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RESEARCH ARTICLE

Genetic Regulation of the Bacterial Omega-3 Polyunsaturated Fatty Acid Biosynthesis Pathway

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ABSTRACT A characteristic among many marine *Gammaproteobacteria* is the biosynthesis and incorporation of omega-3 polyunsaturated fatty acids into membrane phospholipids. The biosynthesis of eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) is mediated by a polyketide/fatty acid synthase mechanism encoded by a set of five genes, *pfaABCDE*. This unique fatty acid synthesis pathway coexists with the principal type II dissociated fatty acid synthesis pathway, which is responsible for the biosynthesis of core 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 showed that expression of the *pfa* operon is downregulated in response to exogenous fatty acids, particularly long-chain monounsaturated fatty acids. This regulation occurs independently of the canonical fatty acid regulators, FabR and FadR, 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 responsible for the observed regulation of the *pfa* operon in *P. profundum* SS9. Gel mobility shift and DNase I footprinting assays confirmed that PfaF binds the *pfaA* promoter and identified the PfaF binding site.

IMPORTANCE The production of long-chain omega-3 polyunsaturated fatty acids (PUFA) by marine *Gammaproteobacteria*, particularly those from deep-sea environments, has been known for decades. These unique fatty acids are produced by a polyketide-type mechanism and subsequently incorporated into the phospholipid membrane. While much research has focused on the biosynthesis genes, their products, and the phylogenetic distribution of these gene clusters, no prior studies have detailed the genetic regulation of this pathway. This study describes how this pathway is regulated under various culture conditions and has identified and characterized a fatty acid-responsive transcriptional regulator specific to PUFA biosynthesis.

KEYWORDS deep-sea bacteria, fatty acids, polyunsaturated fatty acid, regulation

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 control 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 biosynthesis, are regulated by the interplay between FadR and FabR (2–4), as shown in Fig. 1. FadR is a member of the GntR regulator family and acts as a positive regulator of both *fabA* and *fabB*, which encode proteins essential to the biosynthesis of unsaturated fatty acids in this organism

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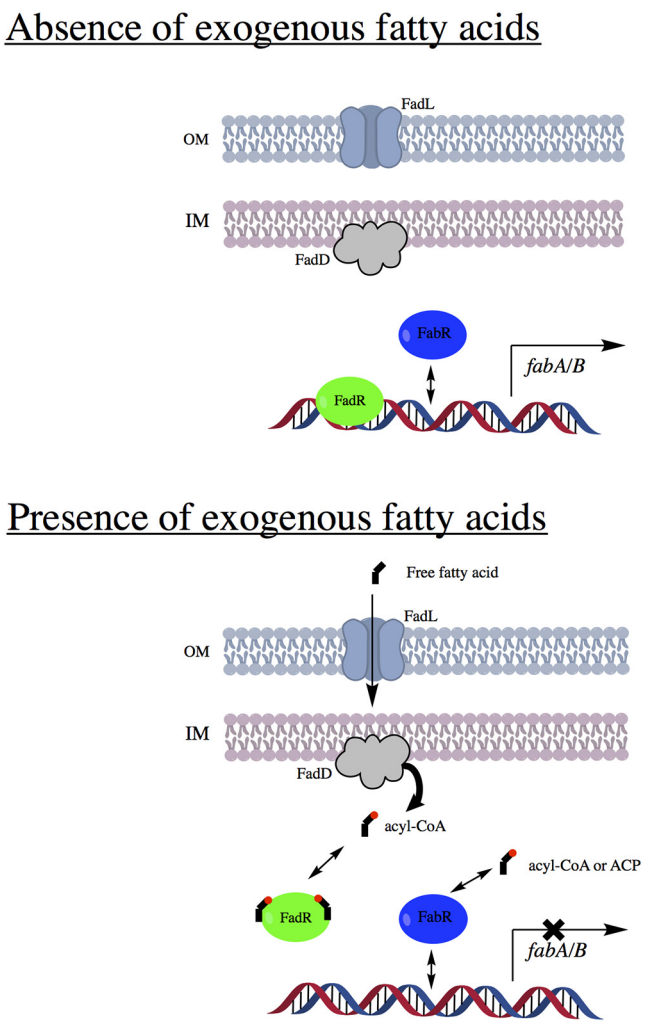


FIG 1 Genetic regulation of monounsaturated fatty acid biosynthesis genes *fabA* and *fabB* in *E. coli*. In the absence of exogenous fatty acids, FadR binds to a site upstream of the *fabA* and *fabB* promoters and acts as an activator of transcription. When present, exogenous fatty acids are transported across the outer membrane by FadL and converted to 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 or 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* and *-B* expression.

(5). Exogenous fatty acids ($>C_{10}$) are imported across the outer membrane via the FadL transporter and subsequently converted into acyl coenzyme A's (acyl-CoAs) by the acyl-CoA synthetase FadD. In the absence of long-chain acyl-CoA, FadR binds to sites upstream of *fabA* and *fabB* and acts as a positive regulator (6, 7). Upon acyl-CoA binding to FadR, it adopts a conformation that is unable to bind to its cognate sites upstream of *fabA* and *-B* promoters, leading to deactivation of transcription. FabR, a TetR family transcriptional regulator, further regulates *fabA* and *-B* by acting as a classical repressor. FabR binds to sites immediately downstream of the FadR site in both *fabA* and *-B* regardless of acyl-CoA being present (3, 8). 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 are 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*, encoding an oxygen-dependent membrane-bound lipid desaturase (9).

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, 10, 11). In this strain, *fabA* and *fabB* form an operon (unlike in *E. coli*), which is regulated by another TetR family regulator, DesT (12). In addition to *fabAB* expression, DesT also modulates the expression of the membrane-bound desaturase DesBC, which catalyzes oxygen-dependent desaturation of saturated acyl-CoA for subsequent incorporation into membrane phospholipids (10, 11).

A subset of marine *Gammaproteobacteria*, particularly strains isolated from cold and/or high-pressure environments, produce long-chain omega-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) (20:5*n-3*) and docosahexaenoic acid (DHA) (22:6*n-3*), which are incorporated into phospholipid membranes (13, 14). The biosynthesis of these unique fatty acids is linked to the *pfaABCDE* operon, which encodes a type I FAS/polyketide synthase (15). In these bacteria, the Pfa synthase pathway coexists with the type II FAS pathway, which produces saturated and monounsaturated fatty acids (14, 16). Given that both pathways utilize the same precursor substrates (15, 17, 18) and that their respective end products are destined for phospholipids (13, 14, 19–22), 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 low temperature (23–28) and/or high pressure (23, 26) leads to increases in PUFA abundance. In the EPA-producing bacterium *Photobacterium profundum* SS9, analyses of transcript abundances of the *pfa* operon at low temperature (4°C) and/or high pressure indicated no significant changes in mRNA abundances relative to those under 15°C or low-pressure conditions (25). 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 possible existence of a *pfa*-specific transcriptional regulator(s) in the strain (25).

