

**Functional analysis of a malonyl-CoA:ACP transacylase-like domain from
polyunsaturated fatty acid synthase in *Thraustochytrium***

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ABSTRACT

Very long chain polyunsaturated fatty acids (VLCPUFAs) such as docosahexaenoic acid (DHA, 22:6-4,7,10,13,16,19) and eicosapentaenoic acid (EPA, 20:5-5,8,11,14,17) have been widely recognized for their health benefits. These omega-3 fatty acids are important because they are essential parts of cell membranes and are involved in mediating various physiological processes in humans and animals. The *de novo* biosynthesis of VLCPUFAs occurs only in certain microorganisms through either an aerobic or anaerobic pathway. In the aerobic pathway, various desaturases and elongases are involved for the introduction of double bonds and the elongation of chain length, while the anaerobic pathway is mediated by a polyunsaturated fatty acid synthase (PUFA synthase). In *Thraustochytrium*, the biosynthesis of VLCPUFAs is catalyzed by a PUFA synthase comprising three large subunits, each with multiple predicted catalytic domains. However, most of these domains are not biochemically characterized and their functions remain unknown. The objective of this project was to functionally characterize a malonyl-CoA:ACP transacylase (MAT)-like domain of a PUFA synthase from *Thraustochytrium* by the heterologous expression of the domain as a standalone protein in *Escherichia coli*.

Bioinformatic analysis was first performed to dissect the MAT-like domain in the subunit-B of the PUFA synthase. After that, this domain was expressed as a standalone protein in *E. coli* mutant and wild type strains for complementation and activity assays. The results showed that the MAT-like domain could not complement a temperature-sensitive mutant ($\Delta fabD^{ts}$) defective in malonyl-CoA:ACP transacylase activity, disallowing growth at a non-permissive temperature (42 °C), while an authentic MAT domain from subunit-A of the synthase could complement the defective phenotype, allowing the growth at both permissive (37 °C) and non-permissive temperature (42 °C). This result indicated that the MAT-like domain does not function as a malonyl-CoA:ACP transacylase. Enzymatic assays showed that the MAT-like domain possessed thioesterase activity towards acyl-CoAs and that DHA-CoA was the preferred substrate among the acyl-CoAs tested. In addition, expression of this domain in an *E. coli* mutant ($\Delta fabD$) defective in acyl-CoA synthetase activity increased the accumulation of free fatty acids (FFAs). Furthermore, site-directed mutagenesis showed that the substitution of two residues, serine at 96 (S96) and histidine at 220 (H220), in

the MAT-like domain with alanine significantly reduced its activity towards DHA-CoA and suppressed the accumulation of FFAs in the *ΔfadD* mutant.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
<i>ΔfabD^{ts}</i>	Malonyl-CoA:ACP transacylase temperature-sensitive mutant
<i>ΔfadD</i>	Acyl-CoA synthetase mutant
<i>ΔtesAtesB</i>	Thioesterase A and B mutant
AA	Arachidonic acid
ACC	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
ALA	α-linolenic acid
AMD	Age-related macular degeneration
BLASTp	Basic local alignment search tool
bp	Base pair
CDV	Cardiovascular disease
CoA	Coenzyme A
COX	Cyclooxygenase
DAG	Diacylglycerol
DH	Dehydratase
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DTNB	5-(3-carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid
EDTA	[[2-(Bis-carboxymethyl-amino)-ethyl]-carboxymethyl-amino] acetic acid
EFAs	Essential fatty acids
EPA	Eicosapentaenoic acid
ER	Reductase
FAMES	Fatty acid methyl esters
FAS	Fatty acid synthase
FatA, B	Plant thioesterase
FFAs	Free fatty acids
g	Gravity
h	Hour(s)

H220	Histidine at position 220
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HP Taq DNA polymerase	High performance <i>Thermus aquaticus</i> polymerase
IκB	Inhibitory subunit of NF- κ B
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IUGR	Intrauterine growth restriction
KS	β -ketoacyl-ACP synthase
L	Liter(s)
LA	Linoleic acid
LB	Luria Bertani
LO	Lipoxygenase
LT	Leukotrienes
MAT	Malonyl-CoA:ACP transacylase
min	Minutes
mL	Milliliter(s)
mM	Millimolar
MUFAs	Mono-unsaturated fatty acids
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor- κ B
ng	Nanogram(s)
OA	Oleic acid
OD	Optical density
ORF	Open reading frame
P	P-value
PA	Palmitoleic acid
PCR	Polymerase chain reaction
PfaB	Polyunsaturated fatty acid B
PG	Prostaglandins
PKS	Polyketide synthase
PL	Phospholipid(s)
PPARs	Peroxisome proliferator activated receptors

PUFA	Polyunsaturated fatty acid
PUFAs	Polyunsaturated fatty acids
rpm	Revolutions per minute
s	Seconds
S96	Serine at position 96
SCFAs	Short chain fatty acids
ScTIOMCAT	<i>Schizochytrium sp.</i> TIO1101 malonyl-CoA:ACP transacylase
SDS	Sodium dodecyl sulfate
TAG	Triacylglycerol
TE	Thioesterase
TesA	<i>E. coli</i> thioesterase I
TesB	<i>E. coli</i> thioesterase II
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor- α
Tris-HCl	Trisaminometano hydrochloride
TX	Thromboxanes
U/μL	Units per microliter
μg	Microgram(s)
μL	Microliter(s)
μM	Micromolar
UV	Ultraviolet
VLCPUFAs	Very long chain polyunsaturated fatty acids
wt/v	Weight/volume
WT	Wild type

1. INTRODUCTION

1.1 Overview

The biosynthesis of very long chain polyunsaturated fatty acids (VLCPUFAs) through the anaerobic pathway is catalyzed by a PUFA synthase in some marine microorganisms. *Thraustochytrium* is a unicellular marine protist that can produce more than 50% of its total fatty acids as DHA, an omega-3 VLCPUFA of nutritional importance, using a PUFA synthase (Meesapyodsuk & Qiu, 2016). The PUFA synthase from *Thraustochytrium* comprises three subunits: subunit-A, -B and -C encoded by three large open reading frames (ORF-A, -B, and -C). The preliminary sequence analysis of the PUFA synthase indicates that ORF-A codes for a protein containing one β -ketoacyl-ACP synthase (KS1) domain, one malonyl-Coenzyme A (CoA):ACP transacylase domain (MAT), eight acyl carrier protein (ACP) domains, one ketoacyl reductase domain and one dehydratase (DH1) domain; ORF-B codes for a protein consisting of two KS domains (KS2, KS3), one MAT-like domain and one enoyl-ACP reductase (ER1) domain; and ORF-C codes for a protein consisting of two DH domains (DH2, DH3) and one ER domain (ER2) (Meesapyodsuk & Qiu, 2016; Zhao, Dauenpen, Qu, & Qiu, 2016). The aim of this study was the bioinformatic dissection and functional characterization of the malonyl-CoA:ACP transacylase (MAT)-like domain of the PUFA synthase from *Thraustochytrium*.

1.2 Hypothesis

The MAT-like domain in the subunit-B of the PUFA synthase from *Thraustochytrium* functions as a thioesterase based on several observations. First, there is already a malonyl-CoA:ACP transacylase (MAT) domain in the subunit-A of the PUFA synthase for the extender-loading function. Second, the MAT-like domain from Subunit-B is divergent from typical MAT domains. Third, VLCPUFAs synthesized by a PUFA synthase are released in free fatty acid form, which must come from internal thioesterase (TE) activity. Furthermore, this MAT-like domain in the PUFA synthase contains a motif GX SXG, which is also conserved in some thioesterases. Therefore, it is possible that this MAT-like domain possesses TE activity releasing synthesized VLCPUFAs from the ACP domains of the PUFA synthase.

1.3 Objectives

The four technical objectives of this research were as follows.

1. To bioinformatically analyze the MAT-like domain of the PUFA synthase from *Thraustochytrium*.
2. To express the MAT-like domain in *E. coli* *fabD* (malonyl-CoA:ACP transacylase), *tesA**tesB* (thioesterase) and *fadD* (acetyl-CoA synthetase) mutants.
3. To determine the enzymatic activity of the MAT-like domain by *in vitro* assays with the MAT-like protein expressed in *E. coli*.
4. To investigate the functional roles of two conserved residues: Serine96 and Histidine220 in the MAT-like domain by site-directed mutagenesis.

2. LITERATURE REVIEW

2.1 Essential fatty acids

Essential fatty acids (EFAs) are those that are required for human growth and development. However, humans cannot *de novo* synthesize these fatty acids and have to rely on the diet for them to stay healthy (Das, 2006a). EFAs are generally polyunsaturated fatty acids (PUFAs) with chain lengths of 18 carbons (18C) or more and double bonds of two or more. Although the terms EFAs and PUFAs are used to refer to the same type of fatty acids; it is important to note that all EFAs are PUFAs, but not *vice versa*. According to the location of the last double bond to the methyl-end, PUFAs can be classified into several families: omega-3 PUFAs, derived from α -linolenic acid (ALA, 18:3-9,12,15); omega-6 PUFAs, derived from linoleic acid (LA, 18:2-9,12); ω -7 PUFAs, derived from palmitoleic acid (PA, 16:1-9) and ω -9 PUFAs, derived from oleic acid (OA, 18:1-9). The first two families are EFAs while the latter two are not (Das, 2006b). Among omega-3 and omega-6 PUFAs, only LA and ALA are considered as strict EFAs as the rest of PUFAs can be synthesized by humans provided with these two fatty acids. The rest of fatty acids derived from the two strict EFAs are considered as conditional EFAs (Das, 2006b).

EFAs, and their derivatives, are biologically active compounds that are involved in various physiological processes and possess many health benefits such as the reduction of cardiovascular disease, improved brain and vision performance, cancer prevention, normal fetus and infant development (Kaur, et al., 2012). Sources for omega-6 EFAs are vegetable oils such as corn, soybean and sunflower oil, cereals and their baked goods, and animal products (Meyer et al., 2003). For the omega-3 EFAs, canola oil, walnuts and marine fish oils are rich sources for these fatty acids (Das, 2006b). Eicosapentaenoic acid (EPA, 20:5-5,8,11,14,17) and docosahexaenoic acid (DHA, 22:6-4,7,10,13,16,19) commonly known as marine omega-3 polyunsaturated fatty acids are the most important EFAs for human health. Modern Western diets are high in omega-6 fatty acids with potential pro-inflammatory properties and low in omega-3 fatty acids with anti-inflammatory properties. Therefore, it is highly desirable for humans to intake more omega-3 fatty acids to prevent inflammatory diseases and promote overall good health (Simopoulos, 2016).

2.2 Very long chain polyunsaturated fatty acids (VLCPUFAs)

According to their chain length, polyunsaturated fatty acids can be classified into two families, long chain PUFAs with the length from 16 carbons to 18 carbons and very long chain polyunsaturated fatty acids (VLCPUFAs) with the length longer than 18 carbons (Ruxton et al., 2004). Omega-3 VLCPUFAs such as EPA and DHA are synthesized from ALA through alternating desaturations and elongations. Omega-6 VLCPUFAs including arachidonic acid (AA, 20:4-5,8,11,14) are synthesized from LA through a similar process. The desaturases and elongases involved in the biosynthesis of omega-3 and omega-6 VLCPUFAs from ALA and LA are shared by the two families; thus a competition for the same set of enzymes between the two family substrates can occur (Koletzko et al., 2008).

Both omega-3 and omega-6 VLCPUFAs are important for the proper growth and development of higher organisms such as humans and animals. They are essential components of cell membranes in the retina and brain, and the lack of these fatty acids impedes brain development and visual function. In addition, the VLCPUFAs are also the precursors of bioactive mediators that are involved in regulating various biological processes. Therefore, supplementation of these fatty acids is encouraged for appropriate development and enhanced performance of our eyes and central nervous system, as well as delayed onset of or treatment of certain chronic diseases (Serhan, et al., 2008), such as cardiac disorders, high blood pressure and diebetics (Adarme-Vega et al., 2012).

2.3 Health benefits of omega-3 VLCPUFAs

The potential health benefits of omega-3 VLCPUFAs were first described in the late 1970s (Mulvad et al., 1996). Later, Danish investigators linked the lower death rate of myocardial infarction in the Inuit population, which they attributed to their fish-based diets rich in omega-3 VLCPUFAs (O'Keefe & Harris, 2000). After that, many health benefits of omega-3 VLCPUFAs particularly DHA on prevention against cardiovascular diseases and brains and eye performance have been examined in animals and humans.

2.3.1 Cardiovascular disease

Cardiovascular disease (CDV) is one of the main causes of death in humans, accounting for almost 50% of all deaths in developed countries and 25% in the developing world. Risk factors, such as poor eating habits, low physical activity, drinking and smoking, high blood pressure, diabetes and being overweight can

contribute to the development of CDV (Sokola-Wysoczanska et al., 2018). All of these factors can result in chronic inflammation, which in turn could lead to the dysfunction of the cardiovascular system (Kromhout et al., 2012).

Several studies suggest that omega-3 VLCPUFAs can prevent myocardial infarction and arrhythmia through the rapid incorporation of these fatty acids in the phospholipids of cell membranes affecting their structure and function, and subsequent anti-inflammatory effects of derivatives synthesized from these fatty acids (Din et al., 2004). Although the precise mechanisms of omega-3 VLCPUFAs on cardiovascular systems are not fully understood; studies have shown that when humans take a diet supplemented with fish oil, a reduction in the platelet-monocyte aggregation, a potential cardiovascular benefit, has been observed (Din et al., 2008).

