UNIVERSITY OF CALIFORNIA, SAN DIEGO

Heterologous expression of an uncharacterized thioesterase in *E. coli* and its relationship to polyunsaturated fatty acid synthesis

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The thesis of Marco Nicholas Allemann is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Heterologous expression of an uncharacterized thioesterase in *E. coli* and its relationship to polyunsaturated fatty acid synthesis

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To interrogate the possible interactions between the polyunsaturated fatty acid (PUFA) and hydrocarbon yielding pathways in *Shewenalla pealeana*, the *pfaA-E* genes along with *oleB*, an annotated thioesterase enzyme, were heterologously expressed in a BL21 DE3 *fadD::kan* strain of *Escherichia coli*. The gene knockout in *E. coli* was performed using lambda red recombination in the TOP 10 strain and P1 phage transduction was used to move the mutation into BL21 DE3. Strains containing various combinations of *pfaA-E* and *oleB* expression constructs were cultured at 16°C and analyzed for fatty acid content using gas chromatography-mass spectrometry (GC/MS). Expression of *oleB* correlated with increases in 18:1 and 16:1 content along with decreases in 16:0 content, which overall contributed to higher unsaturated to saturated

fatty acid ratios (UFA/SFA). Eicosapentaenoic acid (EPA) content varied from 5% to 13% in strains carrying the *pfaA-E* genes and variations in EPA content were not correlated with *oleB* expression. The proposed 16:4 fatty acid precursor of 31:9 polyunsaturated hydrocarbons found in species of *Shewanella* was not detected in any of the strains analyzed in this study.

Introduction

1.1 Lipid biology

Lipids are one of the essential macromolecules for life along with nucleic acids, carbohydrates, and amino acids. Lipids are found in all biological membranes in the form of bilayers where the hydrophobic alkyl chains are associated with one another and hydrophilic components, most often linked through glycerol molecules face outwards. Lipid membranes exist in a semi-fluid state and this state is critical to their function especially as it relates to membrane bound proteins that must move within the membrane to carry out their respective functions. The bilayer maintains its fluidity by incorporating unsaturated fatty acids, which have lower melting points and allow the membrane to assume a semi-fluidic state (Fujita et al., 2007). Maintaining this semi-fluid state is essential to maintaining the physiological functions of the membrane in all forms of life. Environmental stresses such as increased hydrostatic pressure and/or decreased temperature negatively affect the fluidity of the lipid membrane. Fatty acid and hydrocarbon molecules will be referred to by their common name or using a designation such as 20:5 (20 carbons, 5 double bonds).

Production of fatty acids and hydrocarbons by bacteria and other microbes have recently received much attention due to their renewability and possible applications to both nutriceutical and biofuel industries (Ratledge, 2004). In particular, the class of lipids known as polyunsaturated fatty acids (PUFAs), are of particular interest due to

their beneficial role in human brain development (Wainwright, 2007) and cardiovascular health (Jump et al., 2012). Human health relevant PUFAs such as eicosapentaenoic acid (20:5 *n*-3; EPA) and docosahexaenoic acid (22:6 *n*-3; DHA) are commonly known as omega-3 fatty acids because their double bond is located 3 carbons away (*n*-3 designates this) from the alkyl terminus. Such lipids are commonly available as nutritional supplements and are most often derived from fish oils (Kris-Etherton and Hill, 2008). However, with fish stocks in decline and the increasing risks associated with consumption of seafood (Kris-Etherton and Hill, 2008) research towards understanding mechanisms of PUFA synthesis in bacteria is key to providing a renewable and safer source of these biologically important compounds.

1.2 Fatty acid synthesis in *E.coli*

In the case of *Escherichia coli,* at optimal growth temperatures such as 37°C, the lipid membrane is predominantly made up of palmitic (16:0), palmitoleic (16:1) and *cis*-vaccenic (18:1) acid with palmitic acid being the dominant constituent as determined by percent weight (Marr and Ingraham, 1962). When grown at lower temperatures, *E. coli* cells remodel their membrane by the increased production and incorporation of mono-unsaturated fatty acids such as *cis*-vaccenic and palmitoleic acid into the lipid membrane (Marr and Ingraham, 1962). Other changes due to environmental stresses include the incorporation of cyclopropane fatty acids, which are thought to be a response against the

increased acidification within a culture during stationary phase (Chang and Cronan, 1999).

Fatty acids are produced from acetyl-CoA, which can be derived from many metabolic pathways. During synthesis an important factor is the acyl-carrier protein (ACP) that covalently binds the growing acyl chain via a thioester bond. The first step towards fatty acid biosynthesis is the carboxylation of acetyl-CoA to form malonyl-CoA, which is accomplished by acetyl-CoA carboxylase (Magnuson et al., 1993). Malonyl-CoA is then transferred to an ACP moiety by a malonyl-CoA/ACP transferase releasing a free CoA group and reforming a thioester linkage (Magnuson et al., 1993). Decarboxylation catalyzed condensation of malonyl-ACP and one acetyl-CoA or acyl-ACP will generate a β-ketoacyl-ACP intermediate. Within *E. coli* there are three ketoacyl-ACP synthases; FabH, FabB, and FabF (Fujita et al., 2007). FabG will then reduce the 3-ketoacyl-ACP group to 3-hydroxyacyl-ACP, FabZ then dehydrates the 3-hydroxyacyl-ACP group and FabI reduces the enoyl group to yield a saturated alkyl chain (Magnuson et al., 1993). This cycle of condensation, reduction, dehydration, and reduction is cyclically repeated with each cycle increasing the alkyl chain length by 2 carbons. During the synthesis of *cis*-vaccenic (18:1) or palmitoleic acid (16:1) by *E. coli* the incorporation of unsaturations in the alkyl chain is accomplished by *cis* isomerization of the decenoyl-ACP intermediate by FabA (Magnuson et al., 1993). Conversely, if the decenoyl-ACP intermediate is in the *trans* configuration FabI reduces the double bond using NADH and the cycle continues and leads to a saturated fatty acid (Fujita et al., 2007). FabF is responsible for thermal regulation of unsaturated fatty acid content in *E.*

coli. At lower temperatures, the relative activity of FabF increases leading to enhanced palmitoleic acid to *cis*-vaccenic acid conversion (Heath and Rock, 2002).

1.3 Types of fatty acid biosynthesis in Bacteria

There are three known routes for the biosynthesis of fatty acids in bacteria (Shulse and Allen, 2011). The first is known as the Type I fatty acid pathway wherein all of the enzymatic domains for fatty acid biosynthesis are found on a single multi-domain protein complex. The second is known as the Type II or dissociated pathway wherein all of the catalytic enzymes are found on separately encoded genes and therefore separate proteins. A third type of fatty acid biosynthetic pathway found in bacteria is known as the polyketide synthase pathway (PKS) which is associated with the production of unique fatty acids such as PUFAs and a wide variety of other polyketide natural products (Metz et al., 2001). Similar to the Type I pathway, the PKS pathway proteins often contain multiple catalytic domains required for synthesis on one polypeptide chain and these multi-domain proteins are often clustered together to form a larger complex (Metz et al., 2001). In marine bacteria such as *Shewanella* sp. where both Type II and PKS pathways are present, products formed by the PKS pathway, such as DHA and EPA, have been labeled "secondary lipids" to highlight their separation from the "core" fatty acids produced by the Type II pathway (Shulse and Allen, 2011). Despite the unique nature of polyketide products the precursors for the pathway are still derived from acetyl starter units (Metz et al., 2001). Polyketide products are often highly derivatized and/or cyclized

with multiple hydroxyl and alkene functional groups because of the incomplete dehydration/reduction during select reaction cycles (Metz et al., 2001).

1.4 Production of alkenes through the *ole* pathway

The biosynthesis of hydrocarbon molecules is found throughout the bacterial domain of life and the products can range in size from 11 to 35 carbons (Ladygina et al., 2006). To date, there are two broad categories of biosynthetic pathways that are thought to be responsible for the production of bacterial hydrocarbons. The first is known as the condensation/decarboxylation pathway that resembles the synthesis of fatty acids but is followed by a decarboxylation reaction that removes the carboxylic acid functional group yielding an alkyl chain (Ladygina et al., 2006). The other pathway involves a "head-to-head" condensation between two fatty acids or acyl-CoAs (Beller et al., 2010; Frias et al., 2011; Sukovich et al., 2010a).

Hydrocarbon biosynthesis gene clusters involved in the head-to-head condensation pathway have been putatively identified in the genomes of 69 Bacteria genomes and none have been identified in the available Eukaryota or Archaea genomes (Sukovich et al., 2010b). A study using *Shewanella oneidensis* MR-1, a member of the Gamma-proteobacteria, identified a four gene operon that was responsible for production of a unique head-to-head condensation hydrocarbon, hentriacontanonaene (31:9), and designated the four genes *oleA, oleB, oleC,* and *oleD* (Sukovich et al., 2010b). The four genes are usually clustered together in an operon structure, as is the case in *Shewanella* sp.*,* but other arrangements are possible (Sukovich et al., 2010b). One other notable

arrangement of these four functions can be found within *Micrococcus luteus* where the gene cluster contains three genes, one of which is a fusion of *oleB* and *oleC* (Beller et al., 2010; Sukovich et al., 2010b).

The genes encoding the required enzymes for biosynthesis in the head-to-head model were first described in *Micrococcus luteus* as a three-gene cluster that produced long-chain alkenes when heterologously expressed in a fatty acid overproducing strain of *E. coli* (Beller et al., 2010). Orthologous genes from *Stenotrophomonas maltophilia* were also described in an international patent application where expression of these genes in a fatty acid overproducing *E.coli* mutant also led to the production of alkenes that appeared to be derived from head-to-head condensation between known Type II fatty acid constituents (30 November 2008, WO2008/113041). Deletion of the entire *ole* operon or a deletion of *oleA* in *Shewanella oneidensis* MR-1 resulted in a lack of synthesis of hentriacontanonaene (31:9) in *S. oneidensis* MR-1 (Sukovich et al., 2010a, 2010b). Similarly, expression of the gene cluster from *M. luteus* was required for biosynthesis of alkene products in the fatty acid overproducing strain of *E. coli* (Beller et al., 2010).

Based on amino acid sequence data the four genes found in *S. oneidensis* MR-1 were classified into KEGG protein superfamilies (Sukovich et al., 2010b). OleA was identified as a condensing thiolase due to its sequence similarity to FabH, one of the three ketoacyl synthases found in *E. coli* (Sukovich et al., 2010b). OleB is a member of the α/β hydrolase family based on amino acid alignments and is thought to function as an acyl-ACP or acyl-CoA thioesterase at some point in the pathway (Sukovich et al., 2010b). OleC is part of the AMP-dependent ligase/synthase superfamily, which links a CoA

group to a free fatty acid thereby activating it for downstream reactions such as condensation with another fatty acid (Sukovich et al., 2010b). OleD is a member of the short chain dehydrogenase/reductase superfamily and is thought to be responsible for reduction and dehydration of the ketone product produced by the head to head condensation catalyzed by OleA (Frias et al., 2011; Sukovich et al., 2010b).

To date, OleA is the sole protein from this gene cluster that has been purified and functionally assayed (Frias et al., 2011; Sukovich et al., 2010b). OleA from *Xanthomonas campestris* was found to catalyze a non-decarboxylative Claison condensation reaction *in vitro* between two fatty acid-CoA molecules resulting in the loss of a carbon and two CoA groups (Frias et al., 2011). In the *in vitro* assay 8 to 16 carbon length acyl-CoAs were condensed by purified OleA and identification of a β-keto carboxylic acid intermediate indicated that the mechanism did not involve decarboxylation (Frias et al., 2011). The complete pathway and steps of this head-to-head condensation pathway is unknown and the role(s) that the other *ole* gene products play in the pathway is still unclear.