In this work, the transcriptional regulation of the *pfa* operon in *P. profundum* SS9 is further characterized and shown to be downregulated in response to exogenous unsaturated fatty acid supplementation. A genetic screen utilizing a *pfaA*::*lacZY* reporter gene fusion combined with transposon mutagenesis was used to identify a novel transcriptional regulator, herein designated *pfaF*, that positively regulates the *pfa* operon and mediates the regulatory response to exogenous fatty acids. Gel mobility shift assays using purified PfaF demonstrated that PfaF binds to the *pfaA* promoter and that this binding is sensitive to the presence of oleoyl-CoA (18:1). Additionally, DNase I footprinting was used to determine the binding site of PfaF within the *pfaA* promoter. The identification of a novel transcriptional regulator specific to the bacterial synthesis of omega-3 PUFA products expands our understanding of the lipid physiology of a widespread group of marine bacteria that are enriched in permanently cold and deep-sea habitats.

RESULTS

Expression of the *pfa* operon under various culture conditions. To better understand the regulation of the *pfa* operon and to facilitate monitoring of gene expression, a reporter construct was designed to link expression of the *pfa* operon to the *lacZY* operon of *Escherichia coli*. This *pfaA*::*lacZY* strain allows the *pfa* operon promoter to be monitored in single copy with all possible upstream regulatory sequences. As expected from previous work in *P. profundum* SS9 (25), loss of *pfaA* rendered the *pfaA*::*lacZY* strain unable to produce EPA (data not shown). β-Galactosidase assays under standard cultivation conditions (2216 marine broth, 15°C) in mid-log-phase growth produced approximately 48 Miller units of activity, providing a baseline of relative *pfaA* expression (Fig. 2A). The results of 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 Fig. 2A and indicated no changes in LacZ activity, confirming previous results (25). Sequence analysis of the promoter region from EA2, a previously isolated EPA-overproducing strain with increased *pfa* operon transcript levels, also indicated no changes in the promoter sequence of strain EA2 relative to the wild type.

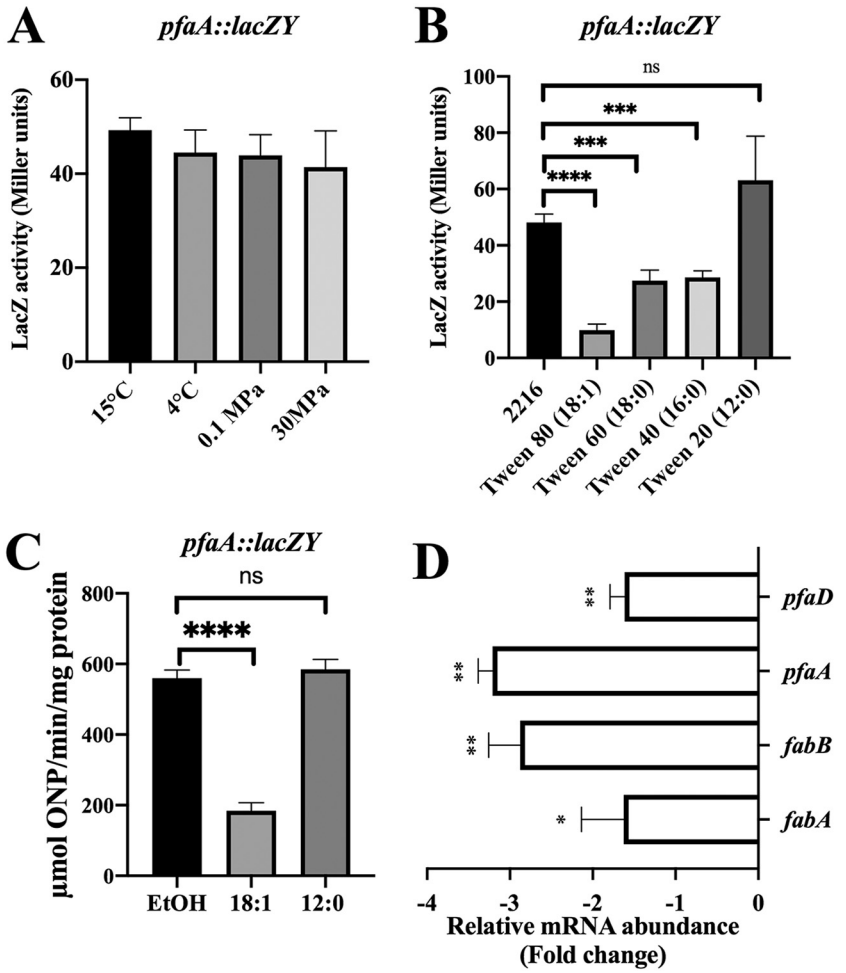


FIG 2 Reporter gene expression in the *pfaA*::*lacZY* strain in response to a variety of culture parameters known to modulate EPA composition. (A) Various temperature and hydrostatic pressure conditions. (B) Cells cultured at 15°C with different Tween compounds added at 0.05%. (C) Effect of 0.05% oleic (18:1) or lauric (12:0) free fatty acid supplementation on *pfaA*::*lacZY* reporter activity. Fatty acid stocks were prepared as potassium salts in 80% ethanol (EtOH). For panels A to C, results from at least six independent experiments are shown as means with error bars representing standard deviation (ns, $P > 0.05$; ***, $P < 0.005$; ****, $P < 0.0001$). (D) 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 qPCRs (*, $P < 0.05$; **, $P < 0.005$).

