UNIVERSITY OF CALIFORNIA SAN DIEGO

Genetic investigation of marine microbial polyunsaturated fatty acid biosynthesis

A dissertation submitted in partial satisfaction of the

Requirements for the degree

Doctor of Philosophy

in

Marine Biology

by

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Professor Doug Bartlett
Professor Bianca Brahamsha
Professor Michael Burkart
Professor Bradley Moore

2019

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

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Chair

University of California San Diego

2019

DEDICATION

For my Mother and Grandma

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LIST OF ABBREVIATIONS

ACP- Acyl carrier protein

CoA- Coenzyme A

CLF- chain length factor

Δ*xxxX*- In-frame deletion of gene *xxxX*

DH/I- β-hydroxy-ACP dehydratase/isomerase

DHA- docosahexaenoic acid (22:6*n*-3)

EPA- eicosapentaenoic acid (20:5*n*-3)

ER- enoyl reductase

FAS- fatty acid synthase

KR- β-ketoacyl-ACP reductase

KS- β-ketoacyl-ACP synthase

MAT- malonyl-CoA acyl transacylase

MPa- Megapascal (0.1MPa = 1 atmosphere)

MUFA- monounsaturated fatty acid

PPTase- phosphopantetheinyl transferase

PUFA- polyunsaturated fatty acid

PUHC- polyunsaturated hydrocarbon (31:9, hentriacontanonaene)

qRT-PCR- quantitative reverse transcriptase PCR

SFA- saturated fatty acid

TE- thioesterase

UFA- unsaturated fatty acid

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ABSTRACT OF THE DISSERTATION

Genetic investigation of marine microbial polyunsaturated fatty acid biosynthesis

by

Marco Nicholas Allemann

Doctor of Philosophy in Marine Biology

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Professor Eric Allen, Chair

Biosynthesis of fatty acids and incorporation into phospholipid membranes is an essential process that underpins all life. The biophysical properties of the phospholipid membrane can be modulated in response to various parameters such as temperature and hydrostatic pressure by the incorporation of unsaturated fatty acids, which act to maintain the fluidity of the membrane by dispersing acyl chain packing. A subset of marine Gamma-proteobacteria utilize a hybrid/fatty acid synthase polyketide pathway to produce polyunsaturated fatty acids (PUFA); eicosapentaenoic (EPA, 20:5*n*-3) or docosahexaenoic (DHA, 22:6*n*-3) and incorporate them into their phospholipid membranes. This dissertation provides new insights into a variety of aspects

of PUFA biosynthesis with particular emphasis on various interactions with other metabolic pathways in the native producing strains.

Chapter 2 examines the relationship between production of EPA and PUHC biosynthetic pathways and describes how these pathways are linked by OleA, a non-decarboxylative ketosynthase, responsible for initiation of the PUHC pathway. The linkage of these pathways is further emphasized by the phenotypic characterization of *pfaT,* a thioesterase, which is required for optimal production of both pathway end products.

Chapter 3 presents new insights into how the *pfa* operon of *Photobacterium profundum* SS9 is controlled at the genetic level. Of the culture conditions tested, addition of exogenous unsaturated fatty acids was shown to dramatically down-regulate the operon. A subsequent transposon screen identified a transcriptional regulator of the *pfa* operon, designated *pfaF,* and subsequent genetic and biochemical experiments validated that PfaF is a fatty acid responsive transcriptional regulator of the *pfa* operon.

Chapter 4 examines the relationship between polyunsaturated and monounsaturated fatty acid biosynthetic pathways and examines how these pathways are functionally related to one another. Derivatives of *P. profundum* SS9, with varying severities of monounsaturated fatty acid synthesis impairments were subjected to high-pressure growth conditions to enrich for suppressor mutants. Strains capable of growth at high pressure and in the absence of exogenous fatty acid supplementation were isolated and sequenced to identify candidate suppressor mutations. Transpositions of insertion sequences into the *fabD* that abolished function or impaired transcription were uncovered. Genetic experiments confirmed that *fabD* is not an essential gene in *P. profundum* SS9 and that *fabD* and *pfaA* constitute a synthetically lethal pair. Heterologous expression of Pfa synthases in *Escherichia coli* complemented a temperature

sensitive *fabD* mutant, suggesting that the malonyl-CoA transacylase (MAT) domain found in PfaA can compensate for loss of FabD activity *in vivo.*

Chapter 1

Characterization and Application of Marine Microbial Omega-3 Polyunsaturated Fatty Acid Synthesis

CHAPTER ONE

Characterization and Application of Marine Microbial Omega-3 Polyunsaturated Fatty Acid Synthesis

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Contents

Abstract

The long-chain omega-3 polyunsaturated fatty acids (n-3 LC-PUFAs) EPA (20:5n-3) and DHA (22:6n-3) are widely recognized as beneficial to human health and development. Select lineages of cosmopolitan marine prokaryotic and eukaryotic microorganisms synthesize these compounds via a unique fatty acid synthase/polyketide synthase mechanism that is distinct from the canonical desaturase/elongase-mediated pathway employed by the majority of eukaryotic single-cell microorganisms and metazoans.

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This "Pfa synthase" mechanism is highly efficient and has been co-opted for the large-scale industrial production of n-3 LC-PUFAs for commercial applications. Both prokaryotic and eukaryotic microbes containing this pathway can be readily isolated from marine environments and maintained in culture under laboratory conditions. Some strains are genetically tractable and have established methods for genetic modification. The discussion and methods presented here should be useful for the exploitation and optimization of n-3 LC-PUFA products from marine microorganisms.

1. INTRODUCTION

Long-chain omega-3 polyunsaturated fatty acids (n-3 LC-PUFAs) such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (Fig. 1) are well known for their beneficial roles in human health and development, and their ameliorative activities in diverse disease conditions, including cardiovascular, inflammatory, autoimmune, and neurodegenerative disorders. Historically, the cardioprotective role of dietary n-3 LC-PUFAs came to prominence in the 1970s during epidemiological studies of Greenlandic Inuits who had drastically reduced occurrence of cardiovascular disease and myocardial infarction rates compared to Western populations (Dyerberg, Bag, & Stoffersen, 1978; Jump, Depner, & Tripathy, 2012). This observation was correlated with high dietary intake

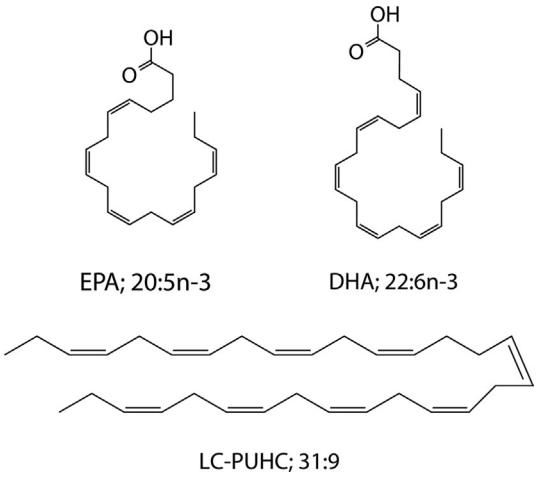

Fig. 1 Chemical structure of long-chain omega-3 polyunsaturated fatty acids and long-chain polyunsaturated hydrocarbon molecules synthesized via the Pfa synthase complex in marine microorganisms.

of marine source n-3 LC-PUFAs from seafood items. Subsequently, the general health benefits of dietary n-3 LC-PUFA consumption has been supported over decades of human health studies (Rizos, Ntzani, Bika, Kostapanos, & Elisaf, 2012; Zárate, el Jaber-Vazdekis, Tejera, Pérez, & Rodríguez, 2017). An expansive body of literature has identified the molecular basis for n-3 LC-PUFA bioactivity, including modulation of the biophysical dynamics of biological membranes (e.g., fluidity) (Calder, 2012), their role as key regulators of gene expression, and as precursors for diverse lipid mediator signaling molecules, such as antiinflammatory eicosanoids, resolvins, protectins, and maresins, that mediate a wide range of homeostatic and inflammatory processes (Serhan, 2014; Serhan, Dalli, Colas, Winkler, & Chiang, 2015; Wall, Ross, Fitzgerald, & Stanton, 2010; Weylandt, Chiu, Gomolka, Waechter, & Wiedenmann, 2012).

The current recommended dietary intake of n-3 LC-PUFAs is $\geq$500 mg per day depending on target population (i.e., age, sex, disease, or at-risk patients) (Global recommendations for EPA and DHA intake, 2014; Stark, Van Elswyk, Higgins, Weatherford, & Salem, 2016). For consumers, marine fish and other seafood are the primary sources of dietary EPA and DHA. While regular consumption of oily fish such as salmon, sardine, and anchovy can meet or exceed these standards, surveys of human blood serum levels have shown that much of the world remains deficient in these beneficial fatty acids (Stark et al., 2016). In recent decades, the recognition of these global dietary deficiencies has spurred a boom in the commercial-scale production of DHA and/or EPA dietary supplements and functional food products, such as DHA additives in infant formula. Historically, commercial production of marine LC-PUFA supplements has been dependent on fish oil; however, ecological concerns over the sustainability of oceanic fish stocks and exposure to toxicants in fish such as methylmercury and polychlorinated biphenyls (Oken et al., 2012) have made the exploitation of alternative sources of these lipids both environmentally and economically attractive (Finco et al., 2017). Specifically, microbial "single-cell oils" derived from heterotrophic marine microalgae such as *Schizochytrium* sp. (Stramenopile; class Labyrinthulomycetes) and *Crypthecodinium cohnii* (Alveolate; class Dinophyceae) have become a major focus for commercial production of n-3 LC-PUFAs in recent years (Borowitzka, 2013) led by companies such as Royal DSM, the dominant supplier of single-cell DHA to US, EU, and Asian markets (excluding China). In addition to nutraceutical applications, microbial n-3 LC-PUFAs are also being exploited to meet demands in the animal nutrition industry, including pet food and aquaculture, as fish oil replacements

(Sprague, Betancor, & Tocher, 2017). Further optimization and diversification of single-cell LC-PUFA products will be the key to establishing new and sustainable sources of these high-value lipid products into the future.

2. DIVERSITY AND DISTRIBUTION OF LONG-CHAIN OMEGA-3-PRODUCING MARINE MICROBES

Production of n-3 LC-PUFAs was once considered a hallmark of eukaryotic, aerobic metabolism. Essential dietary fatty acids such as plant-derived alpha-linoleic acid (ALA; 18:3n-3) can be extended at the carboxyl terminus by fatty acid elongase enzymes, while oxygen-dependent desaturase enzymes incorporate the additional double bonds leading to synthesis of EPA or DHA (Lee, Lee, Kang, & Park, 2016). This pathway is typically found in plants, algae, fungi, and animals, and until the 1980s, it was thought that prokaryotes were unable to produce n-3 LC-PUFAs. Culture-based studies of piezophilic (pressure-loving), psychrophilic (cold-loving) bacteria isolated from deep sea environments demonstrated for the first time that bacteria were capable of producing EPA and DHA (DeLong & Yayanos, 1986). Unlike the canonical elongase/desaturase-mediated aerobic pathway, subsequent work demonstrated that a fatty acid synthase (FAS)/polyketide synthase (PKS) like enzyme system is responsible for synthesis of EPA/DHA in marine bacteria (Allen & Bartlett, 2002; Metz et al., 2001). This enzyme complex is termed the Pfa synthase and the genes encoding the synthase are designated the *pfa* genes (see below) (Allen & Bartlett, 2002). In addition to what now includes a multitude of marine bacterial genera, an orthologous pathway has also been found in several lineages of eukaryotic marine stramenopile protists of the class Labyrinthulomycetes (Metz et al., 2009, 2001). In both instances, the unique FAS/PKS (Pfa synthase) pathway is able to synthesize EPA and/or DHA anaerobically and utilizes malonyl extender units in an iterative process that mimics de novo fatty acid biosynthesis (Metz et al., 2001). In addition to EPA and DHA products, biosynthetic gene clusters homologous to those involved in n-3 LC-PUFA synthesis have also been identified in a broad diversity of bacterial lineages spanning nine bacterial phyla; however, the acyl products associated with these pathways are largely unexplored (Shulse & Allen, 2011b).

2.1 Marine Bacteria

Copiotrophic marine bacteria that synthesize n-3 LC-PUFAs are phylogenetically constrained to select genera within the gamma-proteobacteria,

including EPA-producing *Shewanella*, *Photobacterium*, and *Vibrio* and DHA-producing *Colwellia*, *Moritella*, and *Psychromonas*. Most bacterial lineages that harbor the genetic potential to synthesize LC-PUFAs have been isolated from organic-rich marine habitats such as sediments or homogenates derived from marine invertebrates (e.g., amphipods, squid) or vertebrates (e.g., fish) (Dailey et al., 2016; DeLong & Yayanos, 1986; Nichols, 2003; Okuyama, Orikasa, Nishida, Watanabe, & Morita, 2007; Yano, Nakayama, & Yoshida, 1997). As a result of this close association with marine animals, it has been postulated that these bacteria may be maintained as mutualist symbionts as a source of n-3 LC-PUFAs for these animals (De Carvalho & Caramujo, 2012). Additionally, many of these strains have been isolated from permanently cold (e.g., polar or deep sea) and/or high-pressure (deep sea) habitats where the incorporation of LC-PUFAs into membrane phospholipids is an important adaptation for maintaining optimal membrane fluidity (DeLong & Yayanos, 1986; Valentine & Valentine, 2004; Yoshida et al., 2016).

Mutagenesis experiments with *pfa* mutants have shown that the requirement of EPA for growth at high pressure and/or low temperature is a strain-dependent phenomenon (Allen, Facciotti, & Bartlett, 1999; Kawamoto et al., 2009, 2011; Wang, Xiao, Ou, Gai, & Wang, 2009). From these studies, it has been shown that EPA is generally beneficial under cold temperature/high-pressure conditions (Kawamoto et al., 2011; Sato et al., 2012). In contrast, the piezophile *Photobacterium profundum* SS9 requires only monounsaturated fatty acids such as *cis*-vaccenic acid (18:1n-7) but not EPA, for sustained growth at elevated hydrostatic pressures and/or low temperatures (Allen et al., 1999). While the precise physiological role of these lipid molecules has not been determined, several studies have shown that phospholipids with EPA localize to the sites of cell division and may play a role in outer membrane protein folding (Dai, Kawamoto, Sato, Esaki, & Kurihara, 2012; Sato et al., 2012). Other studies are consistent with n-3 LC-PUFAs having possible membrane-shielding functions against reactive oxygen species (Nishida, Hori, Morita, & Okuyama, 2010; Nishida et al., 2006; Okuyama, Orikasa, & Nishida, 2008) and protecting the cell from membrane-permeable compounds, including antibiotics (Hori, Nishida, & Okuyama, 2011; Yoshida et al., 2016).

2.2 Marine Labyrinthulomycetes and Other Microalgae

While several species of marine bacteria produce LC-PUFAs as components of their membrane phospholipids, large-scale production of bacterial LC-PUFA

products is hindered by the low concentration of LC-PUFAs per cell. In contrast, marine unicellular stramenopile protists of the class Labyrinthulomycetes have been shown to accumulate large quantities of n-3 LC-PUFAs, predominantly as DHA, in the form of triacylglycerides (TAG) that can constitute >50% of total fatty acids and up to 25% of dry cell weight in some strains (Raghukumar, 2002, 2008). The prolific capacity for n-3 LC-PUFA synthesis in Labyrinthulomycete strains is attributed to the Pfa synthase and the commercial exploitation of microbial single-cell LC-PUFAs has largely focused on fermentative growth of these heterotrophic microalgae (Raghukumar, 2008). Moreover, isolated Labyrinthulomycete strains have been shown to utilize a diversity of carbon and energy sources for growth and lipid accumulation, including industrial waste products (Chi, Pyle, Wen, Frear, & Chen, 2007; Scott, Armenta, Berryman, & Norman, 2011), providing economically viable options to diversify feedstock streams in the commercial-scale production of microbial n-3 LC-PUFAs from single cells.

Broadly, the Labyrinthulomycetes consist of three orders, Labyrinthulida, Amphitremida, and Thraustochytrida, representing over 15 recognized genera (Pan, del Campo, & Keeling, 2017). They have cells that range in size from 2 to 20 μm with cell walls containing sulfated polysaccharides, produce rhizoid-like ectoplasmic net extensions, and possess heterokont biflagellate zoospores (Raghukumar, 2002; Raghukumar & Damare, 2011). Labyrinthulomycetes are ubiquitous in the marine environment and play important ecological roles in the degradation of detritus and the recycling of carbon and nutrients by virtue of their extensive repertoire of hydrolytic enzymes, including proteases, amylases, lipases, ureases, phosphatases, chitinases, alpha-glucosidases, and cellulases (Raghukumar, 2002; Raghukumar & Damare, 2011; Singh, Liu, Li, & Wang, 2014). Their nutritional mode is primarily saprotrophic (osmoheterotrophic) where they associate with detritus and decaying organic matter (Kimura, Sato, Sugiyama, & Naganuma, 2001). In natural systems, it has been estimated that Labyrinthulomycetes are primary contributors to the degradation and mineralization of detritus and dissolved organic material despite representing a relatively minor component of the overall microbial diversity in planktonic and benthic marine habitats (Bongiorni, Pusceddu, & Danovaro, 2005; Raghukumar & Damare, 2011). It is likely a result of these diverse catabolic activities and carbon utilization efficiency that imparts many Labyrinthulomycetes with high n-3 LC-PUFA neutral lipid accumulation potential. These lipids act as storage compounds for energy and carbon demands during motility and ectoplasmic net differentiation as well as energy reserves during starvation (Jain, Raghukumar, Sambaiah, Kumon, & Nakahara, 2007).

The biosynthesis of DHA and other LC-PUFAs has been well characterized in several Labyrinthulomycete strains. Generally, both the elongation/desaturation system and the FAS/PKS (Pfa synthase) pathway are present in strains that accumulate high levels of DHA (Lippmeier et al., 2009; Ren et al., 2017), including evidence of *pfa* gene homologues (designated *PFA1–3*) in diverse genera including *Schizochytrium*, *Thraustochytrium*, *Aurantiochytrium*, and *Ulkenia*. However, genetic experiments in *Schizochytrium* sp. (ATCC 20888) demonstrated that disruption of the *PFA* genes led to a fatty acid auxotroph phenotype revealing that the elongase/desaturase pathway was not responsible for producing DHA (Lippmeier et al., 2009; Metz et al., 2009). A recent genomic study identified the *PFA* gene cluster in the DHA-accumulating strain *Aurantiochytrium* sp. SD116 (Ma et al., 2015). Further transcript analyses of this strain grown under various conditions showed that *PFA* transcripts were upregulated in response to cold temperature during stationary phase of growth. A more complete view of the diversity and distribution of n-3 LC-PUFA lipid accumulation potential in the labryinthulomycetes will require additional emphasis on genome sequencing and experimentation which is currently limited to a small number of strains available through commercial culture collections such as ATCC and DSMZ.

The isolation of Labyrinthulomycete strains is readily performed in the laboratory by the baiting of natural water samples with various organic-rich substrates such as plant material (pollen, algal tissue, cellulosics), compounds containing keratin such as feathers, or chitin compounds such as invertebrate exoskeletons, and plating on nutrient-rich agar in the presence of antibiotics. A brief protocol for the isolation and characterization of Labyrinthulomycetes from marine or estuarine water samples is provided below.

2.2.1 Protocol: Simple Enrichment and Isolation Strategy for Environmental Labyrinthulomycetes

1. Obtain inoculum source environmental sample—typically decaying organic matter, algae, sediment, or seawater/estuarine water
2. Add sample to test tube containing:
   10 mL sterile, 0.2 μm filtered natural seawater
   To inhibit bacterial growth from samples the enrichment culture can be amended with 300 μg/mL penicillin and 500 μg/mL streptomycin
3. Bait enrichment culture with several pollen grains (derived from local trees preferably, e.g., pines) or UV-sterilized feathers
4. Incubate enrichment at 18–27°C for 48–96 h, shaking optional

5. Transfer 200 μL of pollen sample or organic samples using tweezers and spread or streak onto Labyrinthulomycete base media agar plates (see recipe below)
6. Incubate plates for 3–7 days at 18–27°C. Restreak isolated colonies as many times as necessary in order to obtain axenic cultures.
7. Purified colonies should be screened by 18S rRNA gene amplification and amplicon sequencing to verify taxonomic identity of recovered strains.

Base Labyrinthulomycete media recipe, per liter:

1.5 g yeast extract
2.5 g peptone
Add 1 L 0.2 μm filtered natural or artificial seawater and sterilize by autoclave
For solid media, add 15 g agar per L
*Optional: Add glucose to final concentration of 27 m*M* (0.5%, w/v) after autoclaving

In addition to n-3 LC-PUFA-accumulating Labyrinthulomycetes, other marine microalgae harbor homologues of the Pfa synthase mechanism. The DHA-producing coccolithophore *Emiliania huxleyi* (Haptophyte; class Prymnesiophyceae) contains a single 17.6 kbp gene with a domain architecture nearly identical to the combined Labyrinthulomycete *PFA1–3* organization (Fig. 2). However, experimental validation of this unique Pfa synthase and its potential role in DHA synthesis in this globally distributed phytoplankton species has not been performed. It is intriguing to further speculate on the possibility of a Pfa synthase-like mechanism in the heterotrophic marine dinoflagellate *C. cohnii* (Alveolate; class Dinophyceae). This species is an elite platform strain for commercial production of food-grade DHA single-cell oil originally patented by Martek Biosciences Corporation (Martek was acquired by Royal DSM in 2011) (Mendes, Reis, Vasconcelos, Guerra, & da Silva, 2009). While genetic information is currently unavailable from this strain, its prolific DHA accumulation potential is consistent with a Pfa synthase mechanism.

3. THE Pfa SYNTHASE

3.1 Genetic and Catalytic Domain Architecture

The *pfa* gene clusters depicted in Fig. 2 code for the FAS/PKS system responsible for marine microbial n-3 LC-PUFA biosynthesis, hereafter referred to as the Pfa synthase. These genes possess multiple fatty acid

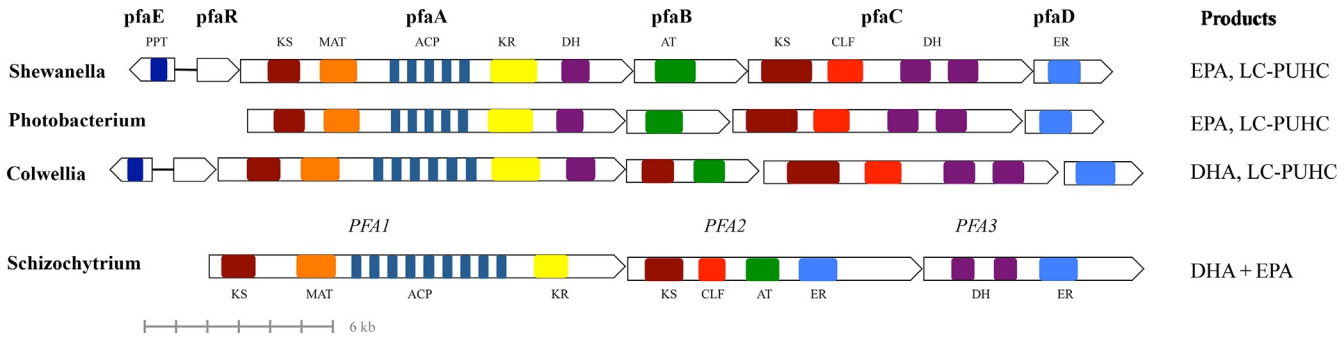

Fig. 2 Diversity and domain architecture of representative Pfa synthase gene clusters from marine gamma-proteobacteria (*Shewanella*, *Photobacterium*, *Colwellia*) and Labyrinthulomycetes (*Schizochytrium*). Domain designations include phosphopantetheinyl transferase (PPT), β-ketoacyl synthase (KS), malonyl-CoA:ACP transacylase (MAT), acyl-carrier protein (ACP), ketoacyl reductase (KR), dehydratase/isomerase (DH), acyltransferase (AT), chain-length factor (CLF), and enoyl reductase (ER).

biosynthetic enzyme activities as integrated domains within the operon-encoded gene products, consistent with classification of the Pfa synthase as an iterative type I FAS/PKS. In the bacterial system, the synthase is coded for by five genes *pfaABCDE* and is typically arranged as an operon with slight domain structural variations depending on species and LC-PUFA product(s). The genetic organization of the biosynthetic gene cluster found in eukaryotic Labyrinthulomycetes (e.g., *Schizochytrium* sp.) is highly similar to the bacterial architecture although the synthase is encoded by only three genes, *PFA1–3*, yet contains the same general domain composition. The highly conserved head-to-tail order and domain organization of *pfa/PFA* genes across disparate marine microbial taxa, from bacteria to eukaryotes, are consistent with horizontal gene transfer having contributed to the broad dissemination of n-3 LC-PUFA biosynthetic processes in marine microorganisms (Metz et al., 2001; Shulse & Allen, 2011b).

3.2 Proposed Biosynthetic Process

The highly conserved enzymatic domains embedded within the Pfa/PFA proteins catalyze the cycle of condensation, reduction, dehydration, and reduction necessary to synthesize the final products as shown in Fig. 3A. Using radiolabeled substrates, it has been shown that the Pfa synthase initiates from acetyl-CoA as a primer and utilizes 2-carbon malonyl units for chain elongation (Metz et al., 2001). The biosynthetic reaction sequence of an elongation cycle includes the following enzymatic steps shown in Fig. 3A inset: (1) β-ketoacyl synthase (KS) catalyzes the condensation of malonyl-ACP with acyl-ACP leading to chain elongation by two carbon atoms; (2) ketoacyl reductase (KR) catalyzes reduction of the β-ketoacyl group to a hydroxyl; (3) dehydratase/isomerase (DH/I) catalyzes dehydration of the β-hydroxyacyl intermediate generated by KR to a *trans*-2-enoyl derivative which can be isomerized to *cis*-3 or *cis*-2 conformations for double bond retention; (4) enoyl reductase (ER) catalyzes reduction of the *trans*-2-double bond generated by DH to complete the chain elongation cycle. Iterative processing of the fatty acyl chains via the above reaction sequence with intermittent selective position-specific *trans–cis* isomerization and enoyl reduction in specific cycles is predicted to result in the ultimate n-3 unsaturation pattern of the final LC-PUFA products.

The Pfa synthase contains at least two KS domains that catalyze the Claisen condensation of malonyl-ACP with acyl-ACP substrates to yield a 3-keto-acyl-ACP product. An additional KS domain appears in the PfaB

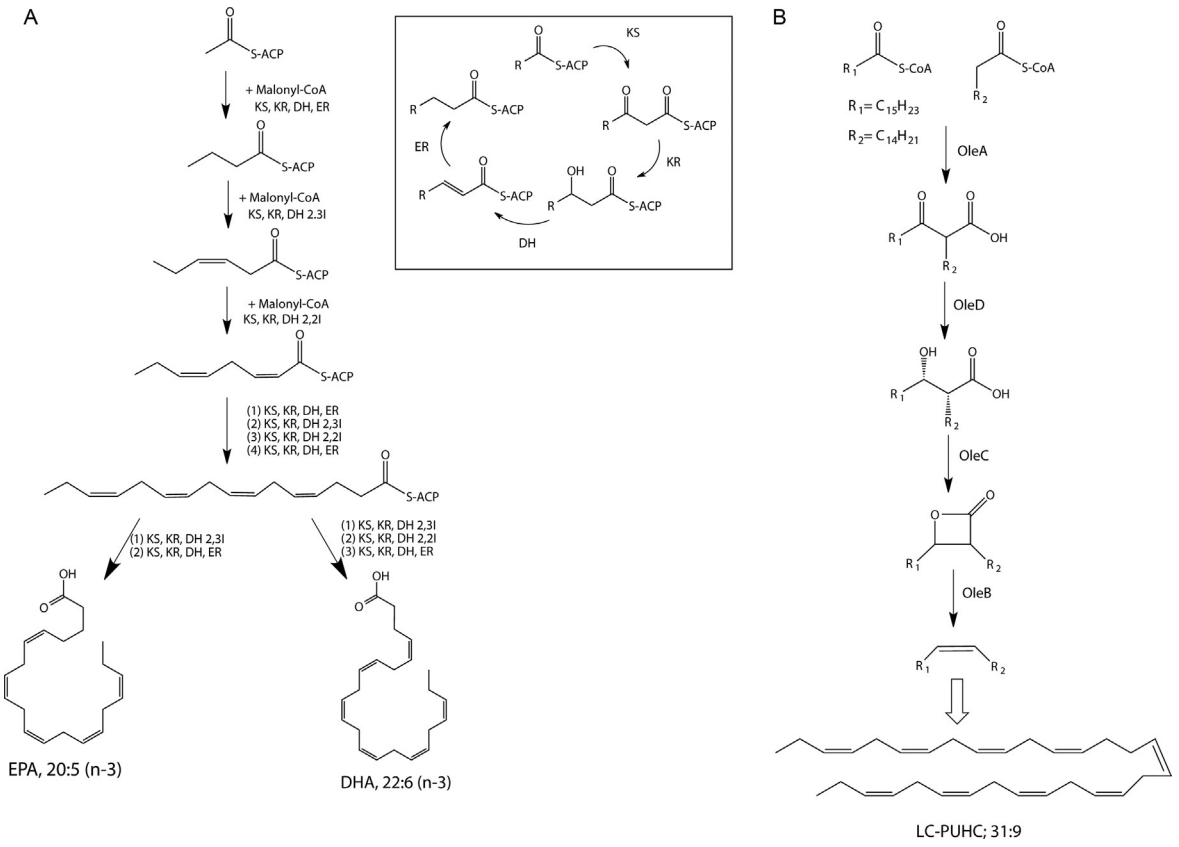

Fig. 3 Biosynthetic reaction cycles mediated by the Pfa synthase. (A) Proposed mechanism for EPA and DHA biosynthesis. (B) Head-to-head condensation of two Pfa synthase-derived intermediate fatty acid molecules catalyzed by OleABCD to yield the LC-PUHC product.

homologue responsible for bacterial DHA biosynthesis and has been implicated in the additional condensation reaction required to elongate EPA to DHA (Orikasa et al., 2009). Both DHA and EPA synthase types contain a KS domain lacking the catalytic cysteine in PfaC, which is thought to be a so-called chain-length factor domain, which in concert with an active KS domain determines the final chain length of the product. While the exact roles of the KS domains in PfaC and PfaA are not known, site-directed mutagenesis of the active site cysteine in the PfaC KS domain of *Shewanella pealeana* led to a complete loss of EPA production in recombinant *Escherichia coli* strains (M.N. Allemann & E.E. Allen, Unpublished data). Highly conserved regions within the KS domain of PfaA have been used as a probe to find other Pfa synthases from diverse lineages and environments, the majority of which representing novel synthase types with uncharacterized fatty acyl products (Shulse & Allen, 2011a, 2011b).

A notable feature of the domain architecture of Pfa synthase complexes is the tandem acyl-carrier protein (ACP) domains seen in PfaA/PFA1. This is a defining feature of the Pfa synthase and is found in both eukaryotic and prokaryotic forms of the synthase. Prior studies have shown that decreasing the number of functional ACP domains via site-directed mutagenesis hindered the productivity of the synthase in vivo (Jiang et al., 2008). Another observation in this study was that any of the ACP domains are able to support LC-PUFA biosynthesis, suggesting that each ACP is catalytically equivalent. Further structural analysis of the ACP domains has indicated that they adopt a "beads-on-a-string" conformation, which is thought to allow for multiple biosynthesis sites, which can be accessed by the other catalytic domains of the synthase (Trujillo et al., 2013). The Pfa synthase characterized from *Schizochytrium* sp. (ATCC 20888) also contains a tandem ACP array with a total of nine ACP domains in *PFA1* (Fig. 2). Further work on this feature demonstrated that increasing or decreasing the number of ACP domains led to increased and decreased yields of DHA, respectively (Hayashi, Satoh, Ujihara, Takata, & Dairi, 2016).