1.5 Physiological Roles of PUFAs and *ole* pathway hydrocarbons

There has been much speculation as to the physiological role of PUFAs in marine bacteria, but what is readily apparent is that PUFAs are a remarkable example of multifunctional adaptation. The co-occurrence of piezophilic organisms with PUFA production led to the hypothesis that these polyunsaturated fatty acids play a critical role in maintaining membrane fluidity at high hydrostatic pressures and/or low temperatures

(Delong and Yayanos, 1986). Studies using the marine bacterium *Photobacterium profundum* SS9, a psychro-tolerant piezophile, showed that monounsaturated fatty acids are required for growth at high pressures and low temperature (Allen, Facciotti, & Bartlett, 1999). One *P. profundum* mutant, which contained higher PUFA content and lower monounsaturated fatty acid content, was able to grow at lower temperatures and/or higher pressures at a slower rate indicating that PUFA production does play some role in high pressure and/or cold temperature adaptation (Allen et al., 1999). More recent studies have shown that *Shewanella* sp. deficient in PUFA synthesis display abnormal cell physiology when grown at higher pressures (Kawamoto et al., 2011) or lower temperatures (Kawamoto et al., 2009). In particular, the loss of EPA production in the high pressure adapted *Shewanella violacea* led to filamentous cells that contained multiple FtsZ rings, which is indicative of a blockage in the final stages of cell division (Kawamoto et al., 2011). While membrane fluidity dynamics might be partly responsible for this effect, another explanation is that PUFAs play an essential role in the recruitment of key membrane bounded proteins similar to lipid rafts. Another plausible role for these lipids given their high level of unsaturation is that they form a protective shield against oxidative damage caused by reactive oxygen species and other harmful free radicals (Nishida et al., 2007). Experimental work on this effect showed that both *Shewanella marinintestina*, a known producer of EPA, as well as recombinant *E. coli* that produced EPA were both resistant to oxidative stress from hydrogen peroxide and that this was dependent on the production of EPA (Nishida et al., 2006, 2007).

The role that hydrocarbons play in the cellular physiology of their respective producers is also not yet known. The 31:9 hydrocarbon, produced by select *Shewanella* sp., was first chemically indentified in an Antarctic marine bacterium which is capable of PUFA production and therefore was proposed as a possible biomarker for PUFA production (Nichols et al., 1995). The production of this polyunsaturated hydrocarbon was thought to be an additional adaptation to the extreme cold temperatures found in the Antarctic (Nichols et al., 1995). While the cold adaptation explanation does seem plausible considering the high degree of unsaturation seen in the compound, the same compound has been discretely identified in two mesophilic organisms; the tropical isolate *Shewanella amazonensis* (Motoigi and Okuyama, 2011) and *Shewanella oneidensis* MR-1 (Sukovich et al., 2010a). In both instances, lower than optimal growth temperatures led to an increase in the amount of hydrocarbon being produced. One possible explanation for this data is that both *S. oneidensis* MR-1 and *S. amazonensis* produce no significant amounts of EPA (Kato and Nogi, 2001) and the 31:9 hydrocarbon is simply a substitute for EPA (Motoigi and Okuyama, 2011).

The physiological role(s) of non-PUFA derived head to head condensation products in other organisms is also not well understood. RT-qPCR analysis using *Micrococcus luteus,* which produces a 29:1 hydrocarbon, found that expression of the *ole* genes begins during late exponential phase and continues through stationary phase (Beller et al., 2010). The high level of saturation seen in these compounds indicates that a similar role in promoting membrane fluidity might not be applicable and that these compounds serve another yet to be determined purpose.

1.6 Interactions between hydrocarbon and fatty acid producing pathways

How Type II and/or the polyketide synthase pathways and the olefin pathway interact with each another has yet to be determined. Recent work studying the production of the 31:9 hydrocarbon found that the ability to produce EPA and/or DHA was correlated with the presence of the 31:9 hydrocarbon (Sugihara et al., 2010). Supporting this hypothesis is the finding that a *pfaA* knockout in *S. oneidensis* MR-1 eliminated production of the 31:9 hydrocarbon (Sukovich et al., 2010a). *Shewanella oneidensis* MR-1 is of particular interest in that significant EPA production is absent (Kato and Nogi, 2001; Sugihara et al., 2010) despite the presence of the *pfaA-E* gene cluster (Shulse and Allen, 2011). Even more puzzling is that despite the low amount of EPA production; the 31:9 hydrocarbon is still produced in *Shewanella oneidensis* MR-1(Sukovich et al., 2010a).

Production of hydrocarbon compounds using the *oleA-D* pathway does not appear to be exclusively dependant on "secondary lipids" however. An international patent application (18 September 2008, WO2008.113041) demonstrated that condensation of native fatty acids from a fatty acid overproducing strain of *E. coli* that heterologously expressed the *ole* genes from *Stenotrophomonas maltophilia* could yield hydrocarbons that appeared to be the product of head to head condensations between known fatty acids found in *E. coli.* Additionally, head to head condensation products from *E.coli* fatty acids

were found when the *oleA-D* gene cluster from *Micrococcus luteus* was heterologously expressed in a fatty acid overproducing strain *E. coli* (Beller et al., 2010). While the source of fatty acid constituents seems to be variable what remains to be determined is what, if any, protein from the *oleA-D* gene cluster is responsible for feeding fatty acids into the condensation pathway. If OleB is functioning as a thioesterase as per its annotation (Sukovich et al., 2010b) it might play a role in releasing specific ACP bound acyl intermediates from the PUFA biosynthetic pathway in *S. oneidensis* MR-1. Principle coordinate analysis comparing known products and amino acid sequences of each *ole* gene found that OleB varied the least in relation to the downstream product (Sukovich et al., 2010b) indicating that OleB might have a conserved function in this pathway. If OleB does have thioesterase activity it could be employed to cleave ACP linked fatty acids at the outset of the pathway or it could cleave either acyl-CoA or acyl-ACP moieties at multiple points in the pathway.

In an attempt to elucidate the possible interactions between these pathways the *pfaA-E* gene cluster and *oleB* were co-expressed in the heterologous host *E. coli.* Both the *pfaA-E* gene cluster and the *oleB* gene were derived from *Shewanella pealeana* and they were carried on a low copy number fosmid (1F12) and high copy number plasmid (pMA-1), respectively. OleB was chosen due to its predicted thioesterase function, which in theory would hydrolyze predicted precursors such as a 16:4 ACP-bound fatty acid from the Pfa synthase complex. In theory, two 16:4 fatty acids would be required for a condensation yielding a 31:9 hydrocarbon where loss of CO_2 and addition of one double bond formed by dehydration account for the loss of one carbon and the addition of one

unsaturation, respectively. FIGURE 1-1 is an illustrated diagram that outlines this proposed interaction between the EPA and hydrocarbon producing pathways. To investigate whether a 16:4 free fatty acid is in fact the precursor to biosynthesis of the 31:9 hydrocarbon, the heterologous host expressing these pathways would have to be genetically altered so that it cannot metabolize the free fatty acid. In order to accomplish this in *E. coli* a disruption of *fadD*, the fatty acid-CoA synthase, which begins the first step of β-oxidation, was performed using the lambda red gene knockout protocol (Datsenko and Wanner, 2000) in the cloning strain TOP 10 (Invitrogen Inc.). From there the *fadD*::Kan mutation in the TOP 10 strain was transferred into the BL21 DE3 strain of *E. coli* using P1 phage transduction (Thomason et al., 2007). Combinations of 1F12 and pMA-1 were introduced into the BL21 DE3 *fadD::kan* strain using electroporation. Strains were verified using colony PCR and cultured under a variety of conditions. Cultures were harvested and lyophilized before being derivatized to fatty acid methyl esters (FAMEs) for GC/MS analysis.

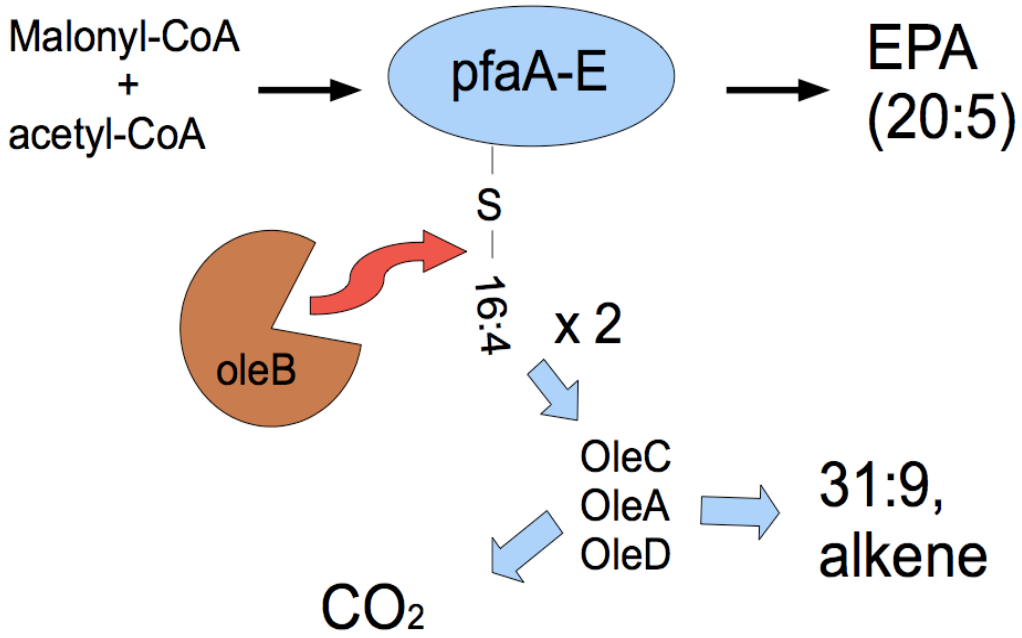

FIGURE 1-1: Putative model showing interaction between the polyunsaturated fatty acid and hydrocarbon yielding biosynthetic pathways.

Materials and Methods

2.1 Generation of an *E.coli fadD* mutant in TOP 10 strain

The *fadD* knockout strain was generated by using the Datsenko and Wanner protocol for in frame chromosomal insertions of antibiotic resistance cassettes (Datsenko and Wanner, 2000). Plasmids that carried the lambda functions and antibiotic template were kindly provided by the Saier Lab (UCSD).

The Datsenko-Wanner protocol involves electroporation of a linear double-stranded DNA construct that contains homology arms flanking an antibiotic resistance marker into *E.coli* that express the lambda phage proteins Exo, Bet, and Gam from the low-copy number plasmid pKD46. The lambda proteins required for recombination are under the control of an arabinose induced promoter system. The antibiotic resistance marker neo^r was amplified from the template plasmid pKD4 using Datsenko kan (pKD4) primers found in TABLE 2-1. The underlined bases denote the homologous segments to the template plasmid and were used to amplify the neo^r gene from the pKD4 plasmid. The non-underlined bases represent 50 base-pair homology regions that flank *fadD* and were obtained from supplemental information accompanying the Keio collection of *E. coli* single gene knockouts (Baba et al., 2006). A two-step PCR protocol was used to amplify the resistance gene and the product was run on a 1.0% agarose gel to confirm proper amplification. Gel purification using a kit (Qiagen Inc.) was performed to remove residual template plasmid. After gel purification the product was used as a template for

amplification using the same primers and the product was purified using the UltraClean PCR Cleanup kit (MO-BIO). The DNA was additionally concentrated before electroporation by using a standard ethanol precipitation protocol (Sambrook et al., 2001).

The pKD46 plasmid was introduced into electro-competent TOP 10 *E.coli* (Invitrogen Inc.). Cells were recovered in Luria Bertani medium (LB) at 30°C for two hours and plated onto LB plates containing ampicillin (100µg/ml) and incubated overnight at 30°C. A single colony was picked and used as the strain for the knockout (Top10λ). Electro-competent cells were prepared by diluting an overnight culture of Top10λ 100-fold into fresh LB with ampicillin (100µg/ml) and growing this strain at 30°C until an OD_{600} of approximately 0.2 was reached. At this point L-arabinose was added to final concentration of 10mM to induce the lambda functions for 2 hours. The cell culture was transferred onto ice for 20 minutes and centrifuged. The supernatant was decanted and the cell pellet was resuspended and washed in ice cold 10% glycerol solution. The cells were pelleted and resuspended a total of 5 times at 4°C in 10% glycerol. The cell pellet was then resuspended in 160µl of 50% glycerol for storage. A measure of 80µL of cells was mixed with 8µL of ethanol precipitated linear DNA previously generated and electroporation was carried out using the following parameters; 1750V, 200Ω, 25µF. Cells were recovered in SOC media (Sambrook et al., 2001) at 37°C for one hour and spread on LB plates containing kanamycin (10µg/mL). The next day 24 colonies were selected for screening using the kan::*fadD* primers specific to the insertion (primers listed in TABLE 2-1). Positive clones were re-streaked onto LB plates

containing 50μg/mL kanamycin. A second PCR screening was performed on re-streaked colonies using the same primers as before and internal primers specific to the *fadD* gene (DH10B_fadD_int) to ensure gene disruption. Upon confirming *fadD* disruption, colonies were cultured at 37°C to cure the temperature sensitive pKD46 plasmid. Colonies were replicated onto LB plates with ampicillin (100μg/ml) to ensure curing of pKD46. The TOP 10 *fadD*::kan strain was designated "MA 8" as seen in TABLE 2-2. Cured colonies were cultured for long-term storage at -80°C.