Given its role in producing fatty acids destined for phospholipid biosynthesis, we hypothesized that the *pfa* operon might be regulated in a manner similar to the prototypical *fab* regulon involved in saturated and monounsaturated fatty acid synthesis. To circumvent the previously noted solubility issues of free fatty acids in 2216 marine growth medium (23), cultures were supplemented with exogenous fatty acids in the form of polysorbate esters, i.e., Tween 20 (12:0), 40 (16:0), 60 (18:0), and 80 (18:1). As shown in Fig. 2B, significant decreases in β-galactosidase activity were observed in the *pfaA*::*lacZY* strain in response to all Tween compounds except for Tween 20 (12:0). Given the various degrees of downregulation observed with the addition of Tween compounds that differ only in their fatty acid component, the possibility of this response being due to the polysorbate component can be eliminated. Due to the insolubility of free fatty acids in 2216 marine medium noted above, β-galactosidase activities were calculated as a function of cellular protein concentrations. As seen in Fig. 2C, these assays yielded results qualitatively similar to those of the Tween supplementation assays in the case of 18:1 and 12:0 supplementation. This downregulation

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli		
MG1655	Wild type, source of *lacZY* operon	*E. coli* Genetic Stock Center
DH5α*pir*	Cloning strain for *pir* vectors	54
S17-1λ*pir*	Biparental mating strain	49
KJ1C	Bacterial one-hybrid reporter strain, *hisB463*	Addgene 21874
Photobacterium profundum		
SS9R	Rifampin-resistant derivative of strain SS9	46
EA2	EPA overproducer, *pfa* regulatory mutant	23
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*::Tn*5*	This study
Plasmids		
pRE118	Allelic exchange vector, Kan^r	47
pFL122	Broad-host-range complementation vector	55
pRK2073	Conjugation helper plasmid	56
pMUT100	Suicide plasmid for insertional inactivation	48
pKJ1712	B1H reporter plasmid, *HIS3-aadA* cassette	Addgene 21870
pB1H2	B1H, RNAPα fusion expression	Addgene 12613
pRL27	Mini-Tn*5* transposon delivery vector	29
pET28	T7 expression plasmid	Novagen
pMA21	pRE118 with Δ*pfaA* allele	This study
pMA29	pRE118 with Δ*lacZ* allele	This study
pMA38	pRE118 with Δ*fabR* allele	This study
pMA39	pRE118 with Δ*fadR* allele	This study
pMA41	pRE118 with Δ*pfaA*::*lacZY* allele	This study
pMA61	pMUT100 with *pfaF* internal fragment	This study
pMA62	pFL122 with *pfaF*	This study
pMA66	pET28 with PfaF N-terminal 6-His tag	This study
pMA75	pB1H2 with PfaF N-terminal fusion to RNAPα	This study
pMA77	pKJ1712 with *pfaA* promoter region	This study

was also shown to occur in the parental strain SS9R, with *pfaA* and *pfaD* transcript abundances reduced ~3-fold and ~2-fold, respectively, in response to Tween 80 (18:1) supplementation (Fig. 2D). Transcript abundances of *fabA* and *fabB* were also decreased under these conditions (Fig. 2D), indicating that the monounsaturated fatty acid biosynthesis genes are also downregulated in the presence of exogenous unsaturated fatty acids, a result expected based on prior studies in *E. coli* (2–4, 8). Fatty acid profiling of SS9R grown in the presence of Tween 80 supplementation indicated a nearly 4-fold increase in 18:1 composition and an approximately 10-fold decrease in EPA, consistent with the downregulation of *pfaA* noted above (see Table S2 in the supplemental material). A novel fatty acid, tentatively identified as 18:2, was also observed in cultures supplemented with Tween 80 and may be representative of further metabolic processing of the incoming 18:1 acyl chain.

Role of FabR/FadR in response to exogenous fatty acids. Given the strong exogenous fatty acid supplementation phenotype observed, it was suspected that FadR and/or FabR, which are known to regulate *E. coli fabA* and *fabB* in response to exogenous fatty acids, may be responsible for this regulatory phenomenon. Homologs of *fabR* (locus tag PBPRA3467) and *fadR* (locus tag PBPRA2608) were identified in the SS9 genome via homology searches, and deletion mutants of both SS9R and the *pfaA*::*lacZY* reporter strain were generated (Table 1). As shown in Fig. 3A, strains containing the Δ*fadR* mutation displayed decreased β-galactosidase activities. The

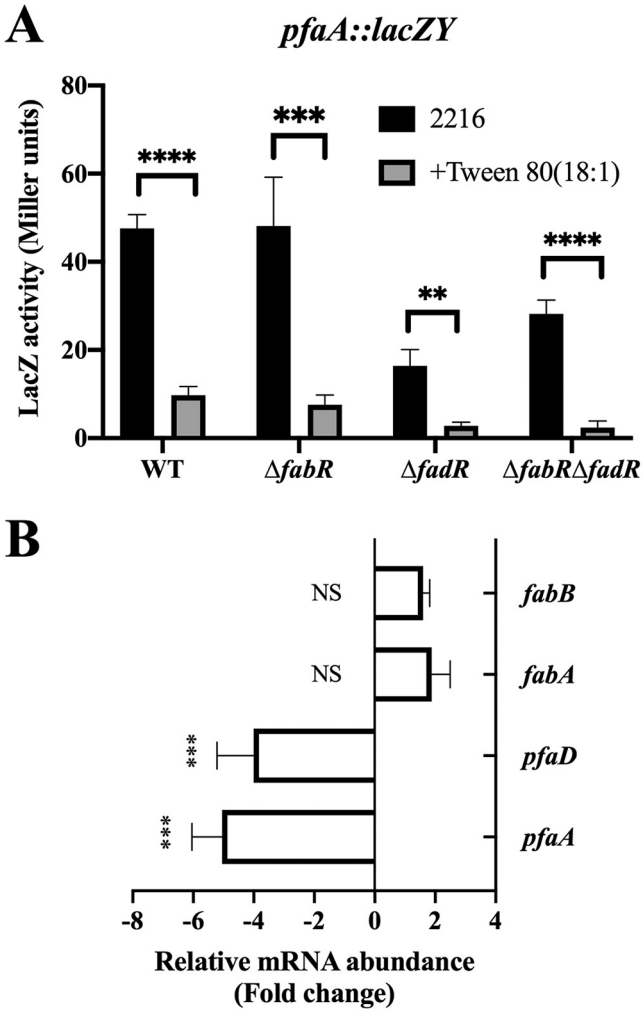
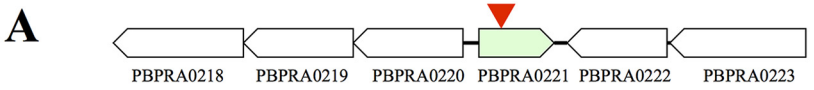


FIG 3 Influence of FadR and FabR on expression of the *pfa* operon. (A) LacZ activities of strains carrying Δ*fabR* and/or Δ*fadR* mutations in the presence or absence of 0.05% Tween 80 (18:1). Results of at least six independent experiments are shown as means with error bars representing standard deviation (**, $P < 0.05$; ***, $P < 0.005$; ****, $P < 0.0001$). (B) Relative transcript abundances of *pfaA*, *pfaD*, *fabA*, and *fabB* in the Δ*fabR* Δ*fadR* mutant grown in the presence or absence of Tween 80 (18:1). Cells grown without supplement represent the calibrator condition. Error bars represent the standard deviations based on at least three independent biological replicates with duplicate qPCRs (NS, $P > 0.05$; ***, $P < 0.005$).