The ACP domains on the Pfa synthase are activated for biosynthesis by the posttranslational addition of phosphopantetheine to a conserved serine residue within the ACP (Beld, Sonnenschein, Vickery, Noel, & Burkart, 2014). This modification is carried out by a phosphopantetheinyl transferase (PPT) encoded by *pfaE*. PfaE is classified as a type II PPT which is typically associated with the activation of secondary metabolite ACP domains (Beld et al., 2014). While PfaE is considered the cognate PPT for the Pfa synthase, other type II PPTs, such as EntD from *E. coli* and Sfp from *Bacillus subtilis*,

have been shown to successfully modify the ACP domains either in vivo or in vitro (Jiang et al., 2008; Sugihara, Orikasa, & Okuyama, 2010; Trujillo et al., 2013). While a cognate PPT for the Pfa synthase in *Schizochytrium* has yet to be identified, another type II PPT, HetI from *Nostoc* sp., was shown to successfully modify the synthase in vivo (Hauvermale et al., 2006). While *pfaE* is typically clustered with the operon as shown in Fig. 2, in certain strains, such as *P. profundum* SS9, the *pfaE* homologue is located elsewhere in the genome (Sugihara, Orikasa, & Okuyama, 2008; Vezzi et al., 2005).

Based on the predicted biosynthetic scheme, the Pfa synthase has the unusual capability to catalyze both 2,3 and 2,2 *trans/cis* isomerizations. The 2,3 *trans/cis* isomerization of the double bond is typical of the reaction carried out by FabA, the DH/I responsible for unsaturated fatty acid biosynthesis in the canonical bacterial type II FAS (Heath & Rock, 1996; Leesong, Henderson, Gillig, Schwab, & Smith, 1996). Based on the primary amino acid sequence homology, the Pfa synthase appears to contain five possible DH domains, four of which are in PfaC and a fifth in PfaA. At the amino acid level, the four domains in PfaC are most similar to FabA, while the domain in PfaA is most similar to DH domains found in PKSs. A fragment containing the four domains within PfaC has been cloned, expressed, purified, and shown to have DH activity in vitro and in vivo (Oyola-Robles, Rullán-Lind, Carballeira, & Baerga-Ortiz, 2014). Mutagenesis of catalytic residues within the four DH domains within PfaC demonstrated that only two of the four domains in PfaC had significant DH activity in vitro. The remaining DH domain found at the C-terminus of PfaA has significant similarity to other PKS DH domains, yet its role in the biosynthetic reaction sequence is unknown at this time.

Unlike most PKS and type I FAS, the Pfa synthase does not contain a thioesterase (TE) domain responsible for cleavage and release of the final product from the synthase. However, sequence analysis of the *pfa* gene neighborhood in *P. profundum* SS9 identified an ORF upstream of *pfaA* encoding a putative TE enzyme. Subsequent biochemical assays showed that this ORF, designated *orf6*, had TE activity in vitro on a variety of long-chain acyl-CoA substrates (Rodríguez-Guilbe, Oyola-Robles, Schreiter, & Baerga-Ortiz, 2013). Homologues of *orf6* have been identified in multiple lineages of n-3 LC-PUFA-producing bacteria leading to the suggestion that Orf6 participates in the biosynthesis of EPA/DHA. Subsequent genetic studies of *orf6* in *P. profundum* SS9 indicate that it is not required for EPA biosynthesis; however, its involvement in optimal n-3 LC-PUFA synthesis

was confirmed as *orf6* mutants produced severalfold less EPA (M.N. Allemann & E.E. Allen, Unpublished data). This is further supported by repeated experiments wherein EPA/DHA biosynthesis in *E. coli* has been achieved in the absence of coexpression of an *orf6* homologue. From this observation, it is proposed that Orf6 participates in maintaining optimal turnover rate of the synthase in vivo by acting as a type II TE. In other PKS systems, type II TE domains function in the removal of products that have been improperly processed or decarboxylated extender units that are catalytically inactive (Heathcote, Staunton, & Leadlay, 2001; Kotowska & Pawlik, 2014). Unlike the Pfa synthase in marine bacteria, the *Schizochytrium* synthase releases its fatty acid product(s) as free fatty acids and requires acyl-CoA synthetase activity for incorporation of DHA into phospholipids and TAG (Metz et al., 2009).

4. RELATIONSHIP BETWEEN LC-PUFA AND LC-PUHC BIOSYNTHESIS

Chemical intermediates of the Pfa synthase are also utilized by a separate bacterial pathway involved in the production of a long-chain polyunsaturated hydrocarbon (LC-PUHC). Bacterial strains containing the *pfa* gene cluster responsible for EPA or DHA synthesis and the *oleABCD* gene cluster produce a single LC-PUHC product, hentriacontanonaene (31:9) (Sukovich, Seffernick, Richman, Hunt, et al., 2010; Sukovich, Seffernick, Richman, Gralnick, & Wackett, 2010). Genetic evidence has shown that disruption of either *pfa* or *ole* gene functions results in complete loss of LC-PUHC synthesis in *Shewanella* species (Sukovich, Seffernick, Richman, Hunt, et al., 2010). Based on the structure of the LC-PUHC and the known biochemical functions of OleABCD homologues (Bonnett, Papireddy, Higgins, del Cardayre, & Reynolds, 2011; Christenson, Jensen, et al., 2017; Christenson, Richman, et al., 2017; Frias, Richman, Erickson, & Wackett, 2011; Goblirsch, Jensen, Mohamed, Wackett, & Wilmot, 2016), a predicted biosynthetic scheme for the LC-PUHC is shown in Fig. 3B. In this scheme, two 16:4n-3 acyl-CoA or ACPs are condensed by OleA and subsequently processed by OleD, OleC, and OleB. Despite the presence of the type II FAS in all LC-PUHC-producing strains, the OleA–D pathway exclusively utilizes Pfa synthase-derived fatty acids to generate a single 31:9 product (Sukovich, Seffernick, Richman, Gralnick, et al., 2010). In contrast, strains harboring *oleA–D* and the type II FAS alone produce multiple hydrocarbon products consistent with saturated or monounsaturated fatty acid substrates

(Beller, Goh, & Keasling, 2010; Sukovich, Seffernick, Richman, Gralnick, et al., 2010). Interestingly, *Shewanella* strains that produce trace to no EPA, such as *Shewanella oneidensis* MR-1, produce the PUHC product, suggesting that all Pfa synthase products are diverted to PUHC biosynthesis (C.N. Shulse & E.E. Allen, Unpublished data). The mechanistic details connecting the Pfa synthase to the Ole pathway awaits future characterization. While the exact physiological role for the PUHC is unknown, studies in various members of the *Shewanella* genus suggest that the PUHC has a minor role in cold-adapted growth (C.N. Shulse & E.E. Allen, Unpublished data; Sukovich, Seffernick, Richman, Hunt, et al., 2010).

5. CULTIVATION AND GENETIC OPTIMIZATION OF NATIVE STRAINS

Isolation of n-3 LC-PUFA-producing bacteria from marine samples can be readily achieved using standard Difco 2216 marine broth or comparable rich media for the cultivation of heterotrophic marine bacteria. A novel screening method utilizing growth media supplemented with 2,3,5-triphenyltetrazolium chloride (TTC) has also proven to be a valuable tool in the isolation of LC-PUFA-producing bacteria from the marine environment (Ryan, Farr, Visnovsky, Vyssotski, & Visnovsky, 2010). Briefly, TTC is a colorless reagent that can be reduced to a bright triphenyl red formazan and has been used as a colorimetric indicator for reductive metabolism. Inclusion of TTC in isolation media has been shown to be a quick method of screening for potential LC-PUFA-producing bacteria from environmental samples. It should be noted however that TTC reduction is not directly linked to LC-PUFA biosynthesis as strains containing deletions of the *pfa* operon still reduce TTC in both solid and liquid media (M.N. Allemann & E.E. Allen, Unpublished data). Our work has also shown that TTC at higher concentrations can inhibit the growth of n-3 LC-PUFA producers such as *Colwellia* and *Photobacterium*.

Bacteria that contain Pfa synthase also contain the canonical type II FAS responsible for intermediate chain saturated, monounsaturated, and, in some strains, branched chain fatty acids (Cronan & Rock, 2008). Given that both the type II FAS and the Pfa synthase produce fatty acids destined for incorporation into membrane phospholipids, how these pathways interact is an intriguing physiological question that has not been investigated. Inhibition of monounsaturated fatty acid biosynthesis using the FabB/FabF inhibitor cerulenin, a fungal antibiotic, can significantly boost EPA/DHA production

in native producing strains as well as recombinant production strains (Allen et al., 1999; Morita, Nishida, Tanaka, Yano, & Okuyama, 2005). This is perhaps not surprising given that EPA/DHA typically associate at the *sn*-2 position of glycerol 3-phosphate, a position that is occupied by monounsaturated fatty acids (Cho, Kasai, Kawamoto, Esaki, & Kurihara, 2012; Yazawa, 1996). Consistent with this phenomenon, disruption of monounsaturated fatty acid biosynthesis by cerulenin treatment or via targeted *fabF* (Allen & Bartlett, 2000) or *fabB* mutations in *P. profundum* SS9 can elicit dramatically higher EPA levels similar to culturing the strain at low temperature or elevated hydrostatic pressure, as seen in Fig. 4. For example, *fabB* deletion in *P. profundum* SS9 results in a fivefold increase in relative EPA content (up to 25% total fatty acids) compared to the wild-type parental strain (M.N. Allemann & E.E. Allen, Unpublished data).

Several native strains of n-3 LC-PUFA-synthesizing marine bacteria have proven to be important model organisms owing to their genetic tractability. *P. profundum* SS9 is a well-established model organism for elevated hydrostatic pressure adaptation and has contributed to the study of bacterial n-3 LC-PUFA biosynthesis (Bartlett, 1999; Vezzi et al., 2005). *P. profundum* SS9 can be grown over a wide range of temperatures (4–20°C) and hydrostatic pressures (0.1–50 MPa) and produces EPA at approximately 5%–10% of total fatty acid content depending on cultivation parameters. A variety of plasmids can be mobilized into the strain using conjugal donor strains for gene knockouts, complementation, overexpression, and transposon

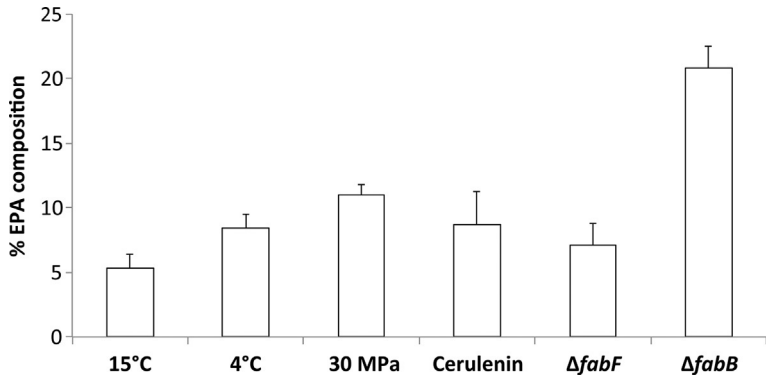

Fig. 4 EPA composition of *Photobacterium profundum* SS9 lipids under varying culture conditions and genetic backgrounds. For the indicated genetic modifications, mutant strains were grown aerobically at 15°C.

mutagenesis approaches (Lauro, Eloe, Liverani, Bertoloni, & Bartlett, 2005). Other LC-PUFA-synthesizing bacteria such as members of the *Shewanella* have also been shown to be amenable to genetic manipulation using similar methods to those described for SS9 and have been used to interrogate the physiological roles of LC-PUFA production in vivo (Kawamoto et al., 2009, 2011; Wang et al., 2009). Likewise, several types of transformation systems have been developed for diverse genera of Labyrinthulomycetes that allow for expression of transgenes and gene-targeted mutagenesis providing new opportunities to genetically engineer optimized n-3 LC-PUFA synthesis in these strains (Cheng et al., 2012; Kobayashi et al., 2011; Sakaguchi et al., 2012).

For genetic manipulation studies in marine bacteria, DNA is typically introduced into cells by conjugation using *E. coli* strains carrying the RK2/RP4 conjugal transfer system (Brahamsha, 1996; Lauro et al., 2005). Widely utilized conjugal donor strains such as S17-1, SM10, and WM3064 can be used to introduce transferable plasmids into recipient strains. Both recipient and donor strains are grown under appropriate conditions and mixed together in empirically determined ratios and spotted onto non-selective agar media. Once the cell mixture has dried into a film, the agar plate is incubated overnight at a temperature favorable to both the donor and recipient strains. The next day cells are removed from the plate and plated on selective media, which is incubated under optimal conditions for the recipient strain. A more detailed protocol for conjugation experiments is provided below.

5.1 Protocol: General Conjugation Protocol for Marine Bacteria

1. Inoculate a 5 mL liquid culture of donor *E. coli* strain in LB medium with appropriate antibiotics. Grow overnight at 37°C or 30°C
2. Inoculate a 5 mL liquid culture of desired recipient strain in appropriate rich media and incubate at optimal temperature. Most PUFA-producing marine bacteria grow well in 2216 marine broth (Difco). While growth temperature optima can vary, 15–20°C is a good range for most PUFA-producing marine bacteria.
3. Once both donor and recipient strain have reached stationary phase, centrifuge a volume of donor *E. coli* and discard supernatant. Wash cells by resuspending the cell pellet in an equal volume of 50% sea salt solution (16 g/L) (Sigma-Aldrich). Repeat centrifugation and wash step.

4. In a separate tube, centrifuge 1 mL of recipient culture and discard supernatant. Add 0.5 mL washed *E. coli* cells to recipient cell pellet and centrifuge to obtain a cell pellet.
5. Resuspend the donor–recipient cell pellet in a minimal volume of 2216 marine broth media (Difco) or LB and spot cell mixture onto a sterile Millipore 0.45 μm HA 25 mm filter (or equivalent) that has been placed onto a 2216 or LB agar plate. Alternatively, the cell mixture can be spotted directly onto the agar surface. Allow spotted cell mixture to dry into a film and incubate the plate for 16–24 h at room temperature (~20–22°C).
6. If filters were used, carefully remove them using sterile tweezers and place into tubes containing 1 mL 2216 broth and resuspend cells off filter by gentle pipetting or vortexing. Remove filter and plate resuspended cells onto appropriate selective media. If spotted directly to agar, scrape cell spots up using a sterile loop and resuspend cells from filters into 1 mL of 2216 liquid broth. Gently pipet cells to resuspend the conjugation mix evenly.
7. Plate aliquots of resuspended cells onto the appropriate selective media.

For the recipient strain *P. profundum* SS9, the above protocol utilizing filters on 2216 plates will yield conjugation efficiencies of $\sim 10^{-4}$ and $\sim 10^{-7}$ exconjugants per recipient cells for replicating and suicide plasmids, respectively.

To obtain exconjugants, an adequate selection must be placed on the recipient cells as well as an adequate counterselection against the donor *E. coli*. The use of spontaneously derived rifampicin-resistant recipient cells is one route to achieve selection advantage against the donor strain. To obtain such cells, a dense culture of wild-type recipient cells is grown to stationary phase and plated on media containing 50–100 μg/mL rifampicin. Spontaneously derived mutant colonies can be further purified from this initial selection by restreaking onto rifampicin media. Another method of counterselection against donor *E. coli* utilizes a diaminopimelic acid (DAP) auxotroph donor strain such as WM3064. Conjugations occur on media supplemented with DAP and the resuspended cell mixture is rinsed several times in rich media or in 50% sea salt (16 g/L) solution before plating to selective media lacking DAP. Depending on the recipient strain being utilized, the appropriate selective marker and the minimum inhibitory concentration of the antibiotic are determined empirically. In our experience, antibiotics such as ampicillin are poor selective markers because many environmentally derived marine strains are resistant. Other antibiotics such as kanamycin, streptomycin, gentamicin, and chloramphenicol are often highly effective selective markers in our experience.

For genetic knockout experiments, suicide vectors, plasmids that cannot replicate in the recipient strain, are commonly employed in marine bacteria. Most often, these plasmids are from the R6K group, which require the *pir* locus for replication (Edwards, Keller, & Schifferli, 1998). Using this strategy, either insertional inactivation or allelic exchange mutagenesis is possible. Other replicons such as the ColE01 plasmid pBR322 have been shown to be useful as suicide vectors in various marine bacteria as well (Allen et al., 1999; Brahamsha, 1996). Expression or complementation studies require stable replicating plasmids in the recipient strain. While each strain is unique in terms of what types of plasmids will propagate successfully, in our experience broad host range plasmids belonging to the IncQ/RSF1010 and pBBR1 replicon families are good candidates for establishing a replicating vector system in marine bacteria (Bagdasarian et al., 1981; Kovach et al., 1995).

6. RECOMBINANT PRODUCTION OF N-3 LC-PUFAs IN HETEROLOGOUS HOSTS

Given the large size of the *pfa/PFA* gene clusters (approximately 20 kbp in marine bacteria and Labyrinthulomycetes) standard cloning techniques are unlikely to succeed in cloning the entire *pfa* operon. Indeed, early studies on the *pfa* genes utilized genomic cosmid or fosmid libraries to clone the *pfa* operon in its entirety (Okuyama et al., 2007; Yazawa, 1996). While fosmid/cosmid cloning approaches have been repeatedly demonstrated successfully, the wealth of genomic information available for n-3 LC-PUFA-producing strains allows the efficient use of more directed cloning approaches. To this end, our lab has employed transformation-associated recombination cloning (Yamanaka et al., 2014) in yeast to capture the *pfa* operon responsible for DHA biosynthesis from *Colwellia psychrerythraea* 34H (M.N. Allemann & E.E. Allen, Unpublished data). Other methods such as Gibson assembly can also be used to assemble the operon from multiple PCR amplicons or the independent cloning of individual genes using multiple compatible constructs to carry the complete operon. Once cloned into *E. coli* a broad suite of tools is available for further genetic manipulation of the EPA/DHA biosynthesis genes. In particular, the use of lambda red-based genetic manipulations has been most useful in our experience. Using these recombineering approaches (Sharan, Thomason, Kuznetsov, & Court, 2009; Warming, Costantino, Court, Jenkins, & Copeland, 2005) specifically designed for fosmid/cosmid manipulations, we have successfully generated single nucleotide changes as well as in-frame deletions/insertions within *pfa* operons.

Heterologous production of EPA and/or DHA in *E. coli* has been achieved through cloning of both bacterial and eukaryotic Pfa synthases. Depending on the source organism of the *pfa/PFA* genes, production of LC-PUFAs in *E. coli* has been achieved at temperatures ranging from 10°C to 25°C (Okuyama et al., 2007). It should also be noted that this temperature range is often within the optimal growth temperature for the majority of native LC-PUFA-producing marine strains. Other optimization strategies, such as promoter substitutions and deletion of repressors, have also been successful in optimizing recombinant yields. The regulatory gene *pfaR* in *Shewanella* species, shown in Fig. 2, was removed from the cluster and a *lac* promoter cloned in its place. This construct was shown to increase EPA production in *E. coli* by 11-fold compared to the native promoter (Lee et al., 2008). Additional research has shown that targeted mutagenesis of the DNA-binding domain within *pfaR* alone is sufficient to cause a twofold increase in EPA production in recombinant *E. coli*, while heterologous *pfaR* expression *in trans* leads to a fivefold decrease in EPA (M.N. Allemann & E.E. Allen, Unpublished data). However, subsequent genetic experiments in native strains of *Shewanella piezotolerans* WP3 and *S. oneidensis* MR-1 demonstrated that in-frame deletion of *pfaR* does not lead to elevated EPA; thus the relevance of *pfaR* in vivo remains to be evaluated (M.N. Allemann & E.E. Allen, Unpublished data).

In addition to *E. coli*, the Pfa synthase has been successfully expressed in a variety of other organisms. Heterologous expression of the *pfa* operon from *Shewanella baltica* MAC1 in recombinant *Lactococcus lactis*, a relative of well-known food-grade lactic acid bacteria, produced both EPA and DHA (approximately 1.5 mg g^{-1} cell dry weight) (Amiri-Jami, Lapointe, & Griffiths, 2014). This work opens the possibility of using genetically modified food-grade microbes as a strategy for supplementing fermented food products with n-3 LC-PUFAs. Recombinant n-3 LC-PUFA synthesis mediated by the Pfa synthase has also been accomplished in photosynthetic organisms, including EPA synthesis in the marine cyanobacterium *Synechococcus* sp. NKBG15041c (Yu et al., 2000) and DHA + EPA synthesis in engineered *Brassica napus* (canola) seeds (Walsh et al., 2016). Introduction of the Pfa synthase genes from *Schizochytrium* in transgenic canola seeds resulted in significant production of DHA (3.7%) and EPA (0.7%) in field-produced canola oil while maintaining commercial quality attributes. Importantly, expression of the synthase in canola did not significantly impact the fatty acid profile of the oil beyond the enrichment in n-3 LC-PUFAs and did not require supplementation of fatty acid precursors. The versatility and

efficiency of the Pfa synthase enzyme system for the production of n-3 LC-PUFAs in transgenic oil seed crops and phototrophic microorganisms provide immediate opportunities for the recombinant production of n-3 LC-PUFAs as alternatives to marine fish oil.

7. CHEMICAL ANALYSIS METHODS

For lipid analysis, cell cultures are harvested by centrifugation and cell pellets are lyophilized. While other lipid extraction protocols such as Bligh and Dyer (1959)) can be applied, the following protocols have been used routinely and have been shown to yield equivalent results with less handling time (Lewis, Nichols, & McMeekin, 2000). For routine profiling, fatty acids are derivatized into methyl esters (FAME) and run on a GC–MS as described below. Representative GC chromatograms of n-3 LC-PUFA-producing marine microorganisms and recombinant *E. coli* are shown in Fig. 5. Hydrocarbon extractions also utilize freeze-dried biomass and a protocol is also included below.

7.1 Protocol: Whole-Cell Fatty Acid Methyl Esterification Derivatization Protocol

1. Harvest cells by centrifugation and freeze dry cell pellet
2. Crush dried cell pellet into a fine powder and transfer 10–20 mg to 1.8 mL borosilicate glass sample vial with PTFE-lined cap
3. Add approximately 0.5 mL 5% H_2SO_4 in methanol to dried biomass in vial
4. Cap vial tightly and incubate at 90°C for 90 min and allow to cool
5. Add approximately 0.4 mL of hexane and mix by inversion
6. Add approximately 0.8 mL 10% NaCl in water and mix thoroughly
7. Allow mixture to separate into two layers; brief centrifugation may be necessary to achieve good separation
8. Remove upper hexane layer and transfer to new vial, repeat hexane extraction on remaining aqueous layer, and pool hexane phases
9. If the sample needs to be concentrated, evaporate hexane under gentle N_2 stream
10. Analyze via GC–MS or GC-FID as desired
11. For sample storage add N_2 or argon gas layer before capping sample vial and store at −20°C or −80°C

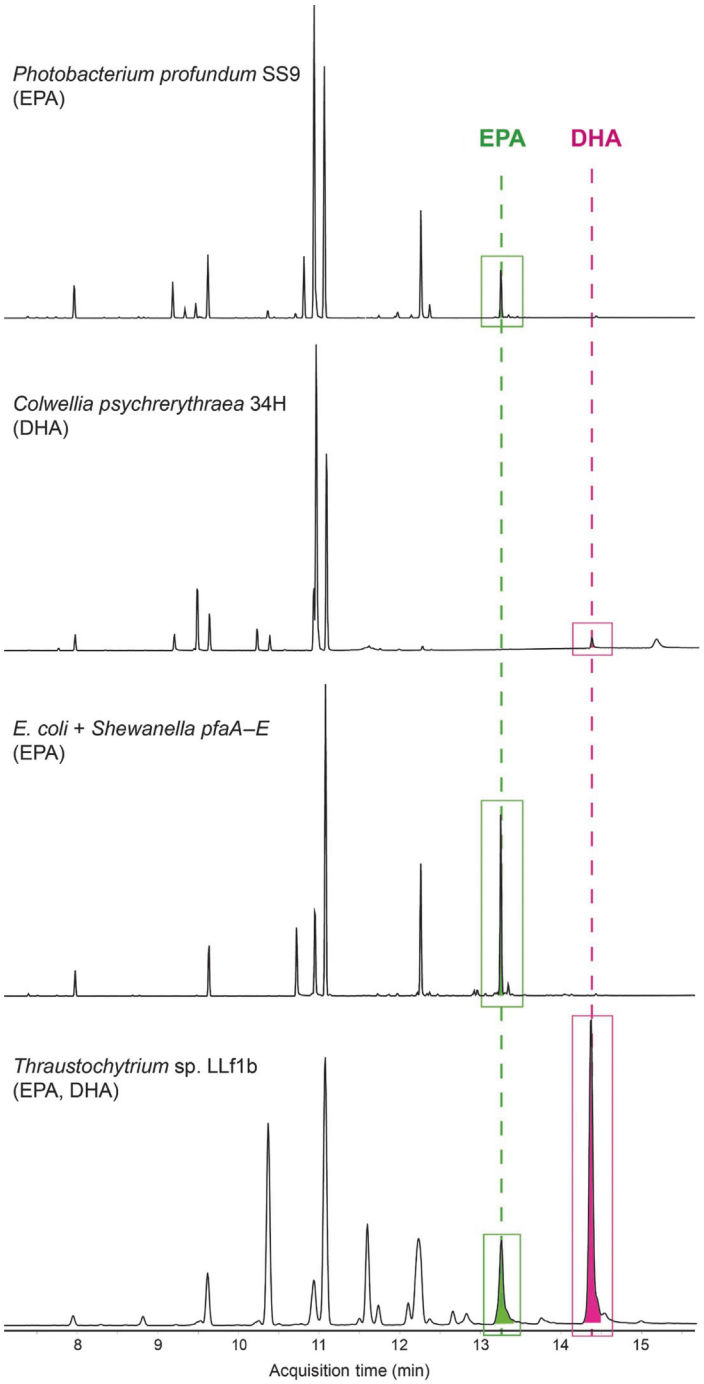

Fig. 5 Representative GC chromatograms of whole-cell fatty acid methyl esters derived from EPA- and DHA-synthesizing microorganisms.

7.2 Protocol: Long-Chain Hydrocarbon Extraction Protocol

1. Harvest cells by centrifugation and freeze dry cell pellet
2. Crush cell pellet into a fine powder and transfer approximately 50 mg to 8 mL borosilicate glass sample vial with PTFE-lined cap
3. Add 2 mL of a 2:1 (v:v) dichloromethane:methanol solution and stir mixture overnight
4. Filter the solvent biomass mixture through a glass wool/Celite-packed Pasteur pipette to remove cell debris and collect the liquid extract in a new sample vial
5. Dry down extract completely under a gentle N_2 stream; slight heating to 40–50°C will aid in evaporating the methanol
6. To dried extract, add 2 mL methanol and 2 mL hexane to yield a two-phase mixture; mix well and allow phases to separate
7. Remove the upper hexane layer carefully to a GC–MS sample vial; repeat hexane extraction on the methanol phase and pool hexane layers
8. Analyze via GC–MS or GC-FID
9. For sample storage add N_2 or argon gas layer before capping sample vial and store at −20°C or −80°C

For GC–MS analysis, we favor the use of an Agilent DB5-MS 30 m × 250 μm diameter column or equivalent. Both FAME and hydrocarbon compounds can be analyzed. The following GC–MS run parameters are a good starting point for method development and have been extensively used for routine analysis of EPA, DHA, and PUHC in microorganisms.

7.2.1 FAME GC–MS Method

FAME samples are injected at 110°C in splitless mode with a 30-s venting time. After 3 min the oven is temperature programmed from 110°C to 270°C at a rate of 15°C per minute. The final temperature of 270°C is held for 2 min. Helium is used as carrier gas and the injector and the detector both maintained at 260°C. MS-operating conditions were as follows: 3-min solvent delay, 50–500 mass unit range, and 230°C source temperature.

7.2.2 PUHC GC–MS Method

Samples are injected in splitless mode at 50°C with a 30-s venting time. The oven is temperature programmed from 50°C to 320°C at 10°C per minute. The final temperature of 320°C is held for 10 min. Helium is used as carrier gas and the injector and the detector are maintained at 250°C and 320°C, respectively. MS-operating conditions were as follows: 6-min solvent delay, 50–500 mass unit range, and 230°C source temperature.

8. SUMMARY

The Pfa synthase is a remarkable example of FAS/PKS biosynthetic logic to produce a specific class of molecules with broad applications in human and animal nutrition industries. While commercial exploitation of single-cell microbial n-3 LC-PUFAs is well established, future developments will necessitate a more complete understanding of the genetic regulation, biochemistry, and enzymology of the Pfa synthase system. Many fundamental questions relating the macromolecular structure, catalytic turnover, and intracellular localization of the Pfa synthase complex remain unanswered. Some insights into the physiological roles that these lipid products mediate in native producing strains have been gained through targeted genetic manipulation experiments; however, a unified concept for microbial n-3 LC-PUFA synthesis has not emerged. In addition to the archetypal Pfa synthase involved in EPA and DHA synthesis, several homologous FAS/PKS types with varied domain architectures have been discovered in diverse bacterial lineages, yet the chemical products associated with these pathways have not been identified. Looking forward, the Pfa synthase paradigm underscores the versatility of microbial lipogenic processes and will continue to be a valuable biosynthetic template for the production of healthy, sustainable n-3 LC-PUFA products.

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Chapter 2

Linkage of Marine Bacterial Polyunsaturated Fatty Acid and Long Chain Hydrocarbon Biosynthesis

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Linkage of Marine Bacterial Polyunsaturated Fatty Acid and Long-Chain Hydrocarbon Biosynthesis

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Various marine gamma-proteobacteria produce omega-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (20:5, EPA) and docosahexaenoic acid (22:6, DHA), which are incorporated into membrane phospholipids. Five genes, designated *pfaABCDE*, encode the polyketide/fatty acid synthase necessary for production of these long-chain fatty acids. In addition to *de novo* biosynthesis of EPA and DHA, the "Pfa synthase" is also involved with production of a long-chain polyunsaturated hydrocarbon product (31:9, PUHC) in conjunction with the *oleABCD* hydrocarbon biosynthesis pathway. In this work, we demonstrate that OleA mediates the linkage between these two pathways *in vivo*. Co-expression of *pfaA-E* along with *oleA* from *Shewanella pealeana* in *Escherichia coli* yielded the expected product, a 31:8 ketone along with a dramatic ∼10-fold reduction in EPA content. The decrease in EPA content was independent of 31:8 ketone production as co-expression of an OleA active site mutant also led to identical decreases in EPA content. We also demonstrate that a gene linked with either *pfa* and/or *ole* operons in diverse bacterial lineages, herein designated *pfaT*, plays a role in maintaining optimal production of Pfa synthase derived products in *Photobacterium* and *Shewanella* species.

