretion based on the data in figure 4.3c, where an *E. coli* strain expressing ‘TesA that has a functional β-oxidation pathway does not have elevated fatty acid levels suggesting the cells are viable and able to degrade the fatty acids being overproduced. Secreted fatty acids were also observed by Cho and Cronan, where 90% of the fatty acids were secreted into the medium but the cells remained intact based on turbidity, colony-forming ability, and metabolic activity (Cho and Cronan, 1995). To further validate this in a future experiment, enzyme activity of a normally cytoplasmic enzyme could be assayed and compared between the ‘TesA expressing strain and vector control to determine if there is significant lysis. If activity is determined to be due to secretion, fatty acid loss can be minimized by optimizing expression of FadD and OleA so accumulating free fatty acids are quickly converted to the activated CoA forms by FadD and subsequently condensed by OleA to hydrocarbons.

Hydrocarbons were produced in both *S. oneidensis* and *E. coli,* but expressing genes targeted to overproduce hydrocarbons yielded no increases, and in the case of *E. coli*, actually caused a decrease in hydrocarbons. These results further support that the substrates for OleA are most likely not LCFA-CoAs.

Chapter 5

Conclusions and future directions

The ultimate goal of overproducing hydrocarbons from *S. oneidensis* proved a difficult task, but a significant amount of information was gained from this work. *S. oneidensis* has two acyl-CoA dehydrogenase homologues that are capable of degrading long chain fatty acids (C12 and longer), and when both are disrupted, the strain can no longer oxidize long chain fatty acids. This was an important step in engineering a hydrocarbon overproducing strain so that the fatty acid precursors being overproduced and needed for hydrocarbon synthesis were not siphoned off into the β-oxidation pathway. Disruption of these homologues and subsequent analysis revealed redundant specificity for fatty acid substrates. This was an interesting finding, and it is possible that the expression of each homologue is regulated differently. Additionally, a third acyl-CoA dehydrogenase appears to exist for medium chain fatty acids that has yet to be identified. Future experiments include determining under what conditions each long chain acyl-CoA dehydrogenase homologue is upregulated and identifying the third homologue responsible for medium chain acyl-CoA dehydrogenase activity.

Questions are raised as to the substrate for OleA in the condensation of fatty acids. Initially, it was believed that long chain acyl-CoAs were the substrate for the OleA condensation reaction. However, disruption of the two fatty acyl-CoA ligase homologues responsible for activating fatty acids with CoA still leads to the production of hydrocarbons. Additionally, the acyl-CoA ligase double knockout strain is incapable of growing on long chain fatty acids because long chain fatty acids cannot be activated with CoA and thereby targeted for β-oxidation. It was also found that the two acyl-CoA ligases have fatty acyl-CoA chain length specificity: FadD-1 was a better activator of long chain fatty acids (C16 and C18) and FadD-2 of shorter chain fatty acids (C6), but both homologues are equally capable of degrading C12 and C14 fatty acids. It is most likely that long chain fatty acyl-ACPs or potentially some other fatty acid derivative are the preferred substrate. Surprisingly, complementation of the *E. coli fadD* mutant that is also unable to oxidize fatty acids with FadD-1 and FadD-2 shows that FadD-2 is unable to restore growth on fatty acids as it does in the *S. oneidensis* complementation. The

different chain length specificity for the FadD homologues could have potential biotechnological applications such as in the synthesis of fatty acid ethyl esters of different chain lengths to make biodiesel and other platform chemicals (Steen et al., 2010).

Deregulating fatty acid synthesis by methods developed in *E. coli* proved unsuccessful when applied to *S. oneidensis*. The method itself was validated by deregulating fatty acid synthesis in an *E. coli fadE* mutant deficient in β-oxidation. This data indicated that the thioesterase 'TesA is active in both *S. oneidensis* and *E. coli,* and both strains exhibit a similar lag in growth, most likely associated with competition for long chain acyl-ACPs with the phospholipid synthesis pathway. One possible explanation is that the cytoplasmic concentration of long chain acyl-ACPs in *S. oneidensis* does not cause feedback inhibition of enzymes involved in fatty acid synthesis as occurs in *E. coli*. By this explanation, when long chain acyl-ACPs are cleaved in *S. oneidnesis*, no change in fatty acid synthesis occurs where in *E. coli* this allows synthesis to continue unabated because the allosteric inhibitor, long chain acyl-ACPs, has been removed. Perhaps in *S. oneidensis*, feedback regulation of fatty acid synthesis occurs further downstream from intermediates in the phospholipid synthesis pathway.

When plasmids designed to overproduce hydrocarbons are used to transform *S. oneidensis,* there is no effect on hydrocarbons, and when those same plasmids are used to transform *E. coli,* it results in a decrease in hydrocarbon production. This information coupled with the result that the *S. oneidensis fadD* double mutant expressing OleA still synthesizes hydrocarbons suggests that the substrate for OleA is actually not long chain acyl-CoAs. Future directions should focus heavily on identifying other fatty acid derivatives that could be substrates for OleA. It would also be worth exploring the regulation of genes involved in fatty acid synthesis in *S. oneidensis* since this seems to differ from *E. coli.* A great amount of information was gained in *S. oneidensis* physiology pertaining to fatty acid metabolism and the resulting potential biotechnological applications related to fuel production provide many research opportunities to explore.

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