pfaA::*lacZY* reporter strains containing either Δ*fadR* or Δ*fabR* Δ*fadR* mutations had similar β-galactosidase activities, suggesting that FabR is not involved in the regulation of the *pfa* operon. Interestingly, β-galactosidase assays of the Δ*fabR* Δ*fadR* mutant indicated that the downregulation of the *pfaA*::*lacZY* gene fusion in the presence of Tween 80 (18:1) was independent of both FadR and FabR (Fig. 3A). Gene expression analysis performed on RNA from the SS9 Δ*fabR* Δ*fadR* mutant grown in the presence or absence of Tween 80 revealed that both *pfaA* and *pfaD* transcripts were downregulated in response to supplementation, while *fabA* and *fabB* transcripts were essentially equivalent under the two conditions (Fig. 3B). Fatty acid analyses of the corresponding single and double mutant derivatives of SS9R are shown in Table S2 in the supplemental material. Strains containing the Δ*fadR* mutation had decreased EPA levels, consistent with the reduced β-galactosidase activities noted. As predicted, given the role of FadR as a positive regulator of *fabA* and *-B*, a decrease in monounsaturated fatty acids was noted in strains containing the Δ*fadR* mutation (Table S2).

Identification of a regulator specific to the *pfa* operon. The FadR/FabR-independent downregulation of the *pfa* operon in response to Tween 80 (18:1) observed here



Locus tag	Pfam hit	Function	E-value
PBPRA0218	Pfam4932	O-antigen ligase	3.2e-18
PBPRA0219	Pfam03279	Lipid A acyltransferase	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

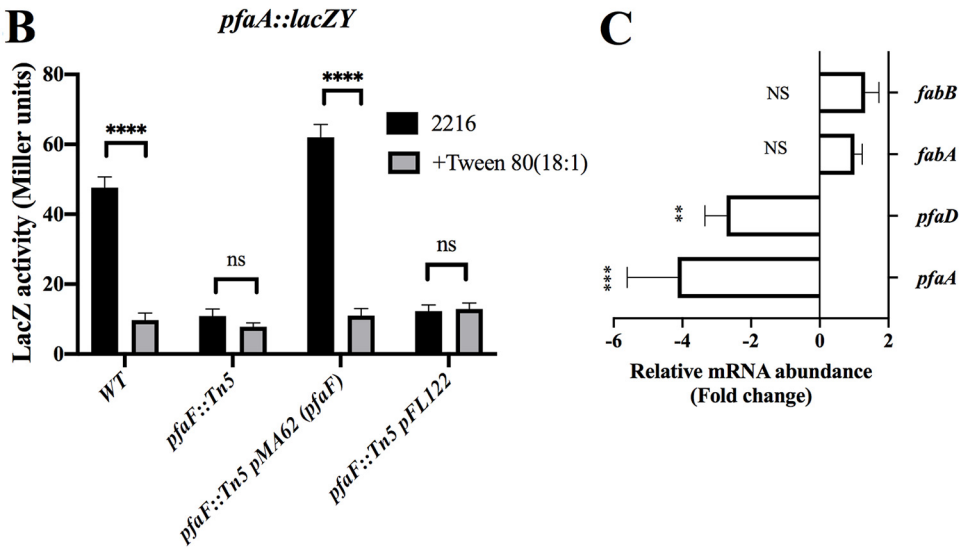


FIG 4 The *pfaF* genetic locus and regulatory phenotypes associated with its disruption. (A) Genetic organization of PfaF locus. The arrowhead indicates the relative position of the mini-Tn*5* insertion site in the *pfaA*::*lacZY* regulatory mutant. (B) LacZ activity of the *pfaF*::Tn*5* mutant and the complemented strains carrying pMA62 (pFL122 *pfaF*) or vector (pFL122) only with or without 0.05% 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 the SS9R *pfaF* mutant relative to SS9R. Error bars represent the standard deviations based on at least three independent biological replicates with duplicate qPCRs (ns, $P > 0.05$; **, $P < 0.005$; ***, $P < 0.001$).

suggested that another regulator(s) for the *pfa* operon might exist in *P. profundum* SS9. To search for additional regulators, the *pfaA*::*lacZY* strain was subjected to transposon mutagenesis using the mini-Tn*5* delivery vector pRL27 (29, 30). Of the approximately 10,000 mutants screened, several mutants with reduced LacZ activity or loss of LacZ activity were identified and saved for further analysis. Arbitrary PCR was performed to identify the sites of mini-Tn*5* insertion. Excluding mutants that had Tn*5* insertions in the reporter gene or *fadR*, we identified mutants from independent libraries that contained unique transposon insertions in the same gene, PBPRA0221 (TetR bacterial transcriptional regulator, pfam13972) (Fig. 4A). One of these mutants displayed a 5-fold decrease in LacZ activity and no longer responded to exogenous Tween 80 (18:1) supplementation (Fig. 4B). The PBPRA0221 gene, herein designated *pfaF*, encodes a protein that is a member of the TetR family of transcriptional regulators and is clustered with genes related to lipopolysaccharide synthesis. To verify that this locus is involved in *pfa* gene regulation, single-crossover insertion mutants were generated in the parental *pfaA*::*lacZY* reporter strain and the EPA-producing SS9R strain. The LacZ activity of the resulting strain was identical to that seen in the *pfaF*::Tn*5* mutant, thereby verifying this relationship and excluding the possibility of additional transposon insertion events

TABLE 2 Fatty acid compositions of SS9R and various *pfaF* mutant strains at 15°C

Fatty acid	Mean % by wt[a] in:			
	SS9R	SS9R *pfaF*	SS9R *pfaF*/pFL122	SS9R *pfaF*/pMA62
12:0	4.16 ± 1.53	2.84 ± 0.33	3.72 ± 0.45	3.73 ± 0.10
14:0	4.33 ± 1.21	5.25 ± 0.94	9.46 ± 0.31	8.03 ± 0.22
14:1	3.19 ± 1.11	3.90 ± 0.73	6.04 ± 0.19	4.90 ± 0.16
16:0	21.07 ± 1.84	19.03 ± 0.67	19.39 ± 3.64	19.50 ± 2.82
16:1	45.09 ± 1.68	50.95 ± 0.53	45.33 ± 2.06	39.59 ± 1.58
12-OH	1.99 ± 1.23	2.22 ± 0.28	2.66 ± 0.14	2.96 ± 0.27
18:0	0.71 ± 0.11	0.94 ± 0.08	1.37 ± 0.15	1.74 ± 0.09
18:1	12.26 ± 2.26	12.92 ± 0.42	9.38 ± 0.97	12.02 ± 2.82
20:5	4.52 ± 1.55	0.82 ± 0.23	1.14 ± 0.17	5.14 ± 0.30