Another mechanism of omega-3 VLCPUFAs could be due to their anti-arrhythmic properties. Studies report that this type of fatty acid exerts anti-arrhythmic effects by modulating of myocyte electrophysiology, reducing the activity of membrane sodium channels in the cardiomyocyte, and regulating the activity of L-type calcium channels, thereby stabilizing the myocyte electrical excitability and preventing arrhythmia (Endo & Arita, 2016). However this is controversial as other studies also show that supplementation with omega-3 VLCPUFAs for a short period of six months to one year did not have any anti-arrhythmic effects (Bianconi et al., 2011; Macchia et al., 2013) indicating that the health promoting benefits of these molecules occurs through alternative and as-of-yet unidentified mechanisms. In addition, the anti-inflammatory properties of omega-3 VLCPUFAs can provide cardiovascular protective effects. Omega-3 VLCPUFAs, particularly EPA, decrease the production of inflammation-related cytokines such as tumor necrosis factor- α (TNF- α). TNF- α expression is regulated by nuclear factor- κ B (NF- κ B). I κ B, the inhibitory subunit of the NF- κ B complex is kept inactive in the cytoplasm under non-stress conditions. The phosphorylation of I κ B by an I κ B kinase leads to the activation of NF- κ B and subsequently up-regulating the expression of TNF- α . Other studies also revealed that omega-3 VLCPUFAs can exert anti-inflammatory effects through the suppression of the NF- κ B and TNF- α expression (Gomaa & Abd El-Aiz, 2016; Y. Zhao et al., 2004).

Omega-3 VLCPUFAs anti-inflammatory properties (Figure 2.1) have been attributed to the prevention of arachidonic acid (AA) conversion into pro-inflammatory 2-series prostaglandins (PG₂) and 4-series leukotrienes (LT₄) as catalyzed by

phospholipase A₂, cyclooxygenase and 5-lipoxygenase (Simopoulos, 2016). These enzymes are also involved in the production of anti-inflammatory prostaglandins such as 3-series PG₃ and thromboxanes (TX₃) and leukotrienes 5-series LT₅ (Endo & Arita, 2016) using EPA as a substrate. DHA can also serve as a precursor of anti-inflammatory mediators such as resolvins, protectins and maresins, which have pro-resolving and regenerative properties (Serhan, 2014).

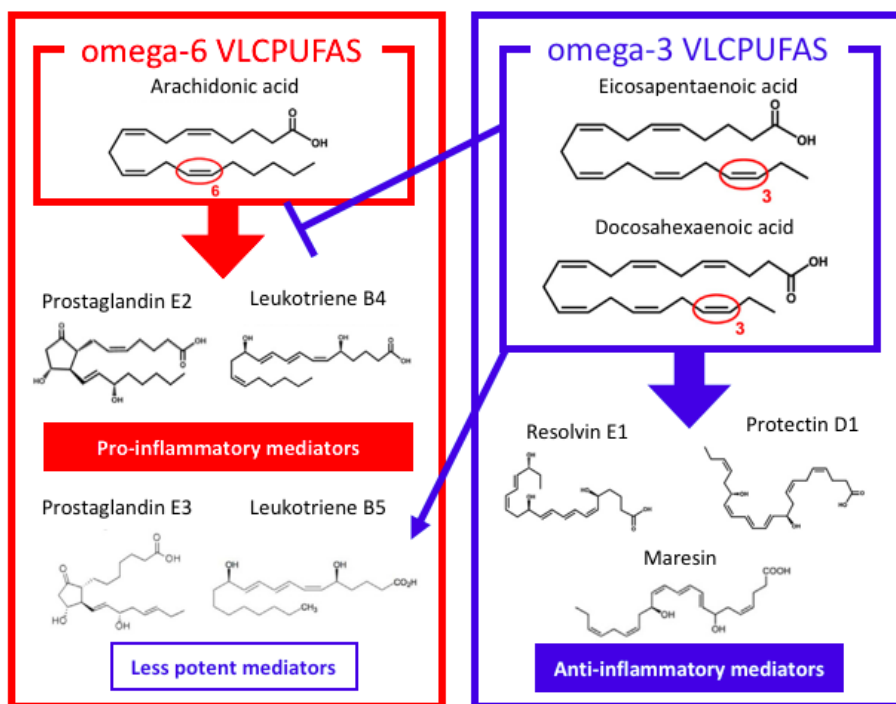


Figure 2.1 Pro-inflammatory and anti-inflammatory products of omega-6 and omega-3 VLCPUFAs. Adapted from Endo & Arita, 2016.

2.3.2 Brain function

Fatty acid composition of the brain consists of high levels of palmitic acid (16:0), AA, DHA and a lower level of EPA. DHA is 250-300 times higher than EPA (Chen et al., 2009). This ratio is probably conserved in higher animals (Crawford et al., 1976).

Omega-3 VLCPUFAs are essential components of cell membranes in human neurons, and play an important role in the fluidity modulation, permeability and protein activity in the neuronal membrane (Stillwell et al., 2005). It has been observed that when DHA was administered in rat neural stem cells, it promote neurogenesis, a process by which neural stem cells differentiate into new neurons, and the damaged brain tissue is self-repaired (Dyall, 2015). Neurogenesis promoted by DHA could be due to a

decreased level of Hes1 (hair and enhancer of split-1), a transcriptional repressor that inhibits neuronal differentiation (Katakura et al., 2013). However, it is still unclear if the omega-3 VLCPUFAs exert these effects by themselves or through their derived mediators.

In the aging brain, the anti-inflammatory properties of omega-3 VLCPUFAs have positive effects on age-related neural impairments such as decreased learning and memory (McGahon et al., 1999). In particular, docosapentaenoic acid (DPA, 22:5-7,10,13,16,19) and EPA have been shown to decrease the age-related microglial activation, reverse age-related impairment in spatial learning and reduce the associated oxidative stress when 200 mg/kg/day of these fatty acids are supplemented in rats (Kelly et al., 2011).

2.3.3 Retina function

DHA is an essential component of the membrane of photoreceptor cells in retina and helps to maintain membrane flexibility. Thus, it plays an important role in retina function (Garelli et al., 2006). Another important function of omega-3 VLCPUFAs is their anti-inflammatory effects in retina. The presence of high levels of omega-3 VLCPUFAs inhibit the formation of pro-inflammatory omega-6 AA derivatives through competition for cyclooxygenase (COX) and lipoxygenase (LO) (Calder, 2003). In addition, DHA prevents the induced apoptotic death of photoreceptors by reducing oxidative damage of the mitochondrial membrane (Rotstein et al., 2003). Treatment with algae oil high in omega-3 VLCPUFAs has been shown to reduce light-induced apoptosis and angiogenesis in rabbits retina through the suppression of the expression of interleukin-1 β , TNF- α and COX2 (Deng et al., 2018).

Although vast scientific evidence suggests that omega-3 VLCPUFAs can aid in the maintenance of retina health, research results are not conclusive on the benefits of a diet high in these fatty acids for the prevention of age-related macular degeneration (AMD) (Souied et al., 2015), a medical condition produced by the loss of vision in the central region of the retina known as the macula, which is one of the principal causes of blindness in the elderly (Tan et al., 2018). Therefore, more research would be required to establish the benefits of omega-3 VLCPUFAs against macular degeneration.

2.3.4 Fetal development

Supplementation with omega-3 VLCPUFAs during pregnancy and maternal

stages provides positive effects on the development of fetal and child brains, and cognitive activity (Saccone et al., 2016). The report by Olsen et al (1986) demonstrated that the consumption of omega-3 VLCPUFAs prolonged gestation in human beings by reducing the production of PG2 (Olsen et al., 1986). It is noteworthy that the fetus and neonate can synthesize short chain fatty acids (SCFAs) and mono-unsaturated fatty acids (MUFAs), but their ability to produce VLCPUFAs is limited. Therefore, it is important that they obtain these fatty acids from breast milk which is in turn derived from mother's diet (Mennitti et al., 2015).

Supplementation of DHA during pregnancy can activate the peroxisome proliferator activated receptors (PPARs), which are nuclear receptors regulating the expression of anti-inflammatory genes through the ligand-binding with VLCPUFAs; this activation has been beneficial to the lung development of fetus. When there is condition of intrauterine growth restriction, supplementation of omega-3 VLCPUFAs can ameliorate its effects and facilitate the normal development of baby lungs (Joss-Moore et al., 2011). Another benefit of the consumption of omega-3 VLCPUFAs during pregnancy is the reduction of fat accretion and adiposity in the offspring. Sardinha, et al (2013) fed rats with a diet with 8% of fish oil during day 1 to 12 of pregnancy and found fat accretion in male offspring was reduced, although this effect was not seen in female offspring (Sardinha et al., 2013). Furthermore, the supplementation of omega-3 PUFAs can help ameliorate fetus damage caused by a diabetic condition in the mother. The incorporation of flax seeds rich in an omega-3 fatty acid in a diet administered to diabetic pregnant rats resulted in a decrease in the activity of antioxidant enzymes such as catalase and superoxide dismutase, and the malondialdehyde and glutathione levels, indicators of oxidative stress, suggesting that omega-3 fatty acids could provide prevention against the negative effect of diabetes mothers on their offspring (Makni et al., 2011).

2.4 Sources of VLCPUFAs

The most common source of VLCPUFAs for humans and animals are fish, such as salmon, mullet and mackerel (Adarme-Vega et al., 2012); however, the increasing demand for these fatty acids in dietary supplementation has placed high pressure on this source. First, as the fish population in oceans is over-exploited, the sustainability of this source is under pressure. Second, farmed fish require omega-VLCPUFAs for normal growth and development, but are unable to synthesize these compounds, and must rely

on feed supplementation. Finally, as marine fish species rich in omega-3 VLCPUFAs are at the top of the food chain in oceans, the availability of effective VLCPUFAs, particularly DHA, varies with growth seasons and changes of upstream species in the food chain (Mohanty et al., 2016; Walsh & Metz, 2013). Therefore, there is a need for seeking alternative and sustainable sources for these fatty acids.

One possible alternative source for VLCPUFAs is the native microbial VLCPUFA producers such as *Schizochytrium* and *Thraustochytrium* that can accumulate a very high level of DHA (36% and 50% of the total fatty acids, respectively) (Meesapyodsuk & Qiu, 2016; B. Zhao et al., 2018). Another possible source for these fatty acids is transgenic oilseeds producing these fatty acids by metabolic engineering of the biosynthetic pathways of microbial origin in plants.

2.5 Fatty acid biosynthesis

De novo biosynthesis of long chain fatty acids in yeast and vertebrates is catalyzed by a large enzyme called Type I fatty acid synthase (FAS). However, the same biosynthesis of these molecules in plants and bacteria is catalyzed by a protein complex comprising multiple discrete enzymes known as Type II fatty acid synthase. Although the structure and organization of these fatty acid synthases are different, the biochemical process for fatty acid biosynthesis is similar (Verwoert et al., 1994).

The biosynthesis of long chain fatty acids comprises four basic reactions (Figure 2.2). The first biochemical reaction is the condensation of acyl-ACP with malonyl-ACP by 3-keto-acyl-ACP synthase (keto synthase or KS) producing 3-keto-acyl-ACP. Next, 3-keto-acyl-ACP is reduced by 3-keto-acyl-ACP reductase (KR), which is then dehydrated by 3-hydroxy-acyl-ACP dehydratase (DH) to a 2,3-trans-enoyl-ACP. Finally, the enoyl-ACP is reduced to saturated acyl-ACP with the extension of two carbons by 2,3-trans-enoyl reductase (ER).

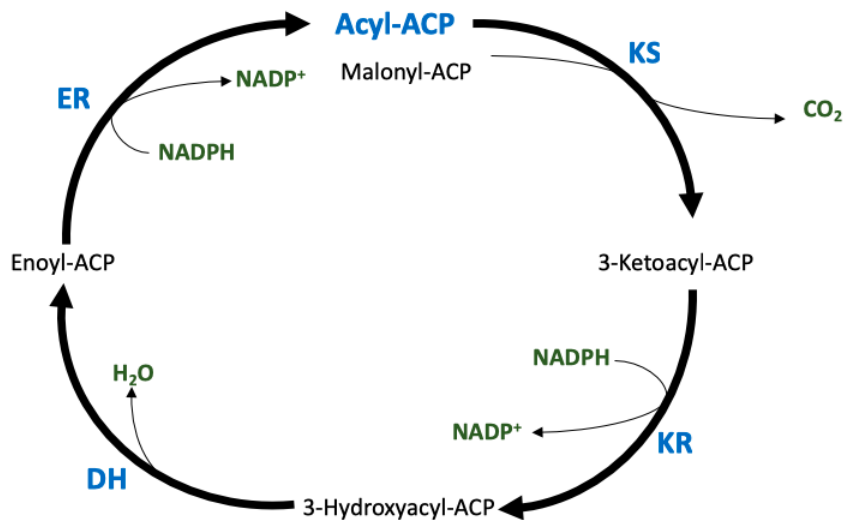


Figure 2.2 Biosynthesis of long chain fatty acids. Adapted from Meesapyodsuk & Qiu, 2016.