2.2 Generating *oleB* expression construct pMA-1

The *oleB* gene was amplified from purified genomic DNA isolated from *Shewanella pealeana* and then cloned into pETDUET (Novagen) vector using restriction enzyme based cloning. Briefly, *S. pealeana* was cultured in 2216 Marine broth media prepared as is recommended by the manufacturer (Difco) and cultured with shaking at 18°C. Genomic DNA was isolated using the UltraClean Microbial Genomic DNA Isolation Kit (MO-BIO Laboratories Inc.). The *oleB* gene was amplified using *Taq* polymerase (New England Biolabs) with oleB_NdeI and oleB_PacI primers listed in TABLE 2-1. The forward primer contains an NdeI site on its 5' end (underlined) and the reverse primer contains a PacI site on its 5' end. Once proper gene amplification was confirmed on a gel, both the NdeI/PacI *oleB* PCR product and pETDUET vector were subjected to an overnight double restriction digest using NdeI and PacI using manufacturer protocols (New England Biolabs). Once digested the reaction was heat killed at 68°C and XhoI (New England Biolabs) was added to the reaction to remove

residual vector fragments that might interfere with cloning. After the XhoI digestion was heat-killed, both vector and gene insert digestions were run through a PCR cleanup kit (MO-BIO) to remove any residual fragments. Ligation reactions were conducted as per manufacturer recommendations using T4 ligase (Thermo Fisher). Ligation reactions were then transformed into chemically competent TOP 10 cells (Invitrogen) using manufacturer protocols.

2.3 Confirmation of *oleB* expression

OleB expression from the pETDUET vector in BL21 DE3 was confirmed using SDS-PAGE. Strains were cultured with aeration in Terrific Broth (12g/L tryptone, 24g/L yeast extract, 0.4%(v/v) Glycerol) with the appropriate antibiotics at 37°C until an OD600 of approximately 0.2 was reached. Samples of each culture were taken before induction (t=0) and treated as negative controls. IPTG was added to a final concentration of 0.1mM and 1mL samples were taken 1 hour after induction and incubation at 37°C (t=1). After the first samplings were collected, cultures were shifted to 16°C for overnight incubation after which second samples were taken (t=2). Culture samples were centrifuged at 13000 rpm for 1 minute and supernatant was aspirated and discarded. Cell pellets were then frozen and later resuspended in Laemmli buffer (Laemmli, 1970). Samples were boiled for 5 minutes and allowed to cool. Samples were run on a SDS-PAGE gel and stained with Coomasie blue as described . Gels were decolorized in a solution of 40% methanol 10% glacial acetic acid. Once de-stained, gels were preserved between cellophane sheets.

2.4 P1 Transduction of *fadD*::Kan mutation into BL21 DE3

P1 transduction was performed to move the *fadD*::kan chromosomal cassette into *E.coli* strain BL21 DE3 because this strain could not be transformed using the Datsenko gene knockout protocol. Transduction was performed using P1*vir* phage (kindly provided by Saier Lab). Overnight cultures of both BL21 DE3 and MA8 strains were grown in LB with the appropriate antibiotic.

The lysate was made from the MA8 strain which was diluted 1:250 into LB+ 0.2% glucose + 10mM $CaCl_2$. Once an OD600 of approximately 0.1 was reached, 100ul of P1 phage stock was added. Lysis was allowed to proceed for approximately 3 hours at 37°C with aeration. A few drops of chloroform were added to the lysate and the lysate was centrifuged at 8000rpm for 10 minutes at 4°C to pellet cell debris and chloroform. Lysate supernatant was removed and stored at 4°C.

Recipient strain BL21 DE3 was grown overnight in LB. Cells were pelleted the next day and resuspended in LB+ 10mM $CaCl_2$ and 5mM $MgSO_4$. Varying amounts of lysate (0, 10, 100µl) respectively were added to 100µl of resuspended cells and allowed to sit on the bench for 30 minutes. To stop infection, 200µl 1M sodium citrate was added to the cells along with 1mL of LB. Cells were shaken at 37°C for 1-2 hours and plated on LB plates containing 10µg/mL kanamycin. The lysate was also plated to ensure it was free of residual donor strain bacteria. Colonies that formed were subsequently re-streaked onto LB plates containing 30µg/mL kanamycin. A total of four re-streakings were performed to remove residual P1 phage. Colony PCR using the primers that previously verified MA8 was used to confirm a successful transduction.

2.5 Creation of "MAE" designated strains

These strains were generated by electroporation of constructs containing the *pfa* gene cluster previously generated and/or the pMA1 plasmid construct. Strains were made electrocompetent as before and isolated colonies were re-streaked and later verified using colony PCR. Strain designations and their relevant genotypes are listed in TABLE 2-2. Additionally, vector only control sets of strains containing the original pETDUET vector were created.

2.6 Culturing Strains for chemical analysis

Each strain was streaked onto LB plates containing the appropriate antibiotic(s). Single colonies were used to inoculate 3mL cultures and these cultures were used to inoculate (1:100 dilution) larger 50mL cultures. Larger cultures were grown in Terrific broth; 12g/L tryptone, 24g/L yeast extract, 0.4%(v/v) glycerol. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cultures after 24 hours of growth at 16°C along with ampicillin (200μg/mL) to minimize any potential plasmid curing. L-arabinose was added where appropriate to induce a higher copy number of the 1F12 fosmid. The induced cultures were incubated at 16°C again and harvested approximately 24 hours after induction. Cultures were harvested using centrifugation at late exponential or stationary phase. Cell pellets were frozen at -80°C and lyophilized using a VirTis Benchtop Freeze Dryer running at -75°C and ≈100mT.

2.7 Derivatization of fatty acid methyl esters (FAMES)

Dried cell pellet was crushed and approximately 12 mg ± 3mg was weighed out and transferred to a 1.8ml glass sample vial with Teflon lined caps (Wheaton). Approximately 0.5ml of 5% H_2SO_4 in methanol was added to the dry biomass and the mixture was incubated at 90°C for 90 minutes for refluxing. Samples were taken off the heat block and allowed to cool to room temperature. Once cooled, approximately 0.4ml of hexane was added along with approximately 0.8ml of 10% NaCl in water solution. The vial was capped and mixed and allowed to sit for proper phase separation. If required, separation was aided by centrifugation at 1000rpm for 1 minute. An upper hexane layer was extracted three times from the mixture and evaporated under a gentle stream of N_2 gas. Samples were stored at -20°C for later analysis.

2.8 GC/MS analysis of FAMES

GC/MS was run on an Agilent 7890A GC system connected to a 5975C VL MSD quadropole MS (EI). Samples were separated on a 60m DB23 Agilent GC/MS column using helium as a carrier gas and a gradient of 110°C to 200°C at 15°C/minute, followed by 20 minutes at 200°C. Peaks were assigned as compounds based on mass spectrum comparisons to the NIST database. Peaks were integrated and the area values were used to determine percent weight of each fatty acid. Unsaturation index was calculated by adding percentages of unsaturated fatty acids and multiplying each percentage by the number of double bonds found in the molecule.

TABLE 2-1: Primers used in this Study

Primer Name	Sequence 5'-3'
Datsenko kan (pKD4) fwd	CATTTGGGGTTGCGATGACGACGAACACGCATTTTA GAGGTGAAGAATTGGTGTAGGCTGGAGCTGCTTC
Datsenko kan (pKD4) rev	TAACCGGCGTCTGACGACTGACTTAACGCTCAGGCT TTATTGTCCACTTTATGGGAATTAGCCATGGTCC
kan::fadD_rev	CATATCACGCCAGGCATCTT
kan::fadD fwd	ATCAGGATGATCTGGACGAA
DH10B_fadD_int fwd	CGTTAAAACCGGAAACCAGA
DH10B_fadD_int rev	GCTGGATTTCTCCAGTCTGC
oleB_NdeI fwd	CGCGCATATGCTAGACACCCTGCAC
oleB_PacI rev	CGCGCTTAATTAATTAGGCTACCGATTCAGTAGG
pET T7 terminator	GCTAGTTATTGCTCAGCGG
pET upstream 2	TTGTACACGGCCGCATAATC

TABLE 2-2: Strains and Plasmids used in this Study

Strain	Genotype and characteristics	Source
BL21 DE3	F– ompT hsdSB(rB–, mB–) gal dcm (DE3)	Lab stock
TOP 10	F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu) 7697 galU galK rpsL nupG λ–	Invitrogen
MA 8	TOP 10, *fadD*::kan	This study
MAE 1	BL21 DE3, IF12	This study
MAE 2	BL21 DE3, 1F12, pMA-1	This study
MAE 3	BL21 DE3, pMA-1	This study
MAE 4	BL21 DE3, *fadD*::kan	This study
MAE 5	BL21 DE3, *fadD*::kan, 1F12	This study
MAE 6	BL21 DE3, *fadD*::kan, pMA-1	This study
MAE 7	BL21 DE3, *fadD*::kan, 1F12, pMA-1	This study
MAE 8	BL21 DE3, *fadD*::kan, pETDUET	This study
MAE 9	BL21 DE3, *fadD*::kan, pcc2fos	This study
MAE 10	BL21 DE3, *fadD*::kan, 1F12, pETDUET	This study
Plasmids	Characteristics	
pETDUET	Amp^r	Novagen
pcc2fos	Cm^r	Epicentre
1F12	pcc2fos; contains PfaA-E cluster from *S. pealeana*	Lab stock
pMA-1	pETDUET; *oleB* from S. *pealeana,* T7 inducible	This study

Results

3.1 Creation of *fadD*::kan in *E. coli* TOP 10

A total of 24 colonies were recovered and screened after overnight incubation at 37°C on LB plates with kanamycin (10μg/ml). Colonies were screened using colony PCR with primers specific to the *fadD*::kan locus generated. One primer was nested inside the kanamycin resistance gene and the other primer was located in the ribonuclease D gene upstream of the *fadD* gene on the *E. coli* genome. Of the 24 colonies screened initially, 23 had bands of the expected size, approximately 1.3kb, and those colonies were restreaked and later rescreened (results not shown). For the second round of PCR screens the same primer set was used along with *fadD* internal primers that were nested inside the *fadD* gene. The results of the second screening are shown in FIGURE 3-1 and all six colonies screened were positive for the *fadD*::kan insertion as seen by the 1.3kb bands and none had a product corresponding to the *fadD* gene. This strain was cultured and archived and given the designation MA8 as shown in TABLE 2-2.