[a]Data represent the averages from at least three independent cultures ± standard deviations.

being responsible for the observed phenotype (data not shown). Transcript abundance analysis of the *pfaF* mutant strain indicated significant downregulation of both *pfaA* and *pfaD* transcripts relative to SS9R (Fig. 4C), consistent with the LacZ activity data. Additionally, quantification of *fabA* and *fabB* transcripts indicated no major differences between SS9R and the *pfaF* mutant derivative. Comparison of the fatty acid profiles of SS9R and the *pfaF* mutant grown at 15°C shows that the mutant displays an approximate 4-fold reduction in EPA content relative to the wild type (Table 2). The minor difference in the abundances of other fatty acids in the *pfaF* mutant is consistent with the minimal changes in *fabA* or *fabB* transcription. In both SS9R (EPA^+) and the *pfaA*::*lacZY* strain (EPA^-), genetic disruption of *pfaF* did not result in observable growth defects at high pressure or low temperature (data not shown).

The *pfaF* gene was cloned onto the broad-host-range complementation plasmid pFL122, and the resulting construct, pMA62, was introduced into *pfaF*::Tn*5* and SS9R *pfaF* mutant strains. As shown in Fig. 4B, expression of *pfaF* was able to fully restore LacZ activities to that seen the parental *pfaA*::*lacZ* strain relative to the vector-only control. Similarly, the ability to downregulate the operon in response to exogenous Tween 80 (18:1) was restored in the complemented strain and not in the vector-only control (Fig. 4B). Fatty acid analysis of the complemented strains indicated that expression of *pfaF* from this construct was able to fully restore EPA levels back to wild-type levels (Table 2). Similarly, transcript abundances of *pfaA* and *pfaD* were also restored to wild-type levels in the complemented strain (data not shown).

Characterization of PfaF binding to the *pfa* promoter. To verify that regulation by PfaF was direct, electrophoretic mobility shift assays (EMSA) were performed. The *pfaF* gene was cloned onto pET28 with an N-terminal 6-His tag to yield the construct pMA66 (Table 1), expressed in *E. coli*, and purified to homogeneity by Ni affinity chromatography (see Fig. S1 in the supplemental material). The DNA probe used in the EMSA was generated by PCR using a 6-carboxyfluorescein (6-FAM)-labeled primer set and was designed to cover 400 bp directly upstream of the translational start of PfaA. Recombinant PfaF was able bind to the *pfaA* promoter probe in a concentration-dependent manner, forming a single discrete complex (Fig. 5A).

To further verify the interaction between PfaF and the *pfaA* promoter, bacterial one-hybrid assays were conducted (31). In this assay, the promoter of interest is fused to a reporter cassette (HIS3-*aadA*) on one plasmid and the DNA binding protein partner is fused to the N-terminal RNA polymerase alpha subunit ($RNAP\alpha$) on a compatible separate plasmid. Both plasmids are transformed into an *E. coli* reporter strain (KJ1C) which cannot grow on minimal medium with glucose as a sole carbon source due to a *hisB463* mutation. Interaction between the regulator and cloned promoter pair activates transcription of the HIS3-*aadA* cassette on the reporter plasmid complementing this auxotrophy in the presence of the HIS3 inhibitor 3-amino-1,2,4-triazole (3-AT) (see Fig. S3A in the supplemental material). The *aadA* gene confers resistance to streptomycin and provides an additional means of confirmation. As seen in Fig. S3B, the *pfaA* promoter/PfaF pairing allowed for growth on selective minimal media, indicative of PfaF binding to the *pfaA* promoter *in vivo*.

