Ketoacylsynthase Domains of a Polyunsaturated Fatty Acid Synthase in *Thraustochytrium* sp. Strain ATCC 26185 Can Effectively Function as Stand-Alone Enzymes in *Escherichia coli*

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ABSTRACT *Thraustochytrium* sp. strain ATCC 26185 accumulates a high level of docosahexaenoic acid (DHA), a nutritionally important ω -3 very-long-chain polyunsaturated fatty acid (VLCPUFA) synthesized primarily by polyunsaturated fatty acid (PUFA) synthase, a type I polyketide synthase-like megaenzyme. The PUFA synthase in this species comprises three large subunits, each with multiple catalytic domains. It was hypothesized that among these domains, ketoacylsynthase (KS) domains might be critical for catalyzing the condensation of specific unsaturated acyl-acyl carrier proteins (ACPs) with malonyl-ACP, thereby retaining double bonds in an extended acyl chain. To investigate the functions of these putative KS domains, two segment sequences from subunit A (KS-A) and subunit B (KS-B) of the PUFA synthase were dissected and then expressed as stand-alone enzymes in *Escherichia coli*. The results showed that both KS-A and KS-B domains could complement the defective phenotypes of both *E. coli fabB* and *fabF* mutants. Overexpression of these domains in wild-type *E. coli* led to increases in total fatty acid production. KS-B produced a higher ratio of unsaturated fatty acids (UFAs) to saturated fatty acids (SFAs), while KS-A could improve the overall production of fatty acids more effectively, particularly for the production of SFAs, implying that KS-A is more comparable to FabF, while KS-B is more similar to FabB in catalytic functions. Successful complementation and functional expression of the embedded KS domains in *E. coli* are the first step forward in studying the molecular mechanism of the PUFA synthase for the biosynthesis of VLCPUFAs in *Thraustochytrium*.

IMPORTANCE Very-long-chain polyunsaturated fatty acids (VLCPUFAs) are important for human health. They can be biosynthesized in either an aerobic pathway or an anaerobic pathway in nature. However, abundant VLCPUFAs in marine microorganisms are primarily synthesized by polyunsaturated fatty acid (PUFA) synthase, a megaenzyme with multiple subunits, each with multiple catalytic domains. Furthermore, the fundamental mechanism for this enzyme to synthesize these fatty acids still remains unknown. This report started with dissecting the embedded KS domains of the PUFA synthase from marine protist *Thraustochytrium* sp. strain ATCC 26185 and then expressing them in wild-type *E. coli* and mutants defective in condensation of acyl-ACP with malonyl-ACP. Successful complementation of the mutants and improved fatty acid production in the overexpression experiments indicate that these KS domains can effectively function as stand-alone enzymes in *E. coli*. This result has paved the way for further studying of molecular mechanisms of the PUFA synthase for the biosynthesis of VLCPUFAs.

KEYWORDS polyunsaturated fatty acid synthase, ketoacylsynthase domain,

Received 21 November 2016 Accepted 3 February 2017

Accepted manuscript posted online 17 February 2017

Citation Xie X, Meesapyodsuk D, Qiu X. 2017. Ketoacylsynthase domains of a polyunsaturated fatty acid synthase in *Thraustochytrium* sp. strain ATCC 26185 can effectively function as stand-alone enzymes in *Escherichia coli*. Appl Environ Microbiol 83: e03133-16. <https://doi.org/10.1128/AEM.03133-16>.

Editor M. Julia Pettinari, University of Buenos Aires

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docosahexaenoic acid (DHA), *Thraustochytrium*, fatty acid biosynthesis, *Escherichia coli*, docosahexaenoic acid