In plants, these four reactions are catalyzed by four individual enzymes constituting a Type II fatty acid synthase and the end products of biosynthesis are 16:0 and 18:0 fatty acid thioesters (fatty acyl-acyl carrier protein (ACP)). After synthesis, these fatty acids can be desaturated by a soluble stearoyl-ACP desaturase in chloroplast stroma, to produce 16:1-ACP and 18:1-ACP, respectively. The two monounsaturated fatty acids are then moved out of chloroplast to cytosol where two membrane-bound desaturases (Δ 12 and Δ 15 desaturase) introduce two additional double bonds sequentially on 18:1-9, producing 18:2-9,12 (LA) and 18:3-9,12,15, respectively (Wallis et al., 2002).

In animals, the reactions involved in fatty acid biosynthesis are catalyzed by four main catalytic domains (KS, KR, DH and ER) in a Type I fatty acid synthase. Besides the four catalytic domains, this large enzyme also contains other important domains involved in fatty acid synthesis such as ACP, malonyl-CoA:ACP transacylase (MAT) and thioesterase (TE). In PUFA synthases, an ACP domain can be present several times in tandem, which carries and delivers growing fatty acyls to the different catalytic sites of the fatty acid synthase (Jiang et al., 2009). The malonyl-CoA:ACP transacylase domain selects an extender unit from acyl-CoAs and discharges it to ACP. TE hydrolyzes acyl-ACP, releasing free fatty acids (Keatinge-Clay, 2012).

2.5.1 Malonyl-CoA:ACP transacylase (MAT)

Malonyl-CoA is a two-carbon donor molecule, product of the carboxylation of acetyl-CoA through the activity of acetyl-CoA carboxylase (ACC). This molecule serves as a control point between lipogenesis and β -oxidation and is important for fatty-acid biosynthesis (Cheng et al., 2013). The malonyl moiety of malonyl-CoA is transferred to a holo-ACP, producing malonyl-ACP, which serves as an extender substrate for the biosynthesis of fatty acids (Voelker & Davies, 1994). This transfer is catalyzed by malonyl-CoA:ACP transacylase (MAT) either from a discrete enzyme in a Type II fatty acid synthase complex or from a MAT domain in a Type I fatty acid synthase (Figure 2.3, Liu et al., 2006).

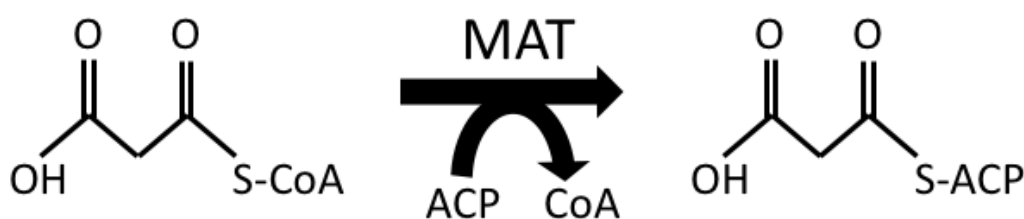


Figure 2.3 Reaction of the MAT. Adapted from Yu et al., 2011.

The MAT protein, also known as FabD in *E. coli*, is essential for fatty acid biosynthesis (Cheng et al., 2013). The genetic inactivation of *fabD* is lethal in the bacterium (Sun et al., 2012). The crystal structure analysis of FabD shows that it has the core structure of a α/β hydrolase with β strands connected by α helices and a catalytic triad: Ser-His-Asp (Liu et al., 2006; Serre et al., 1995). The catalytic reaction comprises two steps. First, a MAT binds a malonyl-CoA to form a malonyl-MAT intermediate. Second, the MAT transfers the malonyl moiety from the intermediate to a holo-ACP (Sun et al., 2012).

In marine VLCPUFA producers Thraustochytrids, including *Schizochytrium* and *Thraustochytrium*, a discrete MAT from *Schizochytrium* was recently identified and functionally characterized. It contains two conserved motifs: FPGQG involved in catalysis and GHSLGE involved in ACP-binding (Cheng et al., 2013). Two putative MAT domains in a PUFA synthase responsible for the biosynthesis of VLCPUFAs in *Thraustochytrium* have also been identified. One MAT domain resides in Subunit-A

while the other is located in Subunit-B (Meesapyodsuk & Qiu, 2016). However, their exact functions are currently unknown.

2.5.2 Thioesterase (TE)

Thioesterases or thioester hydrolases, are a large group of enzymes that release acyl moieties from the phosphopantetheine of ACP or CoA (Smith & Tsai, 2007) through hydrolysis of the thioester bond between a carbonyl group and a sulfur atom. Some thioesterases can also release the acyl moiety from glutathione and ubiquitin (Cantu et al., 2010). *E. coli* has two major acyl-CoA thioesterases, thioesterase I and II, encoded by *TesA* and *TesB* genes, respectively; however, neither is essential for the bacterial growth and development. The thioesterase I (*TesA*) consists of 182 amino acids (Cho & Cronan, 1993) and is a monomeric enzyme located in the cell periplasm for hydrolyzing acyl-thioester bonds to produce free fatty acids (Lennen & Pflieger, 2012). The active site of thioesterase I is found on the motif Gly-Asp-Ser-Leu-Ser-Ala-Gly with Ser being the active residue. As a major thioesterase in *E. coli*, one of its possible roles is to modulate the chain length of fatty acids synthesized. However, this function has been questioned, as the thioesterase is a periplasm enzyme, while fatty acid synthesis occurs in the cytosol. In addition, the overexpression or inactivation of the thioesterase does not change the fatty acid profile (Cho & Cronan, 1993). Nevertheless, it has substrate specificity towards C12 to C18 acyl chains and may play a role in fatty acid synthesis (Naggert et al., 1991; Zheng et al., 2004). *E. coli* thioesterase II (*TesB*) is a 286 polypeptide and forms a homo-tetramer for the function (Naggert et al., 1991). It can cleave C6 to C18 acyl-CoA esters (Zheng et al., 2004). Thus, it seems unlikely that this thioesterase is a chain-terminating enzyme in the fatty acid synthesis.

Thioesterases have been classified into 23 families based on their tertiary structures. Families 14 and 19 correspond to thioesterases that hydrolyze acyl groups and ACP (Figure 2.4) (Cantu et al., 2010). The structure of acyl-ACP thioesterases in bacteria and plants possesses α/β -hydrolase folds that are in charge of fatty acid or polyketide synthesis termination. In plants, two major thioesterases FatA and FatB have been identified and they hydrolyze C8-C18 acyl-ACP thioesters (Cantu et al., 2010).

Like other α/β -hydrolases, thioesterases employ a nucleophile (Ser, Cys or Asp)-His-acid triad for catalysis. The first residue of the triad is often a serine as a nucleophile, the second residue is always a histidine, and the third is an acidic amino acid. The nucleophile is located in “nucleophile elbow” and can be reached by a

substrate and a water molecule for hydrolysis (Nardini & Dijkstra, 1999). Commonly, the acid residue stabilizes the His, which is a basic amino acid and accepts a proton from the nucleophile to form an intermediate with the substrate to be further attacked by a water molecule (Cantu et al., 2010).

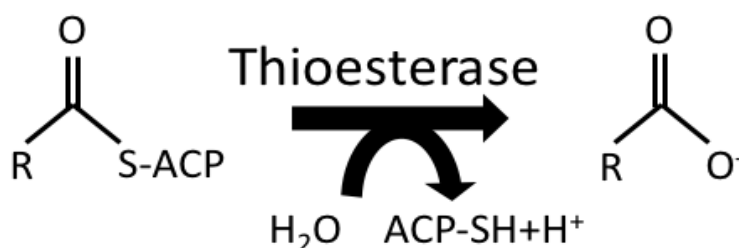


Figure 2.4 Reaction catalyzed by thioesterase. Adapted from iGEM, 2017.

Acyl-ACP thioesterase is hypothesized to be required for releasing VLCPUFAs from an ACP domain of a PUFA synthase at the end of the VLCPUFA biosynthesis, which possibly is catalyzed by internal thioesterase activity of the synthase (Metz et al., 2009). However, a domain inside a PUFA synthase responsible for this activity has not been identified in Thraustochytrids. *Photobacterium profundum*, a deep-sea bacterium, can also produce omega-3 VLCPUFAs using a PUFA synthase. However, no thioesterase-like domain is present in the synthase. Rodríguez-Guilbe et al., (2013) identified a discrete thioesterase near the PUFA synthase cluster in the bacterium and showed that it has preferential activity towards long-chain fatty acyl-CoA substrates.

2.6 VLCPUFAs biosynthesis

Omega-3 VLCPUFAs are mainly *de novo* synthesized in marine microorganisms and subsequently go through the food chain from zoo-plankton to carnivorous fish (Walsh et al., 2016). There are two different biosynthetic pathways to produce the VLCPUFAs in these microorganisms, aerobic and anaerobic (Qiu, 2003; Swanson et al., 2012).

2.6.1 Aerobic pathway

The aerobic pathway to produce VLCPUFAs occurs in the endoplasmic reticulum of eukaryotic cells where double bonds are introduced by desaturases (Qiu et al., 2011), and fatty acid chain lengths are determined by elongases (Figure 2.5). As mentioned previously, fatty acids are synthesized from acetyl-CoA and malonyl-CoA

through a series of reactions performed by the fatty acid synthase complex or enzymes. The resulting stearic acid (18:0) is then desaturated by a $\Delta 9$ desaturase to form OA, which further can be desaturated to produce the two PUFAs families: omega-3 and omega-6. the biosynthesis of VLCPUFAs in the aerobic pathway usually starts with the omega-6 LA (18:2-9,12) which can be desaturated by a $\Delta 15$ desaturase to form omega-3 ALA (18:3-9,12,15). LA and ALA are substrates of the same enzymes to produce VLCPUFAs. The first step is a desaturation by $\Delta 6$ desaturase that introduces a double bond in the $\Delta 6$ carbon. This desaturation is followed by an elongation. Subsequently, a $\Delta 5$ desaturation and a new elongation takes place, finally a $\Delta 4$ desaturation occurs to form DPA and DHA, respectively (Ratledge, 2004).

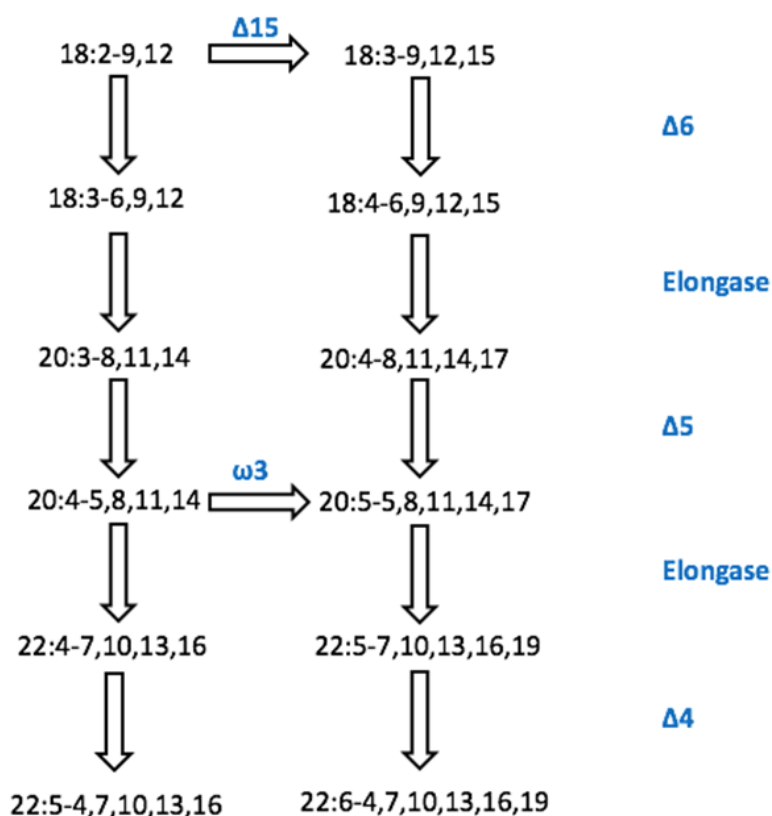


Figure 2.5 Aerobic pathway for the biosynthesis of VLCPUFAs in *Thraustochytrium*. Adapted from Qiu, 2003.

2.6.2 Anaerobic pathway

The anaerobic pathway mostly occurs in prokaryotic and eukaryotic microbes where the biosynthesis of VLCPUFAs is catalyzed by a polyunsaturated fatty acid (PUFA) synthase. Similar to polyketide synthase (PKS) and Type I fatty acid synthase (FAS), PUFA synthase is a mega-enzyme comprising more than one modules or

subunits, each with multiple catalytic domains such as ketosynthase (KS), malonyl-CoA:ACP transacylase (MAT), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), enoylreductase (ER) and acyl carrier protein (ACP) (Jiang et al., 2008) (Figure 2.6). These domains can be arranged in different orders, but they are well conserved in PUFA synthases across marine microorganisms (Shulse & Allen, 2011).

The biosynthetic process takes place through assembling a two-carbon unit precursor from malonyl-CoA, and double bonds are introduced during the chain extending process (Walsh et al., 2016). The synthesis of one DHA molecule requires one primer acetyl-CoA and ten extenders malonyl-CoA and as well as sixteen nicotinamide adenine dinucleotide phosphate (NADPH) molecules, with no intermediate precursors involved (Walsh & Metz, 2013). A distinguish feature of PUFA synthases is the presence of multiple (5-10) tandem ACP domains, which increases the biosynthetic capacity of VLCPUFAs (Jiang et al., 2008). The final products are in free fatty acid form (Walsh & Metz, 2013).