Keywords: hydrocarbon, thioesterase, omega-3 polyunsaturated fatty acid, *Shewanella*, *Photobacterium*

INTRODUCTION

The pathway for biosynthesis of omega-3 polyunsaturated fatty acids (PUFAs) in certain marine bacteria occurs via a polyketide synthase type mechanism encoded by five genes *pfaABCDE* (Metz et al., 2001; Allen and Bartlett, 2002; Shulse and Allen, 2011; Yoshida et al., 2016; Allemann and Allen, 2018). The "Pfa synthase" has been identified in several bacterial lineages and shown to synthesize a variety of PUFA products, most notably the long-chain omega-3 PUFAs, such as eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic (22:6*n*-3) acids (Shulse and Allen, 2011; Yoshida et al., 2016; Allemann and Allen, 2018). The Pfa synthase multienzyme complex contains all of the required enzymatic domains for lipid biosynthesis and these activities reside on either multi-domain or stand-alone proteins (**Figure 1**). Recombinant production of EPA or DHA in *Escherichia coli* has been demonstrated by heterologous expression of *pfaA-E* from various marine

bacterial strains (Metz et al., 2001; Okuyama et al., 2007; Amiri-Jami and Griffiths, 2010). Akin to canonical fatty acid biosynthesis, PUFAs are synthesized from 2C malonyl extender units (Metz et al., 2001) and the final product is incorporated into membrane phospholipids of the producing strain (Yazawa, 1996; Yoshida et al., 2016).

The biosynthesis of long-chain fatty acid-derived olefin hydrocarbons has been previously shown to be the result of the *oleABCD* pathway in various marine and non-marine bacterial lineages (Beller et al., 2010; Sukovich et al., 2010a,b). In this pathway, hydrocarbons are generated by the condensation of two fatty acyl chains via a "head-to-head" Claisen condensation mechanism, resulting in an intermediate β-keto acid product (**Figure 1**; Frias et al., 2011; Goblirsch et al., 2016). After condensation, the β-keto acid produced by OleA is processed by OleD, a NADPH-dependent reductase, to form a β-keto alcohol (Bonnett et al., 2011). OleC, a β-lactone synthetase, then generates a β-lactone moiety (Christenson et al., 2017b) which is subsequently de-carboxylated by OleB, a β-lactone decarboxylase, yielding the final olefin product (Christenson et al., 2017c). Recent work has demonstrated that OleBCD together form a large multimeric enzyme complex that processes the β-keto intermediate formed by OleA activity (Christenson et al., 2017a). Weak interactions between the OleBCD complex and OleA *in vitro* suggests that OleA condenses the precursor acyl groups and possibly transfers the β-keto acid product directly to the OleBCD complex for further processing (Christenson et al., 2017a). In the absence of downstream processing by OleBCD, the OleA catalyzed β-keto acid intermediate undergoes spontaneous decarboxylation to form a ketone product (Sukovich et al., 2010b; Frias et al., 2011; **Figure 1**).

The precursor fatty acids for olefin biosynthesis are derived from the cellular fatty acid biosynthesis pathway(s) in the producing host (Sukovich et al., 2010a). In strains such as *Xanthomonas campestris* and *Stenotrophomonas maltophilia*, the C27–C31 olefin products are derived from the core Type II fatty acid synthase (FAS), which produces saturated, monounsaturated, or branched chain fatty acids (Beller et al., 2010; Sukovich et al., 2010a). However, marine bacteria that synthesize EPA and/or DHA via the Pfa synthase mechanism produce a unique polyunsaturated hydrocarbon (Nichols et al., 1995), hentriacontanonaene (31:9, PUHC), via the OleABCD pathway (Sugihara et al., 2010; Sukovich et al., 2010a,b). Previous genetic experiments in *Shewanella oneidensis* MR-1 verified that PUHC biosynthesis is dependent on the Pfa synthase, as mutations in the *pfa* operon led to loss of PUHC production (Sukovich et al., 2010b). Interestingly, despite the presence of the Type II FAS in PUFA-producing bacteria, OleA exclusively condenses a predicted 16:4*n*-3 acyl chain derived from the Pfa synthase in these species.

Given the biosynthetic linkage of the *ole* and *pfa* pathways, it is not surprising that in some strains such as *Photobacterium profundum* SS9, the two operons are located adjacent to one another on the chromosome (Allen and Bartlett, 2002; Vezzi et al., 2005). Intriguingly, a previously characterized gene encoding an acyl-CoA thioesterase, previously designated *orf6*, sits between the two operons in *P. profundum* SS9. Previous structural and biochemical characterization of Orf6 revealed a "hot dog" fold topology with thioesterase activity on various long-chain acyl-CoA substrates *in vitro* (Rodríguez-Guilbe et al., 2013). Based on its activity and its conservation among EPA/DHA producing bacteria, it was speculated that this thioesterase may be involved with product release from the Pfa synthase (Rodríguez-Guilbe et al., 2013). However, modest rate enhancement of thioesterase activity *in vitro* raised doubts as to the role of Orf6 in product release (Rodríguez-Guilbe et al., 2013).

In this work we establish the linkage between PUFA and PUHC biosynthesis and show that OleA is responsible for mediating the linkage between the two pathways. Our results indicate that OleA can interact with the Pfa synthase directly, most likely with the acyl carrier protein (ACP) domains that shuttle acyl intermediates, including the 16:4*n*-3 PUHC precursor molecules, among catalytic domains during *de novo* PUFA biosynthesis. We also investigated the *in vivo* role of the *orf6* gene in *P. profundum* SS9 and *S. oneidensis* MR-1, demonstrating that it is required for optimal biosynthesis of EPA or PUHC in each strain, respectively. Given our results, we have re-designated *orf6* as *pfaT* (*pfa*-associated thioesterase). Together, these results provide new insight into the genetic and enzymatic determinants involved in the bacterial synthesis of long-chain fatty acid and hydrocarbon products of biotechnological interest.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

A list of strains used in this study is shown in **Table 1**. *E. coli* and *S. oneidensis* MR-1 strains were cultured in Luria Bertani media at 37 and 30°C, respectively, unless noted otherwise. *P. profundum* SS9R, a rifampin-resistant derivative of wild-type SS9, and *Shewanella pealeana* strains were cultured in 75% strength 2216 marine broth media (BD Difco, 28 g/L) at 15°C unless noted otherwise. For high hydrostatic pressure growth studies, SS9R strains were grown in heat-sealed bulbs as described previously (Chi and Bartlett, 1993). For analysis of heterologous production of EPA in *E. coli*, relevant strains were grown at 15°C for 48 h. For solid medias, agar was included at 15 g/L. The antibiotics kanamycin (50 μg/ml for *E. coli* and *S. oneidensis* MR-1; 200 μg/ml for *P. profundum*), chloramphenicol (15 μg/ml), carbenicillin (100 μg/ml), and rifampicin (100 μg/ml) were used as required.

Gene Disruption Mutagenesis

To generate in-frame deletions of genes, an allelic exchange approach was used similar to previous work (Eloe et al., 2008). Briefly, upstream and downstream regions of the gene of interest were amplified with the appropriate primer combinations (5′O, 5′I) and (3′O, 3′I), respectively (**Supplementary Table 1**). Purified PCR products were assembled using overlap PCR and subsequently amplified with 5′O and 3′O primers. Assembled fragments were then cloned into the suicide vector pRE118 (Edwards et al., 1998) using standard methods (Sambrook et al., 1989). Colony PCR and subsequent DNA sequencing were used to verify constructs.

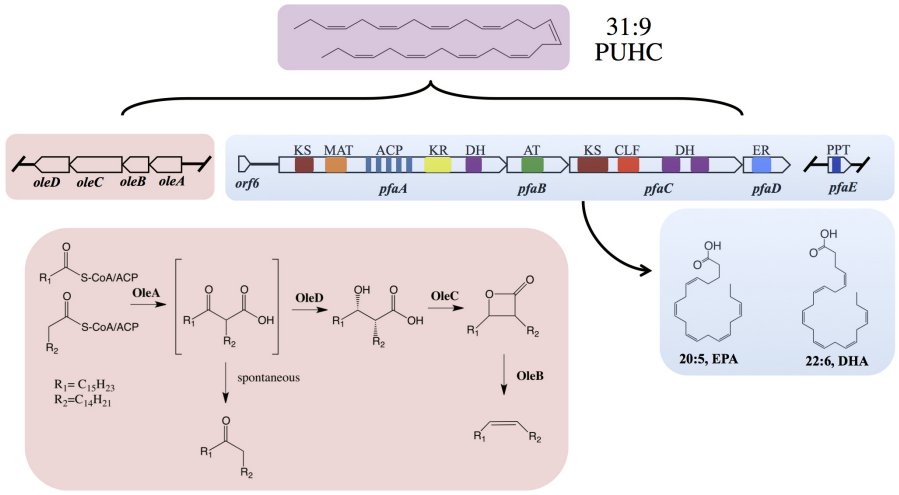

FIGURE 1 | Biochemical and genetic aspects of PUFA and PUHC biosynthetic pathways. Domain designations within the Pfa synthase are; phosphopantetheinyl transferase (PPT), β-ketoacyl synthase (KS), malonyl-CoA:ACP transacylase (MAT), acyl-carrier protein (ACP), ketoacyl reductase (KR), dehydratase/isomerase (DH), acyltransferase (AT), chain-length factor (CLF), and enoyl reductase (ER). Both *pfaE* and/or *oleABCD* can be clustered with the *pfa* operon or found elsewhere in the genome depending on the host organism.

For conjugation into *P. profundum*, biparental matings were performed using the *E. coli* donor strain S17-1λ*pir* containing the desired plasmid to be mobilized into SS9R. Selection for exconjugants was performed on 2216 agar containing rifampicin and kanamycin as described previously (Lauro et al., 2005; Allemann and Allen, 2018). After colony purification of exconjugants, colonies were grown without selection and dilutions plated onto 2216 agar supplemented with 5% sucrose to select for a second recombination event. Colony PCR was used to screen sucrose resistant clones for the targeted deletion.

For conjugation into *S. oneidensis* MR-1, biparental matings were performed using an *E. coli* diaminopimelic acid (DAP) auxotroph, WM3064, on LB agar supplemented with DAP, as described previously (Yang et al., 2015). Exconjugants were selected on LB plates containing kanamycin without DAP. Colony purified exconjugants were grown in LB without NaCl for several generations and subsequently plated onto LB supplemented with 5% sucrose for counter-selection. Sucrose resistant colonies were screened for kanamycin sensitivity and colony PCR was used to screen kanamycin sensitive clones for the targeted deletion.

Cloning/Expression Procedures

The *pfa* operon from *S. pealeana* was cloned into the pre-linearized pCC2FOS vector following manufacturer guidelines (Epicentre, Madison, WI, United States). Briefly, a fosmid library was constructed from *Swa*I digested genomic DNA from *S. pealeana*. Colony PCR using primers listed in **Supplementary Table 1** specific to *pfaD* and *pfaE* was used to screen clones for presence of the *pfa* operon. A single clone, which contained the entire cluster was identified and designated 1F12R. Other plasmids were constructed by PCR amplification of indicated genes using the appropriate primer pair containing restriction sites (i.e., pBAD24 SS9 orf 6 F/R) listed in **Supplementary Table 1**. PCR products were cloned into various plasmids at restriction sites given in **Table 1** using standard procedures (Sambrook et al., 1989). Site-directed mutagenesis of OleA was accomplished using PCR mutagenesis primers listed in **Supplementary Table 1**. Briefly, the plasmid pMA63 was used as a template for PCR using mutagenesis primer pair "OleA C123A mut F/R." A restriction digest using *Dpn*I removed the original plasmid template and the resulting DNA was transformed into competent cells. DNA sequencing was used to verify the introduced mutation. For expression of genes cloned onto pBAD24 (Guzman et al., 1995) L-arabinose was added. Expression of OleA and Orf6 (PfaT) homologs was confirmed by SDS-PAGE of whole cell lysates followed by Coomassie staining (Sambrook et al., 1989).

Fatty Acid/Neutral Lipid Extraction and Analysis

Late log phase cultures were harvested by centrifugation and cell pellets rinsed once with 50% Sigma Sea Salts solution (16 g/L) and stored at −80°C. Cell pellets were lyophilized prior to fatty acid or hydrocarbon analysis.

For fatty acid analysis, lipids were converted to fatty acid methyl esters (FAME) by adding 5% H_2SO_4 in methanol directly to lyophilized biomass and refluxing at 90°C for 90 min. After cooling, hexanes were added and non-esterified fatty acids were saponified by addition of 10% NaCl. The hexane extraction was repeated twice and pooled fractions were evaporated completely under a gentle N_2 stream and re-dissolved in 1 ml of hexane. Samples were stored at −80°C until analysis.

TABLE 1 | Strains and plasmids used in this study.

Strain	Genotype or relevant characteristics	Source
***Shewanella* strains**		
S. pealeana ATCC 700345	Wild type, EPA^+, $PUHC^+$	American Type Culture Collection
S. oneidensis MR-1	Wild type, EPA^-, $PUHC^+$	Heidelberg et al., 2002
S. oneidensis Δ*ole*	Δ*oleABCD*, EPA^-, $PUHC^-$	Sukovich et al., 2010a
S. oneidensis MAS1	Δ*orf6*	This study
***Photobacterium* strains**		
SS9R	Rifampicin resistant, EPA^+, $PUHC^-$	Chi and Bartlett, 1993
MAP1	SS9R, Δ*pfaT*	This study
***E. coli* strains**		
DH5α*pir*	Cloning strain, maintaining R6K plasmids	Saltikov and Newman, 2003
WM3064	Conjugal donor strain used for MR1	Saltikov and Newman, 2003
S17-1λ*pir*	Conjugal donor strain used for SS9	Simon et al., 1983
BW25113	Keio collection parental strain	Coli Genetic Stock Center
JW1794	Keio collection Δ*fadD::kan*	Coli Genetic Stock Center
MAE21	JW1794, 1F12R	This study
Plasmids	**Relevant characteristics**	**Source**
pBAD24	Arabinose inducible expression vector, Amp^R	Guzman et al., 1995
pRE118	R6K origin allelic exchange plasmid, Kan^R, SacB	Edwards et al., 1998
pRK2073	Contains *tra* genes for conjugal transfer	Better and Helinski, 1983
pKT231	Complementation plasmid for SS9, Kan^R Sm^R	Bagdasarian et al., 1981
pCC2FOS	Copy control fosmid, Cm^R	Epicentre
1F12R	pCC2FOS containing *pfaA-E* from *S. pealeana*	This study
pOleA	pBBR1MCS-2 with containing *oleA* from *S. oneidensis*	Sukovich et al., 2010a
pMA10	pRE118 containing Δ*pfaT* allele for *S. oneidensis* MR-1, cloned as *Nde*I-*Sac*I fragment	This study
pMA12	pRE118 containing Δ*pfaT* allele for *P. profundum* SS9, cloned as *Kpn*I-*Sac*I fragment	This study
pMA20	pKT231 containing *pfaT* region from *P. profundum* SS9, cloned as *Bam*HI-*Eco*RI fragment	This study
pMA47	pBAD24 containing *pfaT* from *P. profundum* SS9, cloned as *Eco*RI-*Xba*I fragment	This study
pMA48	pBAD24 containing *pfaT* from *S. pealeana*, cloned as *Eco*RI-*Pst*I fragment	This study
pMA63	pBAD24 containing *oleA* from *S. pealeana*, cloned as *Nhe*I-*Pst*I fragment	This study
pMA70	pBAD24 containing C123A OleA, mutant derived from pMA63	This study

Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin; Sm, Streptomycin.

Hydrocarbon/ketones were extracted from freeze-dried biomass by addition of a mixture of dichloromethane/methanol (2:1, vol/vol) and stirred overnight at room temperature. The crude extract was filtered using a Pasteur pipet packed with glass wool and celite and subsequently dried under a gentle stream of N_2 gas. A mixture of hexanes and methanol (4:1, vol:vol) were added to the residue and allowed to form a two-phase mixture. The hexane phase was removed and two additional hexane volumes were added to the methanol phase and subsequently extracted. The hexane fractions were pooled, dried under a stream of N_2 and dissolved in 1 ml of hexane. For quantitative analysis hentriacontane (31:0) (Sigma) was added as an internal standard.

Gas chromatography mass spectrometry (GC-MS) analyses were performed on an Agilent Technologies model 7890A GC connected to a 5975C VL MSL quadrupole MS(EI). Samples were separated on a 30 m HP5ms Ultra Inert Agilent GC-MS column using helium as carrier gas. Fatty acid samples were injected in splitless mode and held at 110°C for 3 min followed by a gradient of 15°C/min, and held at a final temperature of 280°C for an additional 3 min. For analysis of neutral lipid extracts, samples were injected in splitless mode and held at 100°C for 3 min followed by a gradient of 15°C/min and held at a final temperature of 300°C for an additional 10 min. Both injector and detector for the mass spectrometer were maintained at 250°C. Additional MS operating conditions were as follows: mass range 50–500 atomic mass units, 3 min solvent delay. Peak areas were quantified and mass spectra processed using ChemStation software (Agilent Technologies). FAMEs were identified by comparing MS fragmentation patterns to spectra from authentic standards or from spectra on the NIST 2008 Spectral Library.

Phylogenetic Analysis

Homolog amino acid sequences were obtained from a gene neighborhood search on the Joint Genome Institute Integrated Microbial Genome web portal (accessed on October 16, 2018). Sequences were uploaded to the Phylogeny.fr web portal and a maximum likelihood phylogenetic tree was constructed as described previously (Dereeper et al., 2008). For tree construction bootstrap values were generated from 100 re-samplings of the data.

RESULTS

Analysis of EPA and PUHC content in *S. pealeana* and *S. oneidensis* MR-1 as a function of growth temperature displayed the trend of both compounds increasing in abundance as temperature decreased (**Figure 2**). This is consistent with previous findings regarding EPA (Allen et al., 1999; Okuyama et al., 2007; Yoshida et al., 2016) and PUHC (Sukovich et al., 2010b) content as a function of growth temperature. Given the relationship between the *ole* and *pfa* pathways, increased flux through the Pfa synthase is predicted to result in a concurrent increase in PUHC production. Conversely, reducing flux into the *ole* pathway is predicted to lead to an increase in the *pfa* pathway. Given the relationship between the two pathways, it was hypothesized that previously observed differences in EPA content amongst members of the *Shewanella* genus (Kato and Nogi, 2001), may be due to the diversion of acyl substrates from the EPA pathway into the PUHC pathway. Deletion of the entire *ole* operon resulted in a modest ∼twofold increase in EPA content relative to wild-type *S. oneidensis* MR-1 at 15°C (**Supplementary Table 2**).

To gain a better understanding of how the *ole* and *pfa* pathways interact, we performed combinatorial co-expression experiments in *E. coli*. The *pfa* operon was cloned from *S. pealeana* ATCC700345 onto pCC2FOS to yield the construct 1F12R. The *pfa* operon from *S. pealeana* was chosen due to its high EPA and PUHC production phenotypes in the native strain. Transformation of 1F12R into the *E. coli* strain JW1794 yielded strain MAE21 that produced EPA at ∼21% of total fatty acid when cultured at 15°C (**Supplementary Table 3**). The fatty acid profile of a control strain containing pCC2FOS vector only did not produce EPA. Given that OleA catalyzes the first committed step of hydrocarbon biosynthesis in the OleABCD pathway, we speculated that co-expression of OleA from *S. pealeana* might impact EPA production in MAE21. The OleA homolog in *S. pealeana* was successfully cloned under an arabinose inducible promoter (pMA63) and shown to produce a protein of the expected size (38 kDa) in MAE21 upon induction with L-arabinose (**Supplementary Figure 1**). Fatty acid analysis of the MAE21 strain co-expressing OleA (pMA63) indicated a dramatic ∼10-fold decrease in EPA content relative to the vector only control strain (**Figure 3A**). Attempts to titrate this effect with varying amounts of L-arabinose were unsuccessful (data not shown) and are most likely due to our inability to effectively titrate expression of the pBAD promoter under the growth conditions employed. Site-directed mutagenesis of the universally conserved OleA catalytic cysteine residue to alanine (C123A) was employed to determine if catalytically active OleA is required for this reduced EPA phenotype. Under identical conditions, co-expression of mutant $OleA^{C123A}$ (pMA70) in MAE21 yielded nearly identical results as seen for $OleA^{WT}$ (pMA63) (**Figure 3A**).

Previous work in *S. oneidensis* MR-1 had shown that expression of *oleA* alone without *oleBCD* led to production of a polyunsaturated ketone (31:8), which is the result of spontaneous decarboxylation of the OleA β-keto acid product (**Figure 1**; Sukovich et al., 2010a,b; Frias et al., 2011). Neutral lipid extracts of MAE21 containing $OleA^{WT}$ (pMA63) and mutant $OleA^{C123A}$ (pMA70) were analyzed by GC-MS. A peak at 23.1 min corresponding to the 31:8 ketone was found in the wild-type OleA (pMA63) containing strain but not in the mutant $OleA^{C123A}$ (pMA70) containing strain (**Figure 3B**). Mass spectra associated with the peak at 23.1 min also matched the spectra of the 31:8 ketone produced by *S. oneidensis* Δ*ole* strain containing pOleA (**Supplementary Figures 2A,B**). MAE21 is a derivative of JW1794, which contains a Δ*fadD::kan* mutation, rendering the strain unable to produce acyl-CoA from free fatty acids (Cronan, 1997; Black and DiRusso, 2003). The appearance of the 31:8 ketone in the MAE21 strain background indicates that OleA can condense the appropriate acyl products in the absence of acyl-CoA synthetase activity.

Given its clustering with the *pfa* and/or *ole* operons in various EPA/DHA producing strains, *pfaT* (previously *orf6*) and its orthologs could be involved with one or both biosynthetic pathways. A search for homologs of *pfaT* was conducted and a protein sequence phylogenetic tree is shown in **Figure 4**. All of the included species are either known PUFA producers and/or contain both *pfa* and *ole* operons. With the exception of the *Shewanella* and *Psychromonas* species, all *pfaT* homologs were found to be genetically linked with either *pfa* or *ole* operons. All homologs shown in the tree contain the active site aspartate residue previously described to be essential for thioesterase activity (Rodríguez-Guilbe et al., 2013).

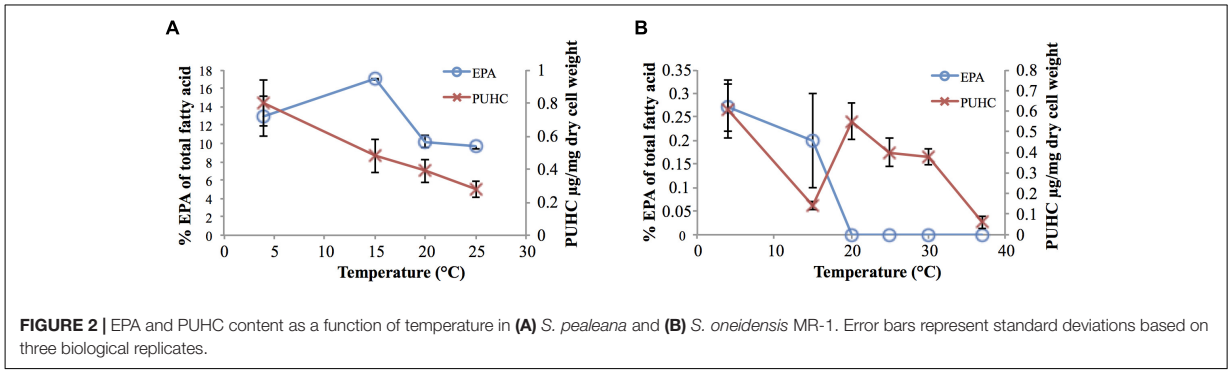

FIGURE 2 | EPA and PUHC content as a function of temperature in **(A)** *S. pealeana* and **(B)** *S. oneidensis* MR-1. Error bars represent standard deviations based on three biological replicates.

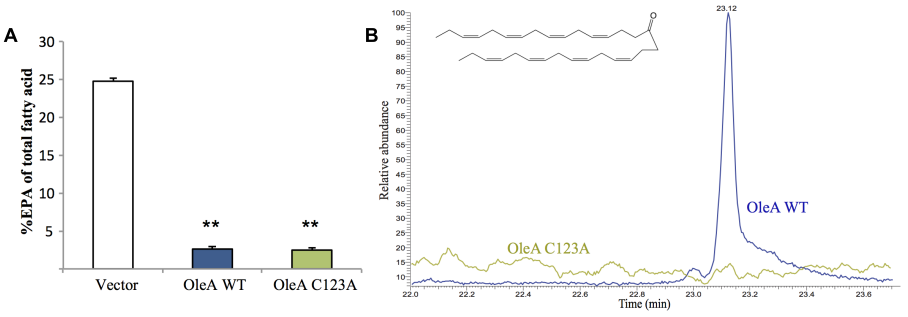

FIGURE 3 | Expression of OleA in *E. coli* MAE21 strain harboring fosmid 1F12R with the *pfa* operon from *S. pealeana* leads to production of a 31:8 ketone and reduced EPA. **(A)** EPA content of MAE21 strains decreases upon induction of $OleA^{WT}$ (pMA63) and $OleA^{C123A}$ (pMA70) with 0.05% L-Arabinose. Bars represent averages of at least three biological replicates with error bars signifying one standard deviation (**$P < 0.005$). **(B)** Total ion chromatograms (TIC) of neutral lipid extracts from MAE21 $OleA^{WT}$ (pMA63 – blue) and MAE21 $OleA^{C123A}$ (pMA70 – green). 31:8 ketone peak seen at 23.1 min.

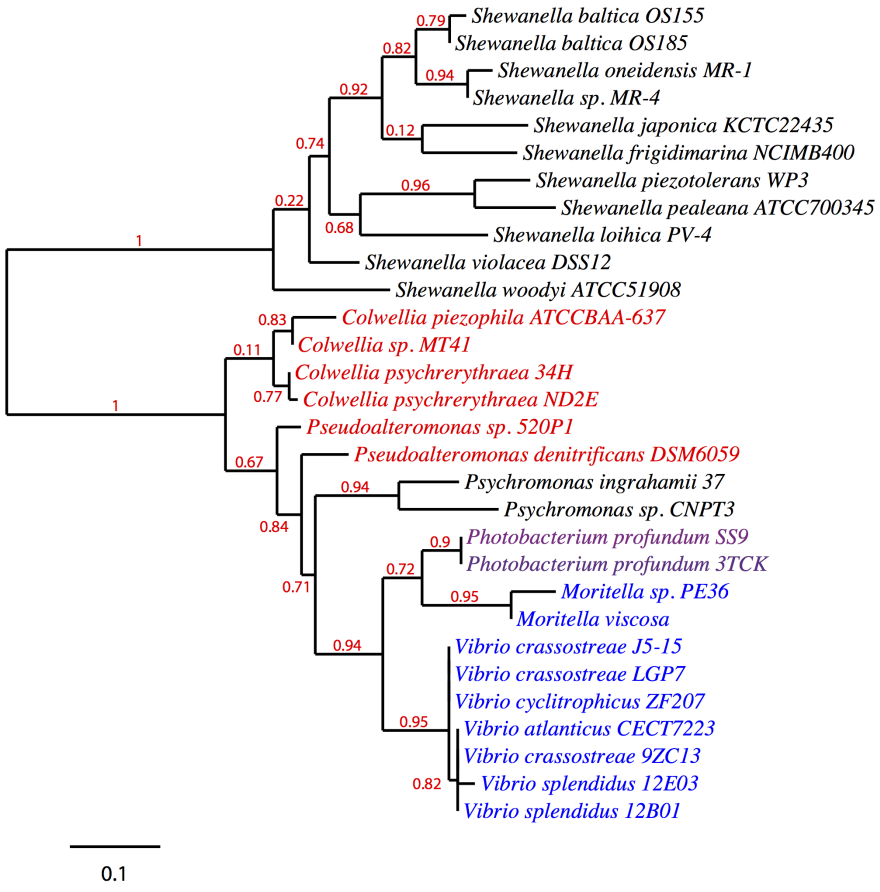

FIGURE 4 | Phylogenetic distribution of PfaT homologs. A maximum likelihood unrooted tree of PfaT homologs found in bacteria with *pfa* and/or *ole* pathways. PfaT homologs clustered with the *pfa* operon are indicated in blue, homologs clustered with *ole* operon in red text, and clustering between the operons in purple. Numbers at branch points indicate bootstrap values based on 100 replicates.

A marker-less in-frame deletion of *pfaT* was generated in *P. profundum* SS9 and the resulting strain, MAP1, was analyzed for EPA content under a variety of culture conditions. As shown in **Figure 5A**, MAP1 produced ∼fourfold less EPA compared to its parental strain SS9R at 15°C. This reduction in EPA content was also seen during growth at low temperature (4°C) and at high hydrostatic pressure (30 MPa) (**Figure 5A**), culture conditions that elicit increased EPA content in wild-type SS9 (Allen et al., 1999). The decreased EPA phenotype was also complemented *in trans* by a construct (pMA20) containing *pfaT* under control of its native promoter (**Figure 5B**). Full fatty acid profiles of SS9R and MAP1 along with the corresponding genetically complemented strains and controls are given in **Supplementary Tables 4, 5**, respectively. The ability to complement the *pfaT* mutation in strain MAP1 also confirms that the observed decrease in EPA is due to the loss of *pfaT* and not a polar effect on transcription of the *pfa* operon. Co-expression of *orf6* from *P. profundum* SS9R (pMA47) or the homolog from *S. pealeana* (pMA48) in the recombinant EPA-producing *E. coli* strain MAE21 did not lead to alterations in EPA content or changes in overall fatty acid profile (**Supplementary Table 3**). Furthermore, expression of both homologs in strain MAE21 or JW1794 did not lead to accumulation of free fatty acids (data not shown).

Surprisingly, neither MAP1 nor SS9R produced PUHC at a detectable level despite the presence of the *oleABCD* operon (data not shown). Given our previous characterization of *S. oneidensis* MR-1 and its genetic tractability, we generated an in-frame deletion of the *pfaT* homolog (locus tag SO1256) in *S. oneidensis* MR-1 (MAS1). Neutral lipid extracts of this strain indicated a drastic ∼10-fold reduction in PUHC content relative to the parental strain under identical conditions (**Figure 5C**). Fatty acid profiles of *S. oneidensis* MR-1 and MAS1 grown at 15°C are shown in **Supplementary Table 2** and a similar fourfold decrease in EPA was observed.