Very-long-chain polyunsaturated fatty acids (VLCPUFAs) are fatty acids with the chain length equal to or greater than 20 carbons and with two or more double bonds. They are classified into two categories, ω -3 and ω -6 fatty acids, depending on the location of the last double bond from the methyl end in an acyl chain (1). VLCPUFAs, particularly docosahexaenoic acid (DHA) (22:6- Δ 4,7,10,13,16,19), are important for human health and wellbeing. DHA is an essential membrane component of neurons and retina and immune cells. In the retina, DHA is a major fatty acid in the photosensitive part of the rod outer segment (2) and is involved in signal transduction, rhodopsin activation, and rod and cone development. In the nervous system, DHA influences synaptic function through the modulation of endocannabinoids in the synaptic system. In the brain, DHA is mainly accumulated in cortical gray matter and has positive effects on sensorimotor integration, attention-executive function, and memory (3, 4). In addition, DHA is a precursor for docosanoids mediating various physiological activities in mammals. For example, neuroprotectin D1 (docosanoid) is a neuroprotective derivative of DHA participating in protection against retina and brain damage (5–8). Furthermore, DHA is a ligand of nuclear transcriptional factors regulating the expression of numerous genes at the transcriptional level by activating transacting factors (9). Therefore, maintaining an optimal level of DHA is crucial for humans to stay healthy, and appropriate dietary supplementation of DHA is encouraged for providing protection against chronic diseases, such as hypertension, arthritis, arteriosclerosis, depression, and thrombosis (10).

Two distinct pathways for the *de novo* biosynthesis of VLCPUFAs have been found in living species (11). The aerobic pathway usually occurs in the cytosolic endoplasmic reticulum of eukaryotes, and double bonds are introduced by an oxygenic desaturation process, while fatty acid chains are elongated through an elongation process. Therefore, in the aerobic pathway, desaturases and elongases are the two types of key enzymes for synthesizing VLCPUFAs. So far, many desaturases and elongases in the pathway have been identified from a wide range of eukaryotes, including animals, plants, fungi, microalgae, and other microorganisms (12, 13). The anaerobic pathway, also called a polyketide synthase pathway, usually occurs only in prokaryotic and eukaryotic microbes. In this pathway, double bonds are introduced during an acyl chain-extending process without the occurrence of oxygenic desaturation. The biosynthesis is catalyzed by polyunsaturated fatty acid (PUFA) synthase, a polyketide synthase (PKS)-like enzyme (14). The PUFA synthase in microorganisms usually comprises one or more subunits; each subunit may contain multiple domains, such as ketoacylsynthase (KS), ketoacylreductase (KR), dehydratase (DH), enoylreductase (ER), acyltransferase (AT), malonyl-coenzyme A (CoA):ACP transacylase (MAT), and acyl carrier protein (ACP). These domains work coordinately for catalyzing the VLCPUFA synthesis through reiterative reactions, including condensation, ketoreduction, dehydration, and reduction.

Thraustochytrium sp. strain ATCC 26185 is a unicellular marine protist in Thraustochytrid (15) and has been explored as a source of very-long-chain polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) (22:6n-3) for industrial use. Our previous research indicated that there was an aerobic pathway in this species for the biosynthesis of VLCPUFAs, and DHA could be synthesized through the final step of Δ 4 desaturation (16). Our recent genome sequencing revealed an alternative anaerobic pathway for the biosynthesis of VLCPUFAs in this species (17). However, the *in vitro* and *in vivo* assays showed that the latter pathway was primarily responsible for the biosynthesis of DHA (18), while the aerobic pathway might be the progenitor and relic system with missing components. Like similar enzymes identified from other marine microbes (19–21), the PUFA synthase from *Thraustochytrium* is comprised of three subunits, each with multiple catalytic domains. Among these domains, two putative KS domains from subunit A and subunit B might be critical for retaining double bonds by

FIG 1 Schematic representation of the structure of the PUFA synthase from *Thraustochytrium* sp. strain ATCC 26185. The borders of the KS domains were denoted by the length (in amino acids). The locations of the N and C termini and the malonyl-CoA:ACP transacylase (MAT), acyl carrier protein (ACP), ketoacylreductase (KR), dehydratase (DH), chain length factor (CLF), acyltransferase (AT), and enoylreductase (ER) subunits are shown.

selectively elongating unsaturated acyl chains (19, 22, 23). In this work, the two embedded KS domains of the PUFA synthase from *Thraustochytrium* were delimited by multiple alignments and homology modeling using protein sequences of ketoacylsynthase from *E. coli* and KS domains from microbial type I PKS and other PUFA synthases. The KS domains were then expressed as stand-alone enzymes for functional analysis through complementation, site-directed mutagenesis, and overexpression in *E. coli*. The results from these studies shed insights into the catalytic functions of these KS domains of the PUFA synthase from *Thraustochytrium*.