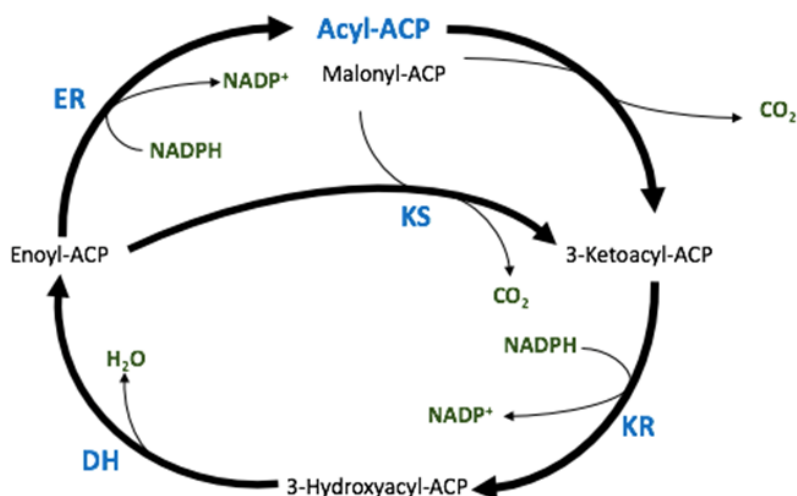


Figure 2.6 Anaerobic pathway for the biosynthesis of VLCPUFAs in *Thraustochytrium*. Adapted from Qiu, 2003.

2.7 *Thraustochytrium*

Thraustochytrium is a unicellular marine protist that belongs to the group of heterokonts called Thraustochytrids. It can produce a higher level of DHA (Zhang et al., 2018) than other species such as bacteria, fish, fungi, and microalgae (Adarme-Vega et al., 2012). The production of DHA can reach up to 56% of the total fatty acids (Meesapyodsuk & Qiu, 2016) in this species. *Thraustochytrium* can be cultivated in

simple media and has been used as a commercial source for DHA. However, the extraction of DHA from the cell requires a high input in energy and is time-consuming in preparing the dried biomass and cell lysis (Lowrey et al., 2016).

2.8 *Thraustochytrium* PUFA synthase

Interestingly, both aerobic and anaerobic pathways for the biosynthesis of VLCPUFAs co-exist in *Thraustochytrium*. The biosynthesis of DHA in the aerobic pathway goes through alternating desaturations and elongations to the final products: DPA and DHA (Qiu et al., 2001). Most desaturases including $\Delta 4$, $\Delta 5$, $\Delta 6$ and $\omega 3$, and elongases including $\Delta 5$ and $\Delta 6$ in this pathway have been identified (Meesapyodsuk & Qiu, 2016). The anaerobic pathway for the VLCPUFA biosynthesis (Figure 2.7) goes through the reiterative cycles of four reactions starting from acetate as the substrate: condensation (KS), ketoreduction (KR), dehydration (DH) and enoylreduction (ER); all are performed by a single PUFA synthase. The synthase is comprised of three subunits or open reading frames (ORF), each with multiple catalytic domains. Based on the presence of characteristic active sites, these catalytic domains were predicted (Figure 2.7, Meesapyodsuk & Qiu, 2016). However, most of these domains are not biochemically characterized.

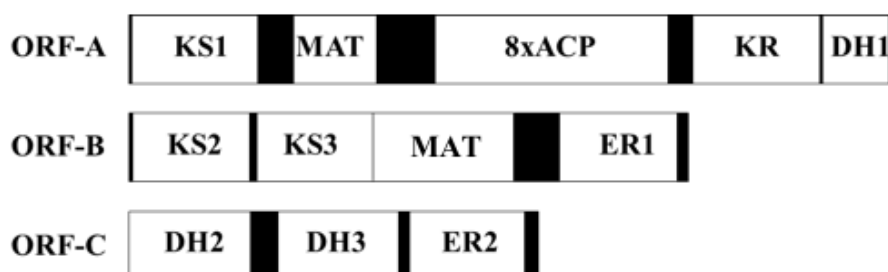


Figure 2.7 Structure of the PUFA synthase from *Thraustochytrium*. Adapted from Meesapyodsuk & Qiu, 2016.

3. MATERIALS AND METHODS

3.1 *E. coli* strains

E. coli Top10 was purchased from Invitrogen Biotechnology Co. (Grand Island, NY, USA). *E. coli fabD* mutants ($\Delta fabD$) were obtained from the Coli Genetic Stock Center from the Yale University (New Haven, CO, USA). An *E. coli tesAtesB* double mutant ($\Delta tesAtesB$) and parental wild type strain were kindly provided by Dr. John Cronan, University of Illinois (Urbana-Champaign, IL, USA). *E. coli $\Delta fabD$* strain was provided by Dr. Pamela Silver, Harvard Medical School (Boston, MA, USA) (Torella et al., 2013). *E. coli* SHuffle strain was acquired from New England BioLabs (Ipswich, MA, USA). *E. coli* BL21 Star (DE3) and Rosetta (DE3) were also purchased from Invitrogen Biotechnology Co. *E. coli* C41 and C43 were obtained from Lucigen (Middleton, WI, USA). The genotypes of the strains are shown in Table 3.1.

Table 3.1 Genotypes of *E. coli* mutant and parental strains.

Strains	Genotype
<i>AfabD</i> parental	<i>araC14, lacY1, tsx-57, glnX44(AS), gltA5, galK2(Oc), Rac-0, rfbC1, rpsL20(strR), xylA5, mtl-1, lldD1, thiE1, tfr-5</i>
<i>AfabD</i>	<i>F⁻, araC14, lacY1, tsx-57, glnX44(AS), gltA5, galK2(Oc), fabD89(ts), rpsL20(strR), xylA5, mtl-1, lldD1, thiE1, tfr-5</i>
<i>AtesAtesB</i> parental	<i>K-12 F⁻ λ^- ilvG⁻ rfb-50 rph-1</i>
<i>AtesAtesB</i>	<i>F⁻, $\Delta tesA::cat$ <i>AtesB</i>, λ^-, λ^- ilvG⁻ rfb-50 rph-1</i>
<i>$\Delta fabD$</i>	<i>F⁻ ompT gal dcm lon hsdSB(rB-mB-) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS) $\Delta fabD$</i>
SHuffle	<i>F' lac, pro, lacIQ / Δ(ara-leu)7697 araD139 fhuA2 lacZ::T7 gene1 Δ(phoA) PvuII phoR ahpC* galE (or U) galK λatt::pNEB3-r1-cDsbC (SpecR, lacIq) ΔtrxB rpsL150(StrR) Δgor Δ(malF)3</i>
BL21 Star (DE3)	<i>fhuA2 [lon] ompT gal [dcm] ΔhsdS</i>
Rosetta (DE3)	<i>F⁻ ompT hsdSB(r_B- m_B-) gal dcm (DE3) pRARE (CamR)</i>
C43	<i>F⁻ ompT hsdSB (r_B- m_B-) gal dcm (DE3)</i>
C41	<i>F⁻ ompT hsdSB (r_B- m_B-) gal dcm (DE3)</i>
Arctic	<i>E. coli B F⁻ ompT hsdS(r_B- m_B-) dcm+ Tetr gal λ(DE3) endA Hte [cpn10 BB cpn60 Gentr]</i>

3.2 Plasmids

The pCDFDuet-1 vector with the ORF-B of the PUFA synthase from *Thraustochytrium sp.* 26185 was obtained from our previous research (Meesapyodsuk & Qiu, 2016). Expression vectors, pBAD, and pET28a were purchased from Invitrogen Co. (Carlsbad, CA, USA). The pBAD vector was used to express foreign gene(s) under the control of an inducible system by arabinose with ampicillin resistance selection. The pET28a vector was used to clone a gene under the control of a strong T7 promoter, induced by isopropyl β -D-1-thiogalactopyranoside (IPTG) with kanamycin resistance selection.

3.3 Luria Bertani (LB) medium and agar plates

LB medium was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and prepared according the manufacturer instruction. For example, 25 g of LB medium powder were dissolved in 1 L of deionized water and then autoclaved at 120 °C for 20 min. For agar plates, 1 % of agar was added to the LB medium, autoclaved at the same condition and plated in 10 cm plastic plates. For antibiotic plates, final concentrations for kanamycin, ampicillin and chloramphenicol were 50, 100 and 25 μ g/mL, respectively.

3.4 Chemical competent cells

A single colony of any *E. coli* strain used was inoculated into 5 mL of LB medium with or without antibiotics and incubated at 37 °C overnight. Three mL of overnight culture were inoculated into 30 mL of LB medium and incubated at 37 °C until an $OD_{600} \approx 0.4$ was reached. After that, the culture was cooled down on ice and then centrifuged at 4 °C for 10 min to pellet cells. The cell pellet was resuspended in 10 mL of ice cold 50 mM $CaCl_2$ solution and incubated at 4 °C for 30 min. The suspension was centrifuge again at 4 °C for 5 min and the cell pellet was resuspended in 1 mL glycerol. Aliquots of 50 μ L or 25 μ L glycerol suspension were prepared and store at -80 °C for future transformation (Ausubel et al., 2003).

3.5 Sequence analysis of the PUFA synthase in *Thraustochytrium*

The sequence of the polyunsaturated fatty acid synthase from *Thraustochytrium sp.* ATCC 26185 (GenBank accession no. AOG21005.1) in the National Center for Biotechnology Information website (NCBI) was analyzed by bioinformatics tools which

included BLASTp (NCBI), MegAlign (DNASTAR), and SWISS-MODEL. The boundaries of the MAT-like domain were determined by sequence alignment and homology modeling. The complete amino acid sequence of the ORF-B of the PUFA synthase was used as a query to search NCBI protein database by BLASTp with the default algorithm parameters to identify the MAT-like domain and its homologous sequences. Two different lengths of the MAT-like domain were used for the protein modelling by SWISS-MODEL, one from amino acid 1048 to 1390 and the other from 1063 to 1390 of the Subunit-B of the PUFA synthase. Three templates with the highest percentage of identity to the domain used for the modelling were the MAT proteins from *Streptococcus pneumoniae* (17.99%), *Coxiella burnetti* (18.56%) and *E. coli* (17.79%). Similar stereospecific structures were used to determine the borders of the MAT-like domain. Sequence alignment of the MAT-like domain with biochemically characterized MAT and TE proteins was performed using MegAlign to identify the conserved motifs. Alignments were done using the ClustalW method with the slow-accurate option to obtain a precise alignment even if the sequences were highly divergent.

3.6 Enzymes, primers and kit for DNA amplification and purification

DNA purification kit was acquired from Bio Basic Inc. (York, ON, Canada). Primers for PCR were synthesized by Sigma-Aldrich (St. Louis, MO, USA) as shown in Table 3.2. Q5 polymerase, restriction enzymes and dNTP were purchased from New England Biolabs (NEB) (Ipswich, MA, USA). HP Taq DNA polymerase was purchased from Bio Basic Inc. T4 ligase was acquired from Thermo Fisher Scientific. EcoRI, HindIII and BglII restriction enzymes were purchased from NEB.

Table 3.2 Primers used for MAT amplifications and site-directed mutagenesis.

Name	Sequence (5' → 3')	Annotation
MAT-like (ORF-B) F	<u>GAATTC</u> ATGTACGGCGAGGGC	EcoRI site (underline)
MAT-like (ORF-B) R	<u>AAGCTT</u> CTAGAGCTTGACGATGGT	HindIII site (underline)
MAT (ORF-A) F	<u>GAATTC</u> ATGTTTCAGCGAGGTC	EcoRI site (underline)
MAT (ORF-A) R	<u>AAGCTT</u> CTACCACTTGTCAAAGCC	HindIII site (underline)
FabD (<i>E. coli</i>) F	<u>AGATCT</u> ATGACGCAATTTGCA	BglII site (underline)
FabD (<i>E. coli</i>) R	<u>GAATTC</u> TAAAGCTCGAGCGC	EcoRI site (underline)
MAT-like S96A F	TTCGGCCTC <u>GCC</u> CTCGGCGAGAT	Mutation site (underline)
MAT-like S96A R	ATCTCGCCGAG <u>GGC</u> GAGGCCGAA	Mutation site (underline)

Table 3.2 Primers used for MAT amplifications and site-directed mutagenesis (continued).

Name	Sequence (5' → 3')	Annotation
MAT-like H220A F	AGGGCATGTGCGGC <u>GCCT</u> GCC	Mutation site (underline)
MAT-like H220A R	GGCAGG <u>CGCC</u> GCACATGCCCT	Mutation site (underline)
pET28a F	GGCCACGATGCGTCCGGCGTAGAGG	For colony PCR
pET28a R	CAGTGGTGGTGGTGGTGGTGCCTCGAGT	For colony PCR
pBAD F	GGGCTAACAGGAGGAATTAACCATGGGGGG	For colony PCR
pBAD R	TTCACTTCTGAGTTCGGCATGGGGTCAGG	For colony PCR

3.7 Construction and transformation of recombinant plasmids

Amplification of the MAT-like domain and control genes was performed using the Q5 High-Fidelity DNA Polymerase from New England Biolabs using primers listed in Table 3.2 under conditions shown in Tables 3.3 (composition) and 3.4 (thermocycler). After amplification, amplicons were digested according to the conditions shown in Table 3.5 with the restriction enzymes specified in Table 3.2. For the digestion of MAT-like (ORF-B), MAT (ORF-A) and MAT-like S96A/H220A mutant domains, EcoRI and HindIII restriction enzymes were used, and for FabD (*E. coli*) digestion, BglIII and EcoRI restriction enzymes were employed at 37 °C overnight. After digestion, the amplicons were ligated to the expression vectors using T4 ligase according to the composition shown in Table 3.6 at 4 °C overnight.