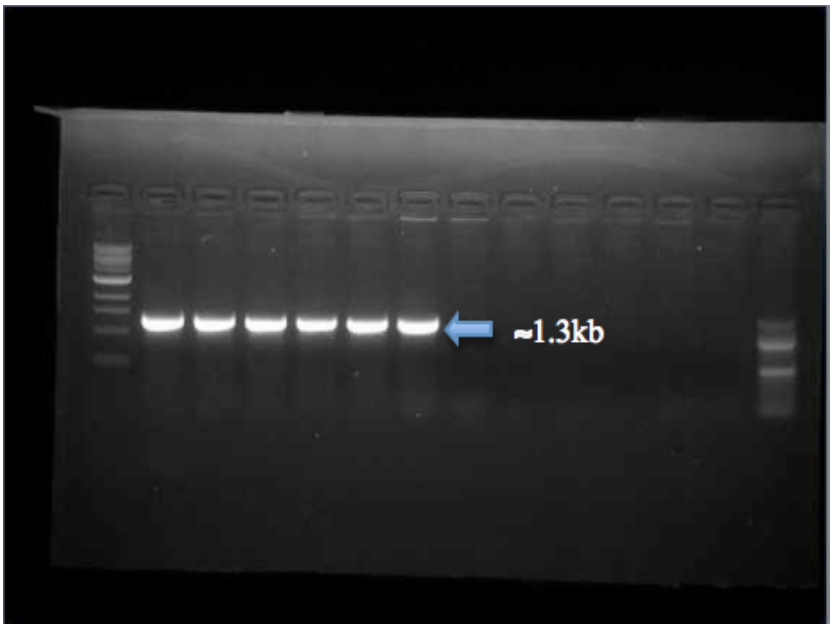

FIGURE 3-1: Lane 1 1kb DNA ladder; Lanes 2-7 amplicon of the expected size confirming *fadD*::kan insertion, Lanes 8-13 internal amplification of disrupted *fadD* gene confirming the *fadD*::kan insertion.

3.2 P1 transduction of *fadD*::kan from TOP 10 to BL21 DE3

P1 transduction was chosen to move the *fadD*::kan mutation into the BL21 DE3 strain because previous attempts to knockout *fadD* in BL21 DE3 using the lambda red protocol were unsuccessful. Between the three transduction plates, each representing a different amount of lysate used (0, 10, 100ul) a total of 5 larger sized colonies were screened using the *fadD*::kan primer set and all 5 colonies contained the *fadD*::kan mutation. Additionally, plating of the lysate alone yielded no colonies indicating a sterile lysate with no residual donor cell background. The same 5 colonies were restreaked a total of 4 times on LB Kan (30µg/ml) plates to remove residual P1 phage left behind. A

final screen was performed using the *fadD*::kan and *fadD* internal primer sets and the results of that screening are shown in FIGURE 3-2. All of the screened colonies displayed the 1.3kb band corresponding to the *fadD*::kan mutation and displayed no bands for the *fadD* internal primer set indicating a deletion of the *fadD* gene. Positive and negative controls for each primer set were included instead of DNA ladders.

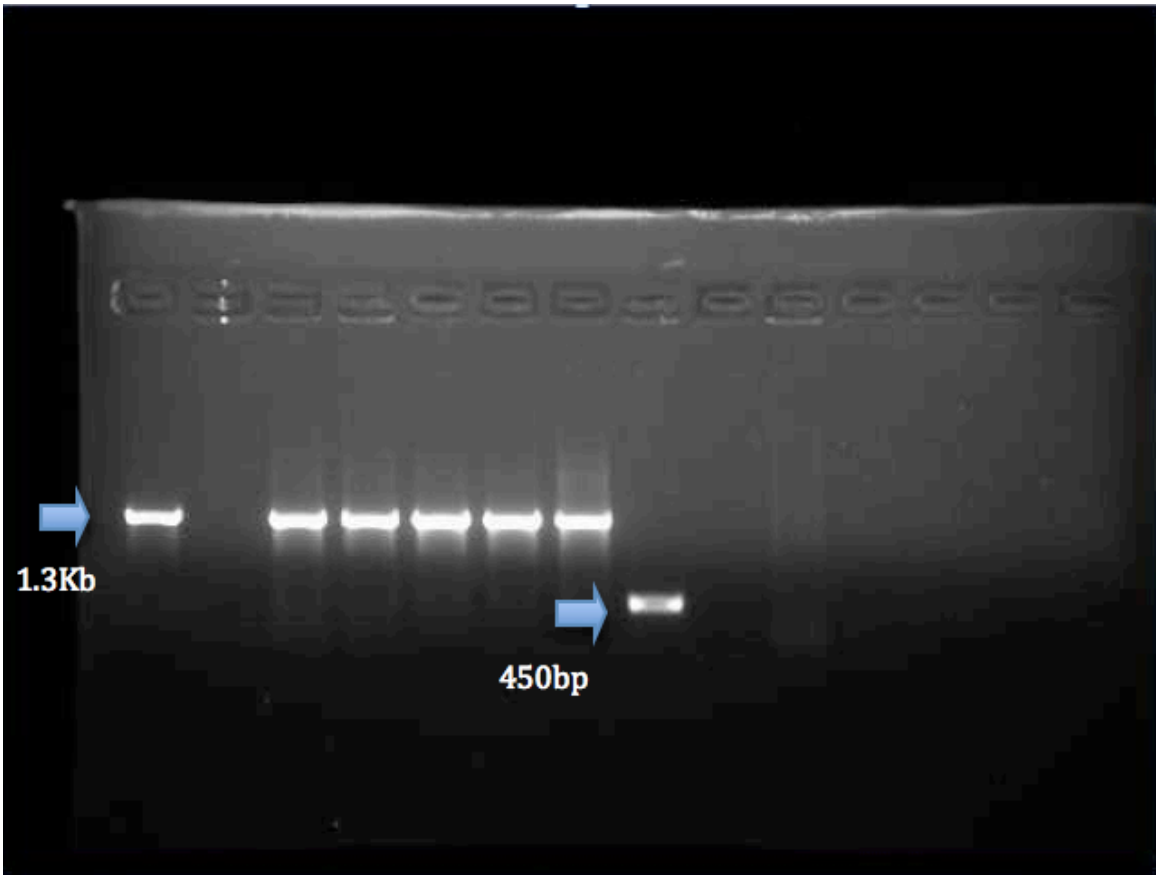

FIGURE 3-2: Lane 1 positive control using MA 8 strain with primers specific to *fadD*::kan insertion, Lane 2 negative control using BL21 DE3 strain with *fadD*::kan primers, Lanes 3-7 five candidate BL21 DE3 *fadD*::kan colonies using *fadD*::kan primers, Lane 8 positive control using BL21 DE3 with *fadD* internal primers, Lane 9 negative control using MA 8 with *fadD* internal primers, Lanes 10-14 same candidate colonies from Lane 3-7 showing *fadD* disruption.

3.3 Cloning of *oleB* into pETDUET vector

Genomic DNA isolated from *Shewanella pealeana* was used a template for amplifying *oleB.* Appropriate PCR conditions were used and the product was run on a 0.8% agarose gel confirming that the NdeI/PacI *oleB* PCR product is the correct size of approximately 1000 base pairs (data not shown). Confirmation of *oleB* ligation into the pETDUET vector was accomplished using colony PCR with the pET upstream 2 and pET T7 terminator primers, which flank the multiple cloning site where *oleB* was inserted. The correct bands seen in FIGURE 3-3 are slightly larger than the 900bp gene amplification and this is due to additional vector sequence flanking the *oleB* insert. Once confirmed by PCR the correct clones were cultured for storage and the new construct was named pMA-1 as shown in TABLE 2-2.

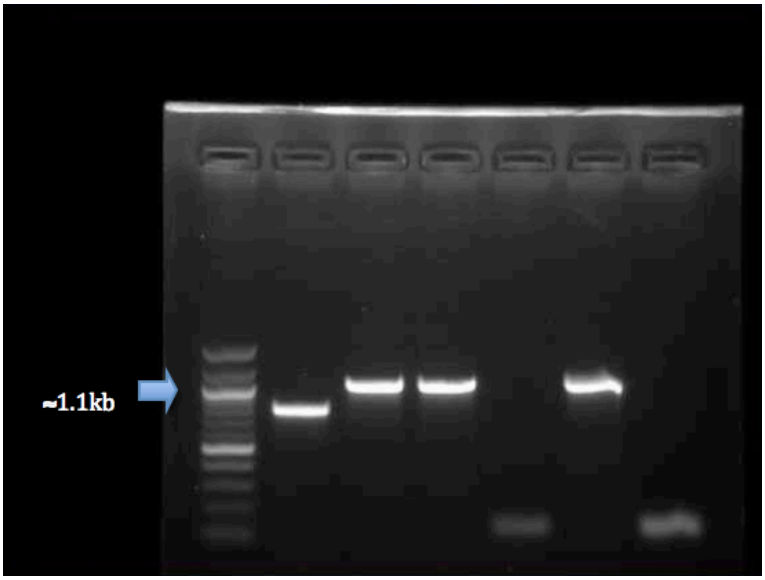

FIGURE 3-3: Lane 1 100bp DNA ladder, Lanes 2-6 colony PCR looking for proper size of the *oleB* insert (Lanes 3,4,5 are the expected size)

3.4 Confirmation of *oleB* expression

To confirm proper expression of *oleB*, SDS-PAGE analysis was used with whole-cell lysates. The picture of the gel shown in FIGURE 3-4 shows a clearly over-expressed protein in Lanes 7, 9, and 10. Lanes 8 through 10 represents the second sampling time. Lane 8 is the BL21 DE3 strain only control and Lanes 9 and 10 represent strains MAE 2 and MAE 3 with 0.1mM IPTG induction. There clearly is a larger band at the approximate size of OleB (≈34kD) indicating a successful induction.

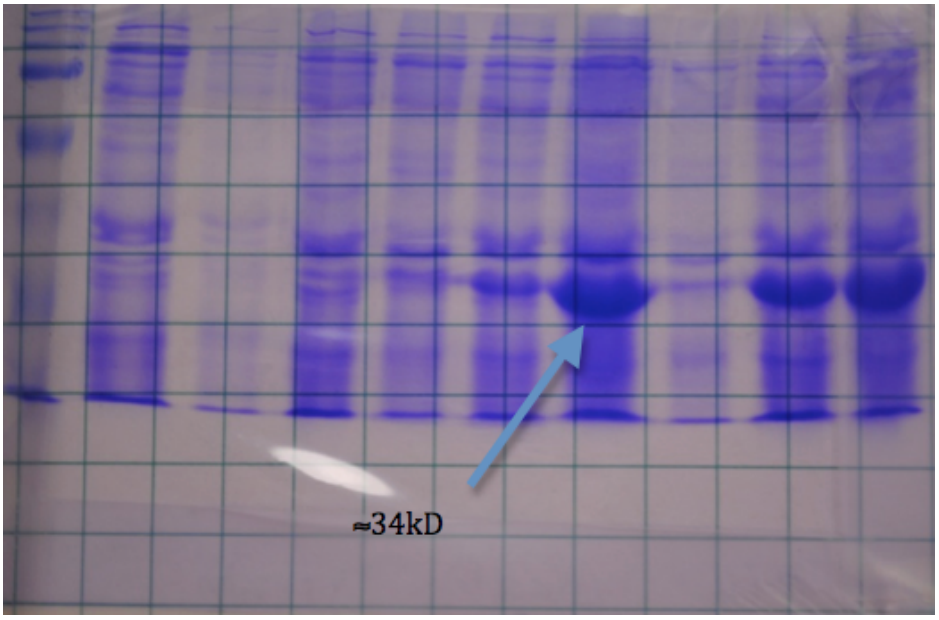

FIGURE 3-4: Lane 1 Molecular weight ladder, Lane 2 t=0 of BL 21 DE3, Lane 3 t=0 of MAE 2, Lane 4 t=0 of MAE 3, Lane 5 t=1 of BL21 DE3, Lane 6 t=1 of MAE 2, Lane 7 t=1 of MAE 3, Lane 8 t=2 of BL21 DE3, Lane 9 t=2 of MAE 2, Lane 10 t=2 of MAE 3 Lanes 7, 9, 10 show an overexpressed protein between 37kD and 25kD markers.

3.5 Confirmation of MAE strains

After electroporation and re-streaking of candidate colonies MAE strains were screened for the presence of vectors using colony PCR with specific primers for each vector. FIGURE 3-5 is a representative screening result from MAE 7 (refer to TABLE 2-2 for strain designations). Lanes 2-5 represents bands from the same primer set used to screen for proper insertion of *oleB* into the pETDUET vector. Lanes 5-8 represent results from the primer set specific to *fadD* and a lack of bands indicates the gene disruption. Lanes 9-13 contain bands specific to the *pfaE* gene, which is indicative of the 1F12 fosmid being present.