RESULTS

Sequence analysis of KS domains of a PUFA synthase in *Thraustochytrium*. To study the biosynthesis of VLCPUFAs in *Thraustochytrium* sp. strain ATCC 26185, we recently sequenced the whole genome of the protist. Analysis of the genome sequence revealed the coexistence of a standard aerobic pathway and an alternative anaerobic pathway for the biosynthesis of DHA in this species (17). Our *in vitro* and *in vivo* assays showed that DHA was primarily synthesized by the PUFA synthase (18), a megaenzyme comprising three large subunits encoded by three open reading frames (ORFs), each with multiple catalytic domains that were strongly predicted by the presence of characteristic active site sequence motifs (Fig. 1). One KS domain (KS-A) was identified in open reading frame A (ORF-A), while the other KS domain (KS-B) was found in ORF-B. The two KS domains had amino acid sequence identity with each other at about 20%, and both domains exhibited similar sequence similarities with FabB and FabF from *E. coli*, two condensing enzymes involved in the biosynthesis of fatty acids in *E. coli*. In addition, a chain length factor (CLF)-like domain was also identified to be adjacent to KS-B in ORF-B. The boundary sites of these domains were determined through multiple-sequence alignments and homology modeling using the protein sequences of prokaryotic ketoacylsynthases and KS domains from microbial type I PKS and other PUFA synthases. The KS-A domain ranged from residues 1 to 492 in ORF-A, while the KS-B domain was from residues 1 to 469 in ORF-B. For the functional characterization of these domains, the DNA regions were amplified and expressed as stand-alone proteins in *E. coli*.

Expression of the KS domains in an *E. coli fabB* mutant. *E. coli* possesses three condensing enzymes, ketoacyl-ACP synthase I (FabB), ketoacyl-ACP synthase II (FabF), and ketoacyl-ACP synthase III (FabH) for fatty acid biosynthesis. FabH catalyzes the initial condensation of acetyl-CoA with malonyl-ACP to ketobutyryl-ACP. FabB and FabF with high sequence similarity to each other catalyze subsequent condensation reactions. However, FabF could specifically elongate long-chain fatty acids, such as palmitic acid (16:0) and palmitoleic acid (16:1–9), while FabB could catalyze elongation of fatty acids with chain length from 4 to 14 carbons (24). As both KS domains from the PUFA synthase showed sequence homology to FabB and FabF, the embedded domains of the PUFA synthase were thus excised and expressed as stand-alone enzymes in *fabB* and *fabF* mutants for functional complementation assays.

FIG 2 Plate complementation assays of the KS domains in *E. coli fabB(Ts)* mutant. The mutant contains empty vector pET28a. KS domains and *E. coli fabB* were inserted behind the T7 promoter of pET28a individually. The recombinant plasmids were transformed into the *fabB(Ts)* mutant. The plates with kanamycin and IPTG were incubated overnight at 30°C and 42°C, respectively. WT, wild type.

fabB is an essential gene for the biosynthesis of unsaturated fatty acids for *E. coli* to maintain homeostasis of the cell membrane (25–29), and no knockout strain of this gene is readily available for the complementation test. Therefore, the complementation assays of KS domains were carried out in a *fabB(Ts)* mutant where the chromosomal *fabB* gene was chemically mutagenized; as such, the *fabB* mutant became temperature sensitive and could grow at 30°C, but not at a nonpermissive temperature (42°C). However, the *fabB* mutant carrying KS-A and KS-B domains and the wild-type *fabB* strain were all capable of growing at both temperatures (30°C and 42°C), in contrast to the mutant with the empty vector that ceased growth at 42°C on plates (Fig. 2). This result clearly showed that both KS domains from ORF-A and ORF-B could complement the defective phenotype of *fabB* and restore the function for the biosynthesis of unsaturated fatty acids in *E. coli*.