Table 3.3 Q5 polymerase PCR reaction setup.

Reagent	20 µL reaction	Final Concentration
5X Q5 reaction buffer	4 µL	1X
5X Q5 High GC Enhancer	4 µL	1X
10 mM dNTPs	0.4 µL	200 µM
10 µM Forward Primer	1 µL	0.5 µM
10 µM Reverse Primer	1 µL	0.5 µM
Template DNA		1 ng
Q5 High-Fidelity DNA Polymerase	0.2 µL	0.02 U/µL
DMSO		5%
Nuclease-Free Water	Up to 20 µL	

Table 3.4 Q5 PCR thermocycling conditions.

Step	Temperature (°C)	Time
Denaturation	98	30 s
Annealing for MAT-like	68	30 s
Annealing for MAT	64	30 s
Annealing for fabD	62	30 s
Extension	72	2 min

Table 3.5 Enzymatic digestion reaction setup.

Reagent	50 μL reaction
DNA	Up to 1 μ g
10X Reaction Buffer	5 μ L
Enzyme(s)	10 units
Nuclease-Free Water	To 50 μ L

Table 3.6 Ligation reaction setup.

Reagent	20 μL reaction
5X ligase reaction buffer	4 μ L
Insert:Vector Molar Ratio	3:1
Total DNA	0.1 μ g
T4 DNA ligase	Up to 20 μ L
Nuclease-Free Water	1 μ L

Two plasmids pBAD, and pET28a were used to express the MAT-like domain as a standalone protein in *E. coli*. pBAD (4092 bp) was used to express the MAT-like domain from ORF-B and a MAT domain from ORF-A of the PUFA synthase as well as *E. coli* FabD gene under an arabinose-inducing promoter for complementation assays in *E. coli* mutants (Figure 3.1). pET28a (5369 bp) was used to express the MAT-like domain under a T7 promoter in *E. coli* SHuffle strain for activity assays (Figure 3.2).

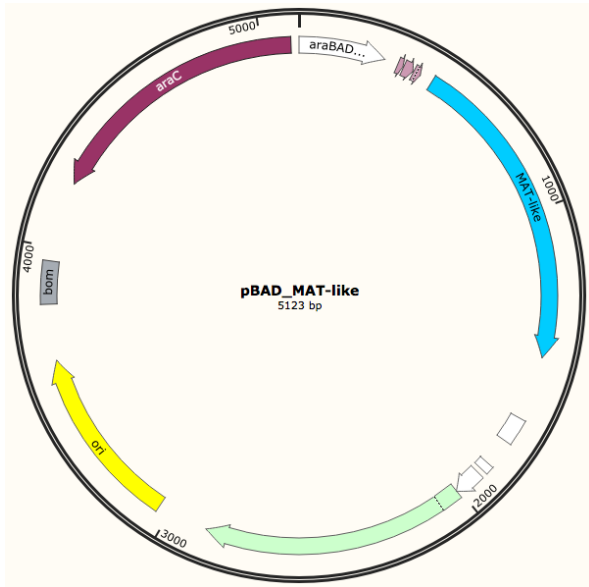


Figure 3.1 Physical map of recombinant plasmid pBAD/MAT-like.

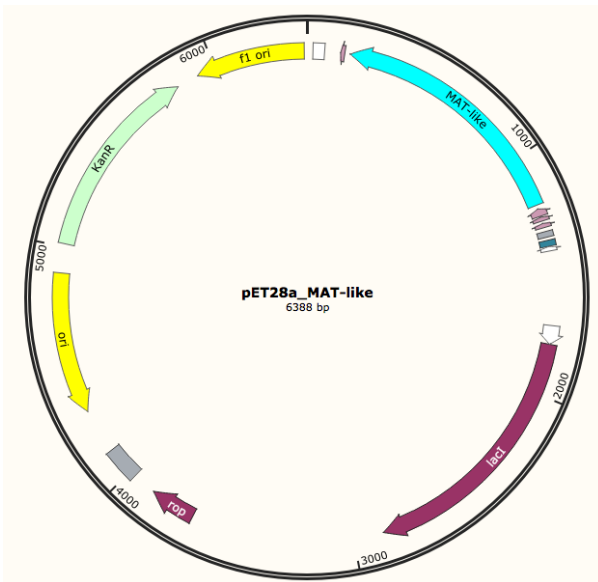


Figure 3.2 Physical map of recombinant plasmid pET28a/MAT-like.

The recombinant plasmids were transformed into *E. coli* strains by a chemical method. Approximately 10 ng of each construct was pipetted into 100 μ L of chemical competent cells. The mixture was placed on ice for 10 min, and then heat-shocked at 42 $^{\circ}$ C for 45 seconds. After that, 1 mL of LB broth was added, and the cells were incubated at 37 $^{\circ}$ C for 1 h with shaking. The cell culture was spread on LB agar plates with appropriate antibiotic and incubated at 37 $^{\circ}$ C overnight to select the transformants (Ausubel et al., 2003).

A colony PCR method was used to screen for authentic transformants. A single colony of *E. coli* transformants was scraped from an overnight plate and dispersed into

the PCR master mix. The remainder was inoculated into 2 mL of LB medium (with antibiotics if necessary) and incubated at 37 °C overnight (Bergkessel & Guthrie, 2013). The colony PCR was performed using HP Taq DNA polymerase and primers listed above. The reaction setup was shown in Table 3.7 and the thermocycling conditions were the same as in Table 3.4. After colony PCR, the plasmids from the positive colonies were extracted using plasmid extraction kit from Bio Basic Inc. and digested using appropriate restriction enzymes to confirm structures. The positive plasmids were sent to the National Research Council Canada (NRC, Saskatoon, SK, Canada) for sequencing to confirm the correct of recombinant sequences.

Table 3.7 HP Taq DNA polymerase PCR reaction setup.

Reagent	20 μL reaction	Final Concentration
10X Taq reaction buffer	2 μ L	1X
20 mM MgSO ₄	2 μ L	2 mM
10 mM dNTPs	0.4 μ L	200 μ M
10 μ M Forward Primer	1 μ L	0.5 μ M
10 μ M Reverse Primer	1 μ L	0.5 μ M
Template DNA	0.5 μ L of overnight culture	
Taq DNA polymerase	0.08 μ L	0.4 U/ μ L
DMSO		5%
Nuclease-Free Water	Up to 20 μ L	

3.8 Complementation assays in *E. coli fabD* (Δ *fabD*) mutant with MAT-like domain

An *E. coli* Δ *fabD* mutant used for the complementation assay contains a thermolabile malonyl-CoA:ACP transacylase (MAT) resulting in defective fatty acid synthesis and temperature-sensitive growth (Semple & Silbert, 1975). Specifically, it could grow only at 37 °C (permissive temperature), but not at 42 °C (non-permissive temperature) (Simon & Slabas, 1998). This conditional mutant was used for the complementation test because the complete genetic inactivation of the *MAT* gene was lethal in *E. coli* (Liu et al., 2006).

Two positive controls, pBAD_FabD expressing wild type FabD and pBAD_MAT expressing the MAT domain from the ORF-A of the PUFA synthase were also constructed and used in the complementation assays. For the assay, a single colony of each transformant was first grown in an ampicillin LB liquid medium at 37 °C until an OD₆₀₀ = 0.4 was reached. After that, 2 µL of each culture were dotted on ampicillin LB plates containing 0.01% (wt/v) of L-arabinose (Shafiee et al., 2015; Terpe, 2006). The plates were cultured overnight at 37 °C and 42 °C, respectively.

3.9 Complementation assays in *E. coli tesAtesB* mutant (*ΔtesAtesB*) with MAT-like domain

The *E. coli tesAtesB* mutant (*ΔtesAtesB*) is a double-gene knockout generated by gene deletion of thioesterase I and II with chloramphenicol resistance marker (Baba et al., 2006; Cho & Cronan, 1993). According to Cho & Cronan (1994), the thioesterase double mutant did not produce detectable growth phenotype nor different fatty acid profile when compared to the parental wild type (Cho & Cronan, 1994).

For the assay, the pBAD_MAT-like plasmid expressing the MAT-like domain and the empty pBAD were introduced in the mutant *ΔtesAtesB* and the parental wild type strain shown in Table 3.1. The mutant transformants were selected on chloramphenicol and ampicillin LB plates and the parental wild type transformant was selected on ampicillin plates. The positive mutant transformants and wild type clones were then grown in chloramphenicol and ampicillin and only ampicillin LB liquid medium, respectively, for lipid analysis.

3.9.1 Lipid analysis

For lipid analysis, single colonies of the wild type and *ΔtesAtesB* transformants were inoculated to 10 mL of chloramphenicol and ampicillin LB and grown at 37 °C until an OD₆₀₀ = 0.4 was reached. After that, expression was induced with 0.01% (wt/v) of L-arabinose and the strains were grown until an OD₆₀₀ = 1 was reached. Subsequently, the cultures were centrifuged at 2200 g for 10 min, the total lipid from the biomass was extracted and analyzed.

The lipids in the cells were extracted based on the method described by Folch et al., (1957). For the preparation of fatty acid methyl esters from the total lipids, 2 mL of 1% (v/v) sulfuric acid in methanol was added to the total lipid extract and the mixture was incubated at 80 °C for 1 hour. After that, the samples were cooled down on ice, 2

mL of hexane and 1 mL of potassium chloride 0.88% (wt/v) were then added. The mixture was centrifuged at 2200 g for 10 min and the hexane phase was transferred to a new tube and dried with N₂ gas. After drying, 50 µL of hexane was added to solubilize the transmethylated fatty acids and analysed by gas-chromatography (GC) in an Agilent Technologies 7890A System with a column DB-23. The GC program started with 160 °C and ended at 240 °C. Ten µL of 0.1 mg/L of heptadecaenoic (17:0) was used as an internal standard. For lipid class analysis, the total lipid was dissolved in 100 µL of chloroform and loaded on a Whatman K6 silica gel plate. The plate was developed in a mixture of hexane, diethyl ether and acetic acid (70:30:1) and stained with 0.005% (wt/v) primulin in acetone-water (80:20). The lipid class bands were visualized under UV light.

3.10 Expression of the MAT-like domain in the *E. coli fadD* mutants ($\Delta fadD$)

After confirmation of the sequence, pET28a_MAT-like was transformed into the *E. coli* $\Delta fadD$ strain. The transformant with the empty vector was used as a negative control. The transformants were selected on kanamycin LB plates. A single colony from the plate was inoculated to 5 mL of kanamycin LB liquid medium and incubated overnight at 37 °C. An aliquot of 300 µL of the overnight culture was inoculated into 30 mL of LB medium and incubated at 37 °C. After an OD₆₀₀ of the culture reached 0.5, IPTG at the final concentration of 1 mM was used to induce the expression at 16 °C (Terpe, 2006). To determine the expression of the protein, the cell pellet of 200 µL culture was resuspended in 20 µL of SDS-PAGE loading buffer (60 mM Tris-HCL pH 6.8, 2% SDS, 0.1% bromophenol blue, 25% glycerine, 14.4 mM β -mercaptoethanol) and analyzed by electrophoresis (Xie et al., 2017). Free fatty acids in the sample after 24 h of induction were analyzed.

3.10.1 Free fatty acid analysis

Ten mL of the culture was centrifuged at 10000 rpm for 10 min for collecting cell pellets and supernatant. Total lipids from cell pellets were extracted following the protocol of Folch et al., (1957). Cells were disrupted using 1 mL of isopropanol at 80 °C for 10 min. After the sample was cooled down to room temperature, 2 mL of chloroform and 1 mL of methanol were added. The mixture was centrifuged at 2200 rpm for 10 min and the lower phase was transferred to a clean tube. Two mL of

chloroform was added again to extract the residual lipids left in the sample. The lower phase was combined with the previous one. After that, 1 mL of KCl 0.88% (wt/v) was added to extract any contaminated hydrophilic compounds in the lipid extract. The mixture was centrifuged at 2200 rpm for 10 min, and the aqueous phase was removed as much as possible. The organic solvent phase of extractions was dried under N₂ gas. Free fatty acids were selectively transmethylated to fatty acid methyl esters (FAMES) using 20 µL of diazomethane (1:8, diazomethane:hexane) and 10 µL of methanol at room temperature for 5 min. After the sample was dried under N₂ gas, 50 µL of hexane were added to solubilize the FAMES and analyzed by gas-chromatography (Folch et al., 1957).

Free fatty acids were extracted from the culture by mixing 2 mL of the supernatant with the same volume of hexane and 500 µL of NaCl saturated water (26.47 %, w/w). The organic solvent phase of extractions was dried under N₂ gas. Free fatty acids were selectively transmethylated as described previously for cell pellets. After the sample was dried under N₂ gas, 50 µL of hexane were added to solubilize the FAMES and analyzed by gas-chromatography. Ten µL of 0.1 mg/L of heptadecaenoic (17:0) was used as an internal standard. All analyzes were performed in triplicate.