DISCUSSION

In this work we have demonstrated that bacterial PUFA and PUHC biosynthesis are linked and this linkage is mediated by OleA. Our initial work with *S. pealeana*, which produces both PUFA and PUHC products, indicated that culture conditions which lead to increases in PUFA lead to commensurate increases in PUHC. While the physiological role of PUHC remains obscure, this result suggests that PUHC may play an additional role in adaptation to cold and/or high-pressure environments, conditions that impact membrane physical structure, e.g.,

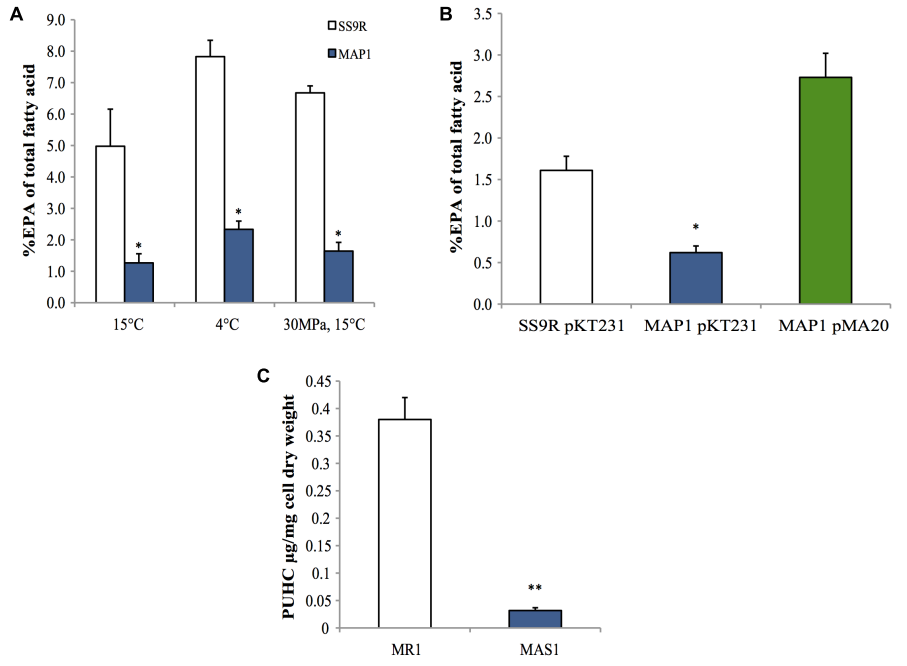

FIGURE 5 | Phenotypes of *pfaT* deletion mutants in *P. profundum* SS9 and *S. oneidensis* MR-1. **(A)** An approximate fourfold decrease in EPA composition in *P. profundum* strain MAP1 harboring an in-frame deletion of *pfaT* relative to SS9R under various culturing conditions. **(B)** Complementation analysis. Comparison of the amount of EPA as a percentage of total fatty acids among parent strain SS9R harboring empty complementation plasmid pKT231, *P. profundum* strain MAP1 harboring empty complementation plasmid pKT231, and *P. profundum* strain MAP1 harboring plasmid pMA20 containing *pfaT* under control of its native promoter. Cells grown at 15°C as in **A**. Bars represent averages of at least three experimental replicates and error bars represent one standard deviation. **(C)** PUHC content of *S. oneidensis* strain MAS1 (harboring an in-frame deletion of the *pfaT* homolog) and parent strain *S. oneidensis* MR-1 at 30°C. Bars represent averages of three biological replicates and error bars represent one standard deviation (*$P < 0.05$; **$P < 0.005$).

fluidity. The modest EPA content in the Δ*ole* strain (<1% of total fatty acid) indicates that other factors in the biosynthesis and incorporation of EPA into membrane phospholipids are responsible for the previously observed differences in EPA production amongst members of the *Shewanella* genus (Kato and Nogi, 2001).

Our results also indicate that the *in vivo* OleA substrate is an acyl-ACP and not an acyl-CoA as described previously (Frias et al., 2011; Goblirsch et al., 2016). Our heterologous expression system in *E. coli* demonstrated that co-expressing *oleA* along with *pfaABCDE* was sufficient for producing the expected 31:8 ketone previously observed in *S. oneidensis* MR-1 mutants (Sukovich et al., 2010a). The nearly identical reduction in EPA content associated with co-expression of wild-type OleA or the $OleA^{C123A}$ mutant suggests that OleA interacts directly with the ACP domains of the Pfa synthase and that this reduction is not a result of acyl groups being removed from the synthase. Rather, the reduction in EPA production may instead reflect steric competition for the ACP domains between OleA and the various catalytic domains on the Pfa synthase. From a biosynthetic standpoint, such direct ACP interactions would be the most efficient method for obtaining acyl groups for OleA condensation. Other routes of diverting substrates such as thioester cleavage would require acyl-CoA synthetase activity and would consume ATP to regenerate the acyl-CoA needed for condensation by OleA. Previous work indicated that OleA and its homologs specifically act on acyl chains derived from either the Type II FAS or the Pfa synthase (Sukovich et al., 2010a; Frias et al., 2011). Protein–protein interactions between OleA and its cognate ACP may be the mechanism for this substrate specificity.

In this work the *in vivo* role of the previously characterized *orf6* thioesterase, now designated *pfaT*, was investigated. While *pfaT* was not completely essential to EPA biosynthesis in *P. profundum* SS9, it was required for wild-type production levels. Similarly, the *pfaT* homolog in *S. oneidensis* MR-1 was not essential to PUHC biosynthesis; rather it was required for optimal biosynthesis of PUHC. Reduction in both end products of the Pfa synthase indicates that PfaT is not involved in a process specific to either pathway alone. Rather, these results suggest that PfaT may function as a Type II thioesterase with activity upon the Pfa synthase. Type II thioesterases serve accessory roles in removing aberrant intermediates or starter units from polyketide synthases and their genetic disruption typically leads to a reduction in the final polyketide product (Kotowska and Pawlik, 2014). Intriguingly, co-expression of various *pfaT* homologs in the EPA-producing *E. coli* strain MAE21 did not lead to modulation in EPA production. Previous studies in *E. coli* have described accumulation of free fatty acids in the culture media or intracellularly in response to expression of acyl-CoA/ACP thioesterases, particularly in strains in which β-oxidation is non-functional (Cho and Cronan, 1995; Lennen et al., 2011; Zhang et al., 2011). In all instances, expression of *pfaT* homologs in MAE21 or JW1794, did not lead to any free fatty acid accumulation in the media or the appearance of novel fatty acids. This discrepancy between results from the heterologous host *E. coli*, wherein omission or co-expression of PfaT led to no changes in EPA production, and native strains in which genetic disruption of PfaT led to decreases in both EPA and PUHC suggests that PfaT performs a function specific to biosynthesis of PUFA and/or PUHC in the native producing strains only. The relatively low *in vitro* thioesterase activity of PfaT (Orf6) reported previously (Rodríguez-Guilbe et al., 2013), along with phenotypic data presented herein is suggestive of PfaT acting as a type II thioesterase.

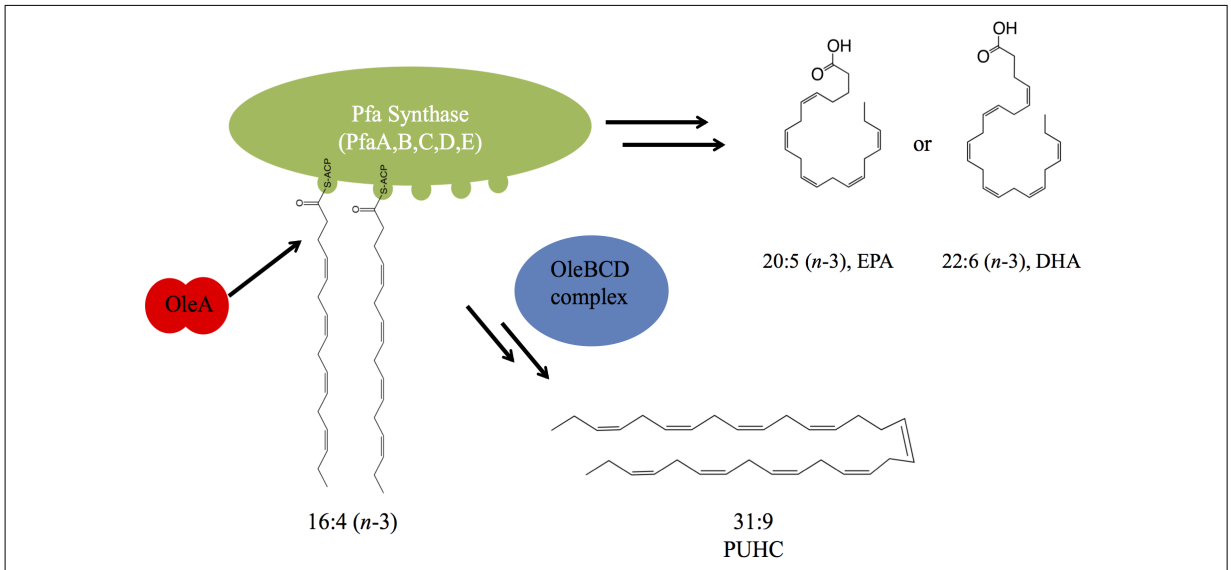

FIGURE 6 | A model depicting the interaction between the Pfa synthase and Ole biosynthetic pathways. OleA interacts with the ACP domain(s) carrying a 16:4 acyl group. Condensation of two fatty acids is followed by further biosynthetic processing by the OleBCD complex to yield the 31:9 PUHC.

From the data presented, a model depicting the interaction between PUFA and PUHC biosynthesis is depicted in **Figure 6**. During EPA biosynthesis, a 16:4*n*-3 acyl group, a predicted intermediate of the biosynthetic pathway, is processed by OleA via direct interaction with the ACP domains on PfaA. After condensation, the β-keto acid product is further processed by the activities of the OleBCD complex to form the final 31:9 PUHC product (**Figure 1**). The absence of a significant increase in EPA production in the *S. oneidensis* Δ*oleABCD* strain relative to other members of the *Shewanella* genus indicates that diversion of intermediates to PUHC biosynthesis is not strictly responsible for the differences in EPA phenotypes observed previously (Kato and Nogi, 2001). The relative activities of the Pfa synthase between strains might be a possible mechanism for the observed differences in EPA production potential among *Shewanella* species.

OleA is a member of the thiolase protein family which have been shown to utilize either acyl-CoA and/or acyl-ACP substrates (Cronan and Rock, 2008; Goblirsch et al., 2012). Patent literature describing OleA from *S. maltophilia* demonstrated that OleA utilizes both acyl-CoA and acyl-ACP substrates *in vitro* (Friedman and Da Costa, 2008). Detailed examination of previously published crystal structures of OleA (Goblirsch et al., 2012, 2016) from *X. campestris* showed that the pantetheine channel entrance contains a cluster of positively charged residues, which form a "positive patch." This positive patch feature is found in many fatty acid biosynthesis enzymes, which are known to interact with ACP (Zhang et al., 2001; Finzel et al., 2015). While there is no structural data corresponding to OleA homologs from any PUHC producers it is expected that a similar positive patch feature would be present.

Polyunsaturated fatty acids and PUHC biosynthesis are intrinsically linked and the results of this work have identified that OleA mediates this linkage *in vivo*. Notably, the results presented here suggest that OleA is capable of interacting with the Pfa synthase *in vivo* and that the acyl substrates are derived directly from the ACP domains of the synthase. This work has also more clearly defined the role of PfaT, not as a thioesterase for final product release, but rather as an accessory enzyme whose activity is required for optimal production of Pfa synthase derived end products including omega-3 PUFAs and long-chain olefin hydrocarbons.

AUTHOR CONTRIBUTIONS

MA, CS, and EA conceived the idea, designed the project, and contributed to the discussion and critical writing. MA and CS carried out the experiments and collected and analyzed the data. MA wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00702/full#supplementary-material

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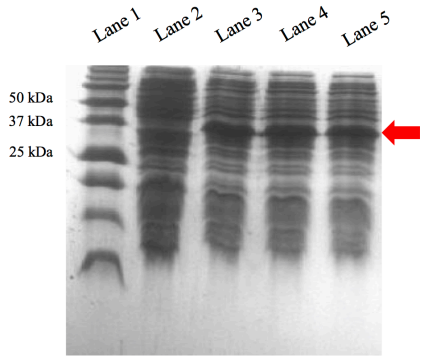
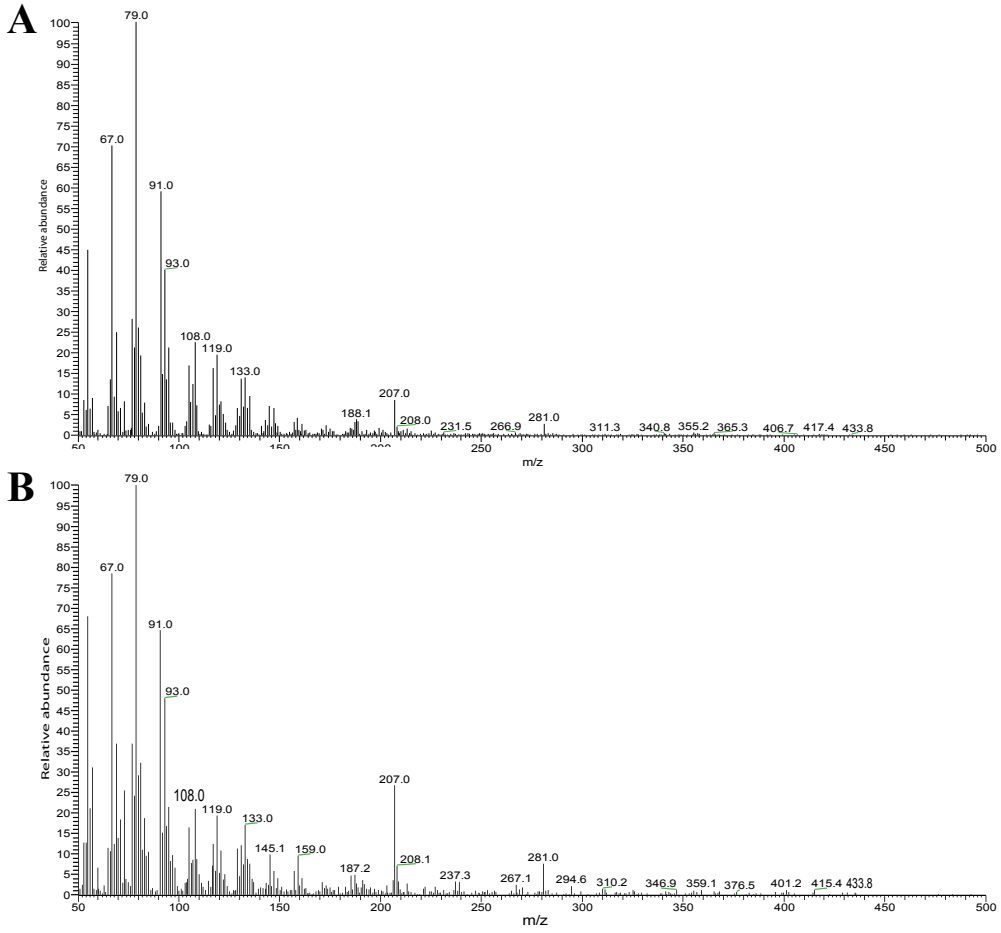


Supplementary Figure 1 SDS-PAGE of whole cell lysates of MAE21 containing pMA63 grown at 15°C; Lane 1, protein standard ladder; Lane 2-5; 0, 0.001, 0.05, and 0.1% L-Arabinose. Arrow indicates OleA band at approximately 38kDa.



Supplementary Figure 2 Mass spectra of 31:8 ketone product produced by OleA. (A) Spectra of 31:8 ketone being produced by *Shewanella oneidensis* MR-1 *ΔoleABCD* with pOleA. (B) Spectra associated with peak at 23.1min from FIGURE 3B in main text.

Supplementary Table 1 - Primers used in this study, restriction sites underlined

Primer name	Sequence 5'→3'
MR1 SO1256 5'O	TGACTCATATGGCCCACGTGATATGGGTTAC
MR1 SO1256 5'I	GACTGGCTTAGGTCGTCTGGCGGCTAAATTGCGGCATAAAA
MR1 SO1256 3'I	GCCAGACGACCTAAGCCAGTCCCCATGCCTGCAGAGTTAAT
MR1 SO1256 3'O	TGACTGAGCTCGCGGGAATTTCTGTTTGTGT
MR1 orf6 del ver F	GCTCAGAAGGCTTGATTTGG
MR1 orf6 del ver R	CTGCAACGTCACAGAACCAT
SS9 orf6 5'O	TGACTGGTACCCCCGAAAGTACGCCTAAACA

SS9 orf6 5'I	GACTGGCTTAGGTCGTCTGGCTTGCACTGGGTGGTGATAAA
SS9 orf6 3'I	GCCAGACGACCTAAGCCAGTCTGGCTTTTAGAGCGTTTTCC
SS9 orf6 3'O	TGACTGAGCTCAGGAGGCACTTACCATCCCT
SS9 orf6 del ver F	ATCCATAATGCCCATGGAAA
SS9 orf6 del ver R	TCGCAATTGGCATGTCTTTA
pKT231 orf6 F	GGGGCTAAACGGAATTCTAACT
pKT231 orf6 R	TACGATGGATCCAGGGCTATCAATTGGTTGGA
pBAD24 Spea oleA F	CGATCGCTAGCAGGAGGGCAGTTTCATGAAATATTCCCG
pBAD24 Spea oleA R	ACGATCCTGCAGGTAGGCTGGTTTCAATTACC
pBAD24 SS9 orf6 F	TACGATGAATTCAGAGTTGTTGCAGCAATGAG
pBAD24 SS9 orf6 R	TATAGTCTAGATTAATCAGCCATCATCGAAG
pBAD24 Spea orf6 F	ACGATCGAATTCACTATGTCGCAAACTCATAC
pBAD24 Spea orf6 R	ACGATCCTGCAGAAGGTAAGTCGGTTATGTCG
OleA C123A mut F	GATATCAGCAACGCTGCTCTTGGTGTGCTTTCA
OleA C123A mut R	TGAAAGCACACCAAGAGCAGCGTTGCTGATATC
Spea pfaE F	ATGGAGGCTGTTGAGTTTGG
Spea pfaE R	GGCTAAGCCCAATCCCTTAG
Spea pfaD F	CGAGATGGGCGTTAAGCTAC
Spea pfaD R	GCGCTCATTAAAGTGTGCAA

Supplementary Table 2- *S. oneidensis* MR-1 fatty acid profiles at 15°C

	MR1[a]	MAS1[a]	Δ*oleABCD*[a]
12:0	2.17 ± 0.28	2.26 ± 0.13	2.28 ± 0.05
13:0	3.66 ± 1.05	3.12 ± 0.34	3.07 ± 0.42
13:0iso	0.59 ± 0.13	0.56 ± 0.09	0.53 ± 0.03
12:0 3-OH	1.61 ± 0.24	1.72 ± 0.05	1.76 ± 0.48
13:0an	1.01 ± 0.26	0.77 ± 0.08	0.79 ± 0.13
14:1	0.36 ± 0.02	0.37 ± 0.02	0.35 ± 0.00
14:0	1.92 ± 0.37	1.91 ± 0.19	1.52 ± 0.03
14:0 3-OH	2.12 ± 0.30	1.82 ± 0.11	1.91 ± 0.53
15:0iso	30.29 ± 2.28	28.20 ± 3.11	27.15 ± 2.43
15:0an	2.80 ± 0.14	3.05 ± 0.78	2.61 ± 0.42
16:1	25.40 ± 0.85	30.43 ± 4.88	29.35 ± 0.65
16:0	8.78 ± 2.73	7.89 ± 2.11	8.51 ± 1.32
17:0	1.43 ± 0.27	1.18 ± 0.08	1.31 ± 0.02
17:0cyc	8.73 ± 1.29	8.20 ± 1.21	9.14 ± 0.64
17:0iso	1.11 ± 0.38	1.15 ± 0.58	1.11 ± 0.45
18:1	7.46 ± 1.17	7.02 ± 0.27	7.69 ± 1.07
18:0	0.30 ± 0.12	0.29 ± 0.02	0.35 ± 0.08
20:5	0.26 ± 0.04	0.06 ± 0.01	0.55 ± 0.04
UFA/SFA	0.50	0.61	0.61

[a] Values are the mean ± standard deviation from at least three independent replicate cultures

Supplementary Table 3-Fatty acid profiles of *E.coli* strain MAE21 grown at 15°C

	JW1794 pCC2FOS[a]	MAE21[a]	MAE21 pMA47[a]	MAE21 pMA48[a]
12:0	1.68 ± 0.74	1.41 ± 0.13	1.46 ± 0.24	1.35 ± 0.31
14:0	4.67 ± 1.13	4.57 ± 0.06	4.59 ± 0.13	4.64 ± 0.23
14:0 3-OH	1.67 ± 0.42	3.54 ± 0.41	3.49 ± 0.41	3.69 ± 0.46
16:0	29.00 ± 4.86	30.74 ± 0.76	30.31 ± 0.95	29.40 ± 1.06
16:1	19.03 ± 2.32	9.02 ± 1.83	10.77 ± 1.08	9.69 ± 1.11
18:1	43.11 ± 4.10	27.72 ± 0.89	28.51 ± 2.53	27.90 ± 1.07
18:0	0.85 ± 0.23	0.34 ± 0.58	0.33 ± 0.57	0.33 ± 0.57
20:5	0.00 ± 0.00	21.46 ± 2.44	19.93 ± 3.13	22.25 ± 1.03
UFA/SFA	1.64	1.43	1.47	1.52

[a] Values are the mean ± standard deviation from at least three independent replicate cultures

Supplementary Table 4- Fatty acid profiles of SS9R and MAP1 as a function of temperature and pressure

	SS9R[a]			MAP1[a]		
	15°C	4°C	30MPa, 15°C	15°C	4°C	30MPa, 15°C
12:0	4.06 ± 1.47	4.20 ± 1.42	1.96 ± 0.16	3.93 ± 1.29	3.87 ± 1.21	1.79 ± 0.29
14:0	4.17 ± 1.09	3.25 ± 0.62	2.84 ± 0.28	4.02 ± 1.38	2.46 ± 0.46	2.65 ± 0.35
14:1	3.24 ± 1.07	2.69 ± 0.68	0.74 ± 0.04	2.58 ± 0.35	3.05 ± 0.92	0.64 ± 0.02
16:0	23.04 ± 3.34	22.56 ± 2.58	26.45 ± 0.87	21.75 ± 4.13	19.45 ± 0.80	26.48 ± 1.33
16:1	43.29 ± 2.51	41.91 ± 0.29	49.42 ± 1.17	48.48 ± 0.73	50.06 ± 2.37	52.06 ± 2.25
12:0 3-OH	1.79 ± 1.14	2.32 ± 0.87	1.22 ± 0.13	1.52 ± 0.45	1.84 ± 1.15	1.59 ± 0.30
18:0	0.63 ± 0.20	0.07 ± 0.12	10.26 ± 0.39	0.68 ± 0.03	0.07 ± 0.13	12.77 ± 0.52
18:1	11.47 ± 3.23	9.39 ± 0.45	0.44 ± 0.39	13.05 ± 0.66	13.19 ± 1.75	0.38 ± 0.66
20:5	4.98 ± 1.18	7.83 ± 0.52	6.67 ± 0.22	1.27 ± 0.29	2.33 ± 0.26	1.64 ± 0.28
UFA/SFA	1.869	1.908	2.039	2.049	2.478	2.040

[a] Values are the mean ± standard deviation from at least three independent replicate cultures at the indicated culture condition

Supplementary Table 5- Fatty acid profiles of *pfaT* complementation strains at 15°C

	SS9R pKT231[a]	MAP1 pKT231[a]	MAP1 pMA20[a]
12:0	2.73 ± 0.25	2.27 ± 1.13	2.16 ± 0.47
14:0	5.19 ± 0.40	4.36 ± 0.01	3.44 ± 1.88
14:1	3.28 ± 0.21	2.48 ± 0.02	2.10 ± 0.94
16:0	20.20 ± 1.50	21.08 ± 0.22	21.94 ± 1.30
16:1	46.40 ± 3.72	49.44 ± 0.32	51.27 ± 3.46
12:0 3-OH	1.55 ± 0.16	1.61 ± 0.18	0.98 ± 0.49

18:0	1.87 ± 1.41	1.20 ± 0.04	0.82 ± 0.33
18:1	13.23 ± 0.85	15.67 ± 0.39	12.95 ± 0.39
20:5	1.61 ± 0.17	0.62 ± 0.08	2.73 ± 0.29
UFA/SFA	2.05	2.38	2.35

[a] Values are the mean ± standard deviation from at least three independent replicate cultures

Chapter 2, in full, is a reprint of the material. Allemann, M. N., Shulse, C., & Allen, E. E. (2019). Linkage of marine bacterial polyunsaturated fatty acid and long-chain hydrocarbon biosynthesis. *Frontiers in microbiology*, *10*, 702. The dissertation author was the primary investigator and author of this material.

Chapter 3

Genetic regulation of the bacterial omega-3 polyunsaturated fatty acid biosynthesis pathway

Abstract

A characteristic among many marine gamma-proteobacteria is the biosynthesis and incorporation of omega-3 polyunsaturated fatty acids into membrane phospholipids. Biosynthesis of eicosapentaenoic (EPA) and/or docosahexaenoic (DHA) acids is accomplished using a polyketide/fatty acid synthase mechanism encoded by a set of five genes *pfaABCDE.* This unique fatty acid synthesis (FAS) pathway co-exists with the canonical Type II dissociated fatty acid synthesis pathway, which is responsible for the biosynthesis of saturated, monounsaturated, and hydroxylated fatty acids used in phospholipid and lipid A biosynthesis. In this work a genetic approach was undertaken to elucidate genetic regulation of the *pfa* genes in the model marine bacterium *Photobacterium profundum* SS9. Using a reporter gene fusion we identified that the *pfa* operon is down regulated in response to exogenous fatty acids, particularly long chain monounsaturated fatty acids, and this regulation occurs independently of the canonical fatty acid regulators present in *P. profundum* SS9. Transposon mutagenesis and screening of a library of mutants identified a novel transcriptional regulator, which we have designated *pfaF,* to be the primary regulator of the *pfa* genes in *P. profundum* SS9. This relationship was confirmed via complementation studies and gel mobility shift assays, which also revealed that PfaF mediates the observed regulation of the *pfa* operon to exogenous fatty acids.

Introduction

Regulation of fatty acid biosynthesis, particularly the levels of unsaturated fatty acids, has been shown to be a crucial aspect of the bacterial physiological response to a variety of environmental conditions, including temperature, pH, and hydrostatic pressure. Both biochemical and transcriptional regulatory mechanisms exist to regulate the various aspects of

the fatty acid biosynthetic pathway (1). In the model organism *Escherichia coli,* genes that compromise the Type II fatty acid synthase (FAS), and in particular genes related to monounsaturated fatty acid (MUFA) biosynthesis are regulated by the interplay between FadR and FabR (2–4) as shown in Figure 1. FadR is a member of the GntR regulator family and it acts as positive regulator of both *fabA* and *fabB,* which encode for proteins essential to the biosynthesis of unsaturated fatty acids in this organism (5). As seen in Figure 1 exogenous fatty acids (>C10) are imported across the outer membrane via the FadL transporter and subsequently converted into acyl-CoAs by the acyl-CoA synthetase FadD. In the absence of long chain acyl-CoA, FadR binds sites upstream of *fabA* and *fabB* and acts as a positive regulator. Upon acyl-CoA binding to FadR, it adopts a conformation that is unable to bind to its cognate sites upstream of *fabA/B* promoters leading to deactivation of transcription. FabR, a TetR family transcriptional regulator, further regulates *fabA/B* by acting as a classical repressor. FabR binds to sites immediately downstream of the FadR site in both *fabA/B* regardless of acyl-CoA being present (3, 6). While an exact role of FabR has yet to be described, it has been speculated that the opposing actions of FabR and FadR at *fabA* and *fabB* promoters is ultimately responsible for the regulation of these genes (3).

Similar regulatory mechanisms regulating monounsaturated fatty acid biosynthesis have been characterized in other model Gram-negative bacterial systems. In *Shewanella oneidensis* MR-1 the FabR homolog was found to be responsible for the regulation of *fabA* and *desA,* an oxygen dependent membrane bound lipid desaturase (7). Similar regulatory mechanisms controlling unsaturated lipid biosynthesis have been described in other model bacteria such as *Pseudomonas aeruginosa* PAO1*,* which lacks a *fadR* homolog (1, 8, 9). In this strain *fabA* and *fabB* form an operon (unlike *E.coli*), which is regulated by another TetR family regulator, DesT

(10). In addition to *fabAB* expression, DesT also modulates the expression of the membrane bound desaturases DesBC, that catalyzes oxygen dependent desaturation of saturated acyl-CoA that can be incorporated into membrane phospholipids (8, 9).

A subset of marine gamma-proteobacteria, particularly strains isolated from cold and/or high-pressure environments, produce omega-3 polyunsaturated fatty acids (PUFA) such as EPA (20:5*n-3*) and DHA (22:6*n-3*), that are incorporated into their phospholipid membranes (11, 12). The biosynthesis of these unique fatty acids is linked to the *pfaABCDE* operon, which encodes for a type I FAS/polyketide synthase (13). In these bacteria, the Pfa synthase pathway co-exists with the Type II FAS, which produces saturated (SFA) and monounsaturated fatty acids (12, 14). Given that both pathways utilize the same precursor substrates (13, 15, 16) and their respective end products are destined for phospholipids (11, 12, 17–20), an interesting question arises as to how these pathways are physiologically coordinated in the cell. Numerous studies have demonstrated that culturing native PUFA-producing strains at cold temperature (21–26) and/or high pressure (21, 24) leads to increases in PUFA abundance. In the EPA producing bacterium *Photobacterium profundum* SS9*,* analyses of transcript abundances of the *pfa* operon at cold temperatures and/or high pressure indicated no significant alterations mRNA abundances relative to 15°C or low-pressure conditions (23). In the course of those studies, a chemical mutant of *P. profundum* SS9 (EA2), was shown to have increased mRNA abundance relative to its parental strain indicating the possibility of a transcriptional regulator(s) existing in the strain (23).

In this work the transcriptional regulation of the *pfa* operon in *P. profundum* SS9 is further characterized and shown that it is down regulated in response to exogenous unsaturated fatty acid supplementation. Genetic experiments and transcriptional analyses confirmed that this response is due in part to the FadR homolog in SS9. A genetic screen utilizing a reporter gene

fusion combined with transposon mutagenesis was also used to identify a novel transcriptional regulator, herein designated *pfaF,* which positively regulates the *pfa* operon. Gel mobility shift experiments further demonstrate that both PfaF and FadR bind to the *pfaA* promoter confirming the phenotypic data.

Results

To better understand the regulation of the *pfa* operon and to facilitate monitoring of gene expression, a reporter construct was designed linking expression of the *pfa* operon to the *lacZY* operon of *Escherichia coli.* This strain, designated MAP16 (Δ*pfaA::lacZY*), allows the *pfa* operon promoter to be monitored in single copy with all possible upstream regulatory sequences. Assays for β-galactosidase activity indicated that in mid-log phase approximately 48 Miller units of activity were produced (Figure 2A). Further β-galactosidase assays under conditions shown previously to lead to increased EPA content such as high hydrostatic pressure and low temperature are given in Figure 2A and indicated no changes in LacZ activity, confirming previous results from this strain. Sequence analysis of the promoter region from EA2, a previously isolated EPA over producing strain with increased *pfa* operon transcript levels, also indicated no changes in the promoter sequence of strain EA2 relative to the wild-type.

Given its biosynthetic role, i.e. producing fatty acids destined for phospholipid biosynthesis, we hypothesized that the *pfa* operon might be regulated in a similar fashion as the prototypical *fab* regulon. Given the previously noted solubility issues of fatty acids in 2216 growth media (21), exogenous fatty acids in the form of various polysorbate esters (Tween 20 (12:0) , 40 (16:0) , 60 (18:0), 80 (18:1)) were utilized as exogenous fatty acid supplements. As shown in Figure 2B, a significant decrease in β-galactosidase activity was observed in MAP16 in

response to all Tween compounds except for Tween 20 (12:0) (Figure 2B). Given the various degrees of down-regulation noted amongst the various Tween compounds seen in Figure 2B, which differ only in their fatty acid component, the possibility of this response being due to the polysorbate component of these compounds can be eliminated. This down-regulation was also shown to occur in SS9R as both *pfaA* and *pfaD* transcript abundances are reduced ~3-fold and ~2-fold, respectively, in response to Tween 80 (18:1) supplementation (Figure 2C). Transcript abundances of *fabA* and *fabB* were also decreased under these conditions (Figure 3C) indicating that the monounsaturated fatty acid (MUFA) biosynthesis genes are down regulated in the presence of Tween 80 (18:1).