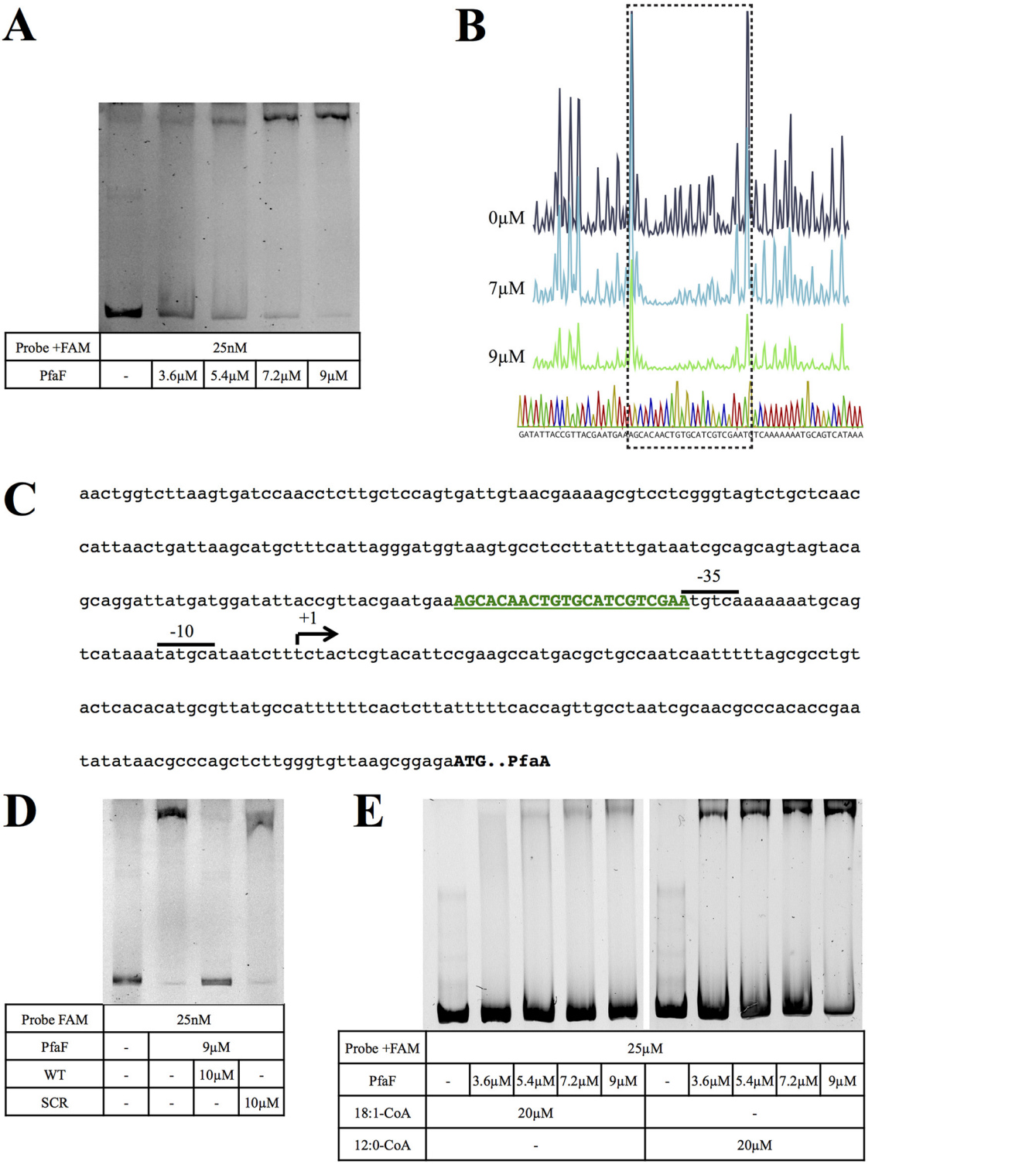


FIG 5 Characterization of PfaF binding to the *pfaA* promoter. (A) Electrophoretic mobility shift assay demonstrating PfaF binding to the *pfaA* promoter in a concentration-dependent manner. (B) DNase I footprinting analysis of PfaF binding to the *pfaA* promoter. Purified PfaF was added at the indicated concentrations and subjected to DNase I digestion as described in Materials and Methods. The chromatograms and sequencing traces shown correspond to the coding strand, and the box indicates the region protected from digestion by PfaF. (C) DNA sequence of the *pfaA* promoter probe used in mobility shift and footprinting assays. The promoter elements (−35 and −10 sites) and transcriptional start site (arrow) were previously determined (25). The region protected by PfaF is indicated by the underlined font. (D) Binding of an FAM-labeled probe by PfaF is reversed by a molar excess of an unlabeled annealed oligonucleotide containing the PfaF binding site (WT). Binding is not affected by a molar excess of a random scrambled oligonucleotide (SCR). (E) Addition of oleoyl-CoA, but not lauroyl-CoA, inhibits the binding of PfaF to the probe. Representative gels at a single concentration (20 μM) of each acyl-CoA with various amounts of PfaF are shown.

To localize the binding site for PfaF in the *pfaA* promoter, nonradioactive DNase I footprinting assays were performed utilizing the 6-FAM-labeled probe used in the EMSA analysis. As shown in Fig. 5B, inclusion of increasing amounts of purified PfaF led to protection of a 22-bp sequence (AGCACAACTGTGCATCGTCGAA) corresponding to positions −182 to −204 of the promoter probe. Bases within the protected DNA sequence (GCACAACTGTGC) form a palindrome, which has been shown to include important elements of the operator sites for TetR family regulators. The identified binding site is positioned directly upstream of the previously identified −35 site within the *pfaA* promoter (Fig. 5C). Gel shift assays with a molar excess of an unlabeled DNA fragment containing this binding site effectively reversed PfaF binding, while a randomly scrambled probe did not affect binding (Fig. 5D).

Further phylogenetic analyses of PfaF indicated that homologs exist in other polyunsaturated fatty acid-producing bacteria, such as members of the genera *Shewanella* and *Colwellia*, and that these homologs are distinct from other well-known transcriptional regulators associated with fatty acid biosynthesis (see Fig. S2A in the supplemental material). One such homolog from *Shewanella amazonensis* SB2B (PDB 3rh2) has an unpublished crystal structure (Fig. S2B). The structure of the *Shewanella* PfaF homolog indicates that its C-terminal domain contains a pocket with an unknown ligand resembling the hydrocarbon "tail" of a fatty acid (Fig. S2C). Given the *in vivo* response to fatty acid supplementation observed, it was suspected that PfaF binding activity could be mediated by acyl-CoA ligands. Mobility shift assays indicated that the addition of oleoyl-CoA (18:1-CoA) inhibited PfaF binding activity in a concentration-dependent manner, while lauroyl-CoA (12:0-CoA) had no effect under identical conditions (Fig. 5E). The results of further assays of binding activity in the presence of various concentrations of oleoyl-CoA and lauroyl-CoA are shown in Fig. S4C and D in the supplemental material and clearly demonstrate that oleoyl-CoA (18:1-CoA) and not lauroyl-CoA (12:0-CoA) modulates DNA binding activity of PfaF.

DISCUSSION

In this work, the genetic regulation of the *pfa* operon has been characterized utilizing a variety of *in vivo* and *in vitro* genetic techniques. While the omega-3 polyunsaturated fatty acid products (13, 14), biosynthetic mechanism (15, 18, 32), and phylogenetic distribution (16, 33) of the bacterial *pfa* operon have 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 high hydrostatic pressure nor low temperature affected the activity of a *pfaA*::*lacZY* reporter is consistent with previous results in *Photobacterium profundum* SS9 (25) and validated our reporter gene fusion approach. We further confirmed the lack of correlation between the expression level of the *pfa* operon and the proportion of EPA found in membrane phospholipids under low-temperature and high-pressure culture conditions. The reasons for this phenomenon are unclear, but it suggests that other factors in the biosynthesis and membrane incorporation of EPA are involved in the increased abundance of EPA at high pressure and/or low temperature.

The finding that the *pfa* operon was downregulated in response to exogenous fatty acids in a FadR/FabR-independent manner indicated that an unknown transcription factor(s) was responsible for regulating this response to fatty acid supplementation. Screening of a transposon library in the *pfaA*::*lacZY* reporter strain identified a novel regulator, designated *pfaF*, whose gene product acts as a positive regulator of the *pfa* operon. Introduction of a *pfaF* null mutation in SS9R resulted in a severalfold decrease in EPA composition with relatively minor changes in the abundance of other fatty acids. Successful complementation confirmed the role of *pfaF* in the 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 (34).