3.11 Expression of MAT-like domain in *E. coli*

The plasmid pET28a_MAT-like was chemically transformed into *E. coli* BL21 Star (DE3). A single colony of transformant was inoculated to 5 mL of kanamycin LB liquid medium and incubated overnight at 37 °C. An aliquot of 200 µL of the overnight culture was then inoculated into 20 mL LB liquid medium and incubated at 37 °C until an OD₆₀₀ = 0.5. Subsequently, 0.5 mM of IPTG was added to induce the expression (Terpe, 2006). The mixture was incubated at 16 °C for 3 hours. After that, the culture was centrifuged at 4 °C for 15 min to pellet cells. The cells were resuspended in 2 mL of ice-cold buffer (50 mM Tris-HCl, pH 8) and disrupted through a beadbeater. The mixture was centrifuged at 15000 rpm at 4 °C for 15 min. Crude proteins in cell pellets and supernatant were separately resuspended in 200 µL of SDS-PAGE loading buffer (60 mM Tris-HCL pH 6.8, 2% SDS, 0.1% bromophenol blue, 25% glycerine, 14.4 mM β-mercaptoethanol) for electrophoresis (Xie, Meesapyodsuk, & Qiu, 2017).

As the first expression of the domain in *E. coli* BL21 Star (DE3) was not successful, several other *E. coli* strains such as Rosetta (DE3), C43, C41, Arctic and

SHuffle (their genotypes described in Table 3.1) were attempted with varying induction conditions. *E. coli* SHuffle was found to be the best strain for the expression under the condition of 13 °C overnight, and 1 mM IPTG induction.

3.11.1 Enzymatic activity

Thioesterases catalyze the release of acyl moieties from the phosphopantetheine of ACP or CoA (Smith & Tsai, 2007). The activity assay using the reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was based on the reduction of DTNB (no colour) to 5-thio-2-nitrobenzoic acid (TNB) (yellow color) (Berge & Farstad, 1981) by CoASH released from acyl-CoA thioesters.

18:0-, 18:1- and 18:2-CoA were purchased from Sigma-Aldrich (St. Louis, MO, USA) and 22:6-CoA was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

The assay mixture (200 µL) contained 30 mM HEPES, pH 7.5, 1 mM EDTA, 0.3 mM DTNB, 100 µM acyl-CoA and 300 µg of total crude proteins. The blank reference for the assay was the same except for missing the proteins. The negative control was the assay using 300 µg of total crude proteins from the strain with empty pET28a as the enzyme source. All the assays took place at 35 °C for 30 min. The absorbance at 412 nm in the reaction was measured by NanoDrop™ 2000/2000c Spectrophotometer. Thioesterase activity was calculated on the reported absorbance (Rodríguez-Guilbe et al., 2013). All assays were done in triplicate.

3.12 Site-directed mutagenesis

Overlapping PCR was employed for the site-directed mutagenesis of two residues S96 and H220 in the MAT-like domain. This method involves two sequential PCR reactions using two flanking primers (a and d) and two internal primers (b and c) to introduce the mutation of interest (+) as shown in Figure 3.3. Both PCR reactions were performed using the same setup and conditions specified in Table 3.3 and 3.4. The internal primers were used for the first PCR to generate two overlapping mutated nucleotide sequences, which were then used as templates for the second PCR to generate the mutated version of a gene product AD (Heckman & Pease, 2007; Reikofski & Tao, 1992). The primers used for the overlapping PCR are listed in Table 3.2.

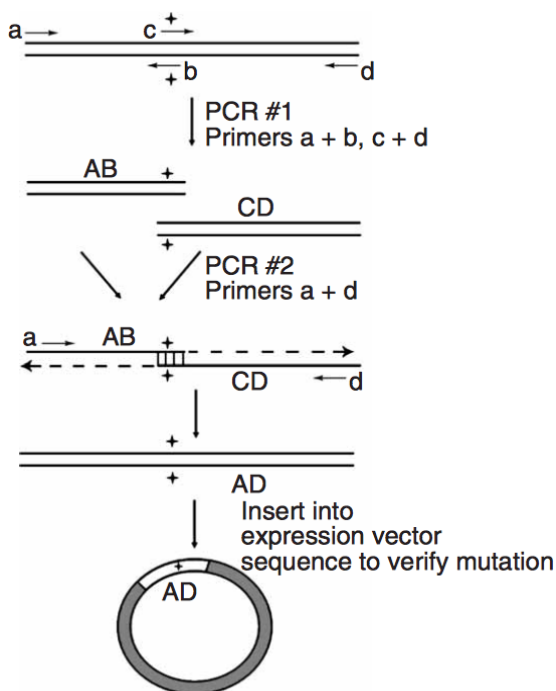


Figure 3.3 Overlapping-PCR for the site directed mutagenesis Adapted from Heckman & Pease, 2007.

3.12.1 Cloning and transformation of mutagenesis plasmids

The amplification of the MAT-like domain with the mutated residues was performed following the same PCR reaction setup and thermocycling conditions as described above (see 3.7). The flanking primers used were pET28a F/R and the annealing temperature for all overlapping PCR was 68 °C. The mutated MAT-like domain (S96A or H220A) was cloned into pET28a vector and the ligation reaction setup was the same as specified in Table 3.6. The recombinant plasmids were chemically transformed into *E. coli* Top10 and positive colonies were confirmed by colony PCR using the same reaction setup as specified in Table 3.7. The mutated sequence was confirmed by restriction digestion and sequencing. The correct constructs were chemically transformed into *E. coli* SHuffle and *E. coli* *ΔfadD* strains (see 3.10).

3.12.2 Expression and enzymatic activity

For the protein expression and enzymatic assay of MAT-like_S96A/H220A, the same procedures were used as described above (see 3.11).

3.12.3 Free fatty acid analysis

The fatty acid analysis of transformants (*ΔfadD*/pMAT-like_S96A/H220A) was performed as described above (see 3.10.1).

4. RESULTS

4.1 Sequence analysis of a MAT-like domain in the PUFA synthase

To dissect the authentic region of the MAT-like domain from the PUFA synthase for its functional analysis, homology search, homology modelling and multiple sequence alignment were employed to delimit the domain in the PUFA synthase. The results showed that the region of the MAT-like domain was located between amino acids 1048 and 1390 in the Subunit-B, belonging to the PfaB group proteins with the functions of hydrolases and transferases including discrete MATs for Type II fatty acid synthesis in bacteria and MAT domains of PUFA synthases in the biosynthesis of VLCPUFAs in pressure and cold-adapted deep-sea bacteria (Orikasa et al., 2009). All these sequences possessed an α/β hydrolase fold with a conserved motif GXSXG in the catalytic site located in a nucleophilic elbow typical for serine hydrolases and transferases. A highly conserved histidine residue at position 220 probably important for the enzymatic activity was also observed in the structure. The MAT-like domain shared a slightly higher amino acid identity to discrete MAT proteins from *E. coli* (Serre et al., 1995), *Coxiella burnetti* (Franklin et al., 2015) and *Streptococcus pneumoniae* (Hong et al., 2010) (19.9%, 17.6% and 19.9%, respectively) than the MAT domain in Subunit-A of the PUFA synthase (13.8%) (Figure 4.1). In addition, this domain also possessed sequence similarity to a group of discrete thioesterases such as an acyl-ACP thioesterases in *Amycolaptosis mediterranei* (August et al., 1998) (16.8 %) and *Streptomyces coelicolor* (Whicher et al., 2011) (16.4 %) (Figure 4.2) with the conserved motif GXSXG.

<i>E. coli</i> FabD	MMA	GHS	LGE	YSAL	VCA	GVI	DFAD	AVRL	VE	MRG	KFM	-	QEAV	PE	GT	GAMA	134		
<i>C. burnetti</i> MAT	VXAG	HSL	GEY	AAL	VCA	GAL	KFEE	AVKL	VEKR	GQY	X-	-	QEAV	PV	GE	GAX	GA	138	
<i>S. pneumoniae</i> MAT	MVAG	L	SLGE	YSAL	VAS	GAL	DFEDA	VAL	VAK	R	GAY	M-	EEAT	P	AD	SG	KMVA	132	
<i>Thraustochytrium</i> MAT	FAAG	HSL	GE	FAAL	YAAG	CVDR	DELY	ELV	CR	RARI	M	GGKD	AP	ATP	K	CC	MAA	136	
<i>Thraustochytrium</i> MAT-like	AAF	GL	SLGE	IT	SML	FSY	SDK	NGK	NS	DRL	TRDL	RA	SRV	WNE	EAL	AI	EFNAL	RE	140
<i>E. coli</i> FabD	ERAG	AA	CKA	AGAKR	-	-	-	-	-	ALPL	PVS	VPS	HCAL	MK	PAAD	KLAVEL	AKIT	TF	221
<i>C. burnetti</i> MAT	DRAL	NXAK	TEGAKI	-	-	-	-	-	-	AKRI	PVS	VPS	HCPL	XQP	AADR	LAQDI	AKISI	SI	225
<i>S. pneumoniae</i> MAT	DRAVEL	LQ	EAGAKR	-	-	-	-	-	-	LIPL	KVSG	PFHTAL	LEPAS	QKLAET	LAQV	SF			219
<i>Thraustochytrium</i> MAT	QAET	AI	LQKEGFR	-	-	-	-	-	-	VVPL	ACESAF	HSPQ	MENASA	AAF	KDVI	SKITSF			216
<i>Thraustochytrium</i> MAT-like	SGKP	DA	CKAAI	ARL	GSKVP	ALP	VS	QGMCGH	CP	EVAP	YAK	EI	AEI	HQI	L	DI			240

Figure 4.1 Partial sequence alignment of the MAT-like domain with discrete MAT enzymes.



Figure 4.2 Sequence alignment of the MAT-like domain with discrete TE sequences.

4.2 Expression of the MAT-like domain in an *E. coli FabD* mutant

To determine the function of the MAT-like domain, this domain was expressed as a standalone protein in an *E. coli FabD* mutant defective in malonyl-CoA:ACP transacylase (MAT) activity. As MAT was essential for fatty acid synthesis, only a temperature-sensitive mutant of this gene was available in *E. coli* for the expression. The wild type strain (WT) with the empty vector could grow at both permission and non-permission temperatures (37 °C and 42 °C), while the mutant with the MAT-like domain from ORF-B (Mutant+MAT), similar to the mutant with the empty vector (Mutant), could grow at 37 °C, but not at 42 °C. On the other hand, the mutant with either wild type discrete *MAT* gene *FabD* (Mutant+*FabD*) or authentic MAT domain from ORF-A (Mutant+MAT) could effectively grow at both 37 °C and 42 °C. These results showed that the MAT-like domain is incapable of complementing the defective MAT phenotype (Figure 4.3), thus it does not function as a malonyl-CoA:ACP transacylase.



Figure 4.3 Plate complementation test of the MAT-like domain in an *E. coli* *fabD* mutant. WT: parental wild type of the mutant.

4.3 Expression of the MAT-like domain in an *E. coli* *tesAtesB* mutant

E. coli possessed two major acyl-thioesterases (TesA and TesB), but neither was essential. No changes on growth phenotype and fatty acid profile could be detected in the double mutant ($\Delta tesAtesB$) (Cho & Cronan, 1993). To further interrogate whether the MAT-like domain could impose any changes on lipid class and fatty acid profile, the domain was expressed as a standalone protein in the double mutant. The total fatty acid analysis did not reveal any difference among three strains: parental wild type, mutant with the empty vector and mutant with the MAT-like domain (Figure 4.4). Lipid class analysis (Figure 4.5) also showed that no difference could be observed either on the type and amount of lipid classes among three strains.

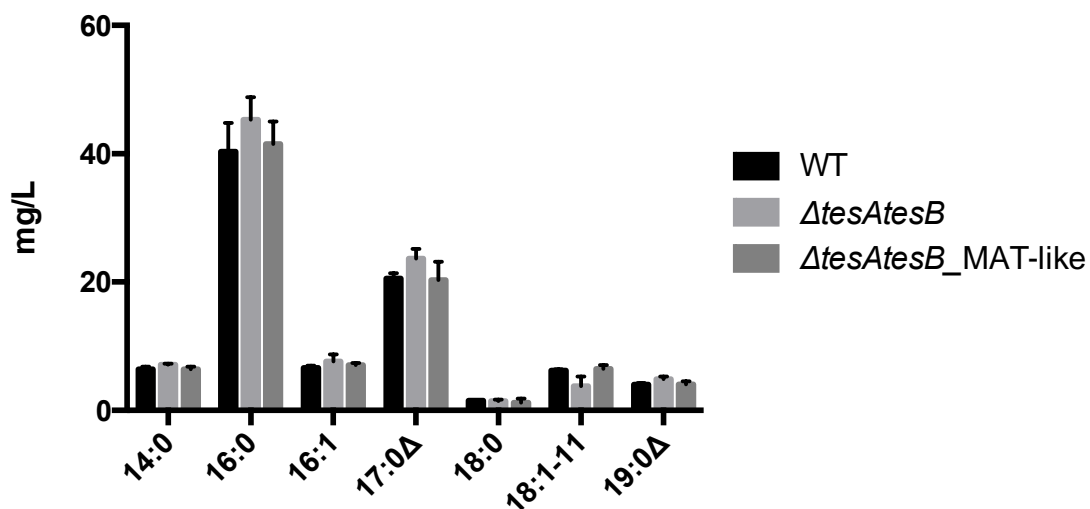


Figure 4.4 Total fatty acid profile of *E. coli* $\Delta tesAtesB$ with the MAT-like domain and the empty vector, and its parental wild type. Values are reported as means \pm standard deviations for three biological replicates.