Given this phenotypic data it was suspected that either FadR and/or FabR, which are known to regulate the *fabA* and *fabB* in response to exogenous fatty acids, were responsible for this regulatory phenomenon. Homologs of *fabR* (locus tag: PBPRA3467) and *fadR* (locus tag: PBPRA2608) were readily identified in the SS9 genome via homology searches and single and double mutants were generated in both SS9R and MAP16 strains respectively (Table 1). As shown in Figure 3A, only deletion of *fadR* led to a significant decrease in β-galactosidase activity. Comparing MAP18 (Δ*pfaA::lacZY*Δ*fadR*) with MAP23 (Δ*pfaA::lacZY* Δ*fabR*Δ*fadR*) indicated that both strains have similar β-galactosidase activities and further ruled out the involvement of FabR in the regulation of the *pfa* operon. Furthermore, β-galactosidase assays on MAP23 indicated that the down regulation of the Δ*pfaA::lacZY* gene fusion in the presence of Tween 80 (18:1) was independent of both FadR and FabR (Figure 3A). Complementation analyses indicated that the decrease in β-galactosidase activity was reversed upon introduction of a plasmid containing *fadR* (Figure 3B). We noted slightly increased β-galactosidase activities in MAP16 containing pMA65 relative to the vector control. Transcript abundance analyses

performed on RNA samples from MAP15 (SS9R Δ*fabR*Δ*fadR*) grown in the presence or absence of Tween 80 (18:1) indicated that both *pfaA* and *pfaD* transcripts were down regulated in response to supplementation while *fabA* and *fabB* transcripts were essentially equivalent between the two conditions (Figure 3C). Intriguingly the magnitude of down-regulation of both *pfaA* and *pfaD* transcripts in MAP15 was greater than that observed in SS9R. Fatty acid analyses of the corresponding single and double mutant derivatives of SS9R are shown in Table 2 and indicate that MAP13 (Δ*fadR*) and MAP15 *(*Δ*fabR*Δ*fadR*) had decreased EPA levels consistent with the β-galactosidase activity data presented. As predicted given its role in regulating *fabA/B,* a significant decrease in MUFA was noted in both strains carrying the Δ*fadR* mutation (MAP13, 15). As expected from the reporter gene fusion data, we were able to complement the Δ*fadR* mutation with the construct pMA65 (Supplemental Table 2). Interestingly, fatty acid profiles of SS9R containing pMA65 also indicated no increases in EPA despite the slightly elevated β-galactosidase activities noted in the gene fusion strains.

The FadR/FabR independent down regulation of the *pfa* operon in response to Tween 80 (18:1) observed here indicated that another regulator(s) for the *pfa* operon might exist in *P. profundum* SS9. To search for additional regulators, MAP16 (Δ*pfaA::lacZY*) was subjected to transposon mutagenesis using the mini-Tn5 delivery vector pRL27(27, 28). Of the approximately 10,000 mutants screened, several LacZ down or loss of LacZ activity mutants were identified and saved for further analysis. Arbitrary PCR was performed to identify the sites of mini-Tn5 insertion in these mutants. Excluding mutants that had Tn5 insertions in the reporter gene and/or *fadR,* we identified mutants from independent libraries that contained unique transposon insertions in the same gene PBPRA0221 (TetR bacterial transcriptional regulator, pfam13972) (Figure 4A). One of these mutants (MAP1603) displayed a five-fold decrease in LacZ activity

and no longer responded to exogenous Tween 80 (18:1) supplementation (Figure 4B). The PBPRA0221 gene, herein designated *pfaF,* encodes for a protein that is a member of the TetR family of transcriptional regulators and is clustered with genes that appear to be related to lipopolysaccharide synthesis. To verify that this locus is involved in *pfa* gene regulation, single crossover insertion mutants were generated in both MAP16 and SS9R strains. The LacZ activity of the resulting exconjugants (MAP26) was identical to that seen in MAP1603 thereby verifying this relationship and excluding the possibility of additional transposon insertion events being responsible for the observed phenotype (data not shown). Transcript abundance analysis of MAP27 (SS9R *pfaF*$^-$) indicated significant down-regulation of both *pfaA* and *pfaD* transcripts relative to SS9R (Figure 4C) consistent with the LacZ activity data. Additionally, quantification of *fabA* and *fabB* transcripts indicated no major differences between MAP27 and SS9R. A comparison of the fatty acid profiles of MAP27 and SS9R grown at 15°C is given in Table 3 and the mutant displays an approximate 4-fold reduction of EPA relative to the wild-type. The minor difference in the abundances of other fatty acids in MAP27 is also consistent with the minimal changes in *fabA* or *fabB* transcription.

The *pfaF* gene was cloned onto pFL122 and the resulting construct (pMA62) introduced into MAP1603 and MAP27 for complementation analyses. As shown in Figure 4B, pMA62 was able to complement the LacZ phenotype seen in MAP1603 relative to the vector only control. Similarly, the ability to down regulate the operon in response to exogenous fatty acids was restored in the complemented strain and not in the vector only control (Figure 4B). Fatty acid analysis of these MAP27 derivatives also indicated that the pMA62 construct was able to restore EPA levels back to wild-type levels compared to the vector control (Table 3). Similarly,

transcript abundances of *pfaA* and *pfaD* were restored to wild-type levels in the complemented strain relative to vector only controls (data not shown).

To verify that regulation by PfaF and FadR was direct, electrophoretic mobility shift assays were conducted. Both PfaF and FadR were cloned, expressed, and purified from *E.coli* as C-terminal and N-terminal 6x-His-tagged proteins, respectively (Supplemental Figure 1). As shown in Figure 5A, recombinant PfaF was able bind to the *pfaA* promoter probe in a concentration dependent manner. A similar result was seen with recombinant FadR and is shown in Figure 5B. A putative FadR binding site was identified at position -232 relative to the start of transcription and based on a previously predicted consensus FadR binding site for FadR homologs from the Vibrionaceae (29). This binding site was within the sequence of the probe used for these mobility shift experiments and its sequence shares identity with 11 of 17 bases of the predicted Vibrionaceae FadR consensus site.

Homology searches were conducted using the amino acid sequence of PfaF and a previously unpublished structure of a TetR regulator from *Shewanella amazonensis* SB2B (PBD: 3rh2) was found to have a high degree of homology to the PfaF protein (Supplemental Figure 2A). Further analysis of the crystal structure indicated that its C-terminal domain contained a ligand pocket containing an unknown ligand that resembles the hydrocarbon “tail” of a fatty acid (Supplemental Figure 2B). Mobility shift assays using purified PfaF indicated that the addition of oleoyl-CoA abolished its *in vitro* DNA binding activity (Figure 5C). As expected from previous studies, FadR exhibited a similar loss of DNA binding activity in the presence of oleoyl-CoA (Figure 5D). In the case of both proteins, the loss of DNA binding activity was dependent on the concentration of oleoyl-CoA in the assay.

Discussion

In this work, the genetic regulation of the *pfa* operon has been extensively characterized utilizing a variety of genetic techniques. While the products (11, 12), biosynthetic mechanisms (13, 16, 30), and phylogenetic distribution (14, 31) of the bacterial *pfa* operon has been extensively studied, there has been little work done describing how the operon is regulated and what gene(s) might be involved. The findings described in this report represent the first systematic investigation into the genetic regulation of the *pfa* operon. The finding that neither pressure nor cold-temperature affected the activity of the Δ*pfaA::lacZ* fusion is consistent with previously reported results in *Photobacterium profundum* SS9 (23) and validated our reporter gene fusion approach. As noted previously (23), we noted the lack of correlation between the expression level of the *pfa* operon and the amount of EPA found in the membrane phospholipids under cold temperature and high pressure culture conditions. The reasons for this phenomenon are unclear but they do suggest that other factors in the biosynthesis and membrane incorporation of EPA are involved in the increased abundance of EPA at high pressures and/or cold temperatures.

The finding that the *pfa* operon was down regulated in response to exogenous fatty acids in a FadR/FabR independent manner indicated that another transcription factor was responsible for regulating this response to exogenous fatty acids. Screening of a transposon library in MAP16 identified a novel regulator, *pfaF,* whose gene product acts as a positive regulator of the *pfa* operon. Reintroduction of a null mutation in *pfaF* in SS9R yielded a mutant (MAP27) that had a specific several fold decrease in EPA composition with relatively minor changes in the abundances of the other fatty acids in the membrane. Successful complementation *in trans* confirmed the role of *pfaF* in positive regulation of the *pfa* operon. Based on amino acid

sequence, PfaF is a member of the TetR transcriptional regulator family, of which several members have been characterized to be involved with regulation of fatty acid biosynthesis and/or degradation in other bacteria (32).

The distance (232bp) between the identified FadR binding site and the transcriptional start site of *pfaA* is consistent with FadR playing an accessory role in regulating the *pfa* operon. Indeed, loss of PfaF alone led to a non fatty acid responsive phenotype and significant decreases in EPA, even in the presence of a functional FadR. The finding that FadR is at least partially involved in the regulation of the *pfa* genes is a significant finding in that it further expands the diversity of genes that are controlled to some extent by this well-known regulator. In particular, FadR homologs, or genes regulated by FadR, have been shown to be involved in processes related to virulence amongst various members of the Vibrionaceae (33–36). Furthermore, many of these virulence genes are regulated in response to exogenous fatty acids (37), similar to the results reported here for the *pfa* operon. Whether the production of EPA or other PUFA is part of a virulence/symbiosis/colonization response is unclear, although it is interesting to note that many PUFA-producing bacteria have been isolated from the gut contents of marine organisms such as fish and various invertebrates (38, 39).

Electrophoretic mobility shift assays using the previously characterized promoter region of *pfaA* indicated that both PfaF and FadR were capable of binding to the promoter *in vitro.* As expected, addition of oleoyl-CoA resulted in a loss of DNA binding activity for FadR. Based on amino acid sequence, an unpublished crystal structure of a homolog of PfaF from *S. amazonensis* was found and close examination of the structure indicated a possible lipid-binding pocket within the C-terminal portion. As seen with FadR, PfaF lost its binding capabilities in the presence of oleoyl-CoA. While the physiologically relevant ligand of PfaF has not been determined, the

binding pocket noted in the homologous structure strongly suggests that a fatty acid or acyl-CoA derivative is the ligand.

At this time it is unclear as to how the various Tween derivatives and their fatty acid components are utilized by *P. profundum* SS9. Various members of the Vibrionaceae genus have been shown to have outer membrane associated lipase enzymes capable of hydrolyzing esterified fatty acids for incorporation into phospholipids or usage as a carbon source via β-oxidation (40, 41). In particular one such enzyme designated VolA, has been well-characterized as being involved with utilization of exogenous esterified fatty acids such as lysophosphotidylcholine (40). Preliminary searches of the *P. profundum* SS9 genome located a homolog (locus tag: PBPRA2574) of *volA* in an operon with a FadL homolog, identical to the genomic context of *volA* in *Vibrio cholerae.* This lipase or other similar lipases may be involved with the utilization of Tween compounds as a lipid source or as a carbon source.

Based on the results in presented here, a proposed model of regulation is shown in Figure 6. In the absence of fatty acids, PfaF binds to a yet to be determined sequence within the *pfaA* promoter region and acts as a positive regulator. In the presence of exogenous fatty acids, which are presumably converted into acyl-CoA, PfaF binds to an acyl-CoA and releases from the promoter region ultimately leading to a lack of transcriptional activation of the *pfa* operon. While FadR does appear to have an effect on the *pfa* operon and is capable of binding to the promoter *in vitro,* it did not mediate the transcriptional response to exogenous fatty acids. Based on the data presented here, the primary role of FadR is similar to its role in *Escherichia coli,* regulating *fabA* and *fabB* transcription in response to exogenous fatty acids.

Homology searches of available genomes indicated that all PUFA-producing marine gamma-proteobacteria contain a *pfaF* homolog in the same relative genomic context. In many

instances, such as in strains of *Shewanella* and *Colwellia*, the *pfa* operon also contains an annotated regulator typically designated as *pfaR* immediately upstream of *pfaA*. Interestingly, the protein sequence of this regulator does not match to any class of bacterial transcriptional regulators and only contains an identifiable N-terminal helix-turn-helix domain, which is most likely involved in DNA-binding activity. Preliminary results using *S. piezotolerans* WP3, a genetically tractable EPA producer, indicated no differences in EPA composition between wild-type and Δ*pfaR* mutants under a variety of temperatures (data not shown). A previous study (42) demonstrated that replacing *pfaR* with an inducible promoter could lead to dramatic increases in EPA production in a heterologous host strain of *E.coli*. Unfortunately, that study lacked adequate data that could be used to ascertain the role of *pfaR* directly. Regulation of the *pfa* operon in strains with *pfaR* may indeed be more complex or otherwise different than in the case of *P. profundum* SS9. The results presented here describe the identification of a novel transcriptional regulator that specifically modulates expression of the *pfa* operon in response to exogenous fatty acids and controls the amount of polyunsaturated fatty acids incorporated into membrane phospholipids. This study adds new insight into the unique lipid physiology of widespread marine bacteria and offers new opportunities for the genetic optimization of microbial omega-3 polyunsaturated fatty acid synthesis.

Materials and Methods

Bacterial strains and growth conditions

Escherichia coli strains were routinely grown at 37°C in Luria Bertani (LB) media unless stated otherwise. *Photobacterium profundum* strains were grown at 15°C in 2216 marine broth (Difco) at 75% strength (28g/L) unless noted otherwise. For solid medias, agar was included at

15g/L. The antibiotics kanamycin (50 μg/ml for *E. coli* and 200 μg/ml for *P. profundum*), chloramphenicol (15μg/ml), ampicillin (100μg/ml), and rifampicin (100μg/ml) were used as required. For high-pressure growth experiments, *P. profundum* SS9 strains were grown in heat-sealed bulbs and incubated in stainless steel pressure vessels as described previously (43).

Targeted Mutagenesis

Vectors for introducing mutations into *P. profundum* were introduced by conjugation using previously described methods with minor alterations (12, 43). In-frame deletions were generated by allelic exchange using the suicide vector pRE118 (44). Insertional inactivation of target genes was accomplished by introduction of the suicide plasmid pMUT100 as described previously (21, 45).

Transposon mutagenesis and screening

Biparental conjugations using *E.coli* S17-1λ*pir* were used to transfer the mini-Tn5 delivery plasmid pRL27 into MAP16 (Table 1) (27, 46). Both recipient and donor strains were grown to stationary phase and conjugations were performed as described above. After ~24hr at ambient temperature (~22°C) cells on filter membranes were resuspended in 2216 broth and plated onto selection media (2216 agar containing 200μg/ml kanamycin and 100μg/ml rifampin) and incubated at 15°C for 5 days. Resulting ex-conjugants were patched to fresh selection plates in grid format. After two days growth at 15°C the arrayed mutants were replica plated to 2216 agar with X-gal (80μg/ml). After two days of growth on indicator media, mutants were screened by eye for differences in blue colony formation. Mutants with differential *lacZ* activity were clonally isolated and further screened by β-galactosidase assays in liquid cultures as described below.

Identification of transposon insertion sites

To identify transposon insertion sites of interest an arbitrary PCR method was utilized similar to the method described previously (28). Primer sequences are given in Supplementary Table 1. In the first round of PCR, a primer specific to one end of the mini Tn5 element (Tn5 ext) in combination with one of three degenerate primers (arb1, 2, or 3) is used with purified genomic DNA as a template. The conditions used for the first PCR were; 95°C 5 min, 6 cycles of 95°C for 30 sec, 30°C for 30 sec, 68°C for 2 min, followed by 30 cycles of 95°C for 30 sec, 45°C for 30 sec, 68°C 2 min, and 68°C 5 min. Two microliters of the first reaction was used as a template for a nested PCR with "Arb clamp" and "Tn5 int" primers. The conditions for the second PCR were 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 68°C for 2 min, followed by 68°C for 10min. PCR reactions yielding single amplicons as judged by agarose gel electrophoresis were purified using a PCR clean up kit (Zymo Research) and sent for DNA sequencing. Sequences were compared to the genome of *P. profundum* SS9 to determine the insertion site of mini-Tn5.

β-galactosidase assays

Cultures of indicated strains were grown at 15°C in aerobic tubes, unless noted otherwise. Typically mid-log phase cultures (OD_{600} = 0.2-0.8) were assayed for changes in LacZ activity from whole cell extracts using the SDS and chloroform lysis modification described previously (47). Activities reported are in Miller units and represent the mean of at least five independent experiments.

RNA isolation and quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was isolated from mid-log phase cells grown under the indicated conditions using Trizol (Invitrogen) following manufacturer guidelines. Crude RNA extracts were further purified and treated with DNase I (Zymo Research) using the RNA Clean and Concentrator kit

(Zymo Research). For cDNA synthesis, the Superscript III First Strand synthesis kit (Invitrogen) was used following manufacturer's recommended protocols. Quantitative PCR's were performed using the Maxima Sybr Green Master Mix (Thermo Scientific) and run on a Stratagene MX3000P qPCR system. For quantification of target transcripts, the *gyrB* gene (PBPRA0011) was used as an internal reference and differences in expression were calculated using the ΔΔCT method. Primers for qPCR experiments are listed in **Supplementary Table 1**.

Expression and Purification of FadR and PfaF

The FadR homologue of SS9 (locus tag: PBPRA2608) and PfaF (locus tag: PBPRA0221) were cloned into pET28 (Novagen) as to generate N-terminal 6xHis tagged FadR and a C-terminal tagged PfaF. After sequence verification, these constructs were transformed into BL21 DE3 Tuner pLysS (Novagen) cells following standard procedures (47). For protein expression, overnight cultures were diluted 1/100 into LB supplemented with chloramphenicol (30μg/ml) and kanamycin (50μg/ml) and grown at 30°C until OD_{600} of ~0.5 at which point IPTG was added to final concentration of 0.5mM and grown for an additional 4hrs at 30°C. Cells were harvested by centrifugation and cell pellets were processed or stored at -80°C. Frozen cell pellets were thawed on ice with the addition of buffer A (50mM Tris-Cl pH 7.5, 200mM NaCl, 10% glycerol). Lysozyme was added and cells incubated on ice for 30 minutes and sonicated on ice to complete the lysis procedure. The lysate was centrifuged at 10,000 rpm, 4°C, for 30 minutes to separate insoluble and soluble fractions. The clarified supernatant was applied to a Ni-NTA column equilibrated with buffer A and mixed gently at 4°C for one hour. The resin was washed with several column volumes of buffer B (buffer A + 30mM imidazole) and proteins were eluted with buffer C (buffer A +300mM imidazole). Eluted fractions were checked by SDS-PAGE for purity, and appropriate fractions were pooled and desalted using PD-10 columns (GE

Healthcare) and exchanged into Buffer D (20mM Tris Cl pH 7.5, 50mM NaCl, 10% Glycerol). Protein samples were pooled and subsequently concentrated by 10,000 kDa centrifugal filter units (Amicon).

Electrophoretic mobility shift assays

Promoter DNA fragments were generated by PCR using a 6-FAM labeled primer set listed in **Supplemental Table 1** and purified *P. profundum* SS9 genomic DNA as template. For mobility shift experiments, binding reactions contained; binding buffer (10mM Tris Cl pH 7.5, 1mM EDTA, 10% glycerol), 1µg poly dI-dC (Thermo Scientific), 20nM promoter probe, and the indicated amount of purified FadR or PfaF. Binding reactions were incubated at 22°C for 30 min and analyzed by electrophoresis using pre-run 6% 0.5X TBE polyacrylamide gels. Gels were visualized and photographed using a GelDoc system (Bio-Rad).

Fatty acid extraction and GC-MS analysis

Late log phase cultures were harvested by centrifugation and cell pellets rinsed once with 50% Sigma Sea Salts solution (16g/L) and stored at -80°C. Cell pellets were lyophilized and fatty acids were converted to fatty acid methyl esters and analyzed by gas chromatography mass spectrometry using previously described protocols and methods (12).

Acknowledgements

We would like to thank Dr. Doug Bartlett for insightful discussions regarding genetic manipulations in *P. profundum* SS9 and Dr. Bianca Brahamsha for the generous gift of the pRL27 mini-Tn5 transposon delivery vector. This work was supported by National Science Foundation Division of Molecular and Cellular Biosciences grant MCB-1149552 to E.E.A.

Table 3.1- Strains and Plasmids used in this study

Strain/Plasmid	Relevant characteristics	Source
E.coli strains		
MG1655	Wild type *E.coli*, source of *lacZY* operon	Coli Genetic Stock center
DH5αpir	Cloning strain for pir vectors	(48)
S17-1λpir	Biparental mating strain	(46)
P. profundum strains		
SS9R	Rifampin resistant derivative of *P. profundum* SS9	(43)
EA2	EPA overproducer, *pfa* regulatory mutant	(21)
MAP7	SS9R, Δ*lacZ*	This study
MAP12	SS9R, Δ*fabR*	This study
MAP13	SS9R, Δ*fadR*	This study
MAP15	SS9R, Δ*fabR*Δ*fadR*	This study
MAP16	MAP7, Δ*pfaA*::*lacZY*	This study
MAP17	MAP16, Δ*fabR*	This study
MAP18	MAP16, Δ*fadR*	This study
MAP23	MAP16, Δ*fadR*Δ*fabR*	This study
MAP26	MAP16, *pfaF*::pMUT100	This study
MAP27	SS9R, *pfaF*::pMUT100	This study
MAP1603	MAP16, *pfaF*::mini-Tn5	This study
Plasmids		
pRE118	Allelic exchange vector Kan^R	(44)
pFL122	Broad host range complementation vector	(49)
pRK2073	Conjugation helper plasmid	(50)
pMUT100	Suicide plasmid for insertional inactivation	(45)
pRL27	Mini Tn5 transposon delivery vector	(27)
pET28	T7 expression plasmid	Novagen
pMA21	pRE118 w/Δ*pfaA* allele	This study
pMA29	pRE118 w/Δ*lacZ* allele	This study
pMA38	pRE118 w/Δ*fabR* allele	This study
pMA39	pRE118 w/Δ*fadR* allele	This study
pMA41	pRE118 w/Δ*pfaA*::*lacZY* allele	This study
pMA61	pMUT100 w/*pfaF* internal fragment	This study
pMA62	pFL122 w/*pfaF*	This study
pMA65	pFL122 w/*fadR*	This study
pMA67	pET28 w/PfaF C-terminal 6xHIS tag	This study
pMA68	pET28 w/FadR N-terminal 6xHIS tag	This study

Table 3.2- Fatty acid compositions of MAP12(Δ*fabR*), MAP13(Δ*fadR*), and MAP15 (Δ*fabR*Δ*fadR*) at 15°C

	Mean % (by weight) fatty acid			
Fatty acid				
	SS9R[a]	**MAP12**[a]	**MAP13**[a]	**MAP15**[a]
12:0	4.16 ± 1.53	3.18 ± 0.32	4.59 ± 0.17	3.88 ± 0.10
14:0	4.33 ± 1.21	4.41 ± 1.32	8.10 ± 0.84	9.66 ± 0.12
14:1	3.19 ± 1.11	1.80 ± 0.39	2.36 ± 0.16	7.95 ± 0.06
16:0	21.07 ± 1.84	17.66 ± 5.23	32.21 ± 2.61	20.30 ± 0.30
16:1	45.09 ± 1.68	35.53 ± 7.06	36.22 ± 0.54	49.64 ± 0.60
12-OH	1.99 ± 1.23	2.89 ± 0.57	4.85 ± 0.96	1.89 ± 0.24
18:0	0.71 ± 0.11	1.13 ± 0.23	0.72 ± 0.09	0.81 ± 0.05
18:1	12.26 ± 2.26	10.77 ± 1.37	3.20 ± 0.60	1.84 ± 0.11
20:5	4.52 ± 1.55	4.58 ± 0.48	1.52 ± 0.11	1.98 ± 0.16

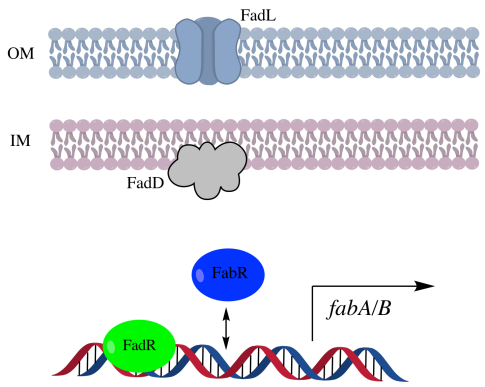
[a] Data represents the average of at least three independent replicates ± standard deviation

Table 3.3- Fatty acid compositions of MAP27 and complemented strains at 15°C

	Mean % (by weight) fatty acid		
Fatty acid			
	MAP27[a]	**MAP27 pFL122**[a]	**MAP27 pMA62**[a]
12:0	2.84 ± 0.33	3.72 ± 0.45	3.73 ± 0.10
14:0	5.25 ± 0.94	9.46 ± 0.31	8.03 ± 0.22
14:1	3.90 ± 0.73	6.04 ± 0.19	4.90 ± 0.16
16:0	19.03 ± 0.67	19.39 ± 3.64	19.50 ± 2.82
16:1	50.95 ± 0.53	45.33 ± 2.06	39.59 ± 1.58
12-OH	2.22 ± 0.28	2.66 ± 0.14	2.96 ± 0.27
18:0	0.94 ± 0.08	1.37 ± 0.15	1.74 ± 0.09
18:1	12.92 ± 0.42	9.38 ± 0.97	12.02 ± 2.82
20:5	0.82 ± 0.23	1.14 ± 0.17	5.14 ± 0.30

[a] Data represents the average of at least three independent replicates ± standard deviation

Absence of exogenous fatty acids



Presence of exogenous fatty acids

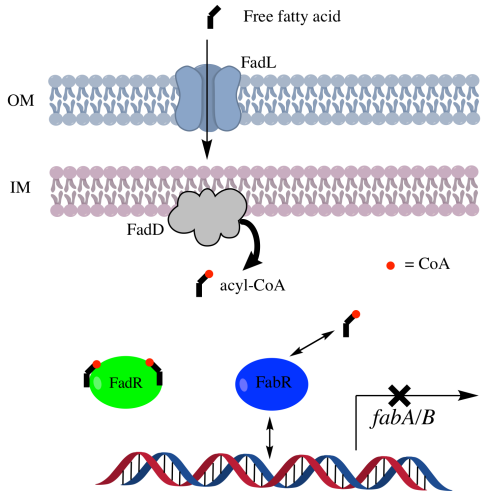


Figure 3.1- Genetic regulation of monounsaturated fatty acid biosynthesis genes *fabA* and *fabB* in *E. coli*. In the absence of exogenous fatty acids FadR binds to a site upstream of the *fabA* and *fabB* promoters and acts as an activator of transcription. When present, exogenous fatty acids are transported across the outer membrane by FadL and converted into acyl-CoA by FadD. Acyl-CoA binding to FadR causes a conformational shift that abolishes the DNA binding capabilities of FadR. In both scenarios FabR binds to a site downstream of FadR and has been shown to bind in the presence/absence of acyl-CoA and/or Acyl-ACP. Loss of FadR activation of transcription presumably allows FabR to act as a better repressor of *fabA/B* expression.

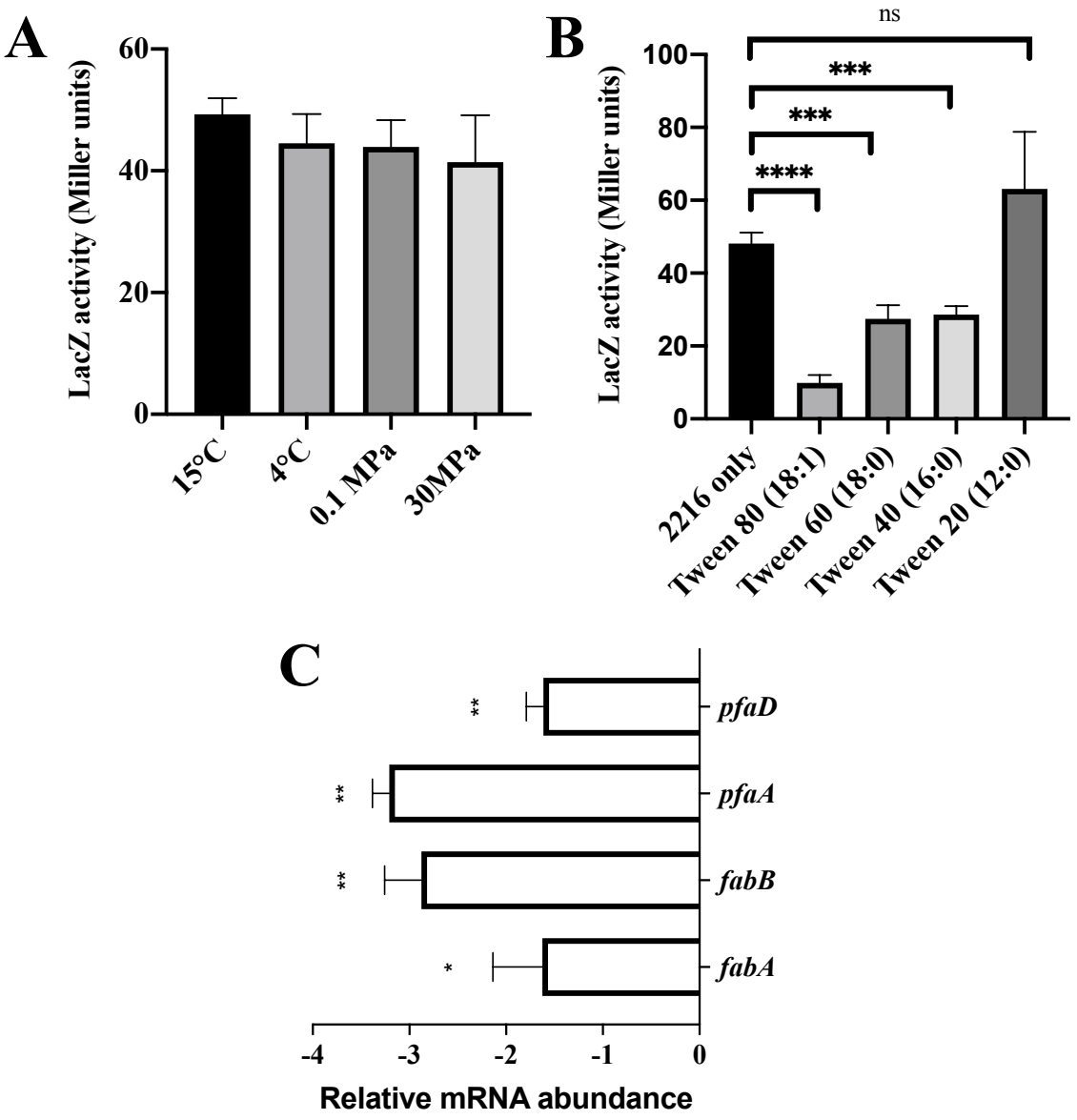


Figure 3.2- Reporter gene expression in MAP16 (Δ*pfaA*::*lacZY*) in response to a variety of culture parameters. (A) MAP16 under various temperature and pressure conditions. (B) MAP16 cultured at 15°C with 0.05% Tween compound supplements indicated. Results of at least six independent experiments shown as means with error bars representing standard deviation (ns, $P > 0.05$; ***, $P < 0.005$; ****, $P < 0.0001$). (C) Effect of 0.05% Tween 80 supplementation on various fatty acid biosynthetic gene transcript abundances in SS9R as determined by qRT-PCR. Cells grown without supplementation represent the calibrator condition. Error bars represent the standard deviations based on at least three independent biological replicates with duplicate qPCR reactions (*, $P < 0.05$; **, $P < 0.005$).