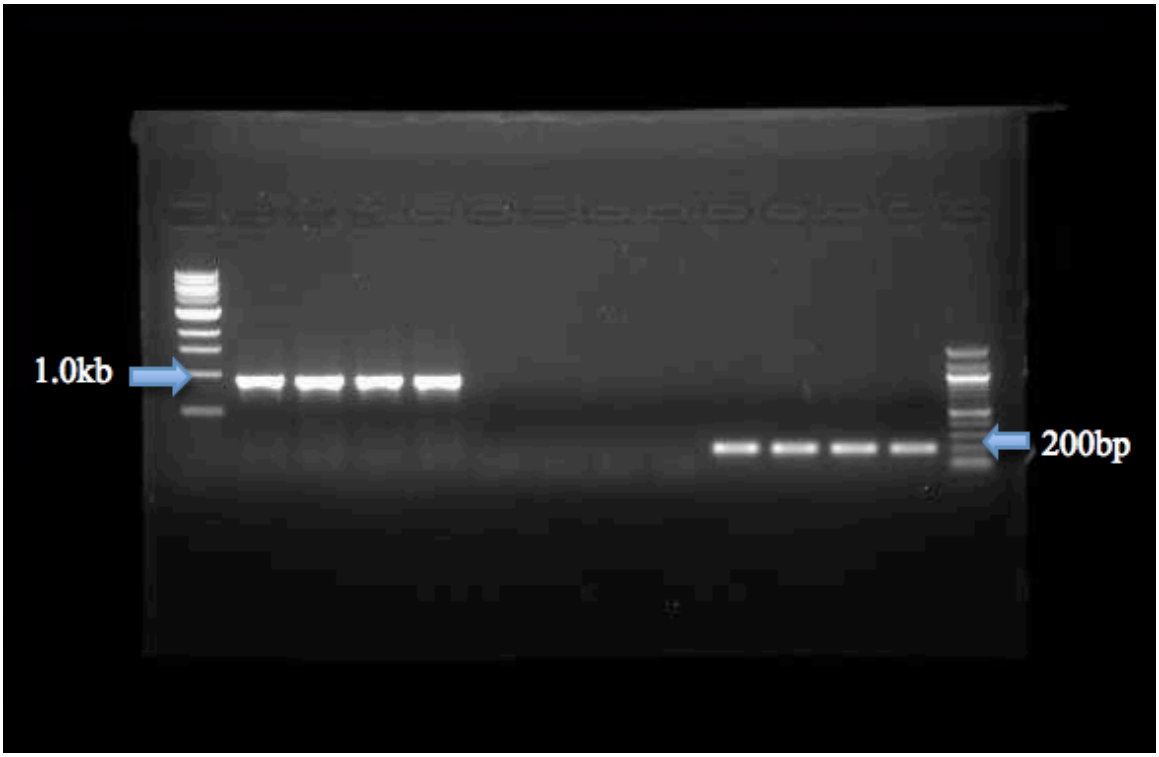

FIGURE 3-5: MAE 7 strain confirmation; Lane 1- 1kb DNA ladder. Lanes 2-5 colony PCR using pMA-1 specific primers. Lanes 5-9 *fadD* internal primers showing disruption. Lanes 9-13 *pfaE* specific primers. Lane 14 100bp DNA ladder.

3.6 Lipid profiles of MAE strains

Product identities were determined by comparing mass spectra of peaks and comparing them to the NIST database. In some of the chromatograms smaller peaks were not assigned product identities because the match found was not consistent with the fatty acids of *E. coli* or intermediates of PUFA biosynthesis. Percentages of each fatty acid and the respective culturing conditions of each strain can be seen in TABLE 3-1. Several GC chromatograms of strains relevant to this study have also been included (FIGUREs 3-6, 7, 8, 9, 10)

The fatty acid profile of MAE 1 contained EPA at about 7-8% of total fatty acid content. Palmitic acid (16:0) and 18:1 were the two most abundant fatty acids at 34% and 20% respectively. One of the interesting aspects of this profile was the appearance of 18:0 fatty acids that contained either single or multiple methoxy groups. Both of these methoxylated products combined to make up approximately 12% of the total fatty acid content.

The profile of MAE 2, which was cultured to induce pMA-1 and have 1F12 at a high copy number, showed a much higher level of unsaturation as seen in an UFA/SFA ratio that exceeds two and an unsaturation index of 95. Notably in this strain, 18:1 is the most abundant fatty acid at approximately 42% while 16:0 dropped to 26%. EPA was also seen in this strain at approximately 7% of total fatty acid content.

The MAE 3 strain, which was cultured to induce *oleB,* displayed a fatty acid profile with a similar drop in 16:0 and rise in 18:1 content to the point where 18:1 becomes the most abundant fatty acid. The UFA/SFA ratio approaches two and the unsaturation index is 64. While the UFA/SFA ratio is similar to MAE 2 the unsaturation index is almost identical to MAE 1.

The *fadD*::kan BL21 DE3 strain, MAE 4, when grown at 16°C displayed a similar membrane profile to MAE 2 and MAE 3 with 18:1 becoming the most abundant fatty acid present. This result indicates that the large increase in 18:1 content seen in other strains analyzed might not be solely due to the action of OleB. As seen in MAE 2 and MAE 3 strains the increase in 18:1 content occurs in parallel with a decrease in 16:0 content as expected given the lower culturing temperature. Surprisingly, the disruption of *fadD* has little to no effect on the fatty acid profile of BL21 DE3.

In the MAE 5 strain, which contains 1F12, eicosapentanoic acid (EPA, 20:5n-3) was detected regardless of whether or not the strain was incubated with L-arabinose. A GC chromatogram is included here in FIGURE 3-7 along with the data in TABLE 3-1. In both trials using different concentrations of L-arabinose EPA was produced at approximately 8% of total fatty acid content. Surprisingly, the culture without L-arabinose, shown in the chromatogram in FIGURE 3-7, produced a greater amount of EPA at around 12% of total fatty acid content. Interestingly, the amounts of 16:1 and 18:1 found in this strain was significantly lower than in the MAE 4 strain. The UFA/SFA ratio in MAE 5 is dramatically less than the MAE 4 strain, however the increase in the unsaturation index indicates that the addition of EPA compensates for the loss of monounsaturated fatty acid production.

The MAE 6 strain is a BL21 DE3 *fadD*::kan strain that contains the pMA-1 plasmid. Data for different culturing conditions of MAE 6 is shown in TABLE 3-1 and FIGURE 3-8 is a representative chromatogram of this strain with *oleB* expression. In this strain, induction of *oleB* resulted in a nearly 3-fold increase in the UFA/SFA ratio indicating a drastic shift in the native Type II acyl-CoA pool. When compared to MAE 4 however, the profile is very similar with respect to 18:1, 16:1 and 16:0 percentages. This result indicates that OleB is not actively modifying the qualitative aspects of the Type II fatty acid pool. Given the acid catalyzed FAME derivitization used, there was no differentiation between free fatty acids and membrane linked fatty acids.

The MAE 7 strain, which contains 1F12, pMA-1 in a BL21 DE3 *fadD*::kan background showed a similar increase in unsaturated fatty acid content upon *oleB* induction with UFA/SFA ratios above 1 in each case. When *oleB* was induced in this

strain the UFA/SFA ratio doubled as compared to the same strain grown without either arabinose or IPTG induction. EPA production did not appear to be significantly affected by *oleB* induction when comparing IPTG induced versus non-induced cultures as seen in TABLE 3-1. Compared to MAE 5, which also contains the 1F12 fosmid in a *fadD*::kan genetic background, a pattern emerges where induction of *oleB* appears to generate an increase in 18:1 production to the point where it becomes the most abundant fatty acid present. While this effect is seen in MAE 4, the *fadD*::kan strain, the effect appears to be abrogated in MAE 5 by the production of EPA. Two cultures of MAE 7 were grown at 37°C to look for any anomalous changes in fatty acid profiles that might occur with maximum *oleB* expression. EPA production was completely absent at 37°C as expected and 16:0 was the most abundant fatty acid in the profile.

None of the MAE 7 strains contained a readily discernable 16:4 fatty acid or anomalous peak not found in any other strain chromatograms as seen in FIGURE 3-9.

The fatty profile MAE 10, which is identical to MAE 7 except for the substitution of the pETDUET vector for pMA-1, when cultured under the same conditions as MAE 7, has a somewhat different profile. Both 16:0 and 18:1 are approximately equal in terms of percentage and EPA is seen at 13% of total fatty acid content. The results shown here indicate that induction of T7 transcription with IPTG is not responsible for the increases in 18:1 content seen in previous strains.

In order to gauge how EPA content and expression of *oleB* modulate the host fatty acid profile, BL21 DE3 was grown at 16°C in the same type of media. The profile of BL21 DE3 can be seen both in TABLE 3-1 and in the representative GC chromatogram seen in FIGURE 3-10. The fatty acid profile of BL21 DE3 contains 18:1 at

approximately 40% of total fatty acid content. Palmitic acid (16:0) is seen at approximately 29% and 16:1 is seen at 17%. The increase in monounsaturated fatty acids is reflected in the UFA/SFA ratio being above one. These results indicate that the BL21 DE3 strain is particularly efficient at modulating its fatty acid profile to lower temperatures by the increased production of 18:1.

The appearance of methoxylated fatty acids in several samples, shown in FIGURE 3-6, 3-7, and 3-10 is interesting in that *E. coli* are not known to make methoxylated fatty acids. Given that these products were found in several samples with no commonality between them, their presence is indicative of possible side products being formed during the esterification process or other contaminant sources such as residual media left behind after lyophilization. The presence of these fatty acids cannot be attributed to the presence of contaminants in the GC column as some profiles do not contain such peaks as shown in FIGURE 3-8 and 3-9.

TABLE 3-1: Fatty acid profiles of MAE strains and their culturing conditions

Strain	Temperature	Arabinose	IPTG	%12:0	%14:0	%16:0	%16:1	%18:1	%18:0	%20:5	UFA/SFA	Unsaturation Index
MAE 1	16°C	1.0mM	0mM	3.040	7.849	34.683	3.687	20.138	2.422	7.856	0.660	63.104
MAE 2	16°C	1.0mM	0.1mM	0.874	3.491	25.873	18.718	41.963	1.119	6.855	2.154	94.955
MAE 3	16°C	0mM	0.1mM	1.105	3.554	27.189	18.157	45.906	1.356	0.000	1.929	64.063
MAE 4	16°C	0mM	0mM	1.798	5.386	24.792	25.618	40.926	0.000	0.000	2.081	66.544
MAE 5	16°C	0mM	0mM	2.973	6.841	33.839	5.654	29.622	0.000	11.977	1.082	95.160
MAE 5	16°C	1mM	0mM	3.594	11.153	38.656	2.724	8.740	3.213	7.544	0.336	49.185
MAE 5	16°C	10mM	0mM	3.264	10.183	37.904	3.152	13.594	7.544	8.065	0.461	57.070
MAE 6	16°C	0mM	0mM	1.519	5.219	33.573	7.236	28.487	0.909	0.000	0.867	35.723
MAE 6	16°C	0mM	0.1mM	1.026	3.371	23.893	21.520	47.661	1.038	0.000	2.359	69.181
MAE 6	16°C	0mM	1.0mM	1.744	5.432	28.432	15.319	39.114	0.592	0.000	1.504	54.433
MAE 7	16°C	0mM	0mM	1.802	5.609	37.482	8.459	27.885	1.444	6.553	0.926	69.107
MAE 7	16°C	1.0mM	0mM	2.778	6.391	30.575	10.977	34.340	0.000	9.366	1.265	92.149
MAE 7	16°C	0mM	0.1mM	1.004	3.209	27.081	14.387	45.004	1.529	7.787	2.047	98.325
MAE 7	16°C	1.0mM	0.1mM	0.954	2.937	25.571	15.543	46.926	1.371	5.874	2.216	91.841
MAE 7	16°C	1.0mM	0.1mM	2.544	5.978	26.893	16.491	35.898	0.000	12.196	1.824	113.367
MAE 7	16°C	10mM	1mM	1.421	4.486	28.133	15.032	41.278	0.877	6.730	1.805	89.961
MAE 7	37°C	0mM	0mM	4.687	8.116	45.415	1.058	13.185	3.735	0.000	0.230	14.243
MAE 7	37°C	10mM	1mM	5.168	9.575	48.655	3.777	9.790	1.673	0.000	0.208	13.567
MAE 10	16°C	1mM	0.1mM	2.379	7.614	29.529	18.643	28.778	0.000	13.057	1.530	112.705
BL21 DE3	16°C	0mM	0mM	2.484	5.405	28.804	16.929	39.699	0.000	0.000	1.389	56.628