Mobility shift and DNase I footprinting analyses verified that PfaF is capable of

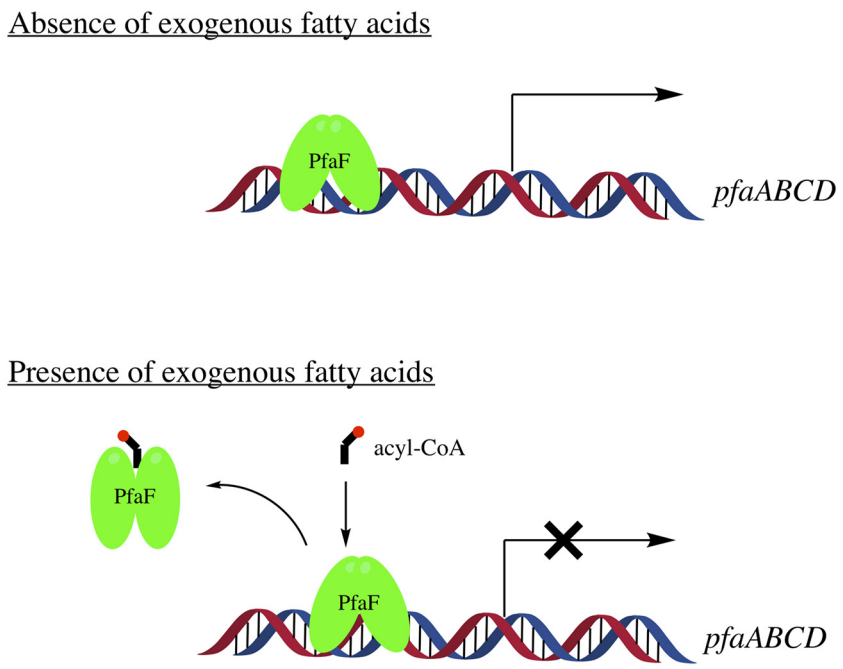


FIG 6 Proposed model of PfaF-mediated regulation of the *pfa* operon. In the absence of fatty acids, PfaF binds upstream of the *pfaA* promoter, acting as a positive regulator. In the presence of exogenous unsaturated fatty acids, PfaF binds to acyl-CoA and releases from the promoter region, leading to downregulation of the *pfa* operon.

binding to the *pfaA* promoter and localized its binding site. The binding site of PfaF and its position relative to the transcriptional start of *pfaA* are typical of other positive transcriptional regulators (35) and further support the role of PfaF as the transcriptional activator of the *pfa* operon. The *in vivo* data indicated that PfaF regulates the *pfa* operon in response to exogenous fatty acid supplementation, with the most robust response mediated by a long-chain monounsaturated fatty acid (18:1). As expected, addition of oleoyl-CoA (18:1-CoA) disrupted PfaF binding activity in a concentration-dependent manner, while a shorter-chain saturated ligand, lauroyl-CoA (12:0-CoA), had no effect under identical conditions. PfaF regulation of the operon in both SS9R (EPA^+) and a *pfaA*::*lacZY* reporter strain (EPA^-) indicates that the final EPA omega-3 fatty acid product of the Pfa synthase is not involved with mediating this regulatory activity.

Based on these results, a proposed model of regulation is shown in Fig. 6. In the absence of exogenous fatty acids, PfaF binds to its cognate sequence within the *pfaA* promoter region and acts as a positive regulator. In the presence of exogenous unsaturated fatty acids, which are presumably converted into acyl-CoA, PfaF binding to an acyl-CoA leads to its dissociation from the promoter, leading to a lack of transcriptional activation of the *pfa* operon. Given its role in producing fatty acids for phospholipid biosynthesis (14) and its utilization of the same precursor metabolites as the type II fatty acid synthase (15), it is not surprising that the Pfa synthase is controlled in a nearly identical fashion, albeit with its own cognate regulatory protein.

Interestingly, genes regulated in a similar fashion, typically by FadR, have been shown to be involved in processes related to virulence within members of the *Vibrionaceae* (36–39). Furthermore, many of these virulence genes are regulated in response to exogenous fatty acids (40), similar to the results reported here for the *pfa* operon. Whether the production of EPA or other PUFA is part of a virulence, symbiosis, or colonization response is unclear, although it is interesting to note that many PUFA-producing bacteria have been isolated from the guts, glands, or other tissues of marine animals such as fish and various invertebrates (41, 42).

At this time, it is unclear how the various Tween derivatives and their fatty acid

components are utilized by *P. profundum* SS9. Various members of the *Vibrionaceae* family have been shown to have outer membrane-associated lipase enzymes capable of hydrolyzing esterified fatty acids for incorporation into phospholipids or use as a carbon source via β-oxidation (43, 44). In particular, one such enzyme, designated VolA, has been well characterized as being involved with utilization of exogenous esterified fatty acids such as lysophosphotidylcholine (43). 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 or carbon source.

Homology searches against all available genomes show that all PUFA-producing marine *Gammaproteobacteria* contain a *pfaF* homolog, many of which reside in similar genomic contexts. Whether *pfaF* is involved with regulating the *pfa* operon in these strains has yet to be determined. In some PUFA-producing genera, such as *Shewanella* and *Colwellia*, the *pfa* operon also contains an annotated regulator, typically designated *pfaR*, immediately upstream of *pfaA*. Interestingly, the protein sequence of *pfaR* does not match to any class of bacterial transcriptional regulators but does contain an identifiable N-terminal helix-turn-helix domain, which may be involved in DNA binding activity. Preliminary results using *Shewanella piezotolerans* WP3, a genetically tractable EPA producer, indicated no differences in EPA composition between the wild type and Δ*pfaR* mutants under different growth temperatures (data not shown). A previous study (45) 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 that in *P. profundum* SS9.

The results presented here describe the identification of a novel transcriptional regulator in the model marine bacterium *P. profundum* SS9 that specifically modulates expression of the *pfa* operon in response to exogenous fatty acids and controls the amount of polyunsaturated fatty acid 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 and synthesis of microbial long-chain omega-3 polyunsaturated fatty acids.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains were routinely grown at 37°C in Luria-Bertani (LB) medium unless stated otherwise. *Photobacterium profundum* SS9 strains were grown at 15°C in 2216 marine broth (Difco) at 75% strength (28 g/liter) unless noted otherwise. For solid media, agar was included at 15 g/liter. The antibiotics kanamycin (50 μg/ml for *E. coli* and 200 μg/ml for *P. profundum*), chloramphenicol (15 μg/ml), ampicillin (100 μg/ml), and rifampin (100 μg/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 (46).

Targeted mutagenesis. Vectors for introducing mutations into *P. profundum* were introduced by conjugation using previously described methods with minor alterations (14, 46). In-frame deletions were generated by allelic exchange using the suicide vector pRE118 (47). Insertional inactivation of target genes was accomplished by introduction of the suicide plasmid pMUT100 as described previously (23, 48).