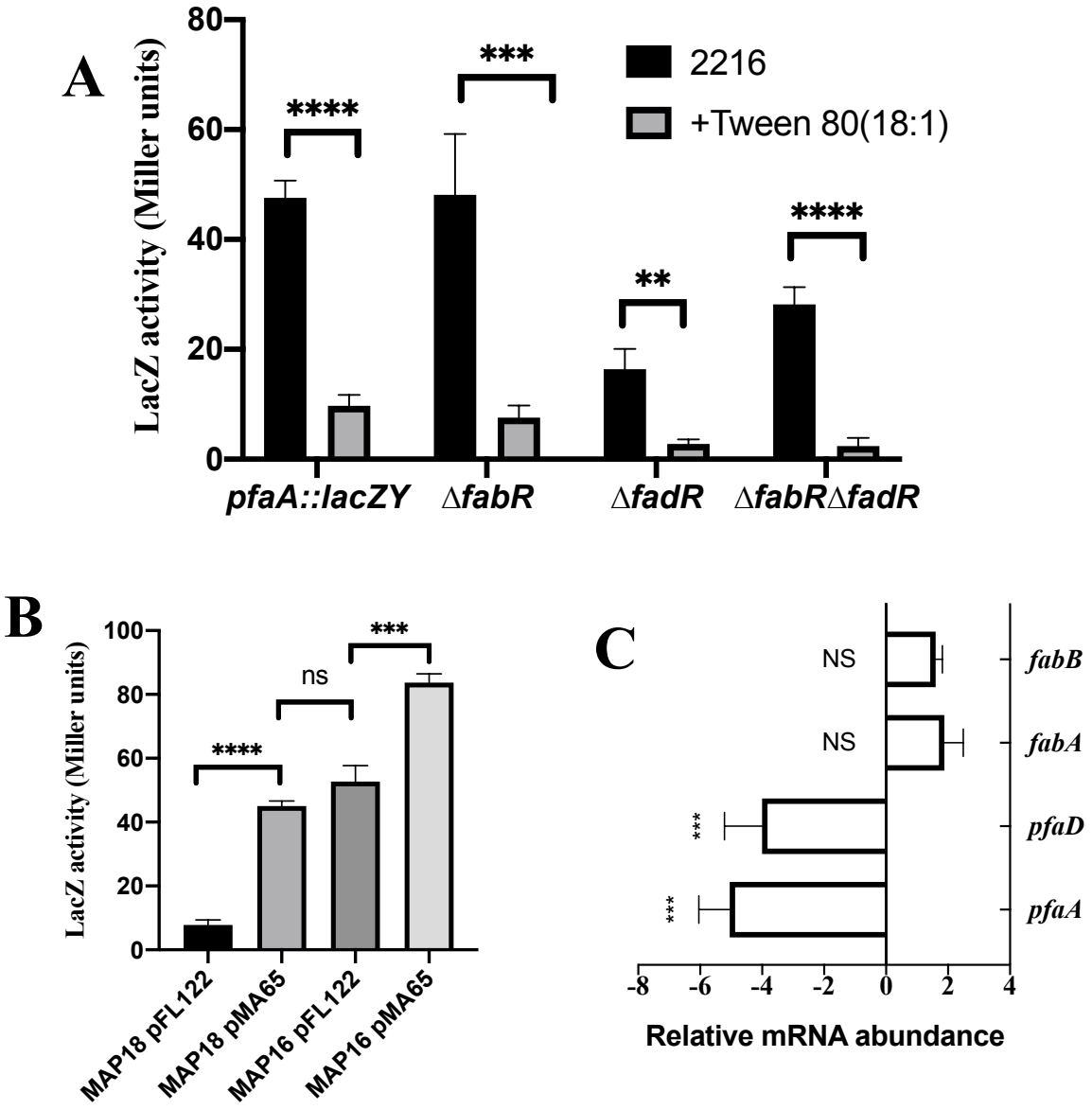
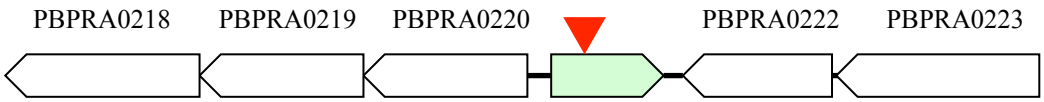


Figure 3.3- Influence of FadR and FabR on expression of the *pfa* operon. (A) LacZ activities of MAP17 (MAP16 Δ*fabR*), MAP18 (MAP16 Δ*fadR*) and MAP23 (MAP16 Δ*fabR*Δ*fadR*) strains in the presence or absence of Tween 80 (18:1). Results of at least six independent experiments shown as means with error bars representing standard deviation. (**, P <0.05; ***, P <0.005; ****, P <0.0001) (B) Complementation of MAP18 strain by construct pMA65. Results of at least six independent experiments shown as means with error bars representing standard deviation. (ns, P >0.05; ***, P <0.005; ****, P <0.0001) (C) Relative transcript abundances of *pfaA, pfaD, fabA,* and *fabB* grown in the presence or absence of Tween 80 (18:1) in MAP15 (SS9R Δ*fabR*Δ*fadR*). Cells grown without supplement represent the calibrator condition. Error bars represent the standard deviations based on at least three independent biological replicates with duplicate qPCR reactions (NS, P >0.05; ***, P <0.005).

A



Locus tag	Pfam hit	Function	E-value
PBPRA0218	PF04932	O-antigen ligase	3.2e-18
PBPRA0219	Pfam03279	Lipid A acytransferase	9.3e-81
PBPRA0220	Pfam01370	NAD dependent epimerase/dehydratase family	1.5e-42
PBPRA0221	Pfam13972	Bacterial transcriptional repressor	3.03e-38
PBPRA0222	Pfam04748	Divergent polysaccharide deacetylase	2.6e-72
PBPRA0223	Pfam01551	Peptidase family M23	2.6e-21

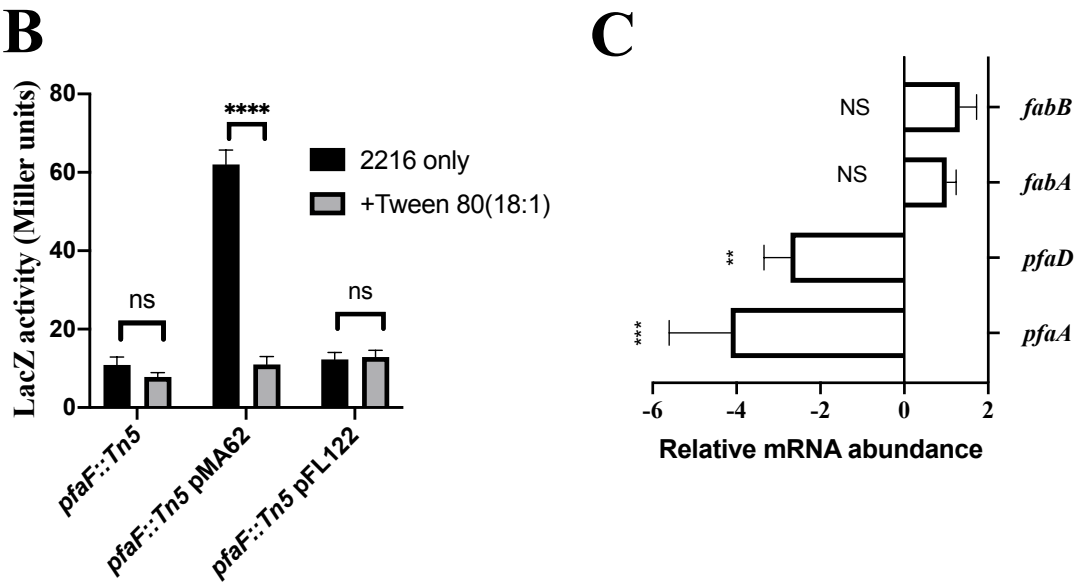


Figure 3.4- The *pfaF* genetic locus and regulatory phenotypes associated with its disruption. (A) Genetic organization of PfaF locus, arrow indicates relative position of mini-Tn5 insertion sites in MAP1603 (MAP16 PBPRA0221::Tn5). (B) LacZ activity of MAP1603 mutants and the complemented strains carrying pMA62 (pFL122 *pfaF*) or vector only ±Tween 80 (18:1) supplementation (ns, $P > 0.05$; ****, $P < 0.0001$) (C) qRT-PCR analysis of *pfaA, pfaD, fabA,* and *fabB* transcript abundances in MAP27 (SS9R *pfaF*$^-$) relative to SS9R. Error bars represent the standard deviations based on at least three independent biological replicates with duplicate qPCR reactions (ns, $P > 0.05$; **, $P < 0.005$; ***, $P < 0.001$).

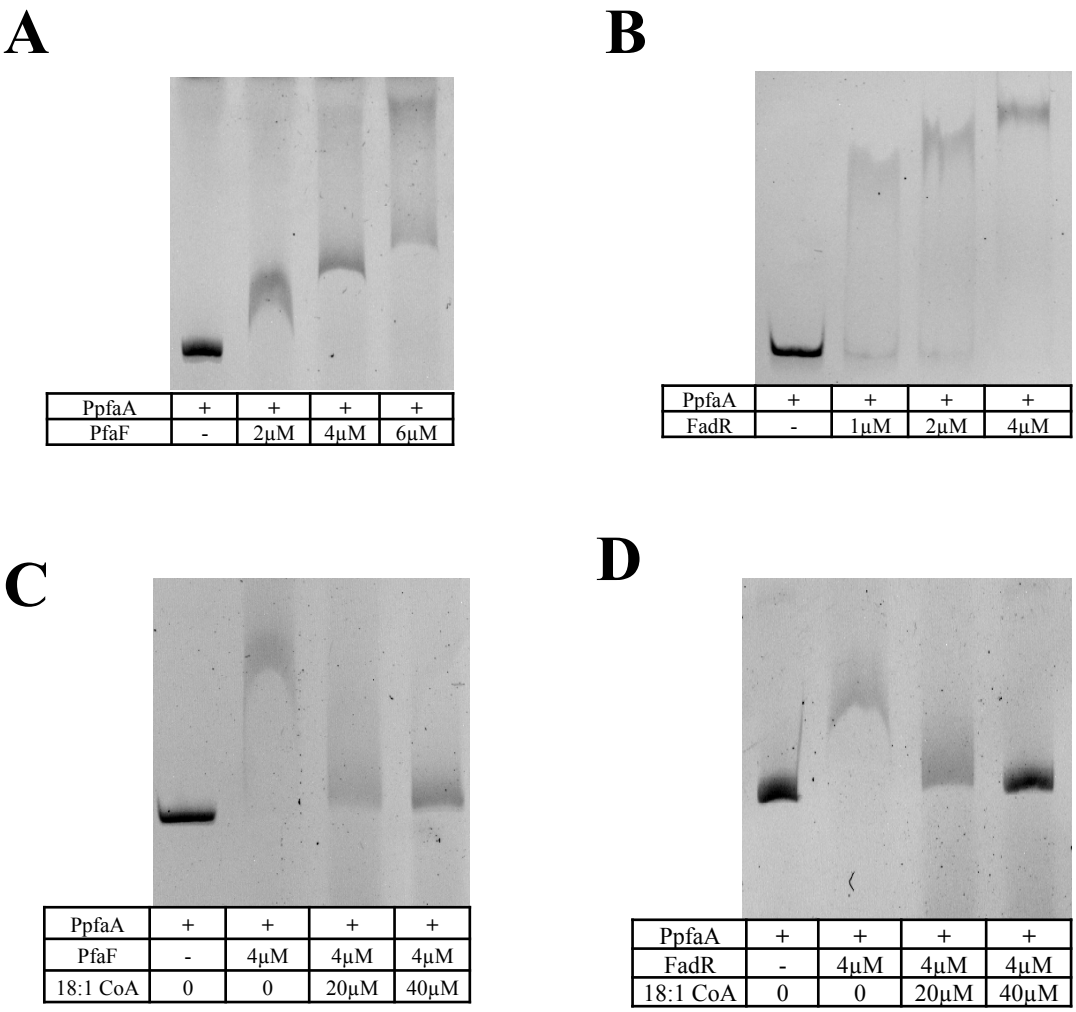


Figure 3.5- Both FadR and PfaF directly bind to pfaA promoter (PpfaA) and binding of both proteins is reversed upon addition of oleoyl-CoA in mobility shift assays. The concentrations of proteins and of added ligand in each reaction are given below each gel image. (A) PfaF binding to the *pfaA* promoter. (B) FadR binding to *pfaA* promoter. Release of PfaF (C) or FadR (D) from *pfaA* promoter probe upon the addition of oleoyl-CoA at indicated concentrations.

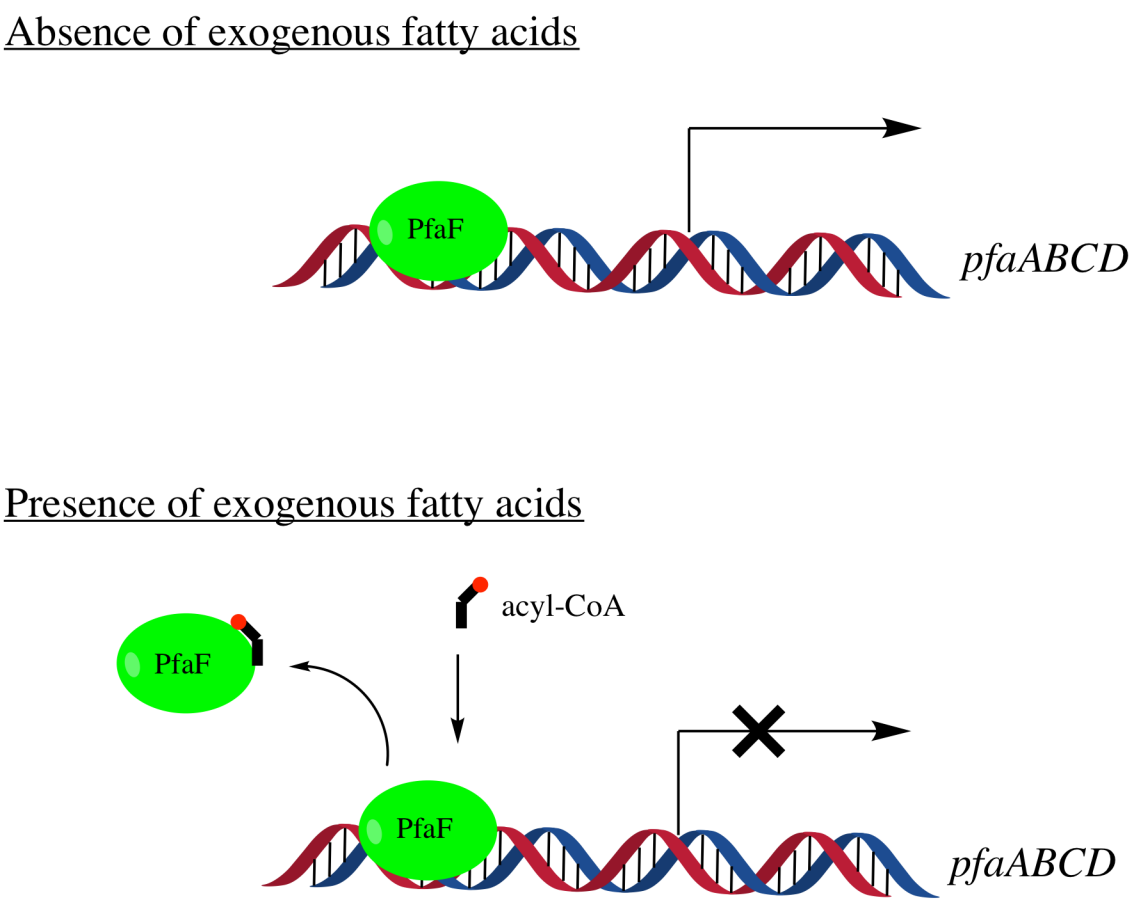


Figure 3.6- Proposed model of regulation of the *pfa* operon as mediated by PfaF. In the absence of fatty acids PfaF binds to the *pfaA* promoter and acts as a positive regulator. In the presence of exogenous fatty acids PfaF binds to acyl-CoA and releases from the promoter region leading to down regulation of the *pfa* operon.

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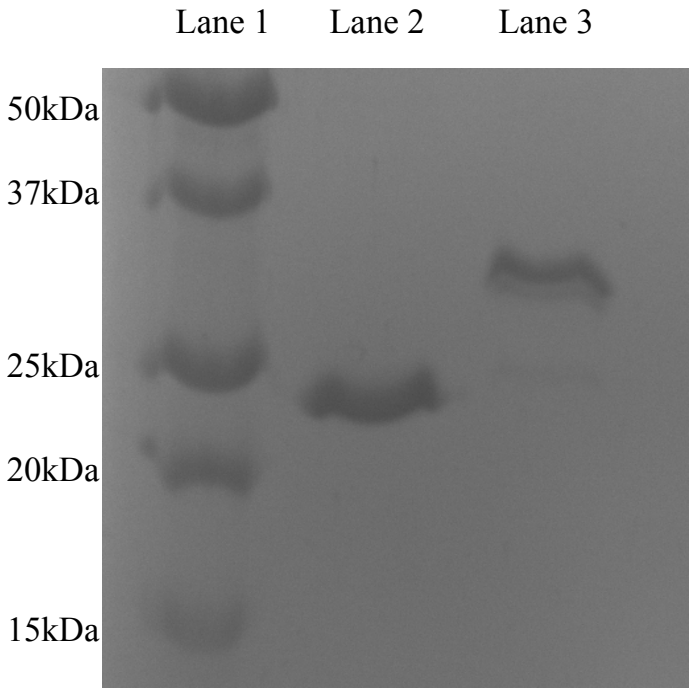
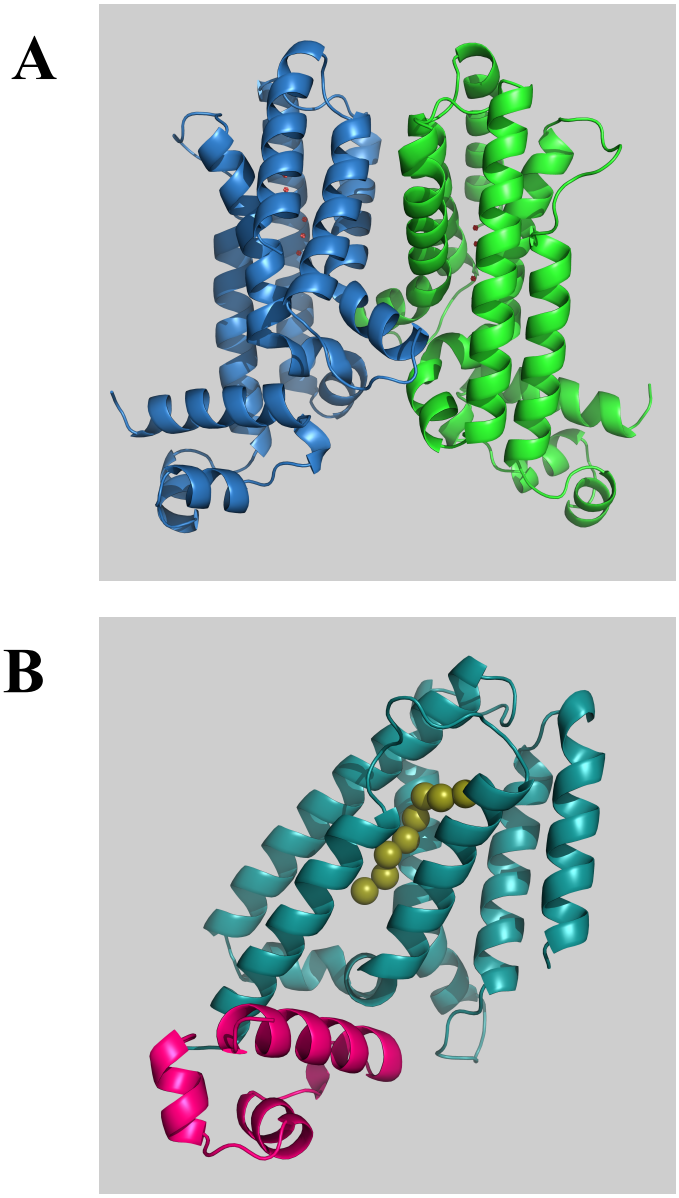


Supplemental Figure 3.1- 12% SDS-PAGE of purified transcriptional regulator proteins used in mobility shift assays. Lane 1, Protein standards. Lane 2, purified PfaF (predicted MW: 25.9kDa). Lane 3, purified FadR (predicted MW: 34.8kDa).



Supplemental Figure 3.2- Structure of the PfaF homolog from *S. amazonensis* (PDB: 3rh2). (A) Structure of PfaF dimer. (B) Detail of PfaF monomer with DNA binding domain colored pink and ligand binding domain in teal. Unknown ligand shown as olive green spheres.

Supplemental Table 3.1- Primers used in this study[a]

ΔlacZ SS9R 5'O	tataggagctcGTGAAGAAACATGTGAAGGG
ΔlacZ SS9R 5'I	gactggcttaggtcgtctggcGCGTCGTTGGATAATGTCTG
ΔlacZ SS9R 3'I	gccagacgacctaagccagtcGCCCCGTCACAACTAAGTAT
ΔlacZ SS9R 3'O	acgatcggtaccGCCATATCGATCGTAGAGTT
lacZ del ver fwd	GGTACCAACAATCTCGTGAA
lacZ del ver rev	CTGTCGCATTCACTCGCTTT
ΔpfaA::lacZY 5'O	tataggagctcTGGCTTTTAGAGCGTTTTCC
pfaA::lacZY 5'I	gatccccatggTCTCCGCTTAACACCCAAGA
pfaA::lacZY 3'I	gatccgcggccgcGTGAAGTCTGAGCCAGAGAA
ΔpfaA::lacZY 3'O	acgatcggtaccTGCCAGGGTATACAGTACCA
lacZY NcoI F	gatccccatggctATGACCATGATTACGGATTC
lacZY NotI R	gatccgcggccgcGCTTAAGCGACTTCATTCAC
pRL27ExtL	AACAAGCCAGGGATGTAACG
pRL27ExtR	TGGCTCCCTCACTTTCTGG
pRL27IntL	CGCACTGAGAAGCCCTTAGA
pRL27IntR	CAGCAACACCTTCTTCACGA
SS9arb1	GACCACGAGACGCCACACTNNNNNNNNNNCATGC
SS9arb2	GACCACGAGACGCCACACTNNNNNNNNNNACTAG
SS9arb3	GACCACGAGACGCCACACTNNNNNNNNNNGATAT
Arb clamp	GACCACGAGACGCCACACT
ΔfabR 5'O	cgatcggtaccTCACAACATCTTGAGCGTGA
ΔfabR 5'I	GCCAGACGACCTAAGCCAGTCTGTTGAGCCCTAATCCCCAT
ΔfabR 3'I	GACTGGCTTAGGTCGTCTGGCGCTCAGGCATAAACTATAACC
ΔfabR 3'O	tataggagctcTGCAAGGCTATGAACGTATT
ΔfabR ver F	CTCGAACGGTATGAGCATCA
ΔfabR ver R	ACGTCGATCTACAGAGCTAC
ΔfadR 5'O	cgatcggtaccACCGATAGCAACAGCTTCAA
ΔfadR 5'I	GCCAGACGACCTAAGCCAGTCGGCTGTCAGCCTTTATAACC
ΔfadR 3'I	GACTGGCTTAGGTCGTCTGGCACGAAGAAGAGCAGTAACTG
ΔfadR 3'O	tataggagctcTGGAGTTCCTCCTCAACCAT
ΔfadR ver fwd	CAGCAGAACCTTAGAGCGAA
ΔfadR ver rev	GGCATTACACCATCATGAAC
PpfaA fwd	AACTGGTCTTAAGTGATCCAACC
PpfaA rev FAM	TCTCCGCTTAACACCCAAGA
pET28 pfaF fwd	cgatccatgggcATGAAGACCCGCGATAGA
pET28 pfaF rev	cgatctcgagGGTTAGATCGTGTTGTACTTG
pET28 fadR fwd	cgatcgctagcATGGTTATAAAGGCTGACAGC
pET28 fadR rev	cgatcctcgagcagttactgctcttcttcgt

pFL122 pfaF F	tgactggtaccCCACTCTGTCGTCGCTGAAC
pFL122 pfaF R	acgatcgaattcCCGCTAGAAGAAGCTGCTAG
pFL122 fadR F	cgatcggtaccACCGATAGCAACAGCTTCAA
pFL122 fadR R	GGCATTACACCATCATGAAC
pMUT100 pfaF F	acgatggatccGATGAGTACGCCCGAGATAT
pMUT100 pfaF R	tacgatgtcgacGCACTTGAGTGGTTTGGTAG
pfaA qPCR fwd	GCGCCCAGAAGATTATTACG
pfaA qPCR rev	CAAGAATGTTAGGCGGAAGC
pfaD qPCR fwd	ATGGCTGATGTTGCAATGGC
pfaD qPCR rev	AGCGCGCATTGGGAATAATG
gyrB qPCR fwd	TGCTGGCATGAAGCTGATTG
gyrB qPCR rev	TTCAACGCAGCGACTAACTG
fabA qPCR fwd	AGGCTTCATTCATGCGGAAC
fabA qPCR rev	TGCCACATTGCATCTAAGCC
fabB qPCR fwd	TCGCAACAAAGGTGTGAAGC
fabB qPCR rev	ACGCCACGGATTTTAAACGG

[a] Restriction sites underlined

Supplemental Table 3.2- Fatty acid profiles of various strains containing pMA65 (pFL122 *fadR*) grown at 15°C

	MAP13 pMA65[a]	**MAP13 pFL122[a]**	**SS9R pFL122[a]**	**SS9R pMA65[a]**
12:0	3.59 ± 1.00	4.48 ± 0.36	4.59 ± 0.28	3.02 ± 0.17
14:0	9.36 ± 0.05	14.94 ± 1.68	4.75 ± 0.12	9.94 ± 2.73
14:1	6.14 ± 0.28	10.17 ± 1.49	2.64 ± 0.27	8.41 ± 0.95
16:0	29.60 ± 1.10	31.48 ± 0.38	27.45 ± 0.62	18.74 ± 0.83
16:1	38.82 ± 0.27	32.43 ± 1.74	43.15 ± 0.15	45.08 ± 1.57
12-OH	3.79 ± 0.49	2.98 ± 0.12	2.23 ± 0.26	2.52 ± 0.55
18:0	0.85 ± 0.02	0.48 ± 0.17	0.52 ± 0.08	0.96 ± 0.36
18:1	2.81 ± 0.19	1.23 ± 0.61	9.25 ± 0.46	0.96 ± 2.87
20:5	5.03 ± 0.78	1.82 ± 1.18	5.42 ± 0.63	3.10 ± 0.58

[a]Data are the mean of at least three independent replicate cultures ± standard deviation

Chapter 3, in full, has been submitted for publication of the material. Allemann M. N., Allen E.E., “Genetic regulation of the bacterial omega-3 polyunsaturated fatty acid biosynthesis pathway”. The dissertation author was the primary investigator and author of this material.

Chapter 4

Genetic suppression of lethal mutations in fatty acid biosynthesis mediated by a secondary lipid synthase

Abstract

The incorporation of monounsaturated (MUFA) and/or polyunsaturated fatty acids into phospholipid membranes has been recognized as unique feature amongst gamma-proteobacteria inhabiting high-pressure/cold temperature environments. The monounsaturated and saturated fatty acids found in the membrane are produced via the classical dissociated Type II fatty acid synthase mechanism. In contrast, polyunsaturated fatty acids such as EPA (20:5*n*-3) and DHA (22:6*n*-3) are produced by a hybrid polyketide/fatty acid synthase. In this work, we show that phenotypes associated with partial or complete loss of MUFA biosynthesis can be compensated for by several-fold increased production of PUFA in *Photobacterium profundum* SS9. One route to suppression of these phenotypes can be achieved by transposition of insertion sequences (IS) within or upstream of the *fabD* coding sequence. Further genetic experiments in this strain indicated that *fabD* is not an essential gene and mutations in *fabD* and *pfaA* are synthetically lethal. Based on these results we speculated that the malonyl-CoA transacylase (MAT) domain within PfaA compensates for loss of FabD activity. Heterologous expression of *pfa* operon from *P. profundum* SS9 and *Shewanella pealeana* in a *fabD* mutant of *E.coli* complemented the temperature sensitive phenotype at the non-permissive temperature.

Introduction

In many cultured lineages of marine gamma-proteobacteria, predominantly from the marine environment, a hybrid polyketide/fatty acid synthase (FAS) pathway exists for the biosynthesis of various long chain polyunsaturated fatty acids (PUFA), in particular eicosapentaenoic (EPA, 20:5*n*-3) and docosahexaenoic (DHA, 22:6*n*-3) acids (1, 2). The genes required for the biosynthesis of these PUFA are designated *pfaABCDE* and have been well

characterized in terms of their distribution and more recently at the enzymatic level (3–5). Typically, these strains produce either EPA or DHA as a small percentage of total fatty acids (~5-10% of total fatty acid content) (1, 6, 7), with the remaining content being a mixture of saturated (SFA) and monounsaturated (MUFA) fatty acids. Numerous studies have also demonstrated that the proportion of PUFA incorporated into phospholipids increases in response to culturing parameters such increased hydrostatic pressure and/or lowered temperature (7–12).

To date, all known bacterial strains that produce PUFA using this Pfa synthase pathway also have a prototypical Type II fatty acid synthase (FAS) similar to that found in the well-studied model *Escherichia coli* (13–15). As shown in Figure 1, SFA and MUFA are synthesized by the Type II FAS, which utilizes the same malonyl-CoA precursors as the Pfa synthase (3, 13, 16) and the acyl products of both pathways are destined for incorporation into phospholipid (17–19). Given the presence of both pathways in these native PUFA producing bacteria, an interesting question arises as to how these pathways interact with one another to maintain membrane homeostasis. Furthermore, each pathway retains the full complement of enzyme activities required for the biosynthesis of fatty acids encoded for by discrete genes (Type II FAS) or by domains within multi-domain genes (Supplemental Figure 1).

Given the presence of two discrete pathways for biosynthesis of unsaturated fatty acids, we hypothesized that genes critical for the biosynthesis of MUFA could be disrupted in the native producing organism and that the PUFA produced by the Pfa synthase could effectively compensate for the loss of MUFA biosynthesis. Previous work utilizing the fatty acid biosynthesis inhibitor cerulenin (7, 20), or genetic disruption of *fabF* (9), had indicated that compensatory increases in PUFA content compensated for loss of MUFA biosynthesis in *Photobacterium profundum* SS9. Additionally, transposon screening for pressure in *P.*

profundum SS9 had revealed that *fabB* was not an essential gene and that its disruption led to a pressure sensitive phenotype (21). Taken together, these results indicated that the products of either pathway could satisfy the cellular demand for unsaturated phospholipids.