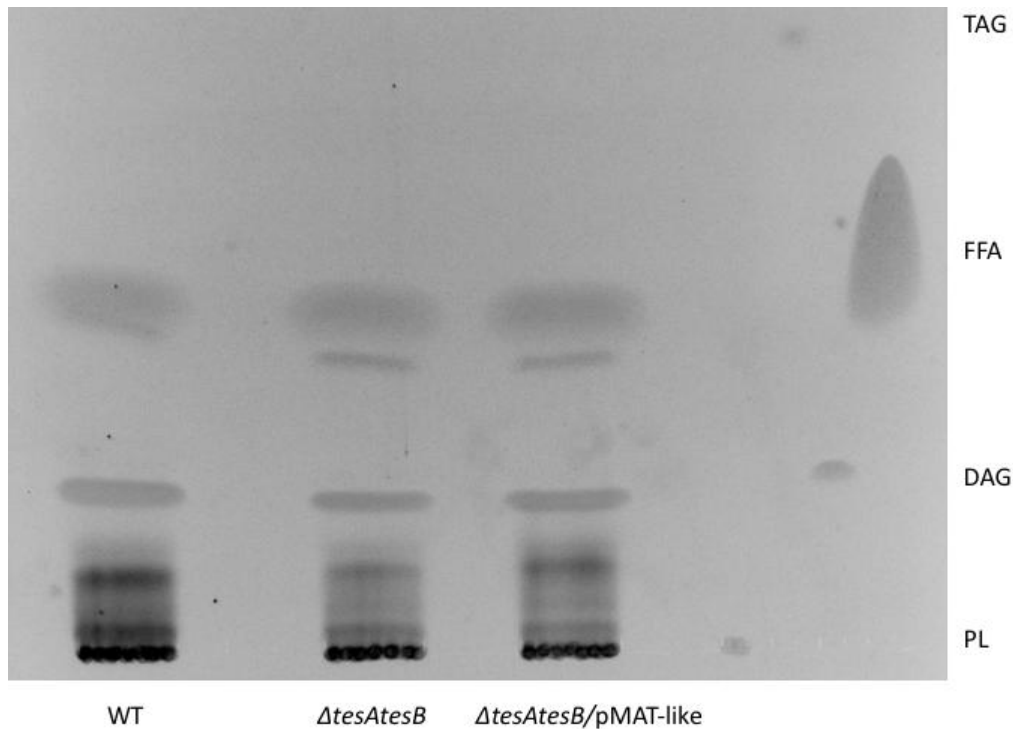


Figure 4.5 Lipid class analysis by TLC. TAG: triacylglycerol, FFA: free fatty acids, DAG: diacylglycerol, PL: phospholipids, WT: parental strain with pBAD empty vector, $\Delta tesAtesB$: mutant strain with the empty vector, $\Delta tesAtesB/pMAT$ -like: mutant strain with pBAD expressing the MAT-like domain.

4.4 Expression of the MAT-like domain in an *E. coli fadD* mutant

To investigate if the MAT-like domain has any impact on the production of free fatty acids, another *E. coli* mutant (*fadD*) defective in acyl-CoA synthetase activity was exploited to express the domain. The rationale was that the MAT-like domain, if functioning as a thioesterase, would increase the accumulation of free fatty acids in the mutant where the defect in acyl-CoA synthetase would prevent fatty acid degradation through β -oxidation. Experimental results showed that the expression of the MAT-like domain in the mutant increased the production of free fatty acids by approximately 50% in cell pellets (inside cells) (Table 4.1, Figure 4.6) and more than 30% in supernatants (secreted outside of cells) relative to the mutant control (Table 4.1, Figure 4.7). Among all the free fatty acids produced, the major increase was observed in 11-cis-octadecenoic acid (18:1-11), a monounsaturated long chain fatty acid (Figures 4.8 and 4.9). The mutant expressing the MAT-like domain produced more than three times of 18:1-11 than the mutant with the empty vector (2.49 vs 0.76 mg/L) inside the cells. These results indicate that the MAT-like domain could function as a thioesterase by increasing free fatty acids production on the background of defective acyl-CoA synthetase.

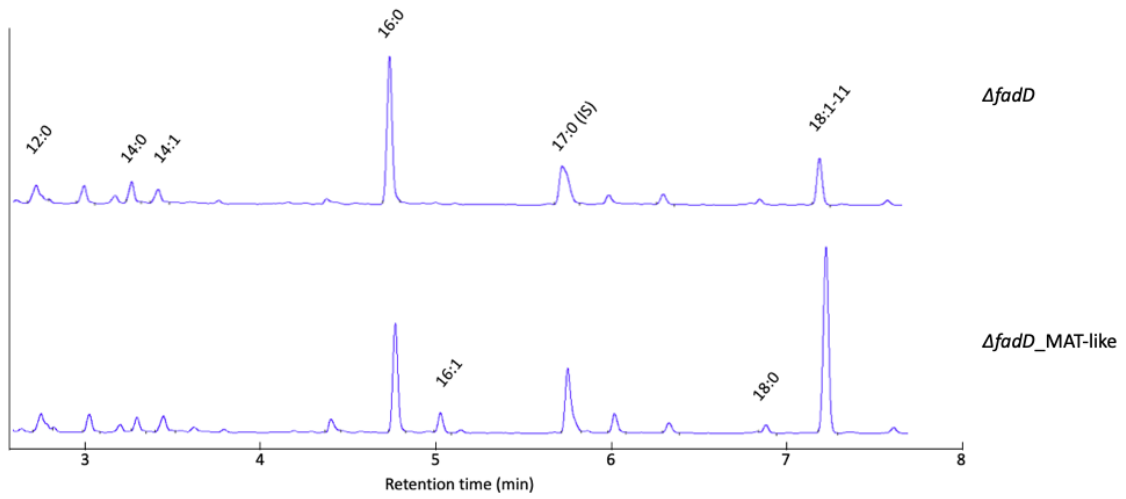


Figure 4.6 GC analysis of FAMES prepared from cell pellets of $\Delta fadD$ and $\Delta fadD_MAT\text{-like}$.

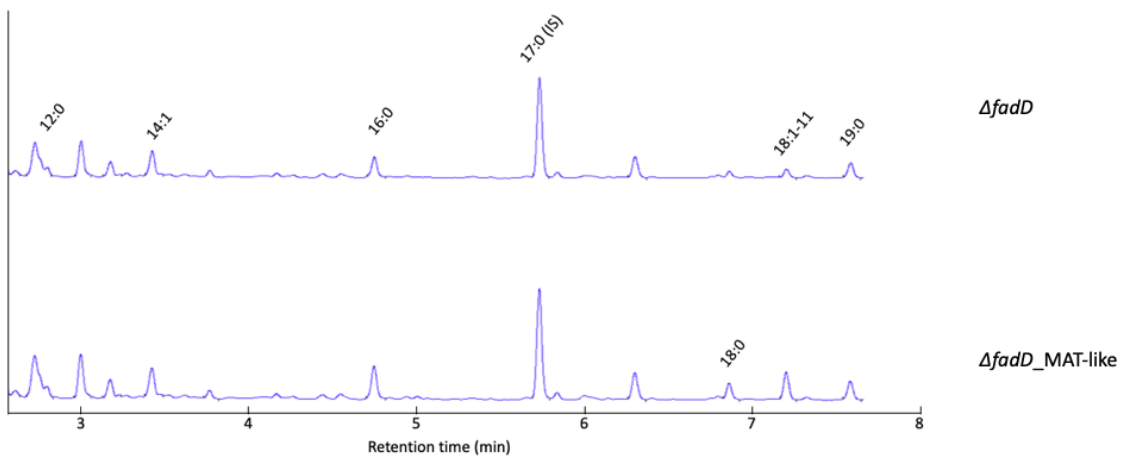


Figure 4.7 GC analysis of FAMES prepared from supernatants of $\Delta fadD$ and $\Delta fadD_MAT\text{-like}$.

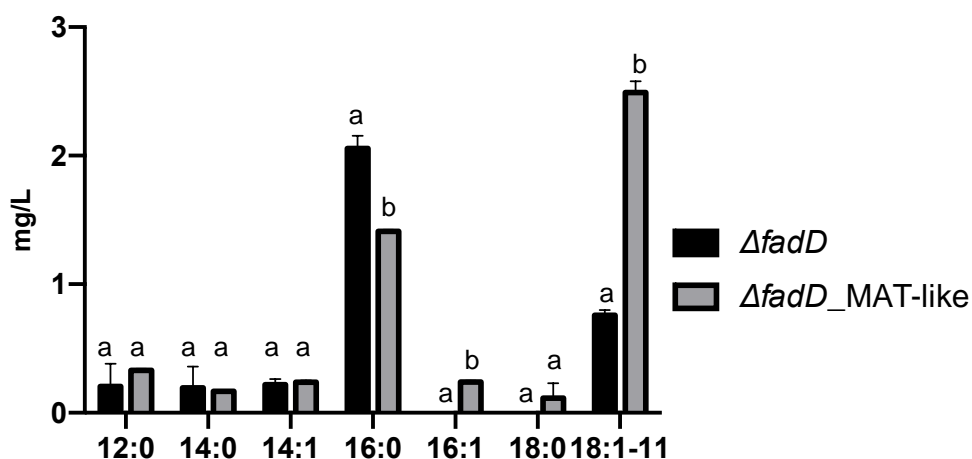


Figure 4.8 Free fatty acid profile in the cell pellets of $\Delta fadD$ mutant with the MAT-like domain. Values are reported as means \pm standard deviations for three biological replicates. Means with different letters were statistically different according to an unpaired t test ($P < 0.05$).

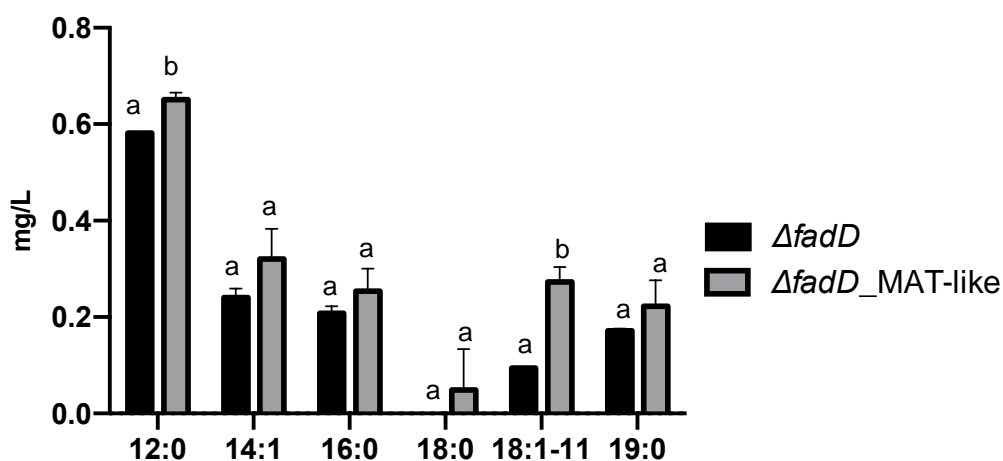


Figure 4.9 Free fatty acid profile in the supernatant of $\Delta fadD$ mutant with the MAT-like domain. Values are reported as means \pm standard deviations for three biological replicates. Means with different letters were statistically different according to an unpaired t test ($P < 0.05$).

Table 4.1 Free fatty acid content in the *E. coli* Δ *fadD* expressing the MAT-like domain. Values are reported as means \pm standard deviations for three biological replicates. Means with different letters are statistically different according to an unpaired t test ($P < 0.05$).

		FFA content [mg/L]	
		Δ <i>fadD</i>	Δ <i>fadD</i> _MAT-like
Source	Cells	3.43 \pm 0.22 ^a	4.98 \pm 0.003 ^b
	Supernatant	1.29 \pm 0.02 ^a	1.77 \pm 0.13 ^b

4.5 *In vitro* activity assays of MAT-like domain

To confirm that the MAT-like domain functioned as a thioesterase, this domain was first expressed as a standalone protein in an *E. coli* BL21 Star (DE3) (Figure 4.10), which was then used as the enzyme source for thioesterase assays using a method based on reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) by CoASH. However, due to the low solubility of the expressed protein in the strain, the enzymatic assay was unsuccessful. To improve the expression, other *E. coli* strains such as Rosetta (DE3), C43, C41, Arctic and SHuffle (Figure 4.10) were subsequently attempted. It appeared that the SHuffle strain with enhanced capacity for appropriate folding provided a slightly better result in expressing the domain.

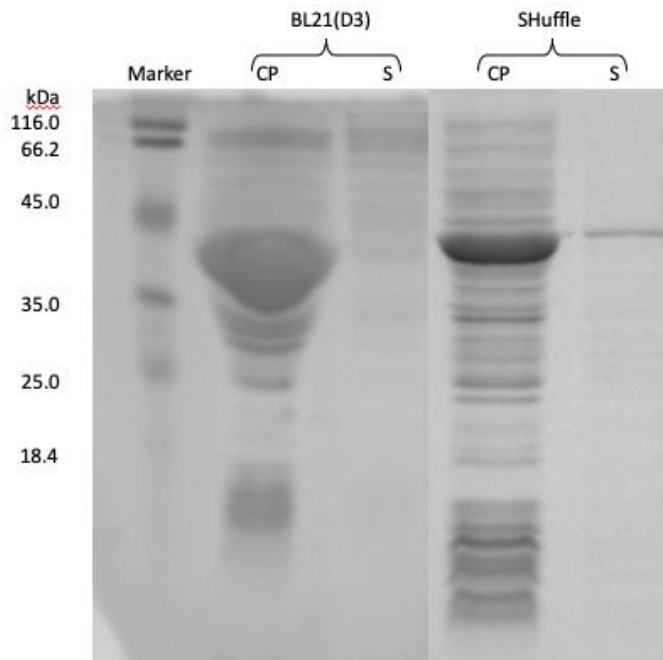


Figure 4.10 SDS-PAGE of protein expression of MAT-like (≈ 38 kDa) in *E. coli* BL21 Star (DE3) and SHuffle. CP: cell pellet, S: supernatant.