Data file Name :C:\msdchem\1\data\Eric Allen\071313_test3\D4.D
Acquired date :13 Jul 2013 11:46
Method Name :C:\MSDCHEM\1\METHODS\EPA_SPLITLESS_DEFAULT_DB23.M
Sample Name :MAE 4 Ara=0 IPTG=0 16C

Compound	RT (min)	Scan numb	Area (Ab*s)	Baseline H	Absolute H	Peak Width	Start Time	End Time (I	Start Heigh	End Height	Peak Type	Fatty acid	%TFA
1	8.971	944	12159351	208591	211857	0.096	8.738	9.085	3120	3337	BB	12:0	1.798328
2	10.685	1505	36414170	1088680	1091768	0.053	10.528	10.803	2576	3469	BB	14:0	5.385535
3	13.135	2307	167630950	4623934	4626355	0.056	12.961	13.348	1622	3389	BB	16:0	24.79206
4	13.719	2498	173217797	4865135	4872906	0.056	13.62	13.833	6507	9198	VV	16:1	25.61834
5	17.947	3882	276719895	5237991	5242625	0.085	17.688	18.153	4388	4826	BB	18:1	40.92595
6	18.797	4160	10005573	178835	180791	0.084	18.52	18.963	2545	1602	BB	18:0 9,10,12-Me	1.479791
			676147736										

%UFA	%SFA	UFA/SFA
66.54429	31.97592	2.081075

Unsaturation index 66.54429

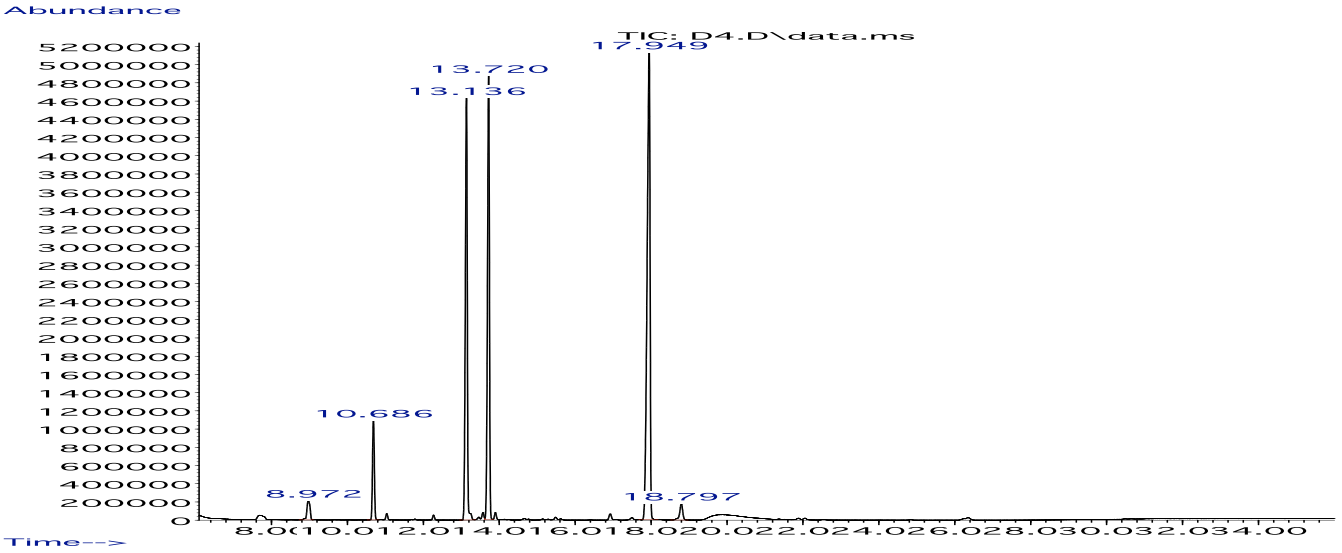

FIGURE 3-6: GC Chromatogram of MAE 4 cultured at 16°C

Data file Name :D:\DATA\Eric_Allen_lab\MARCO\D5C1.D
Acquired date :24 Jul 2013 17:03
Method Name :C:\MSDCHEM\1\METHODS\EPA_SPLITLESS_DEFAULT_DB23.M
Sample Name :MAE 5 Ara=0mM IPTG=0mM 16C

Compound	RT (min)	Scan numb	Area (Ab*s)	Baseline H	Absolute H	Peak Width	Start Time	End Time (	Start Heigh	End Height	Peak Type	Fatty acid	%TFA
1	8.971	944	18470374	324055	328936	0.096	8.774	9.121	5219	4627	BB	12:0	2.972666
2	10.682	1504	42506100	1283048	1285926	0.053	10.528	10.798	2406	3241	BB	14:0	6.841034
3	13.126	2304	210256387	5574021	5576770	0.059	12.956	13.338	1654	4110	BB	16:0	33.83917
4	13.695	2490	35129267	1031344	1039046	0.054	13.614	13.802	8516	6640	VB	16:1	5.653789
5	15.473	3072	10392332	257813	265455	0.063	15.363	15.572	8061	7254	BV		1.672567
6	17.908	3869	184055518	3702489	3707047	0.074	17.689	18.081	2977	5826	BB	18:1	29.62234
7	18.782	4155	31141146	619036	624255	0.081	18.601	18.928	8610	2501	BB	18:0 9,10.12-Me	5.011931
8	26.337	6628	14973138	199859	203030	0.114	26.144	26.532	5548	810	BB	18:0 11-Me	2.409813
9	31.724	8391	74416046	1314066	1331612	0.085	31.479	31.923	14799	19753	BB	20:5	11.9767
			621340308										

%UFA	%SFA	UFA/SFA
47.25282	43.65287	1.082468

Unsaturation index 95.15961

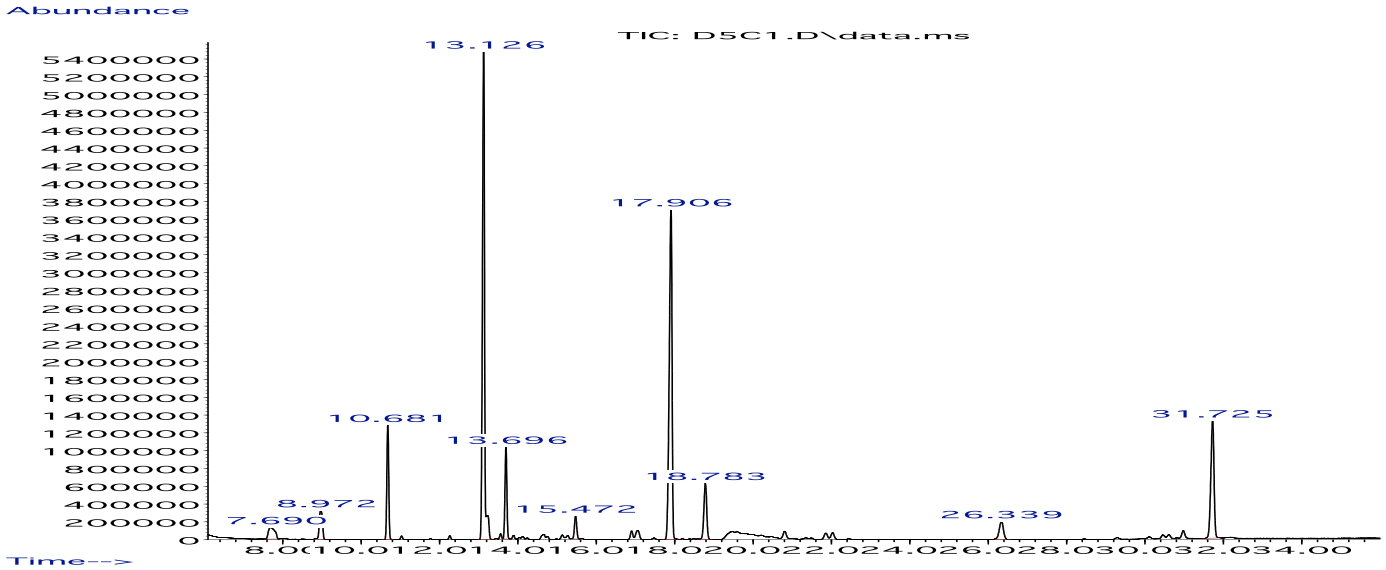

FIGURE 3-7: GC Chromatogram of MAE 5 cultured at 16°C

Data file Name :C:\msdchem\1\data\Eric Allen\071313_test\D63.D
Acquired date :15 Jul 2013 15:13
Method Name :C:\MSDCHEM\1\METHODS\EPA_SPLITLESS_DEFAULT_DB23.M
Sample Name :MAE6 Ara=0 IPTG=0.1mM 16C

Compound	RT (min)	Scan numb	Area (Ab*s)	Baseline H	Absolute H	Peak Width	Start Time	End Time (I	Start Heigh	End Height	Peak Type	Fatty acid	%TFA
1	8.971	944	13812281	239466	243725	0.096	8.794	9.08	5889	3236	BB	12:0	1.025767
2	10.682	1504	45393321	1306236	1308996	0.056	10.513	10.809	2190	3180	BB	14:0	3.371128
3	11.036	1620	4734225	143253	146813	0.054	10.895	11.109	5721	2407	BB		0.351586
4	13.151	2312	321721507	8113518	8115584	0.062	12.956	13.348	1295	2846	BV	16:0	23.8926
5	13.575	2451	8109056	173783	177530	0.07	13.348	13.624	2846	3935	VV		0.602218
6	13.734	2503	289779819	7794942	7799310	0.059	13.624	13.833	3935	4760	VV	16:1	21.52045
7	13.902	2558	7240370	208605	213644	0.055	13.833	14.027	4760	5526	VB		0.537705
8	16.942	3553	13971657	294813	297069	0.074	16.77	17.097	1152	3269	BB	18:0	1.037603
9	18.012	3903	641769864	9464331	9476043	0.1	17.683	18.168	8558	13223	BV	18:1	47.66094
			1346532100										

%UFA	%SFA	UFA/SFA
69.18139	29.3271	2.358958

Unsaturation index 69.18139

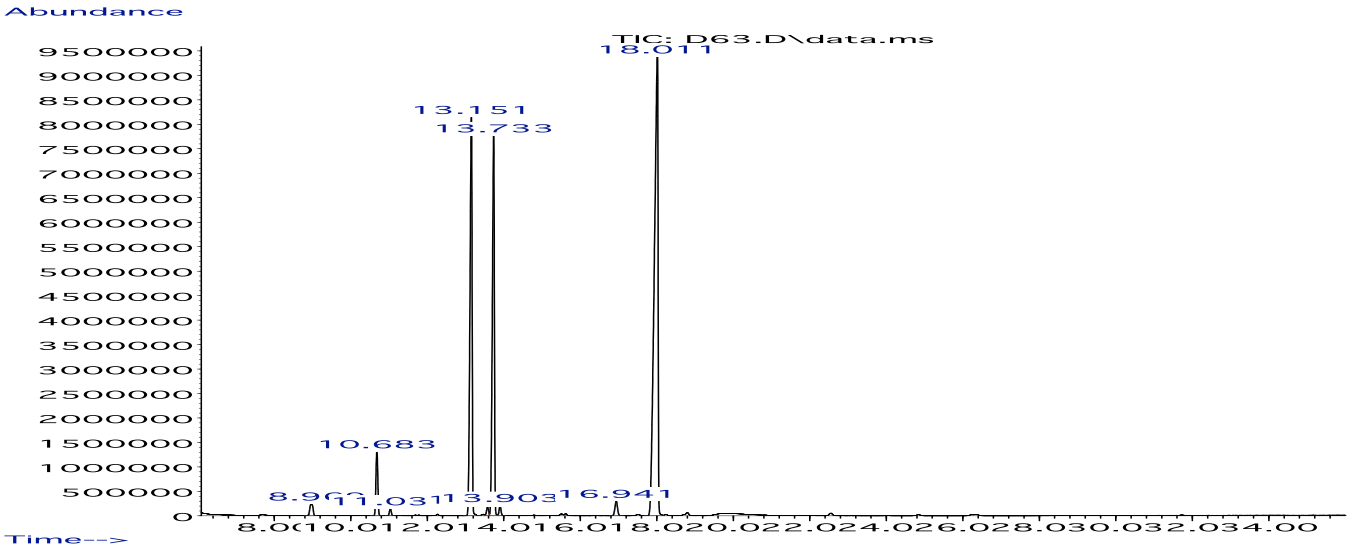

FIGURE 3-8: GC Chromatogram of MAE 6 cultured at 16°C with IPTG induction

Data file Name :C:\msdchem\1\data\Eric Allen\071313_test3\D7E.D
Acquired date :13 Jul 2013 18:53
Method Name :C:\MSDCHEM\1\METHODS\EPA_SPLITLESS_DEFAULT_DB23.M
Sample Name :MAE 7 Ara= 1mM IPTG= 0.1M 16C