To gain greater insight into the interaction of these two fatty acid pathways we engineered mutants of *P. profundum* SS9 with varying levels of MUFA deficient phenotypes and assessed their growth under a variety of conditions. As expected, these strains displayed pressure sensitive growth as well as an auxotrophic requirement for exogenous unsaturated fatty acids. Unexpectedly, extended incubation of these strains led to the appearance of suppressor strains, which no longer required exogenous UFA for growth, and had growth phenotypes similar to that of wild-type *P. profundum* SS9. We isolated and characterized these suppressor strains and subsequently re-sequenced their genomes to determine the genetic basis for this suppressor phenotype. Of the four suppressor strains characterized, three contained mutations resulting in either impaired transcription or loss of function of *fabD,* which encodes for the malonyl-CoA transacylase of the Type II FAS. Further experiments demonstrated that *fabD* is not an essential gene in *P. profundum* SS9, and that the Δ*fabD* mutation is synthetically lethal with mutations in *pfaA,* which contains a homologous FabD domain. We further show that a conditionally lethal mutation in *fabD* of *E.coli* can be complemented by heterologous expression of Pfa synthases from *P. profundum* SS9 and *Shewanella pealeana.*

Results

Fatty acid biosynthesis genes in *P. profundum* SS9

In depth analysis of the previously published genome of *Photobacterium profundum* SS9 led to the characterization of genes associated with a typical bacterial Type II FAS similar to that

found in *Escherichia coli.* A full list of predicted fatty acid biosynthesis genes is given in Supplemental Table 2. In addition to the canonical Type II FAS genes, *P. profundum* SS9 also contains a Type I FAS/polyketide synthase that is responsible for the biosynthesis of the polyunsaturated fatty acid EPA (20:5*n*-3), which is incorporated into phospholipids alongside the acyl products from the Type II FAS. *P. profundum* SS9 contains both *fabA* and *fabB* homologs, which are presumably involved in the biosynthesis of monounsaturated fatty acids. The genome also contains *desA,* a membrane-bound oxygen dependent desaturase, which has been shown to be involved in the production of monounsaturated fatty acids in other gamma-proteobacteria (30–32). Another interesting feature present is a cluster of fatty acid genes present on Chromosome II, which bears a striking resemblance to the O138 genomic island present in pathogenic strains of *E.coli* (33, 34).

Phenotypes associated with loss of MUFA related genes

Given the presence of the Pfa synthase, we predicted that genes related to MUFA biosynthesis in SS9 would be non-essential. Previous work in this strain had already demonstrated that *fabB* was not an essential gene unlike the case for *E.coli* (21). We generated several in-frame deletion strains, which had varying loss of function phenotypes in MUFA biosynthesis. Specifically, we constructed MAP10 (Δ*fabB*) and MAP29 (Δ*fabA*Δ*desA*) mutants (Table 1).

The growth phenotypes of MAP10 (Δ*fabB*) and MAP29 (Δ*fabA*Δ*desA*) under various culture conditions are shown in Figure 2. The MAP10 (Δ*fabB*) strain was capable of limited growth under aerobic conditions (Figure 2A) but displayed virtually no growth in the micro-aerobic bulbs used in pressure experiments (Figure 2C and Figure 2E). Conversely, MAP29 (Δ*fabA*Δ*desA*) displayed no growth under all conditions. It should be noted that the finding that a

fabB mutant was capable of limited growth is consistent with a previous result indicating that *fabB* was not an essential gene in *P. profundum* SS9 (21). Supplementing growth media with 18:1 fatty acid in the form of Tween 80, which has been shown to be an effective means of delivering fatty acids to SS9 (7), fully or partially complemented the growth phenotypes of both mutants (Figure 2B, D, F). The limited growth of strain MAP10 allowed for fatty acid analysis of the strain grown at 15°C and the results of these analyses are shown in Table 2. This strain displayed the expected phenotype of severely limited amounts of MUFA and an approximate 4-fold increase in EPA (20:5) content relative to the wild-type strain. Also of note, were the relatively increased proportions of myristic (14:0) and palmitic (16:0) fatty acids found in this strain relative to SS9R.

As seen in Figure 2, mutant MAP29 (Δ*fabA*Δ*desA*) displayed a strict auxotrophic requirement for exogenous UFA to grow. Under all conditions tested, MAP29 displayed little to no growth unless supplemented with Tween 80 (18:1).

Suppressor mutations in *fabA/B* mutant strains

During the course of our work with these mutants we observed that extended incubation (>2 weeks) of these cultures under a variety of conditions led to prominent growth similar to that of the parental strain SS9R. Serial dilution and plating of these cultures onto solid media indicated the presence of smaller and larger sized colonies. Upon isolation of both colony types it was noted that the smaller sized colonies retained an UFA auxotrophy phenotype while the larger sized colonies grew equally well on UFA supplemented and non-supplemented medias (data not shown). This phenomenon indicated the possibility of second site suppressor mutations that allow for growth in the absence of MUFA biosynthesis. Given our interest in the interplay between MUFA and PUFA biosynthesis in this strain and in particular, how both processes are

affected by parameters such as hydrostatic pressure, we setup a genetic selection scheme outlined in Figure 3A to isolate suppressor mutants from a variety of genetic backgrounds. These mutant backgrounds were chosen due to their varying degree of impairment in MUFA biosynthesis described in the previous section. Given the limited growth of both strains at high-pressure, this condition was chosen for isolation of suppressor mutants. In all, we isolated two independent suppressor strains from each genetic background and subjected them to detailed analyses as described below.

Growth curves of four suppressor strains at high and low pressure and in standard aerobic tubes are presented in Figure 3B-G and indicate that all four strains are capable of growth under conditions which did not support growth of their respective parental strain. Comparing these suppressor growth profiles also indicates that these strains are capable of growth similar to SS9R under the conditions tested. Fatty acid profiles of these strains grown under these conditions are presented in Table 3 and Table 4. One of the universal features shared amongst these strains are the increased amounts of EPA found in phospholipids. With the exception of MAP1002 all of these suppressor strains displayed 3 to 8-fold higher amounts of EPA (20:5) relative to SS9R. It was also noted that MAP10 (Δ*fabB*) suppressors (MAP1002, 1003) were capable of limited MUFA biosynthesis whereas both MAP29 (Δ*fabA*Δ*desA*) suppressors completely lacked any MUFA biosynthesis under all conditions. Intriguingly, some of these suppressors produced significant amounts of DHA (22:6), which had never been observed in *P. profundum* SS9 previously.

Identification of suppressor mutations

Based on the phenotypic data collected, we subjected these suppressor mutant strains to further genetic characterization by genome resequencing. To identify mutations of interest, we

sequenced the genomes of these suppressors using Illumina sequencing technology. After barcode trimming and quality filtering, reads were mapped to the SS9 genome using the breseq pipeline (29). Coverage depth ranged from ~70 to ~250-fold and >99% of the filtered reads mapped to the *P. profundum* genome. As expected, the breseq pipeline identified the *fabB*, *fabA* and *desA* deletion mutations in the respective suppressor strains.

Among the mutations that were found in these suppressors, we noted the presence of insertion sequences, which had been integrated upstream or within the coding sequence of *fabD.* Further homology searches of these IS elements revealed that they resemble an IS66 type sequence found in many gamma-proteobacteria (35). The diagram shown in Figure 4A indicates the relative positions of these IS66 sequences within the *fabHDG* operon. The IS66 element in MAP1003 (-101 to -93) and MAP2902 (-107 to -115) were located at positions upstream of the *fabD* initiation codon. Conversely, the IS66 element in MAP2903 was located within the coding sequence (+129 to +137) of *fabD.* Targeted PCR analyses on EA2, a chemical derived mutant of *P. profundum* SS9, which greatly overproduces EPA while under producing MUFA (7), also revealed a similar integration within the *fabD* coding sequence (+84 to +91). Particularly noteworthy in this context was finding that these mutations in *fabD* were found in suppressors derived from both MAP10 (Δ*fabB*) and MAP29 (Δ*fabA*Δ*desA*) genetic backgrounds.

FabD is not essential in SS9

To date, *fabD* has been considered to be an essential gene in bacteria due its role in producing malonyl-ACP, the substrate used by the various ketosynthases for every round of elongation of fatty acid biosynthesis (13, 36). To confirm that *fabD* is indeed a non-essential gene in SS9R, we attempted to generate an in-frame deletion mutant of *fabD* in SS9R. Much to our surprise, we readily generated this strain, designated MAP37, and this mutant had identical

growth patterns as SS9R under a variety of culture conditions (data not shown). Fatty acid analyses of MAP37, showed that besides the ~2-fold increase in EPA content its fatty acid profile was remarkably similar to that of SS9R (Table 2). Given the locations of IS66 elements directly upstream of *fabD,* it was suspected that these mutations might alter transcription of *fabD.* Quantification of *fabD* transcripts in both MAP1003 and MAP2903 indicated that both strains had several fold decreases in *fabD* transcript abundances relative to SS9R (Figure 4B).

PfaA MAT domain can replace FabD function in vivo

A comprehensive search of the SS9 genome revealed that the MAT domain in PfaA (Figure 1) is the only other protein with the genome of *P. profundum* SS9 that contains the features ascribed to malonyl transacylase (MAT) activity (37, 38). Therefore, it was hypothesized that the MAT domain found within PfaA can replace FabD function *in vivo.* To this end, we attempted to construct a mutant deficient in both *pfaA* and *fabD.* Despite numerous attempts to recover exconjugants in a variety of genetic backgrounds, we were unable to generate mutants with null mutations in both *fabD* and *pfaA* (Figure 4C). Conversely, we were able to routinely recover exconjugants in genetic crosses targeting *pfaD* utilizing the same recipient strain cultures. Given these results we concluded that *fabD* and *pfaA* form a synthetical lethal pair in *P. profundum* SS9.

To further verify this inter-pathway complementation, we attempted complemention of the well-characterized *fabD* mutant strain LA2-89, which contains a temperature-sensitive *fabD89* allele. As shown in Figure 4D, introduction of the previously cloned Pfa synthases from *Shewanella pealeana* (39) and *P. profundum* SS9 (40) complemented this mutation at the non-permissive temperature of 42°C. This complementation occurs in the absence of EPA synthesis as we failed to observe any heterologous EPA production at 42°C from 1F12R (data not shown).

Furthermore, the pFOS8E1 construct lacks the specific phosphopantetheinyltransferase (*pfaE*) required for producing EPA under any condition (40). PCR amplification and sequencing of the *fabD89* allele in strain LA2-89 confirmed that both strains retained the *fabD89* allele and that growth was not due to reversion of the *fabD89* allele. (data not shown).

Other suppressor mutations of interest

Of the high pressure derived suppressor strains obtained, MAP1002 caught our attention due to its unique fatty acid profile which contained increased amounts of palmitoleic acid (16:1) despite the absence of FabB. Re-sequencing of this mutant revealed a non-synonymous mutation (M77T) in *fabA,* the dual-function dehydratase/isomerase, which is primarily responsible for anaerobic generation of MUFA in most Gram-negative bacteria (30, 41, 42). Based on sequence alignments, this methionine residue is highly conserved amongst FabA homologs and is in close proximity to the FabA active site residue. As shown in Supplementary Figure 3, both $fabA^{M77T}$ and $fabA^{WT}$ were cloned onto pFL122 and based on growth; both complemented the Δ*fabA* mutation in the *E.coli* strain MAE30 (Δ*fabA*) equally well.

Given the lack of FabB in MAP1002, another ketosynthase enzyme might substitute for FabB activity in *P. profundum* SS9. A similar study performed in *Shewanella oneidensis* MR-1, indicated that suppression of Δ*fabB* occurred by increased transcription of *fabF* (31). To see if *fabF* from SS9R could indeed replace FabB activity, we generated a Δ*fabB* mutant of *E.coli* (MAE20) and introduced pEA44, a previously generated construct that contains *fabF* from SS9 (9). Based on growth on solid media, both FabB and FabF from *P. profundum* SS9 could complement the Δ*fabB* mutation in *E.coli* (Supplementary Figure 3).

Discussion

In this study, we utilized a genetic approach to gain insights into the metabolic interactions between the two fatty acid biosynthesis pathways in *Photobacterium profundum* SS9. Based on its genome sequence, *P. profundum* SS9 contains the full complement of Type II FAS genes required for the biosynthesis of SFA and MUFA. At the genome level it is interesting to note that in some cases multiple homologs of particular *fab* genes or genes with overlapping activities are present. One such example is the presence of multiple FabH homologs along with a FabY homolog. In *Escherichia coli,* FabH performs the initial condensation of malonyl-ACP and acetyl-CoA, which initiates the reaction cycle of fatty acid biosynthesis (13, 14). In *Pseudomonas aeruginosa,* the first condensation reaction is carried out by an alternate ketosynthase FabY, and the various *fabH* homologs present in the strain are utilized for the production of signaling molecules or for shunting shorter chain fatty acids into the Type II FAS pathway for elongation (43, 44).

Given the presence of the Pfa synthase and its role in producing EPA in this strain, we predicted that loss of MUFA biosynthesis via genetic disruption of either *fabA/desA* or *fabB* would not be lethal. While genetic disruption of either set of genes proved to be possible, the resulting strains displayed varying degrees of auxotrophy for unsaturated fatty acids. As expected from previous work (7), providing exogenous oleic acid in the form of Tween 80 (18:1) reversed this growth impairment. While our work has not addressed how the fatty acid component of Tween 80 (18:1) is incorporated into phospholipids, we have noted the presence of a homolog of an outer membrane lipase, designated *volA,* which was previously characterized in *Vibrio cholerae* (45). VolA was shown to hydrolyze exogenous esterified fatty acids such as lysphosphatidylcholine and the resulting free fatty acids are then imported via the classical fatty

acid degradation (*fad*) pathway and directly incorporated into phospholipid or utilized as a carbon source via β-oxidation.

As described previously (7), loss of MUFA biosynthesis led to unsaturated fatty acid auxotrophy and pressure sensitive growth in SS9. The fatty acid profile of MAP10 (Δ*fabB*) is consistent with FabB acting as the ketosynthase responsible for the majority of MUFA biosynthesis in *P. profundum* SS9. The small amount of MUFA remaining in the fatty acid profile indicates that a bypass for FabB function exists in *P. profundum* SS9. One possibility is that FabF, another ketosynthase, can directly substitute for FabB function. During the course of our work, this phenomenon was observed in *Shewanella oneidensis* MR-1 (31). Another possibility is that the DesA, an oxygen dependent desaturase, acts upon saturated acyl chains within the membrane to generate the MUFA content observed. The differences in growth of MAP10 (Δ*fabB*) observed in aerobic versus micro-aerobic conditions is consistent with the possibility that DesA could be involved in this bypass mechanism. Previous work in *P. aeruginosa* PAO1 has indicated that both FabB and/or FabA function can be bypassed in this manner (30).

One of the other possible routes of suppression we noted was in the strain MAP1002, which contains the $fabA^{M77T}$ allele. While the effect of this mutation has not been extensively explored in this work, our results in the heterologous host *E.coli* showed that the $FabA^{M77T}$ variant produces more MUFA relative to $FabA^{WT}$ of *P. profundum* SS9. One possible scenario is that the $FabA^{M77T}$ has altered isomerase activity, which allows the remaining ketosynthase FabF, to elongate the cis-decenoic (10:1) substrate in the absence of FabB. The finding that FabF of *P. produndum* SS9 can partially complement the loss of *E.coli fabB* is consistent with this idea. While these results cannot definitively rule out the activity of DesA, the increased amounts of

MUFAs in MAP1002 relative to MAP10 (Δ*fabB*), even in micro-aerobic bulbs, is suggestive of at least a partial increase in the anaerobic capacity to produce MUFA.

Extended incubation of these fatty acid auxotroph strains at high pressure led to suppression of this unsaturated fatty acid auxotrophy and growth at high pressure. Isolation and phenotypic characterization of these high pressure derived suppressor strains indicated that various degrees of increased EPA production was a common phenotype amongst these strains. The lack of MUFA biosynthesis in these strains (MAP1003, 2902, 2903) also indicates that high levels of EPA (>15% total fatty acid) are required to compensate for loss of MUFA biosynthesis. Consistent with this finding was that genetic disruption of the Pfa synthase proved to be impossible in all four suppressor strains described here. Given that three of the four suppressor strains analyzed contained mutations mapping to the *fabD* locus, FabD is theorized to play an important role in how the Pfa synthase and Type II FAS interact with one another. Presumably, loss of FabD would render more malonyl-CoA available to the Pfa synthase and allow for the increased levels of EPA production observed in these suppressor strains. Comparing the fatty acid compositions of MAP37 (Δ*fabD*) and SS9R indicates that EPA production can be doubled with only mild perturbations of SFA and MUFA content, indicating that availability of malonyl-CoA might be a factor in determining how much EPA can be produced by the Pfa synthase.

The finding that *fabD* is dispensable in *P. profundum* SS9 marks the first demonstration that this gene is not essential for the growth and viability of a bacterium. Our genetic experiments indicate that the MAT domain found in PfaA is responsible for bypassing the loss of *fabD*. Heterologous complementation of the temperature sensitive *fabD89* allele in *E.coli* using cloned Pfa synthases from both *P. profundum* SS9 and *Shewanella pealeana,* supports this model. During the course of our work, the MAT domain of PfaA was shown to load malonyl

units onto the adjacent ACP domains within PfaA (3). EPA production is not responsible for the complementation in *E.coli* and lends further support to the notion that the MAT domain within PfaA is capable of transferring the malonyl group to the ACP domains in PfaA and the discrete ACP of the Type II FAS. FabD may not be essential in other bacteria containing a Pfa synthase or a homologous secondary lipid synthase. A search of results from a high throughput transposon screen (46) has indicated that *fabD* is not essential to *Shewanella loihica* PV-4, which contains a Pfa synthase (10, 15) and presumably produces EPA. Homologs of the Pfa synthase with uncharacterized products have been noted in numerous distinct lineages of bacteria, and the majority of these synthases contain an embedded MAT domain (15). The findings here are also reminiscent of previous findings in *Streptomyces glaucescans* where the relaxed specificity of FabD allowed for malonyl-CoA loading of both Type II FAS and a Type II polyketide synthase (47).

Materials and Methods

Bacterial strains and growth conditions

Escherichia coli strains were routinely grown at 37°C in Luria Bertani (LB) media unless stated otherwise. *Photobacterium profundum* SS9 strains were grown at 15°C in 2216 Marine broth (Difco) at 75% strength (28g/L). For solid medias, agar was included at 15g/L. The antibiotics kanamycin (50 μg/ml for *E. coli* and 200 μg/ml for *P. profundum*), chloramphenicol (15μg/ml), ampicillin (100μg/ml), and rifampicin (100μg/ml) were used as required. Fatty acid auxotroph strains were grown in medias supplemented with 0.05% Tween 80 as a source of fatty acids. Liquid cultures were maintained in standard glass test tubes in a shaking incubator at 15°C. For pressure related growth experiments, *P. profundum* SS9 strains were grown in heat-

sealed bulbs and incubated in stainless steel pressure vessels as described previously (22). For growth in heat-sealed bulbs, Marine broth was supplemented with 0.4% glucose and 100mM HEPES (pH 7.5).

Mutagenesis in *P. profundum* SS9

In-frame deletions were generated by allelic exchange using the suicide vector pRE118. Briefly, upstream and downstream regions (~500-1000bp) of genes to be deleted were amplified by PCR with primers listed in Supplemental Table 1. PCR products were assembled via 20 base pair homologies using Gibson assembly (New England Biolabs) and cloned into indicated restriction sites of pRE118. Verified constructs were introduced into *P. profundum* strains by biparental conjugation using S17-*1λpir* following previously described protocols (1). Ex-conjugants were selected for on 2216 agar containing rifampicin and kanamycin at 15°C. To select for deletion mutants, ex-conjugants were grown in the absence of antibiotic selection and dilutions plated onto 2216 agar containing 5% sucrose and 0.05% Tween 80 (18:1). Colonies were screened for kanamycin sensitivity, sucrose resistance, and fatty acid auxotrophy. PCR and DNA sequencing using primers flanking the gene(s) of interest were used to confirm deletions. For insertional inactivation, internal fragments of genes to be disrupted were amplified by PCR and cloned into pMUT100 using standard molecular techniques (23). Derivatives of pMUT100 were mobilized into *P. profundum* SS9 using *E.coli* MC1061 containing pRK24 and pRL528 plasmids a described previously (24).

Mutagenesis of *Escherichia coli*

Gene knockouts of E.coli were performed using the lambda red recombineering as described previously (25). Primers used to generate these deletions are listed in Supplemental Table 1. To recover fatty acid auxotrophic mutants of *E.coli,* selections were performed on LB

antibiotic plates supplemented with 0.001% oleic acid (18:1) and 0.005% NP-40 to increase solubility of fatty acid. P1 transductions were used to move mutations into MG1655 following standard protocols (26).

RNA isolation and quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was isolated from mid-log phase cells grown under the indicated conditions using Trizol (Invitrogen) following manufacturer guidelines. Crude RNA extracts were further purified and treated with DNase I (Zymo Research) using the RNA Clean and Concentrator kit (Zymo Research). For cDNA synthesis, the Superscript III First Strand synthesis kit (Invitrogen) was used following manufacturer's recommended protocols. Quantitative PCR's were performed using the Maxima Sybr Green Master Mix (Thermo Scientific) and run on a Stratagene MX3000P qPCR system. For quantification of target transcripts, the *gyrB* gene (PBPRA0011) was used as an internal reference and differences in expression were calculated using the ΔΔCT method. Primers for qPCR experiments are listed in **Supplementary Table 1**.

Fatty acid analysis

Late log phase cultures were harvested by centrifugation and cell pellets rinsed once with 50% Sea Salts (Sigma) solution (16g/L) and stored at -80°C. Cell pellets were lyophilized and fatty acids were converted to fatty acid methyl esters and analyzed by gas chromatography mass spectrometry using previously described methods (1). Fatty acids were identified based on their retention times and mass spectra and compared to authentic standards when necessary.

Isolation of suppressor strains

Fatty acid auxotrophs were grown to late log phase aerobically in 2216 media supplemented with 0.05% Tween 80 (18:1) at 15°C. Cells were harvested by centrifugation and washed three times with 2216 broth to remove the fatty acid supplement. Washed cells were

inoculated into 2216 liquid media which was used to fill heat-sealed polyethylene bulbs and incubated at 30MPa in stainless steel pressure vessels as described previously. Growth was monitored visually by eye or by OD_{600} readings. Culture bulbs displaying high levels of growth were sterilely opened and serial dilutions were plated on 2216 agar. Larger sized colonies were clonally isolated by re-streaking onto 2216 agar plates and screening individual colonies for growth on agar with and without 0.05% Tween 80 (18:1). Growth on plates was assessed by eye and colonies that grew equally well on both medias were designated as putative suppressor strains and saved for further analysis.

Identification of suppressor mutations

High quality genomic DNA was isolated from suppressor mutants using a Wizard genomic DNA kit following manufacturer guidelines for Gram-negative bacteria. Genomic DNA was submitted to Core facilities at UC Davis for Kappa library construction. Genomic libraries were sequenced on a HiSeq4000 Illumina platform to generate paired-end or single-end 150bp reads. Reads were quality checked by fastqc version 0.11.3 (27) Reads that passed the initial quality control were mapped to the published SS9 genome sequence (28) using breseq version 0.33.2 (29) using default parameters. PCR and sequencing of purified products validated mutations of interest.

Table 4.1- Bacterial Strains and Plasmids used in this study

Strain	Characteristics	Source
Escherichia coli strains		
DH5α*pir*	*recA*⁻, Cloning strain used for R6K plasmids	(48)
MG1655	Wild-type *E.coli*	CGSC[a]
LA2-89	*fabD89* (ts)	CGSC[a]
MAE20	MG1655, Δ*fabB::kan*^{R}	This study
MAE30	MG1655, Δ*fabA::cm*^{R}	This study
Photobacterium profundum strains		
SS9R	Rifampin resistant derivative of *P. profundum* SS9	(22)
EA2	Chemically derived MUFA auxotroph	(7)
MAP10	SS9R, Δ*fabB*	This study
MAP16	SS9R, Δ*lacZ,* Δ*pfaA::lacZY*	Unpublished
MAP28	SS9R, Δ*desA*	This study
MAP29	MAP28, Δ*fabA*	This study
MAP1002	MAP10 high pressure derived suppressor	This study
MAP1003	MAP10 high pressure derived suppressor	This study
MAP2902	MAP29 high pressure derived suppressor	This study
MAP2903	MAP29 high pressure derived suppressor	This study
Plasmids		
pRE118	Suicide allelic exchange plasmid, R6K origin, Kan^R, *sacB*	(49)
pMUT100	Suicide plasmid insertional inactivation, Kan^R	(50)
pKT231	Broad host range plasmid, Kan^R, Sm^R	(51)
pFL122	Broad host range plasmid, Sm^R	(52)
pKD46	Arabinose inducible λRed functions, Amp^R	(25)
pKD3	Source of Cm^R cassette	(25)
pKD4	Source of Kan^R cassette	(25)
pCC2FOS	Fosmid cloning vector	Epicentre
1F12R	pCC2FOS w/*pfaABCDE* from *Shewenalla pealeana*	(39)
pFOS8E1	Fosmid clone w/*pfaABCD* from *P. profundum* SS9	(40)
pEA44	pKT231 containing SS9R *fabF*	(9)
pEA30	pMUT100 containing *pfaA* internal fragment	(7)
pEA101	pMUT100 containing *pfaD* internal fragment	(40)
pMA31	pRE118 containing SS9R Δ*fabB* allele	This study
pMA55	pFL122 containing SS9R *fabB*	This study
pMA56	pFL122 containing SS9R *fabA*^{M77T}	This study
pMA57	pFL122 containing SS9R *fabA*^{WT}	This study
pMA58	pRE118 containing SS9R Δ*fabA* allele	This study
pMA64	pRE118 containing SS9R Δ*desA* allele	This study
pMA71	pMUT100 containing SS9R *fabD* internal fragment	This study
pMA79	pRE118 containing SS9R Δ*fabD* allele	This study

[a]Coli Genetic Stock Center, Yale University

Table 4.2- Fatty acid profiles of SS9R, MAP10 (Δ*fabB*) and MAP37 (Δ*fabD*) at 15°C

Mean % fatty acid by weight[a]			
Fatty acid species	SS9R	MAP10 (Δ*fabB*)	MAP37 (Δ*fabD*)
12:0	4.16 ± 1.53	8.19 ± 0.14	2.98 ± 0.21
14:0	4.33 ± 1.21	22.32 ± 0.45	5.64 ± 0.54
14:1	3.19 ± 1.11	0.58 ± 0.50	1.85 ± 0.19
16:0iso	0.00 ± 0.00	0.88 ± 0.03	2.52 ± 0.73
16:0	21.07 ± 1.84	36.01 ± 0.82	28.06 ± 0.72
16:1	45.09 ± 1.68	3.39 ± 0.12	38.97 ± 0.48
12-OH	1.99 ± 1.23	3.01 ± 0.02	1.74 ± 0.56
18:0	0.71 ± 0.11	0.26 ± 0.22	1.07 ± 0.08
18:1	12.26 ± 2.26	0.00 ± 0.00	7.56 ± 0.10
20:5	4.52 ± 1.55	20.84 ± 1.67	9.60 ± 1.97
22:6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[a] Data represent the average ± standard deviations of triplicate samples

Table 4.3- Fatty acid profiles of MAP10 (Δ*fabB*) derived suppressors under indicated conditions [a]

	Mean % fatty acid by weight[a]					
	MAP1002			MAP1003		
Fatty acid species	15°C	0.1MPa[b]	30MPa[b]	15°C	0.1MPa[b]	30MPa[b]
12:0	7.68 ± 1.10	6.60 ± 0.83	4.21 ± 0.79	7.38 ± 0.68	6.48 ± 0.19	4.05 ± 0.14
14:0	14.32 ± 2.84	20.50 ± 1.27	9.35 ± 1.18	20.46 ± 3.32	16.81 ± 0.71	5.55 ± 0.98
14:1	1.69 ± 0.51	1.31 ± 0.51	1.18 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16:0iso	1.67 ± 0.62	0.26 ± 0.45	0.60 ± 0.53	1.00 ± 0.31	0.78 ± 0.68	0.55 ± 0.49
16:0	30.22 ± 3.36	50.12 ± 6.10	47.37 ± 4.54	33.54 ± 1.36	44.66 ± 0.85	40.82 ± 1.06
16:1	24.82 ± 3.03	8.05 ± 0.78	11.58 ± 1.32	15.40 ± 1.53	2.04 ± 0.65	3.71 ± 1.52
12-OH	2.93 ± 0.73	2.37 ± 0.91	2.56 ± 1.31	4.17 ± 0.85	3.66 ± 0.27	3.45 ± 0.08
18:0	0.98 ± 0.01	0.37 ± 0.32	1.25 ± 0.25	0.63 ± 0.08	0.50 ± 0.10	1.58 ± 1.46
18:1	1.45 ± 0.16	0.53 ± 0.45	2.15 ± 0.27	0.33 ± 0.29	0.00 ± 0.00	0.71 ± 0.60
20:5	14.25 ± 3.03	9.91 ± 3.35	19.76 ± 0.65	17.11 ± 0.32	24.95 ± 1.76	37.34 ± 0.87
22:6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.20	2.23 ± 0.16

[a] Data represent the average ± standard deviation of triplicate samples

[b] Pressure incubations were performed in heat-sealed bulbs at 15°C

Table 4.4- Fatty acid compositions of MAP29 (Δ*fabA*Δ*desA*) suppressors under indicated conditions

Fatty acid species	Mean % fatty acid by weight[a]					
	MAP2902			MAP2903		
	15°C	0.1MPa[b]	30MPa[b]	15°C	0.1MPa[b]	30MPa[b]
12:0	4.46 ± 0.14	3.52 ± 0.13	3.69 ± 0.11	4.09 ± 0.18	3.61 ± 0.22	3.90 ± 0.07
14:0	12.86 ± 1.26	19.38 ± 1.15	9.31 ± 0.57	11.91 ± 0.88	18.17 ± 0.83	9.29 ± 0.10
14:1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16:0iso	1.10 ± 0.16	0.42 ± 0.20	0.61 ± 0.24	1.06 ± 0.12	0.00 ± 0.00	0.95 ± 0.19
16:0	37.44 ± 2.14	41.85 ± 0.43	41.32 ± 1.60	37.40 ± 2.47	40.12 ± 0.40	39.07 ± 0.43
16:1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12-OH	3.39 ± 0.69	1.88 ± 0.18	2.21 ± 0.61	3.31 ± 0.61	1.02 ± 0.40	3.14 ± 0.41
18:0	2.95 ± 0.91	0.92 ± 0.02	0.67 ± 0.03	3.12 ± 0.53	0.88 ± 0.05	0.62 ± 0.02
18:1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:5	35.29 ± 1.77	30.82 ± 1.50	40.66 ± 2.48	35.96 ± 1.66	35.18 ± 0.47	41.86 ± 0.10
22:6	2.52 ± 0.74	0.65 ± 0.05	1.28 ± 0.29	3.16 ± 0.89	1.02 ± 0.11	1.17 ± 0.12

[a] Data represent the average ± standard deviation of triplicate samples
[b] Pressure incubations were performed in heat-sealed bulbs at 15°C

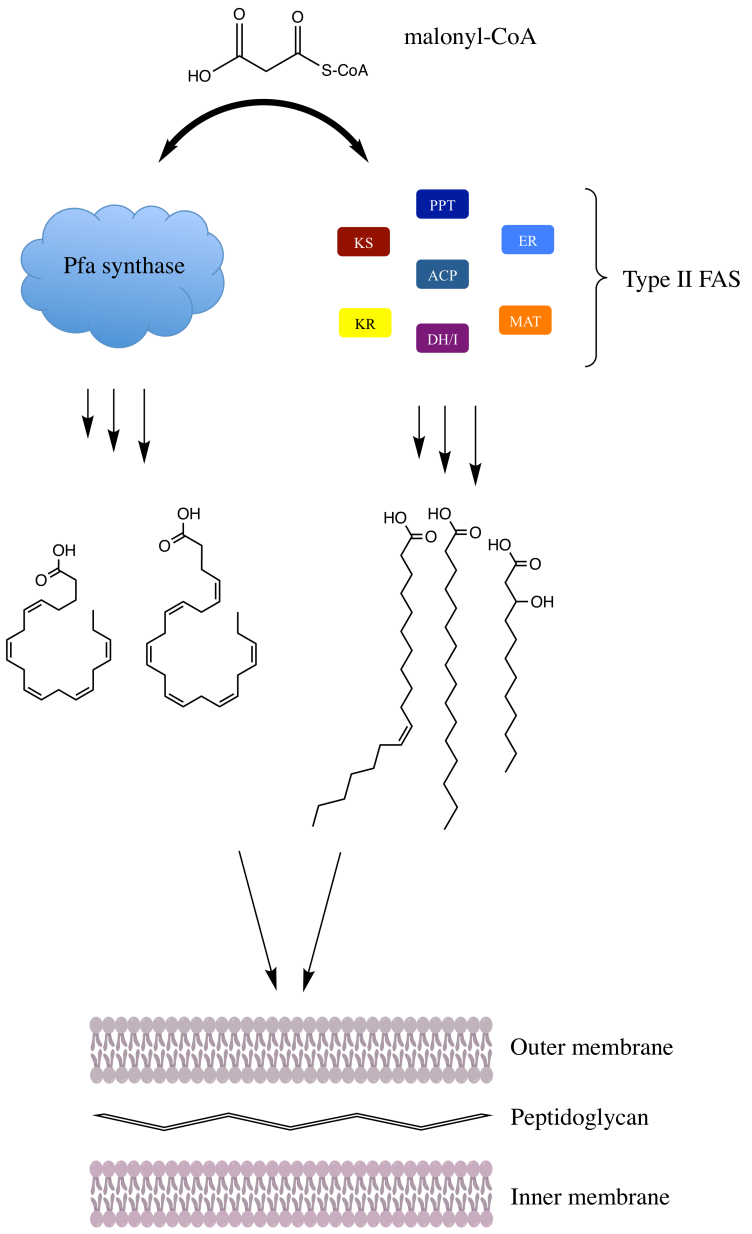


Figure 4.1- Model for interaction of Pfa synthase and Type II FAS in *P. profundum* SS9. Both the Pfa synthase and Type II FAS utilize malonyl-CoA for production of their respective products. End products of both pathways are incorporated into phospholipid membranes.