When grown at the permissive temperature (30°C), the *fabB* mutant, like the parent strain, produced six major fatty acids, including myristic acid (14:0), palmitoleic acid (16:1–9), palmitic acid (16:0), 9,10-methylene-hexadecanoic acid (17:0-cyclopropane), *cis*-vaccenic acid (18:1–11), and 11,12-methylene-octadecanoic acid (19:0-cyclopropane). The two cyclopropane fatty acids were synthesized by cyclopropane fatty acid synthase (CFA) using unsaturated fatty acids 16:1–9 and 18:1–11 as the substrates, respectively (30). However, compared to the wild type, the amounts of these fatty acids in the mutant were substantially reduced, particularly 16:0, 16:1–9, 17:0-cyc, and 18:1–11, which led to drastic reduction in the amount of the total fatty acids in the mutant strain. On the other hand, expression of the KS domains from the PUFA synthase in the mutant, like the expression of wild-type *fabB* in the mutant, could clearly reverse the defective phenotype in fatty acid production. In particular, when KS-A and KS-B were expressed in the mutant, the amounts of fatty acids, such as 16:0, 16:1–9, 17:0-cyc, and 18:1–11 were comparable (KS-B) to or even higher (KS-A) than the amount in the mutant expressing the wild-type *fabB* gene, and the levels were all significantly higher than that of the mutant control with the empty vector (Fig. 3). In addition, although the mutant could not grow at 42°C, the mutant expressing all KS domains could grow at this temperature. Compared to the wild type, however, the amount of the total fatty acids expressed was slightly reduced, particularly 16:0 and 18:0, which led to the slightly higher ratio of unsaturated fatty acids to saturated fatty acids in the mutant expressing KS domains (see Table S1 and Fig. S2 in the supplemental material).

Expression of KS domains in an *E. coli fabF* mutant. Unlike *fabB*, *fabF* is a nonessential gene for *E. coli*; thus, a knockout mutant is readily available for complementation tests. However, the plate assay could not be employed for the test; instead, the complementation tests of the KS domains in a *fabF* mutant were conducted through fatty acid analysis of the mutant and the mutant transformants expressing the KS domains. In comparison with the wild-type control, nearly all fatty acids were significantly reduced in the mutant, leading to a ca. fourfold decrease in the production of total fatty acids (Fig. 4). However, when the KS domains were expressed in the

FIG 3 Amounts of fatty acids produced in the *E. coli fabB* mutant expressing the KS domains from the PUFA synthase at 30°C. Values are reported as means plus standard deviations (error bars) for three biological replicates. The means with the same letters are not statistically significantly different. The statistical analysis of the results from experiments was conducted using one-way analysis of variance ($P < 0.05$).

mutant, the transformants restored fatty acid production to a level similar to that in the wild type. In particular, levels of 16:1–9 and 17:0-cyc were significantly increased in the mutant transformant expressing KS-A domain, which resulted in the amount of total fatty acids being even slightly higher than that in the wild type. This result indicated that these KS domains could also functionally complement the defective phenotype of the *E. coli fabF* mutant.

Functional analysis of mutagenized KS domains. The identity of KS domains in PUFA synthase was predicted based on the presence of characteristic residues at their putative active sites. To examine the authenticity of these residues for the function of KS domains, the cysteine residues at the predicted active sites of both KS domains were

FIG 4 Amounts of fatty acids produced in *E. coli fabF* mutants expressing the KS domains from PUFA synthase. Values are reported as means plus standard deviations for three biological replicates. The means with the same letters are not statistically significantly different. The statistical analysis of the results from experiments was conducted using one-way analysis of variance ($P < 0.05$).

FIG 5 Plate complementation assays of the mutagenized KS domains in *E. coli fabB(Ts)* mutant. The KS domains and *E. coli fabB* gene were individually inserted behind the T7 promoter of pET28a. One of the duplicate plates was incubated at 30°C and the other was incubated at 42°C for complementation testing.

individually replaced by lysine residues using site-directed mutagenesis, and the mutant domains were then expressed as stand-alone enzymes for complementation analysis. Complementation assays of the two mutagenized KS domains in the *fabB* mutant showed that, like the original mutant, the mutant transformants expressing both mutagenized KS domains could grow only at 30°C, but not at the nonpermissive temperature 42°C, which was in contrast to the mutant transformants expressing wild-type KS domains that could grow at both temperatures (Fig. 5). This result reaffirmed the function of the KS domains and the essentiality of the cysteine residues of KS domains for catalytic function as FabB for the biosynthesis of unsaturated fatty acid in *E. coli*.