Transposon mutagenesis and screening. Biparental conjugations using *E. coli* S17-1λ*pir* were used to transfer the mini-Tn*5* delivery plasmid pRL27 into the *pfaA*::*lacZY* reporter strain (Table 1) (29, 49). Both recipient and donor strains were grown to stationary phase, and conjugations were performed as described above. After ~24 h at ambient temperature (~22°C), cells on filter membranes were resuspended in 2216 broth, plated onto selection medium (2216 agar containing 200 μg/ml kanamycin and 100 μg/ml rifampin), and incubated at 15°C for 5 days. Resulting exconjugants were patched to fresh selection plates in grid format. After 2 days at 15°C, the arrayed mutants were replica plated to 2216 agar with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (80 μg/ml). After 2 days of growth on indicator medium, 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 similar to the method described previously was utilized (30). Primer sequences are given in Table S1 in the supplemental material. In the first round of PCR, a primer specific to one end of

the mini-Tn*5* element (Tn*5* 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 as follows: 95°C for 5 min; 6 cycles of 95°C for 30 s, 30°C for 30 s, and 68°C for 2 min; and 30 cycles of 95°C for 30 s, 45°C for 30 s, 68°C for 2 min, and 68°C for 5 min. Two microliters of the first reaction product was used as a template for a nested PCR with "Arb clamp" and "Tn*5* int" primers. The conditions for the second PCR were as follows: 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 2 min; and 68°C for 10 min. Products of PCRs yielding single amplicons as judged by agarose gel electrophoresis were purified using a PCR cleanup kit (Zymo Research) and sent for DNA sequencing. Sequences were compared to the genome of *P. profundum* SS9 to determine the insertion site of the mini-Tn*5* element.

β-Galactosidase assays. Cultures of the indicated strains were grown at 15°C in aerobic tubes, unless noted otherwise. Mid-log-phase cultures (optical density at 600 nm [OD_{600}] = 0.2 to 0.6) were assayed for changes in LacZ activity from whole-cell extracts using the SDS and chloroform lysis modification described previously (50). Activities are reported in Miller units and represent the mean from at least five independent experiments. For experiments using free fatty acid supplementation, LacZ activities were normalized to total protein due to the insolubility of free fatty acids in 2216 marine medium, thus precluding accurate optical density measurements of growth (23). Total protein measurements were obtained with a bicinchoninic acid (BCA) assay kit following the manufacturer's protocol (Thermo Scientific).

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 the manufacturer's 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 PCRs (qPCR) 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 (locus tag PBPRA0011) was used as an internal reference, and differences in expression were calculated using the $\Delta\Delta C_T$ method. Primers for qPCR experiments are listed in Table S1.

Bacterial one-hybrid assays. Plasmids containing the *pfaA* promoter fused to the HIS3-*aadA* cassette and PfaF fused to the RNA polymerase alpha subunit (RNAPα) were constructed using standard methods (51). The plasmids were cotransformed into chemically competent *E. coli* KJ1C and recovered in LB for 1 h at 30°C. Cells were washed three times in 0.9% NaCl to remove nutrients, plated onto M9 glucose agar containing ampicillin (50 μg/ml) and kanamycin (25 μg/ml), and incubated at 30°C for 2 days. Colonies were then patched or streaked onto a selective medium containing 20 mM 3-amino-1,2,4-triazole (3-AT) as described previously (31), and incubated at 30°C for 2 days. Transformants that grew on this selective medium were transferred to 3-AT plates containing 50 μg/ml streptomycin to confirm the interaction.

Expression and purification of PfaF. The SS9 *pfaF* gene (locus tag PBPRA0221) was cloned into pET28 as an NheI-XhoI fragment (Novagen) to generate an N-terminally 6-His-tagged protein. After sequence verification, this construct was transformed into BL21(DE3) Tuner pLysS (Novagen) cells following standard procedures (50). For protein expression, overnight cultures were diluted 1/100 into LB supplemented with chloramphenicol (30 μg/ml) and kanamycin (50 μg/ml) and grown at 30°C to an OD_{600} of ~0.5, at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and cells grown for an additional 4 h 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 (50 mM Tris-Cl [pH 7.5], 200 mM NaCl, 10% glycerol). Lysozyme was added, and cells were incubated on ice for 30 min and sonicated on ice to complete the lysis procedure. The lysate was centrifuged at 10,000 rpm and 4°C for 30 min to separate insoluble and soluble fractions. The clarified supernatant was applied to an Ni-nitrilotriacetic acid (NTA) column equilibrated with buffer A and mixed gently at 4°C for 1 h. The resin was washed with several column volumes of buffer B (buffer A plus 30 mM imidazole), and proteins were eluted with buffer C (buffer A plus 300 mM imidazole). Eluted fractions were checked by SDS-PAGE for purity, and appropriate fractions were pooled, desalted using PD-10 columns (GE Healthcare), and exchanged into buffer D (20 mM Tris Cl [pH 7.5], 50 mM NaCl, 10% glycerol). Protein samples were pooled and subsequently concentrated with 10,000-kDa centrifugal filter units (Amicon).

EMSA and DNase I footprinting. A 400-bp DNA fragment, which included the previously mapped promoter of *pfaA*, was generated by PCR using a 6-carboxyfluorescein (6-FAM)-labeled primer listed in Table S1 in the supplemental material and purified *P. profundum* SS9 genomic DNA as a template. In competition experiments, complementary 60-bp oligonucleotides were annealed in buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA, 50 mM NaCl) by heating to 95°C for 5 min and allowed to cool over several hours to ambient temperature (~22°C). For mobility shift experiments, binding reaction mixtures contained binding buffer (20 mM Tris-Cl [pH 7.5], 0.2 mg/ml bovine serum albumin [BSA], 0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10% glycerol), 2 μg poly(dI-dC) (Thermo Scientific), 25 nM promoter probe, and the indicated amount of purified PfaF. Binding reaction mixtures were incubated at 22°C for 60 min and analyzed by electrophoresis using prerun 6% 0.5× Tris-borate-EDTA (TBE)–1% glycerol native polyacrylamide gels (52). Gels were visualized and photographed using a GelDoc XR+ system (Bio-Rad).

Nonradioactive DNase I footprinting (53) was performed using the same binding buffer and reaction conditions used in the EMSA experiments described above. Digests were initiated by the addition of 0.03 U of DNase I (NEB), and mixtures were incubated at 22°C for 2 min. Digestion reactions were stopped by the addition of DNase I stop buffer (NEB), and mixtures were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) (Thermo Scientific). DNA fragments were further purified by use of a PCR purification kit

(Zymo). Eluted DNA fragments were subjected to fragment analysis by capillary electrophoresis (Eton BioScience). Chromatograms were examined using the microsatellite plug-in within Geneious Prime 2019.2.3 (Biomatters Ltd.). Protected regions were identified and compared to the included LIZ-500 size standards to identify the binding site coordinates and relevant protected bases.

Fatty acid extraction and GC-MS analysis. Late-log-phase cultures were harvested by centrifugation, and cell pellets were rinsed once with 50% Sigma Sea Salts solution (16 g/liter) 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 (GC-MS) using previously described protocols and methods (14).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 7.5 MB.

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