Compound	RT (min)	Scan numb	Area (Ab*s)	Baseline H	Absolute H	Peak Width	Start Time	End Time (I	Start Heigh	End Height	Peak Type	Fatty acid	%TFA
1	8.968	943	13151614	238970	247591	0.093	8.849	9.076	15093	2739	BB	12:0	2.544102
2	10.682	1504	30904082	926741	928802	0.053	10.523	10.799	1743	2299	BB	14:0	5.978212
3	13.126	2304	139024634	3983160	3985045	0.055	12.961	13.318	1210	2661	BB	16:0	26.89349
4	13.704	2493	85248325	2434758	2438685	0.055	13.612	13.833	3606	4384	VV	16:1	16.49078
5	17.923	3874	185571148	3768382	3771796	0.075	17.709	18.132	3736	3097	BB	18:1	35.89764
6	31.739	8396	63045422	1132595	1148001	0.081	31.51	31.953	13730	16962	BB	20:5	12.19576
			516945225										

%UFA	%SFA	UFA/SFA
64.58419	35.41581	1.823598

Unsaturation index 113.3672

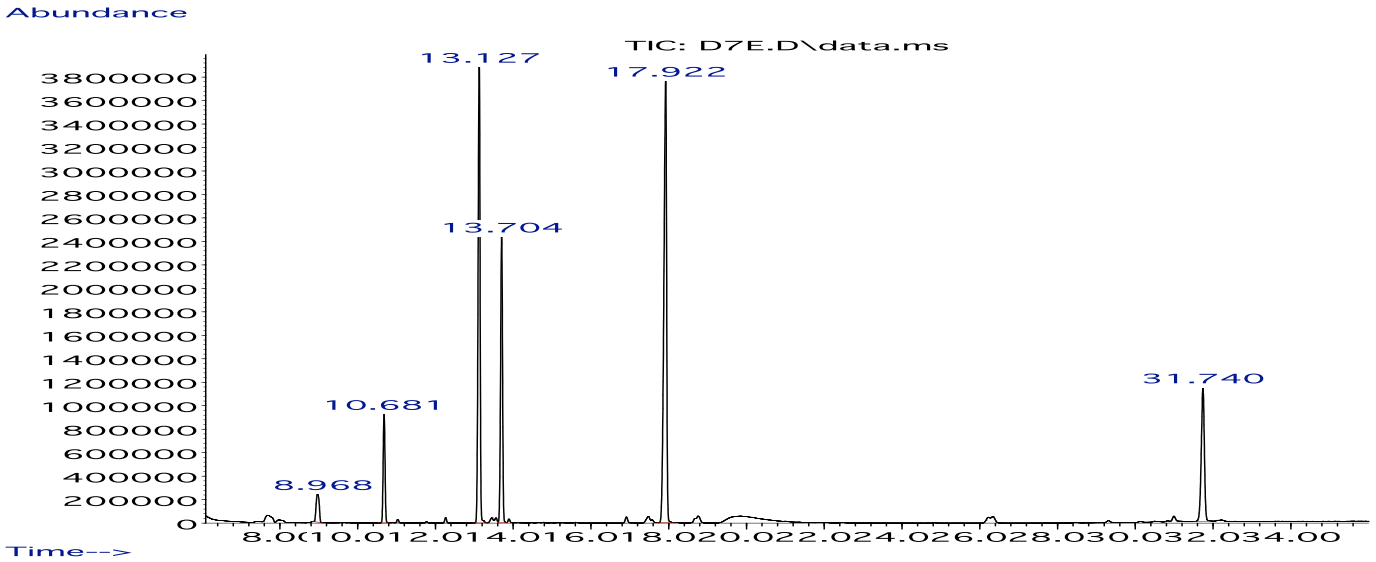

FIGURE 3-9: GC Chromatogram of MAE 7 cultured at 16°C with L-arabinose and IPTG induction

Data file Name :D:\DATA\Eric_Allen_lab\MARCO\BL21.D
Acquired date :24 Jul 2013 13:42
Method Name :C:\MSDCHEM\1\METHODS\EPA_SPLITLESS_DEFAULT_DB23.M
Sample Name :BL21 Ara=0mM IPTG=0mM 16C

Compound	RT (min)	Scan numb	Area (Ab*s)	Baseline H	Absolute H	Peak Width	Start Time	End Time (I	Start Heigh	End Height	Peak Type		%FA
1	8.98	947	16744587	300295	308278	0.093	8.841	9.094	14329	2820	BB	12:0	2.484182
2	10.688	1506	36430891	1095140	1097144	0.053	10.487	10.798	1586	2235	BB	14:0	5.404788
3	13.135	2307	194154589	5399183	5401290	0.057	12.966	13.201	1152	2470	BV	16:0	28.80425
4	13.245	2343	10160041	226545	229265	0.062	13.201	13.348	2470	3301	VB		1.507316
5	13.713	2496	114112256	3294062	3299063	0.055	13.62	13.828	4572	5545	VV	16:1	16.92939
6	15.479	3074	7321666	186226	192402	0.062	15.363	15.567	6366	6036	BV		1.086223
7	17.935	3878	267590446	5205112	5212212	0.078	17.694	18.112	10750	4450	BB	18:1	39.69899
8	18.794	4159	27533961	556870	560493	0.074	18.617	18.948	5606	1870	BB	18:0 9,10,12-Me	4.084864
			674048437										

UFA	SFA	UFA/SFA
56.62838	36.69322	1.543293

Unsaturation Index 56.62838

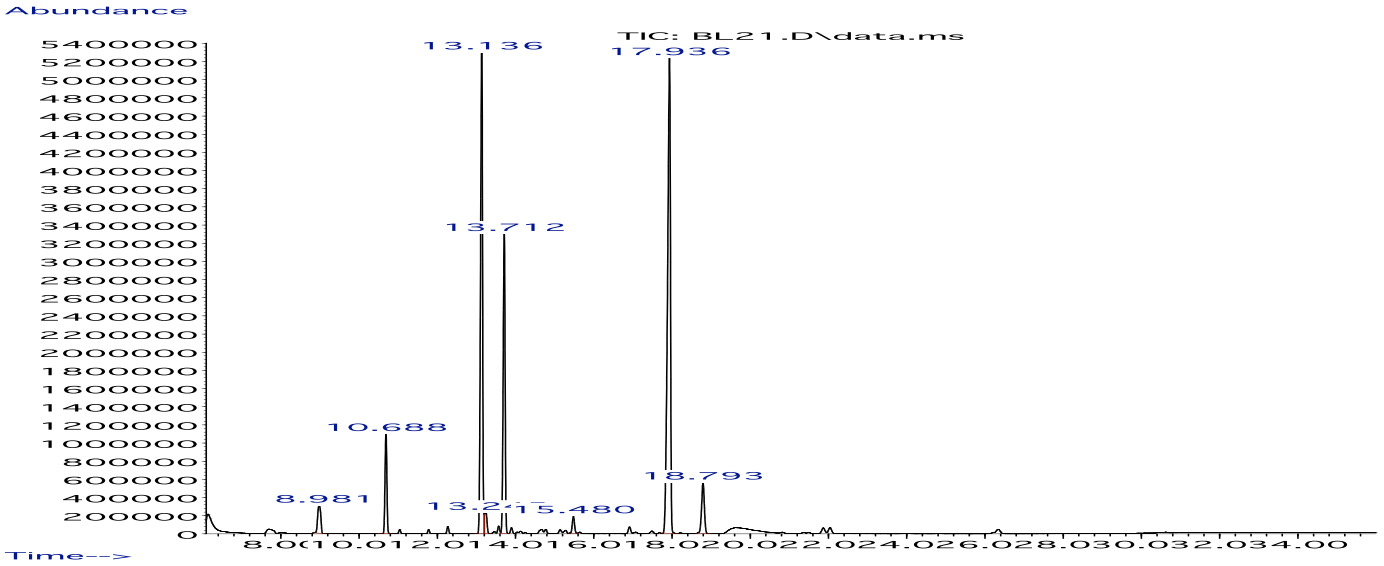

FIGURE 3-10: GC Chromatogram of BL21 DE3 cultured at 16°C

Discussion

The intended goal of isolating a 16:4 fatty acid intermediate was not achieved in this study. Despite having the necessary expression constructs and strains of *E. coli* there was no apparent peak seen in the GC/MS data that corresponded with a 16:4 fatty acid. There are many possible explanations as to why these experiments did not yield such a result.

Expression of *oleB* does not appear to have a clear effect on the qualitative fatty acid profiles of *E. coli.* While variations in profiles are seen between induced and non-induced culturing within the MAE 6 strain, its parental strain MAE 4 had a very similar fatty acid profile despite the absence of OleB activity. While not addressed in this work, differentiating between free fatty acid and phospholipid content might be of interest given that OleB is thought to produce free fatty acids. This is particularly relevant in that acid catalyzed methyl esterification, which was used in this study, acts upon both phospholipids and free fatty acids (Liu, 1994). Given that MAE 6 has the *fadD*::kan insertion, OleB activity should produce a phenotype with a higher level of free fatty acids than without induction. If OleB has broad specificity for different chain length acyl-ACPs it would seem likely that the fatty acid profiles would remain the same qualitatively with the only difference being the increase in free fatty acids due to the activity of OleB and the *fadD*::kan insertion preventing free fatty acid degradation. Using thin-layer chromatography on lipid extracts before their derivatization has been used

previously to separate free fatty acid and phospholipid content in *E. coli* (Cho and Cronan, 1995). Applying this technique to the samples obtained in this study will provide more qualitative data concerning OleB activity and whether or not it affects the free fatty acid content.

Given the current model of 31:9 biosynthesis (Sugihara et al., 2010) there could be other thioesterases involved in cleaving the 16:4 fatty acids required for condensation to form the 31:9 hydrocarbon. One particular thioesterase that has potential to be the key enzyme is Orf 6, a hot-dog fold thioesterase that was originally annotated in the *pfa* gene cluster in *Photobacterium profundum* SS9 (Allen & Bartlett, 2002). A recent study that involved expression and characterization of Orf 6 revealed that it has substrate specificity towards either 16 or 20 carbon fatty-CoA molecules (Rodríguez-Guilbe et al., 2013). Genome sequences of other PUFA produces such as *Shewanella pealeana* and *Moritella marina* revealed that the ortholog of Orf 6 is not always clustered together with the *pfa* gene cluster (Rodríguez-Guilbe et al., 2013).

Given the substrate specificity of Orf 6 for both 20 and 16 carbon chain length fatty acids, a new model could be constructed where Orf 6 releases both 16 and 20 carbon length fatty acids from the *pfa* pathway yielding 20:5 and 16:4 fatty acids. The 20:5 fatty acid is incorporated into lipid membranes and the 16:4 free fatty acid is activated by the addition of a CoA group by OleC, the AMP dependant acyl-CoA synthase. From there OleA condenses two 16:4-CoA groups followed by thioester cleavage of the β-keto-thioester group from the OleA enzyme by OleB. This would then yield a β-keto carboxylic acid that could decarboxylate spontaneously to form a 31:8 ketone. Reduction of the ketone group followed by dehydration by OleD to form an alkene would produce

the final double bond needed to form the 31:9 hydrocarbon. To date, Orf 6 has yet to be expressed in recombinant *E. coli* that produce EPA. Over-expression of Orf 6 in the same strain backgrounds used in this study will undoubtedly provide more information as to how ACP-bound PUFAs and intermediates are released. Expression of such a thioesterase may also improve the yield of EPA in heterologous hosts by inhibiting any feedback control mechanisms that may be present.