The complementation assays of mutagenized KS domains in the *fabF* knockout mutant showed that fatty acid production in the mutant expressing both mutagenized KS domains was comparable to that in the original mutant where the amounts of almost all fatty acids were substantially reduced in comparison to that in the wild-type control, leading to the drastic reduction of total fatty acids in the cells. This result further confirmed that the cysteine residues were also critical for the function of KS domains to complement the activity of FabF in *E. coli* (Fig. 6).

Overexpression of KS domains in *E. coli*. To further investigate functional roles of KS domains, we overexpressed these domains as discrete enzymes in wild-type *E. coli* strains grown at two different temperatures (20°C and 37°C) to see whether they could improve the production of fatty acids. At 37°C, the amount of total fatty acids produced in the wild type and the transformants overexpressing KS domains were relatively low

FIG 6 Amounts of fatty acids produced in the *E. coli fabF* mutant expressing mutagenized KS domains from PUFA synthase. Values are reported as the means plus standard deviations for three biological replicates. The means with the same letters are not statistically significantly different. All experiments were done in triplicate. The statistical analysis of the results from the experiments was conducted using one-way analysis of variance ($P < 0.05$).

FIG 7 Fatty acid production of KS domain overexpressed in wild-type *E. coli* at two different temperatures, 37°C (A) and 20°C (B). Values are reported as the means plus standard deviations for three biological replicates. The means with the same letters are not statistically significantly different. The statistical analysis of the results from experiments was conducted using one-way analysis of variance ($P < 0.05$). EV, empty vector.

compared to those at 20°C. In addition, there was no significant difference in the production of either individual fatty acids or total fatty acids among all strains. However, at 20°C, all transformants overexpressing KS domains produced significantly higher levels of total fatty acids than that in the wild type. In particular, overexpression of KS-A improved the production of total fatty acids significantly over that of the wild-type strain. The amount of total fatty acids produced in the strain overexpressing KS-A reached about 41 mg/liter, while that in the wild type was only about 28 mg/liter, an increase of approximately 45% in the overall fatty acid production largely realized by substantial increases in 16:0, 16:1–9, 17:0-cyc, and 18:1–11 (Fig. 7). Moreover, when the relative level of unsaturated fatty acids (UFAs) and saturated fatty acids (SFAs) produced in these strains was compared, all strains overexpressing KS had a higher ratio of UFAs/SFAs relative to that in wild type. However, the UFA/SFA ratio in the strain overexpressing KS-B was the highest for all strains, even higher than that in the strain overexpressing KS-A, despite the fact that the strain overexpressing KS-A was slightly higher in the production of total fatty acids than the strain overexpressing KS-B (Table 1). The higher production of total fatty acids in the strain overexpressing KS-A was mainly realized by higher production in SFAs, as the level of UFAs produced in the strain overexpressing KS-A was actually slightly lower than that in the strain overexpressing KS-B. This result indicated that overexpression of the two KS domains could significantly improve the overall production of total fatty acids and that the KS-B domain was more effective in the production of unsaturated fatty acids in *E. coli* than the KS-A domain was. The higher production of fatty acids in the overexpression of KS

TABLE 1 Production of saturated and unsaturated fatty acids in the overexpression of KS domains in wild-type *E. coli*^a

Strain	Production of FA (mg/liter) ^b			UFA/SFA ratio
	Total	SFA	UFA	
Wild-type	24.34 ± 1.33 C	9.42 ± 1.09 C	14.92 ± 0.85 C	1.58
Overexpressing KS-A	40.35 ± 2.4 A	14.78 ± 1.77 A	25.56 ± 1.12 A	1.73
Overexpressing KS-B	31.15 ± 1.69 B	10.00 ± 1.02 B	21.14 ± 0.66 B	2.11

^aProduction of saturated (SFA) and unsaturated fatty acids (UFA) in the overexpression of KS domains in wild-type *E. coli*.

^bValues were reported as the means ± standard deviations for three biological replicates. The means with the same letters are not statistically significantly different, while the means with different letters are statistically significantly different according to the one-way analysis of variance ($P < 0.05$).

domains from PUFA synthase than the control strain at the lower temperature is probably due to the improved folding of heterologous KS proteins. As a lower temperature usually favors accumulation of unsaturated fatty acids in cell membrane, the improved production of fatty acids was more substantially found on 16:1 and 18:1 in the *E. coli* BL21 (DE3) strains overexpressing the KS-A or KS-B domain from PUFA synthase.