In vitro thioesterase activity assays using the crude protein extract from the *E. coli* SHuffle expressing the MAT-like domain as an enzyme source showed that the MAT-like domain could hydrolyze the four acyl-CoAs substrates tested, steroyl-CoA (18:0-CoA), octadecenoyl-CoA (18:1-CoA), octadecadienoyl-CoA (18:2-CoA) and docosahexaenoyl(DHA)-CoA (22:6-CoA), and the highest activity was found towards docosahexaenoyl-CoA, followed by 18:2-CoA, 18:0-CoA and 18:1-CoA after normalization against the control (Figure 4.11). This result indicates that the MAT-like domain indeed possesses acyl thioesterase activity.

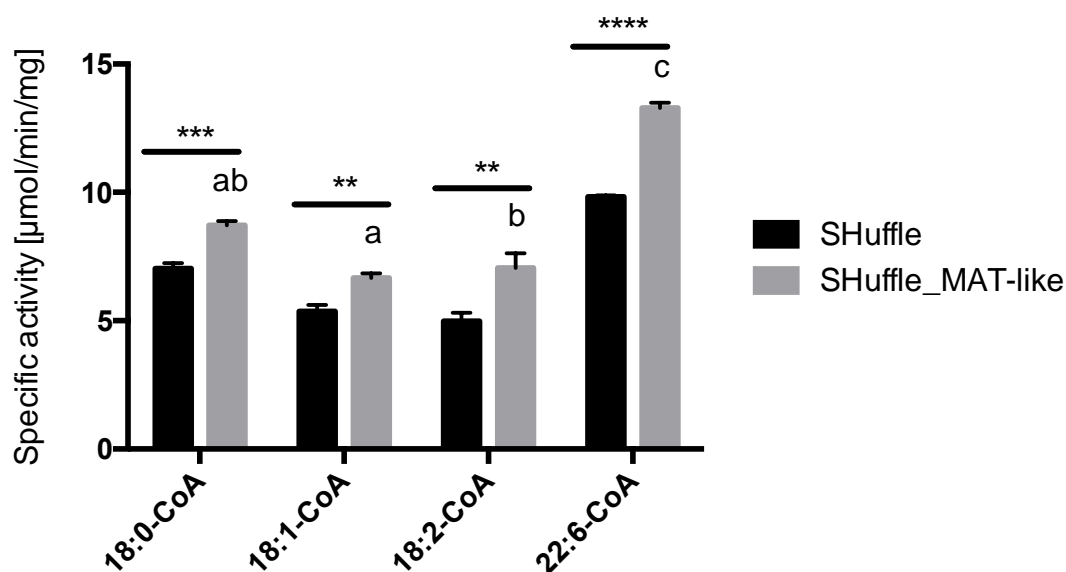


Figure 4.11 Thioesterase activity of MAT-like domain towards four acyl-CoA substrates. Values are reported as means \pm standard deviations for three biological replicates. Means of SHuffle_MAT-like with different letters were statistically different according to a one-way ANOVA ($P < 0.05$). Means between SHuffle and SHuffle MAT-like activities were statistically different according to an unpaired t test.

4.6 Mutagenesis analysis of the MAT-like domain

According to sequence analysis, two conserved residues S96 and H220 were found among the MAT-like domain and related sequences (Figures 4.1 and 4.2). To investigate if the two residues are involved in the catalysis, they were individually replaced by alanine through site-directed mutagenesis. Experimental results showed that the individual substitution of the two residues resulted in the reduction of their thioesterase activities towards DHA-CoA, the most preferred substrate, by about 80% relative to the wild type domain protein (Table 4.2). Furthermore, the expression of the two mutant proteins in the *E. coli* Δ *fadD* strain also resulted in substantial reduction of free fatty acids relative to those of controls expressing the empty vector and wild type domain (Table 4.3). The extent of reduction of free fatty acids in the cells was more obvious in S96A than H220A. These results indicate these two residues indeed play important roles in catalytic activity and support the hypothesis that the MAT-like domain functions as a thioesterase.

Table 4.2 Thioesterase activities of the mutant MAT-like domain towards DHA-CoA. Values are reported as means \pm standard deviations for three biological replicates. Means with different letters were statistically different according to an unpaired t test ($P < 0.05$).

Protein	$\mu\text{mol TNB/min/mg protein}$
MAT-like	3.08 ± 0.17^a
MAT-like S96A	0.68 ± 0.17^b
MAT-like H220A	0.71 ± 0.21^b

Table 4.3 Free fatty acid contents in $\Delta\text{fadD/pMAT-likeS96A}$ and $\Delta\text{fadD/pMAT-likeH220A}$. Values are reported as means \pm standard deviations for three biological replicates. Means with different letters were statistically different according to an unpaired t test ($P < 0.05$).

		Strain FFA content [mg/L]			
		ΔfadD	$\Delta\text{fadD/pMAT-like}$	$\Delta\text{fadD/pMAT-like S96A}$	$\Delta\text{fadD/pMAT-like H220A}$
Source	Cells	3.43 ± 0.22^a	4.98 ± 0.003^b	1.75 ± 0.1^c	2.88 ± 0.18^d
	Supernatant	$1.29 \pm 0.02a$	1.77 ± 0.13^c	1.32 ± 0.03^a	1.05 ± 0.05^b

5. DISCUSSION

PUFA synthases have been identified in marine microorganisms such as *Shewanella sp.*, *Photobacterium sp.*, *Schyzochytrium sp.*, and *Thraustochytrium sp.* (Arts et al., 2009). In *Thraustochytrium*, the PUFA synthase comprises three large subunits each with multiple catalytic domains predicted on the presence of characteristic active sites (Meesapyodsuk & Qiu, 2016). However, the exact boundaries and functions of these domains are not completely known. This study undertook sequence analysis and heterologous expression approaches to investigate the function of a malonyl-CoA:ACP transacylase (MAT)-like domain from the PUFA synthase in *Thraustochytrium sp.* 21685.

Sequence analysis using a combination of bioinformatics tools such as homology search, homology modelling and sequence alignment indicates the MAT-like domain resides between 1048 and 1390 amino acids in the ORF-B. A GX SXG motif, and a histidine residue are conserved among the MAT-like domain and related sequences, such as MAT and TE proteins. The serine at position 96, and the histidine at position 220 are conserved in the sequences (Pazirandehs et al., 1991) and might be part of a catalytic triad in the active site for catalysis, as the substitutions of these two residues with alanine in the domain almost abolished the activity shown by an *in vitro* assay using the crude extract of the expression in *E. coli* as an enzyme source (Kraut, 1977). The residual activity left in the assay could be due to compensated effects of endogenous thioesterases in the host. In fact, the expression of the two mutated MAT-like domains in an *E. coli* mutant (*ΔfadD*) defective in acyl-CoA synthetase activity confirms that the mutated MAT-like domains are not functional, as the level of free fatty acids in the mutant strain expressing the mutated domains is even lower than those in the strains with the empty vector and the wild type domain. The lower production of free fatty acids in the negative control (*ΔfadD/pET28a*) could be due to the dominant negative mutation effect (Herskowitz, 1987), as functional endogenous *E. coli* thioesterases and non-functional mutated MAT-like domains could compete for the same substrate, leading to the reduced production of free fatty acids.

To functionally analyze the MAT-like domain, it was first expressed as a standalone protein in an *E. coli* temperature sensitive MAT mutant (*fabD^{ts}*). This mutant has been previously used to examine the function of predicted MAT proteins by complementation tests (Sun et al., 2012). The result showed that it did not function as a

MAT to complement the defective phenotype of the mutant, although the MAT-like domain shares the highest amino acid identity to malonyl CoA:ACP transacylase proteins. Next, an *E. coli* double mutant (*ΔtesAtesB*) was employed to express the domain, as it has the second highest sequence similarity to thioesterases. However, the expression in the mutant did not produce any detectable changes on growth phenotype and lipid/fatty acid profile, similar to the previous result observed between the mutant and the wild type strain (Cho & Cronan, 1993). Subsequently, the MAT-like domain was expressed in another *E. coli* mutant (*ΔfadD*) where acyl-CoA synthetase gene was deleted. Previously, this mutant has been used for enhancing the production of free fatty acids in bacteria (Lennen & Pfleger, 2012; Steen et al., 2010; Torella et al., 2013; X. Zhang et al., 2011), as inactivation of acyl-CoA synthetase blocks the formation of acyl-CoA from free fatty acids, thereby preventing fatty acid degradation through β -oxidation. *E. coli* produces fatty acids principally for the biosynthesis of membrane lipids and normally does not accumulate a high level of free fatty acids (X. Zhang, Li, Agrawal, & San, 2011). Overproduction of free fatty acids by expressing the MAT-like domain in this mutant is indicative of thioesterase activity. Finally, *in vitro* assays using the protein expressed in an *E. coli* strain with enhanced capacity for appropriate protein folding confirmed the thioesterase activity of the MAT-like domain on acyl-CoAs.

In marine Thraustochytrids, VLCPUFAs are solely synthesized by an anaerobic pathway catalyzed by a PUFA synthase (Metz et al., 2009). After synthesis, VLCPUFAs are released from a PUFA synthase as free fatty acids probably catalyzed by a thioesterase activity of a domain inside the PUFA synthase (Metz et al., 2009). However, identity of this domain has not been determined previously. The complementation test in this study confirms that the MAT-like domain does not function as a MAT. In addition, the increased production of free fatty acids in an *E. coli* acyl-CoA synthetase mutant expressing the domain is indicative of thioesterase activity by releasing free fatty acids from their thioesters. *In vitro* enzymatic assays using the domain protein as an enzyme source demonstrates that this domain can hydrolyze four acyl thioesters with the highest activity towards a thioester of DHA, the most abundant fatty acid produced in *Thraustochytrium*. Collectively, these results support the notion that the MAT-like domain of the PUFA synthase from *Thraustochytrium* possesses thioesterase activity and it might be involved in catalyzing the last step of the VLCPUFA biosynthesis by releasing freshly synthesized VLCPUFAs from their

thioesters attached to ACP domains of the PUFA synthase into a pool of free fatty acids for subsequent metabolisms.

6. CONCLUSION

This research investigated the function of a MAT-like domain of the PUFA synthase in *Thraustochytrium*. Sequence analysis showed that the MAT-like domain was located between amino acids 1048 and 1390 in the subunit-B of the PUFA synthase. A conserved motif GXSXG found in α/β hydrolases was identified in the sequence, and mutation of the serine residue inside the motif almost abolished the catalytic activity in the in vitro assay, indicating this motif is essential for the activity. Expression of the MAT-like domain in an *E. coli FabD* temperature-sensitive mutant defective in MAT activity did not complement the phenotype; while the expression of the MAT from the subunit-A could restore the growth at the non-permission temperature (42 °C), indicating that this MAT-like domain does not function as a MAT. Expression of MAT-like domain in an *E. coli AtesAtesB* double mutant deficient in thioesterase did not produce any change in growth phenotype or lipid profile compared to the wild type, as seen previously. Expression of the MAT-like domain in an *E. coli AfadD* defective in acyl-CoA synthetase activity increased the production of free fatty acids both inside and outside cells, indicating potential thioesterase activity. Finally, *in vitro* assays using four acyl-CoAs and the crude protein extract containing the MAT-like domain showed that it possessed thioesterase activity towards 18:0-CoA, 18:1-CoA, 18:2-CoA and 22:6-CoA, and DHA-CoA is the preferred substrate. These results indicate that the MAT-like domain of the subunit-B of the PUFA synthase in *Thraustochytrium* does not function as a MAT, and it is most likely that it function as a thioesterase. This activity might be employed in releasing synthesized VLCPUFAs attached to ACP domains of the PUFA synthase to free fatty acids in *Thraustochytrium*.

7. FUTURE DIRECTIONS

Through complementation expressions in *E. coli* mutant strains and *in vitro* assays, a malonyl-CoA:ACP transacylase (MAT)-like domain of the PUFA synthase from was shown to possess thioesterase activity, which might be involved in catalyzing the last step of DHA biosynthesis by releasing freshly-synthesized VLCPUFAs from the PUFA synthase in *Thraustochytrium*. To prove that the MAT-like domain is the one responsible for the activity *in vivo*, a knock-out *Thraustochytrium* mutant of this domain in the PUFA synthase is required for assays. If the MAT-like knockout mutant cannot synthesize DHA due to being unable to release DHA from the ACP domains, it would confirm that this domain is essential for the biosynthesis of DHA in *Thraustochytrium*.

VLCPUFAs particularly DHA and EPA are well recognized for their health benefits. As marine fish for these fatty acids are overexploited, metabolic engineering of oilseed crops and oily microorganisms might provide a promising alternative source for the fatty acids. A future direction for implementing this strategy can consider to design new PUFA synthases by recombining essential domains functionally characterized by research such as this one for effective production of VLCPUFAs in heterologous systems.

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