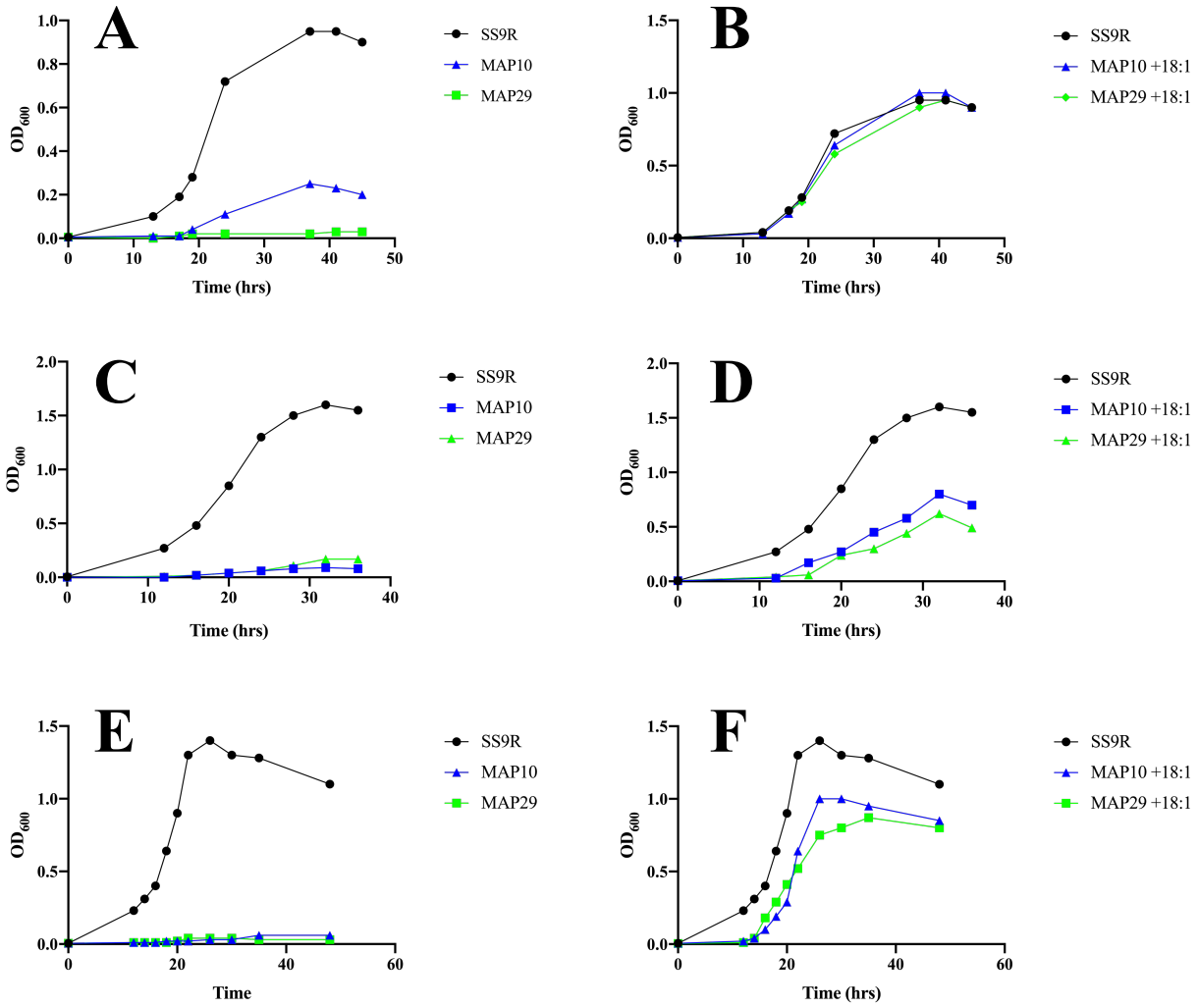


Figure 4.2- Growth phenotypes associated in MUFA gene mutants. Growth of MAP10 (Δ*fabB*) and MAP29 (Δ*fabA*Δ*desA*) strains is severely impaired at (A) 15°C, (C) 30MPa, and (E) 0.1MPa. Growth phenotypes of strains can be complemented by the addition of 0.05% Tween 80 to growth media at (B) 15°C, (D) 30MPa, and (F) 0.1MPa. All pressure experiments were performed at 15°C.

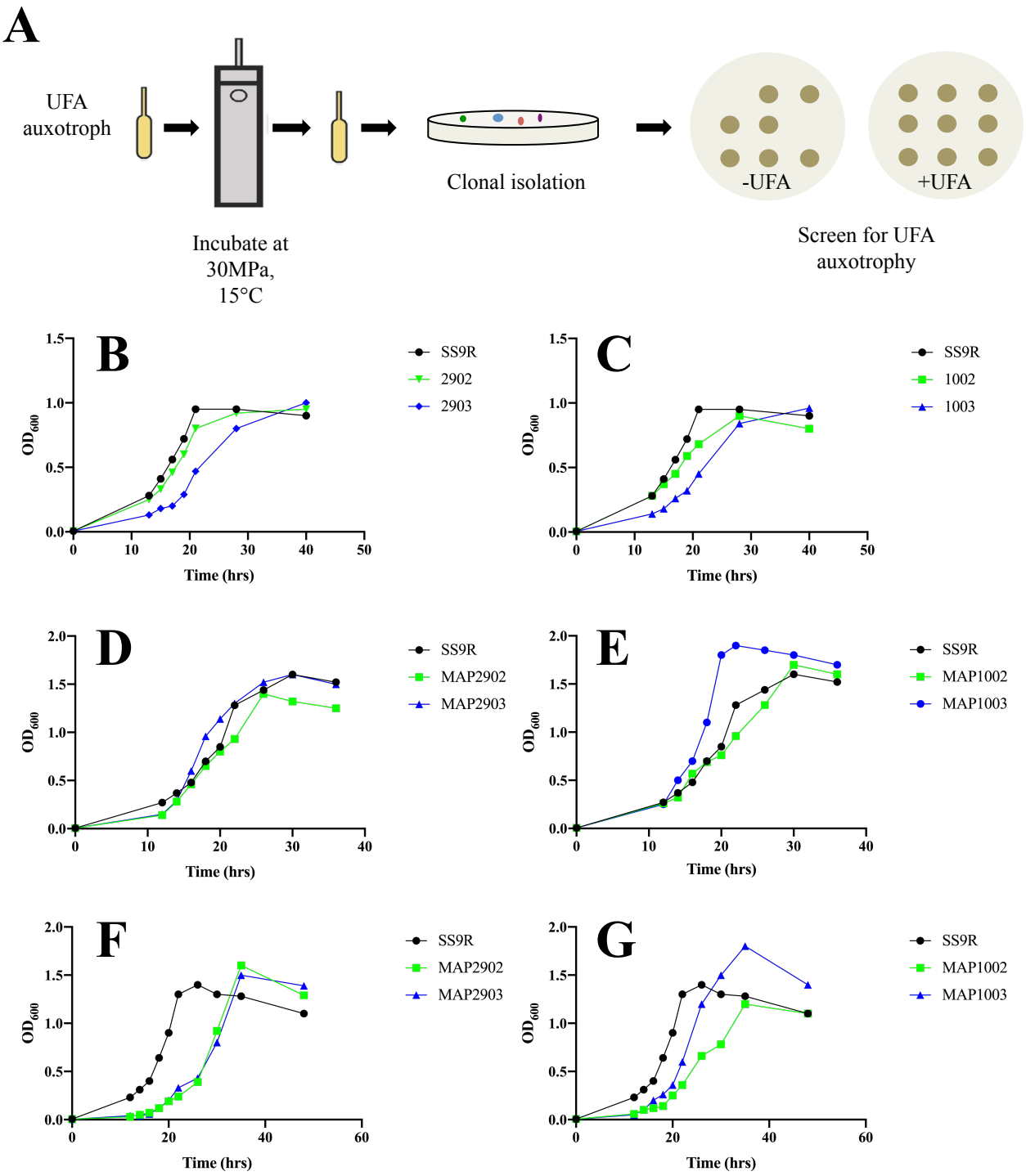


Figure 4.3- Suppression of defects in MUFA biosynthesis. (A) Selection and isolation scheme for the isolation of suppressor strains. Growth of MAP29 derived suppressors (B) and MAP10 derived suppressors (C) at 15°C compared to SS9R. Growth of MAP29 (D) and MAP10 (E) suppressors at 30MPa compared to SS9R. Growth of MAP29 (F) and MAP10 (G) suppressors at 0.1MPa compared to SS9R.

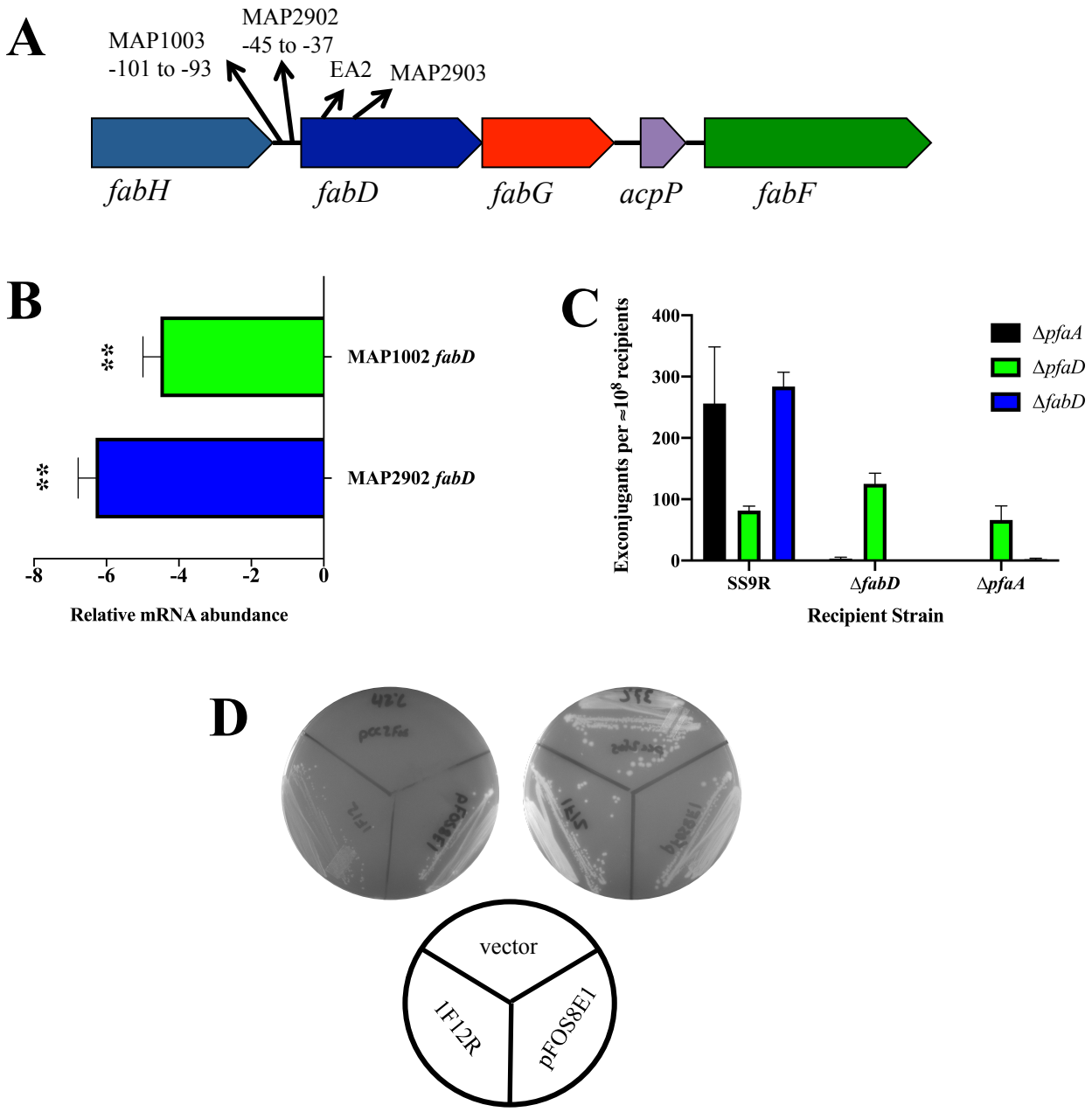


Figure 4.4- Various mutations in suppressor strains mapping to the *fabD* locus. (A) Diagram of *fabHDG* operon of *P. profundum* SS9. Arrows indicate the approximate locations of insertion sequences found in various suppressor mutants and EA2 strain. (B) Abundances of *fabD* mRNA in the indicated suppressor mutants relative to SS9R, as determined by qRT-PCR. (C) Δ*fabD* and Δ*pfaA* mutations are synthetically lethal in *P. profundum* SS9. Conjugation was used to mobilize insertional inactivation vectors into the indicated recipient strains. Values (± standard deviations) represent the average number of exconjugants obtained from three independent experiments. (D) Heterologous expression of the Pfa synthase from either *Shewanella pealeana* (1F12R) or *P. profundum* SS9 (pFOS8E1) was able to complement the *fabD* (ts) mutation in strain LA2-89. Plates were imaged after 24hr at either 42°C (left) or 37°C (right).

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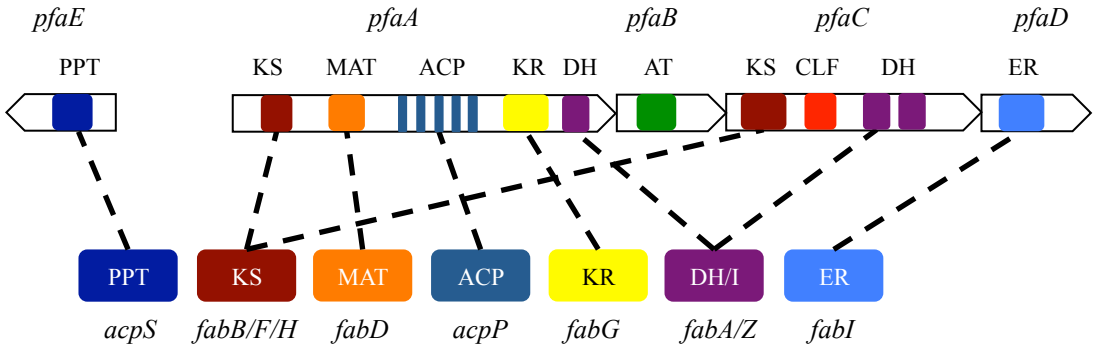
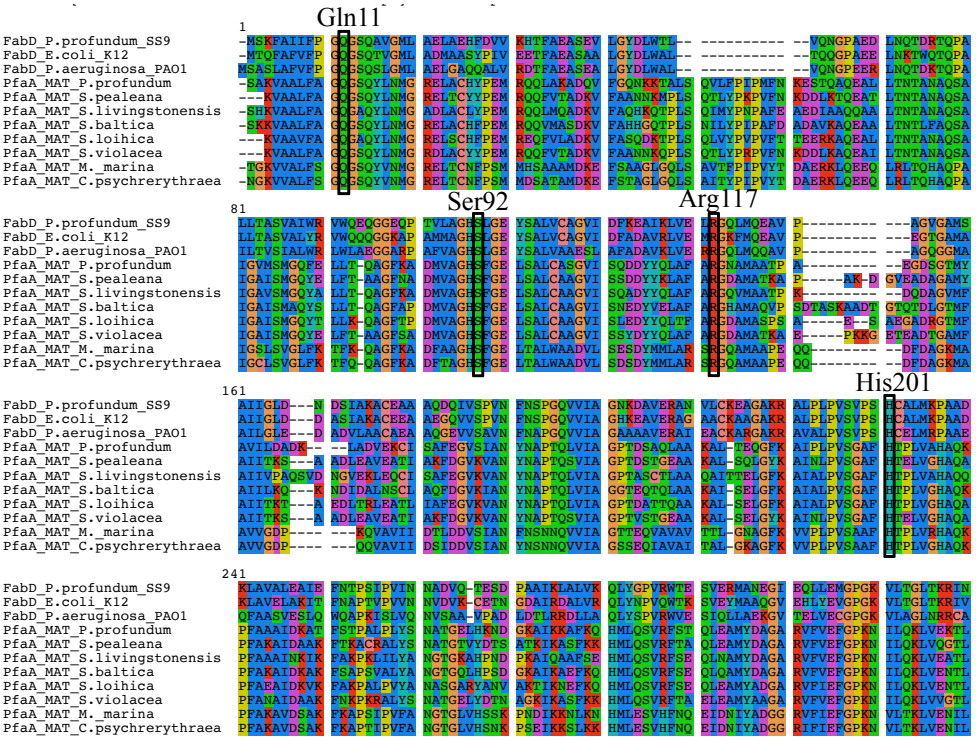
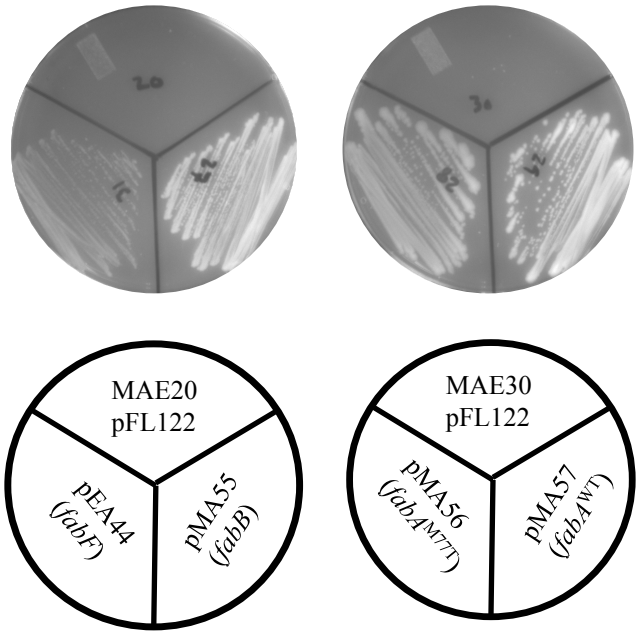


Supplemental Figure 4.1- Fatty acid biosynthesis pathways in *Photobacterium profundum* SS9. Comparison of the genes and functions comprising the Pfa synthase and their respective functional counterparts found in the Type II FAS. Domain designations include: phosphopantetheinyl transferase (PPT), β-ketoacyl synthase (KS), malonyl-CoA:ACP transacylase (MAT), acyl carrier protein (ACP), ketoacyl reductase (KR), dehydratase/ isomerase (DH), acyltransferase (AT), chain-length factor (CLF), enoyl reductase (ER).



Supplemental Figure 4.2- Partial amino acid alignments of FabD and MAT domains from various strains. Previously characterized active site residues of FabD from *E.coli* are boxed including the active site Ser92. The *E.coli* FabD residues; Gln11, Arg117, His201 are involved in substrate recognition and catalysis of malonyl group transfer from CoA to ACP. These residues are universally conserved amongst FabD homologs and within the embedded MAT domains of various PfaA homologs.



Supplemental Figure 4.3- Complementation of MAE20 (Δ*fabB*) and MAE30 (Δ*fabA*) strains with Type II FAS genes from *P. profundum* SS9. Respective E.coli strains were transformed with the indicated vectors and transformants recovered on LB Streptomycin plates supplemented with oleic acid. Individual transformants were restreaked onto plates without oleic acid and incubated at 37°C for 24hr before being imaged.

Supplementary Table 4.1- Primers used in this study

Name	Sequence
fabB 5'O	TACGATTCTAGAGTGATTAAACCCTCATCAAG
fabB 5'I	GCCAGACGACCTAAGCCAGTCGGTTTATTCACAGAAAGTGG
fabB 3'I	GACTGGCTTAGGTCGTCTGGCGGAAGTAAGGGGCTATACGT
fabB 3'O	TACGATGAGCTCTGACAAACTCAGCATCAGCA
ΔfabB ver F	GTGCCTTCAGTGGTTTGATG
ΔfabB ver R	ATCACCATGACGGCATTAGG
fabA 5'O	TGACTGGTACCTTGCCATTACGGGTGCAGTA
fabA 5'I	GATCCGCGGCCGCGCTGGACCATAAAGTTCACC
fabA 3'I	GATCCGCGGCCGcACAAACTTCTAAGTCGTCCC
fabA 3'O	TATAGGAGCTCGGTTGAGGACGAACTATGAA
ΔfabA ver F	TGAGTGCCGCGCTAAACTTA
ΔfabA ver R	TGGTTCCGATCCCAGATGGT
desA 5'O	TGACTGGTACCCTTAGAGGTGTATGCAGAGC
desA 5'I	GACTGGCTTAGGTCGTCTGGCGGTGGTTTAGTGGTCGACAT
desA 3'I	GCCAGACGACCTAAGCCAGTCCTTAGGCGGGATTTAACTCG
desA 3'O	TATAGGAGCTCCAACCGCTGGCAATTCAATG
ΔdesA ver F	GTATGGAGCTTGAAGGTGCT
ΔdesA ver R	CAGCCAGAACTTACTCGCAT
pFL122 fabB F	TACGATGGATCCGTGATTAAACCCTCATCAAG
pFL122 fabB R	TACGATGTCGACTGACAAACTCAGCATCAGCA
pFL122 fabA F	ACGATCGAATTCCCTAACAGGCACTCAAGGTA
pFL122 fabA R	TATAGTCTAGAGGGACGACTTAGAAGTTTGT
pMUT100 fabD F	ACGATGGATCCGCAAGAACAAGGTGGTGAAC
pMUT100 fabD R	ACGATGTCGACTAATTGCAGCAGGATCGCTC
fabD 5'O	TGACTGGTACCGCCTAGCAATACCTGATCAC
fabD 5'I	GACTGGCTTAGGTCGTCTGGCGTTCACCACCTTGTTCTTGC
fabD 3'I	GCCAGACGACCTAAGCCAGTCGAGCGATCCTGCTGCAATTA
fabD 3'O	TATAGGAGCTCCACGGTAATACCACGAGATG
ΔfabD ver F	GTCGTTATTGGTGCAAGTGA
ΔfabD ver R	TTGCTAGTGTAGCAGCACGT
SS9 fabD seq F	GCTGTTGGTATGTTGGCTGA
SS9 fabD seq R	GAGCAACTTTACCTTCCAGG

Supplemental Table 4.2- Type II FAS biosynthetic homologs identified in *P. profundum* SS9

Gene	Locus tag(s)	Notes
fabA	PBPRA1773	Transcriptionally up-regulated at 30MPa (ref)
fabB	PBPRA2658, PBPRB0107	Essential in *E.coli,* not essential in SS9, refer to text
fabD	PBPRA1194	Essential in *E.coli,* not essential in SS9, refer to text
fabF	PBPRA1197, PBPRB0104	Complements E.coli Δ*fabB*
fabG	PBPRA1195, PBPRB0105, PBPRB1106, PBPRB1562	Reduces β-Keto groups after ketosynthase reaction
fabH	PBPRA1193, PBPRA2001	Performs first condensation in Type II FAS
fabV	PBPRA2423, PBPRA2527	Confers resistance to Triclosan in Vibrio sp.
fabY	PBPRB1012	Initiates Type II FAS in *Pseudomonas aeruginosa* PAO1
fabZ	PBPRA2957	Dehydratase primarily involved in SFA production
fabR	PBPRA3467	Regulates *fabA/B* in *E.coli*
fadR	PBPRA2608	Regulates *fabA/B* as in E.coli, partially regulates *pfa* operon (Allemann, Allen; unpublished)
acpP	PBPRA1196	Carries acyl groups in Type II FAS
acpS	PBPRA3085	Cognate PPTase for AcpP
desA	PBPRB0742	Presumably complements loss of *fabA/B* as seen in *P. aeruginosa* PAO1

Chapter 4, in full, is being prepared for submission of the material to a peer-reviewed journal. Allemann M. N., Allen E.E., “Genetic suppression of lethal mutations in fatty acid biosynthesis mediated by a secondary lipid synthase”. The dissertation author was the primary investigator and author of this material.

Chapter 5- Conclusions and suggestions for future research

The work presented in this dissertation sought to gain a greater genetic and physiological understanding of marine bacteria known to produce omega-3 polyunsaturated fatty acids. While much was known regarding the genes required for biosynthesis of PUFA (1, 2), the work in this dissertation sought to expand upon how the PUFA pathway interacts with other lipid biosynthetic pathways in these marine bacteria. The following material will provide a summary of each chapter and suggestions for future research.

Chapter 2 presented genetic evidence concerning how the PUHC and PUFA pathways are linked biochemically through the action of OleA. Co-expression of *oleA* and *pfaA-E* in *Escherichia coli* led to the production of the expected polyunsaturated ketone product and a concomitant decrease in EPA incorporation into phospholipids. The linkage of these two pathways was further demonstrated by genetic investigation of a previously characterized thioesterase, designated PfaT. Phenotypes of Δ*pfaT* mutants in *Shewanella oneidensis* MR-1 and *Photobacterium profundum* SS9 both displayed significantly reduced amounts of PUHC and EPA consistent with the notion that PfaT participates in a process shared by both pathways.

Given the genetic evidence presented regarding OleA and PfaT, further *in vitro* biochemical characterization of these two proteins, with specific emphasis on their interactions with ACP. Homologs of OleA from other bacteria have been successfully expressed and purified in *E.coli* and a similar scheme could be envisioned for the OleA homologs derived from PUFA producing bacteria. Once purified, OleA could be probed for interactions with purified ACP's from the Type II FAS or from PfaA using covalent cross-linking probes (3, 4). Given the unique substrate specificity of the various OleA homologs (5, 6), protein interactions might be the underlying reason for why OleA from a PUFA producing organism does not condense substrates derived from the Type II FAS pathway.

Chapter 3 investigated the genetic regulation of the *pfa* operon in *P. profundum* SS9 and in the process demonstrated that the operon is down-regulated in response to exogenous fatty acids and identified a novel transcriptional regulator, PfaF, specific to this process. Further work using electrophoretic mobility shift assays and bacterial one-hybrid experiments indicated that PfaF is binding to the previously mapped promoter region of the *pfa* operon (7) indicating that this regulation is direct. Further experiments aimed at determining the binding site sequence(s) for PfaF are currently underway in the Allen Lab.

There are several interesting aspects of this work that could be pursued further in future work. Given the genomic context of *pfaF* in *P. profundum* SS9, it is likely that PfaF regulates other genes. A comparison between transcriptomes of a *pfaF* mutant and its parental strain would provide a global assessment of which genes are part of the PfaF regulon. These results could be further supported through the use of other techniques such as DAP-seq (8), which would provide additional data on the location of PfaF binding sites within the SS9 genome. This type of analysis could provide further information as to other cellular processes correlated with EPA production in *P. profundum* SS9.

Further biochemical characterization of PfaF would also be informative. A crystal structure of a PfaF homolog from *Shewanella amazonensis* indicated an unknown ligand being bound in the C-terminal domain. Further structural and biochemical work to identify this ligand and its effect on DNA-binding would provide more mechanistic information as to the function of PfaF and how it regulates the *pfa* operon. Based on previous work concerning transcriptional regulators of fatty acid biosynthesis (9–11) , the ligand is most likely a fatty acid or a derivative thereof (acyl-CoA or acyl-ACP). PfaF may also be capable of binding a variety of acyl moieties

and adjusting its DNA-binding activity similar to what has been observed with DesT from *Pseudomonas aeruginosa* (11, 12).

While the work presented clearly indicates PfaF in the genetic regulation of the *pfa* operon in SS9, it remains to be determined if PfaF homologs found in other PUFA producing bacteria perform the same function. Regulation of the *pfa* operon may be distinctly different in other bacterial lineages such as the *Shewanella* genus, which have a putative regulator, PfaR, clustered with the operon. While PfaR is not homologous to any known transcriptional regulator families, it does contain a N-terminal DNA-binding domain. While deletion of PfaR has been shown to lead to increased EPA production in recombinant hosts, no change in EPA composition was observed in a Δ*pfaR* mutant of *S. piezotolerans* WP3. Given the genetic tractability of members of the *Shewanella* genus (13, 14), a similar investigation into the role of these PfaF homologs in regulating the *pfa* operon could be conducted.

The work presented in Chapter 4 indicated that genetic disruption of MUFA biosynthesis in *P. profundum* SS9, while detrimental to cellular growth, is not lethal and could be suppressed by increased EPA production. Various SS9 derivatives with defects in MUFA biosynthesis were subjected to high pressure growth conditions and suppressor mutants which grew at high pressure similar to SS9 were isolated. Characterization of these suppressor strains indicated that increased EPA production compensates for the loss of MUFA production in SS9. Further genomic characterization of these suppressor strains indicated that insertion sequence (IS) mutations mapping to the *fabD* locus were noted in three of four suppressor mutants. These IS sequences either disrupted the coding sequence of *fabD* or interrupted the predicted promoter leading to decreased transcription of *fabD.* This suggested that *fabD* is a non-essential gene in SS9, and this was further confirmed by the construction of a stable Δ*fabD* mutant in SS9. Using

synthetic lethal phenotypes, it was shown that mutations in *pfaA* and *fabD* are synthetically lethal. A temperature sensitive *fabD* allele in *E.coli* could also be complemented by heterologous expression of Pfa synthases from *P. profundum* SS9 and *S. pealeana.*

Given that the MAT domain of PfaA can complement loss of FabD *in vivo*, and the sequence conservation between the two, it is highly likely that it can interact with ACP of the Type II FAS and have an identical mechanism as that of FabD. Experiments to test if the active site serine is required for the *in vivo* complementation are currently underway in the Allen Lab. Recent mutagenesis studies of FabD from *E.coli* showed that an R117A mutation could expand its substrate specificity (15). An equivalent mutation could be introduced into this conserved residue of MAT using recombineering (16) and the resulting Pfa synthase mutant might produce a different product(s) due the incorporation of different extender units.

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