While not addressed specifically in this study there does appear to be a relationship between EPA and 18:1 content in some of the strains examined here. Comparing MAE 4 and MAE 5, which differ only by the presence of the *pfaA-E* genes a trend can be seen where production of EPA leads to decreases in 18:1 content. A corresponding percentage drop in 16:1, which is preferentially elongated by FabF in response to lower temperatures (Magnuson et al., 1993) indicates that EPA production can compensate for the loss of monounsaturated fatty acids in *E. coli.* This relationship can be further examined by the inclusion of cerulenin, a FabF inhibitor (Heath and Rock, 2002), which should decrease the amount of 18:1 being synthesized. A concurrent increase in EPA should be seen in order to compensate for the loss of monounsaturated fatty acid synthesis. Cerulenin treatment has been previously shown to increase PUFA synthesis in several types marine bacteria (Allen et al., 1999; Morita, Nishida, Tanaka, Yano, & Okuyama, 2005).

Given the results of this study, OleB is not entirely excluded from its role as a thioesterase that cleaves a 16:4 intermediate. Such a polyunsaturated compound may have been produced and be beneath the detection limits of the GC/MS. Another possibility for 16:4 not being detected is that it was oxidized or otherwise degraded

during the derivatization process. Some degradation of polyunsaturated fatty acids during acid-catalyzed methyl esterification has been noted as a drawback compared to base-catalyzed esterification (Eder, 1995; Liu, 1994). In terms of detection limits it is worthwhile to note that isolation of a 16:4*n*-1 isomer from fish oil capsules required approximately 2 grams of concentrated fish oil to detect this lipid and high speed counter current chromatography was required to separate the 16:4*n*-1 and 16:4*n*-3 peaks from one another (Li et al., 2011). This unique fatty acid has also been detected at around 20% of total fatty acid content in the algae genus *Dunaliella* (Zhukova and Aizdaicher, 1995) and derivatization of this algal biomass could provide a standard for this compound. With the *Dunaliella* biomass and its high 16:4 content the processes of derivatization and the protocols used for GC/MS analysis can be further refined for product identification and/or isolation.

While heterologous expression in *E. coli* of the remaining *ole* genes could further enhance our knowledge of each gene product's role in the pathway, studying the pathway in its native host could provide a clearer picture. In particular, *Photobacterium profundum* SS9 and *Shewanella oneidensis* MR-1 are attractive options due to their relative genetic tractability. Both organisms have the *pfa* and *ole* gene clusters and targeted gene knockouts and chemical analysis of the resulting strains might provide more information regarding the essential genes required for hydrocarbon synthesis. *S. oneidensis* MR-1 becomes an attractive host in that it produces trace amounts of EPA and the 31:9 hydrocarbon (Sugihara et al., 2010) indicating that carbon flux through the *pfa* pathway is almost exclusively diverted into the hydrocarbon producing pathway. A knockout strain of *S. oneidenis* MR-1 which contains a deletion of the entire *oleA-D* gene cluster has been

constructed (Sukovich et al., 2010a) and complementation of *oleB* in this strain would be an alternative approach towards defining the role of OleB in hydrocarbon biosynthesis. *P. profundum* SS9 is also of interest in that mutants that greatly overproduce or are deficient in EPA are available (Allen et al., 1999). If *P. profundum* SS9 does produce the 31:9 hydrocarbon an EPA overproducer would theoretically produce more hydrocarbon. To date no chemical characterization looking for the 31:9 hydrocarbon in *P. profundum* SS9 has been done despite the presence of the both *pfa* and *ole* gene clusters in this organism.

Multiple strains of *E. coli* were produced in this study which involved heterologous expression of the *pfaA-E* genes with *oleB,* a predicted thioesterase that is thought to divert a 16:4 intermediate to hydrocarbon biosynthesis via the *ole* gene pathway. While the presumed 16:4 intermediate was not detected in this study, this work is the foundation for further studies aimed at elucidating the interaction between bacterial PUFA and hydrocarbon biosynthetic pathways.

References

Allen, E.E., and Bartlett, D.H. (2002). Structure and regulation of the omega-3 polyunsaturated fatty acid synthase genes from the deep-sea bacterium Photobacterium profundum strain SS9. Microbiology (Reading, England) *148*, 1903–1913.

Allen, E.E., Facciotti, D., and Bartlett, D.H. (1999). Monounsaturated but not polyunsaturated fatty acids are required for growth of the deep-sea bacterium Photobacterium profundum SS9 at high pressure and low temperature. Applied and Environmental Microbiology *65*, 1710–1720.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. a, Tomita, M., Wanner, B.L., and Mori, H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Molecular Systems Biology *2*, 2006.0008.

Beller, H.R., Goh, E.-B., and Keasling, J.D. (2010). Genes involved in long-chain alkene biosynthesis in Micrococcus luteus. Applied and Environmental Microbiology *76*, 1212–1223.

Chang, Y.Y., and Cronan, J.E. (1999). Membrane cyclopropane fatty acid content is a major factor in acid resistance of Escherichia coli. Molecular Microbiology *33*, 249–259.

Cho, H., and Cronan, J.E. (1995). Defective export of a periplasmic enzyme disrupts regulation of fatty acid synthesis. The Journal of Biological Chemistry *270*, 4216–4219.

Datsenko, K. a, and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proceedings of the National Academy of Sciences of the United States of America *97*, 6640–6645.

Delong, E.F., and Yayanos, a a (1986). Biochemical function and ecological significance of novel bacterial lipids in deep-sea procaryotes. Applied and Environmental Microbiology *51*, 730–737.

Eder, K. (1995). Gas chromatographic analysis of fatty acid methyl esters. Journal of Chromatography B: Biomedical Sciences and Applications *671*, 113–131.

Frias, J. a, Richman, J.E., Erickson, J.S., and Wackett, L.P. (2011). Purification and characterization of OleA from Xanthomonas campestris and demonstration of a non-decarboxylative Claisen condensation reaction. The Journal of Biological Chemistry *286*, 10930–10938.

Fujita, Y., Matsuoka, H., and Hirooka, K. (2007). Regulation of fatty acid metabolism in bacteria. Molecular Microbiology *66*, 829–839.

Heath, R.J., and Rock, C.O. (2002). The Claisen condensation in biology. Natural Product Reports *19*, 581–596.

Jump, D.B., Depner, C.M., and Tripathy, S. (2012). Omega-3 fatty acid supplementation and cardiovascular disease. Journal of Lipid Research *53*, 2525–2545.

Kato, C., and Nogi, Y. (2001). Correlation between phylogenetic structure and function: examples from deep-sea Shewanella. FEMS Microbiology Ecology *35*, 223–230.

Kawamoto, J., Kurihara, T., Yamamoto, K., Nagayasu, M., Tani, Y., Mihara, H., Hosokawa, M., Baba, T., Sato, S.B., and Esaki, N. (2009). Eicosapentaenoic acid plays a beneficial role in membrane organization and cell division of a cold-adapted bacterium, Shewanella livingstonensis Ac10. Journal of Bacteriology *191*, 632–640.

Kawamoto, J., Sato, T., Nakasone, K., Kato, C., Mihara, H., Esaki, N., and Kurihara, T. (2011). Favourable effects of eicosapentaenoic acid on the late step of the cell division in a piezophilic bacterium, Shewanella violacea DSS12, at high-hydrostatic pressures. Environmental Microbiology *13*, 2293–2298.

Kris-Etherton, P.M., and Hill, A.M. (2008). N-3 fatty acids: food or supplements? Journal of the American Dietetic Association *108*, 1125–1130.

Ladygina, N., Dedyukhina, E.G., and Vainshtein, M.B. (2006). A review on microbial synthesis of hydrocarbons. Process Biochemistry *41*, 1001–1014.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Li, D., Schröder, M., and Vetter, W. (2011). Isolation of 6,9,12,15-Hexadecatetraenoic Fatty Acid (16:4n-1) Methyl Ester from Transesterified Fish Oil by HSCCC. Chromatographia *75*, 1–6.

Liu, K.-S. (1994). Preparation of fatty acid methyl esters for gas-chromatographic analysis of lipids in biological materials. Journal of the American Oil Chemists' Society *71*, 1179–1187.

Magnuson, K., Jackowski, S., Rock, C.O., and Cronan, J.E. (1993). Regulation of fatty acid biosynthesis in Escherichia coli. Microbiological Reviews *57*, 522–542.

Marr, A.G., and Ingraham, J.L. (1962). Effect of Temperature on the Composition of Fatty Acids in Escherichia Coli. Journal of Bacteriology *84*, 1260–1267.

Metz, J.G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R., Lardizabal, K., Domergue, F., Yamada, a, et al. (2001). Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science (New York, N.Y.) *293*, 290–293.

Morita, N., Nishida, T., Tanaka, M., Yano, Y., and Okuyama, H. (2005). Enhancement of polyunsaturated fatty acid production by cerulenin treatment in polyunsaturated fatty acid-producing bacteria. Biotechnology Letters *27*, 389–393.

Motoigi, T., and Okuyama, H. (2011). Fatty acid and hydrocarbon composition in tropical marine Shewanella amazonensis strain SB2BT. Journal of Basic Microbiology *51*, 484–489.

Nichols, D.S., Nichols, P.D., and McMeekin, T.A. (1995). A new n-C 31:9 polyene hydrocarbon from Antarctic bacteria. FEMS Microbiology Letters *125*, 281–285.

Nishida, T., Orikasa, Y., Ito, Y., Yu, R., Yamada, A., Watanabe, K., and Okuyama, H. (2006). Escherichia coli engineered to produce eicosapentaenoic acid becomes resistant against oxidative damages. FEBS Letters *580*, 2731–2735.

Nishida, T., Morita, N., Yano, Y., Orikasa, Y., and Okuyama, H. (2007). The antioxidative function of eicosapentaenoic acid in a marine bacterium, Shewanella marinintestina IK-1. FEBS Letters *581*, 4212–4216.

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. Biochimie *86*, 807–815.

Rodríguez-Guilbe, M., Oyola-Robles, D., Schreiter, E.R., and Baerga-Ortiz, A. (2013). Structure, activity, and substrate selectivity of the Orf6 thioesterase from Photobacterium profundum. The Journal of Biological Chemistry *288*, 10841–10848.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (2001). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press).

Shulse, C.N., and Allen, E.E. (2011). Widespread occurrence of secondary lipid biosynthesis potential in microbial lineages. PloS One *6*, e20146.

Sugihara, S., Hori, R., Nakanowatari, H., Takada, Y., Yumoto, I., Morita, N., Yano, Y., Watanabe, K., and Okuyama, H. (2010). Possible biosynthetic pathways for all cis-3,6,9,12,15,19,22, 25,28-hentriacontanonaene in bacteria. Lipids *45*, 167–177.

Sukovich, D.J., Seffernick, J.L., Richman, J.E., Hunt, K. a, Gralnick, J. a, and Wackett, L.P. (2010a). Structure, function, and insights into the biosynthesis of a head-to-head

hydrocarbon in Shewanella oneidensis strain MR-1. Applied and Environmental Microbiology *76*, 3842–3849.

Sukovich, D.J., Seffernick, J.L., Richman, J.E., Gralnick, J. a, and Wackett, L.P. (2010b). Widespread head-to-head hydrocarbon biosynthesis in bacteria and role of OleA. Applied and Environmental Microbiology *76*, 3850–3862.

Thomason, L.C., Costantino, N., and Court, D.L. (2007). E. coli genome manipulation by P1 transduction. Current Protocols in Molecular Biology / Edited by Frederick M. Ausubel ... [et Al.] *Chapter 1*, Unit 1.17.

Wainwright, P.E. (2007). Dietary essential fatty acids and brain function: a developmental perspective on mechanisms. Proceedings of the Nutrition Society *61*, 61–69.

Zhukova, N., and Aizdaicher, N. (1995). Fatty acid composition of 15 species of marine microalgae. Phytochemistry *39*.