DISCUSSION

Various PKS systems, including PUFA synthase have been identified in different types of microorganisms, such as bacteria, protists, and algae, for the biosynthesis of a variety of fatty acids (19, 20, 31). However, the exact molecular mechanism of PKS-like PUFA synthase for intricately positioning multiple double bonds in these VLCPUFAs is unknown. In *Thraustochytrium* sp. ATCC 26185, this enzyme comprises three subunits, each with multiple domains predicted by the presence of characteristic active site motifs. However, the authentic roles of these putative domains are not biochemically defined. It is assumed that key activities might be conferred by DH and KS domains of PUFA synthase, where DH domains are used for introducing double bonds while KS domains are for retaining these double bonds during the biosynthesis of VLCPUFAs (3, 23). In this study, we attempted to dissect and express embedded KS domains of PUFA synthase from *Thraustochytrium* in *E. coli* to interrogate the function of these domains.

E. coli is a well-studied model using type II fatty acid synthase to produce both SFAs and UFAs. FabB is a key enzyme for the biosynthesis of unsaturated fatty acids in catalyzing condensation of *cis*-3-decenoyl-ACP (formed by the FabA catalytic reaction) with malonyl-ACP (32), while FabF, despite being a nonessential enzyme, has an important function in elongating long-chain acyl-ACP, particularly for formation of vaccenic acid (18:1–11) (33). Both KS-A and KS-B domains from *Thraustochytrium* PUFA synthase share sequence similarity to FabB and FabF; thus, our first step for the functional characterization of these putative KS domains was to express them as stand-alone enzymes in two *E. coli* mutants. The complementation test of the *fabB* mutant showed that both KS domains could complement the defective growth phenotype at the nonpermissive temperature and the mutant transformants expressing both KS domains produced a high level of UFAs (16:1–9 and 18:1–11) to sustain growth at the high temperature (42°C), as seen in the wild-type strain. In particular, the strain expressing the KS-B domain could accumulate a higher level of UFAs than the strain expressing the KS-A domain. The complementation test of the *fabF* mutant also showed that both KS domains could complement the defective phenotype in fatty acid production. Mutant transformants with the KS-A domain could produce a high level of 18:1–11, as seen in the wild-type strain.

The functions of the KS domains from the PUFA synthase were further reaffirmed by site-directed mutagenesis and overexpression. The cysteines at the predicted active sites of these domains are highly conserved in discrete ketoacylsynthase enzymes and KS domains of fatty acid synthase and polyketide synthase in animals, plants, and microbes (14, 32, 34–38). The thiolate form is believed to serve as an “nucleophilic elbow” necessary for the nucleophilic attack in the transacylation reaction (37). When this residue in the two putative KS domains was changed to the lysine residue, the substitutions could not complement the defective phenotype of the *fabB* mutant and restore the activity of *fabF* in the deletion mutant. Overexpression of FabB and FabF have been previously shown to increase fatty acid production in *E. coli* (39, 40). As KS-A and KS-B can functionally complement the two enzymes, we then overexpress two KS domains in the wild-type strain to see whether they can produce similar effects. Indeed, overexpression of KS-B and KS-A result in 1.4-fold and 1.5-fold increases in total fatty acid production, respectively, relative to the wild-type control at the low temperature. The improvement in total fatty acid production reconfirms the biochemical functions of putative KS domains from PUFA synthase in fatty acid biosynthesis in *E. coli*. The strain carrying KS-B accumulates a high level of UFAs by retaining a double bond, as seen in the strain overexpressing the *E. coli fabB* gene. The KS-A domain has an authentic ability

in elongating 14:0 to 16:0 and 16:1 to 18:1, as seen in the strain overexpressing FabF (41). The fact that KS-B contributes a higher ratio of UFAs to SFAs than KS-A, while KS-A can improve the overall production of fatty acids, particularly for the production of SFAs in the transformants suggests that KS-A might be comparable to FabF, while KS-B might be more similar to FabB in catalytic function.

VLCPUFAs can be synthesized by either an aerobic or anaerobic pathway in marine microorganisms. The latter pathway is advantageous in productivity, as it is a *de novo* fatty acid system where final products are synthesized by a single enzyme complex starting from acetate. On the other hand, the former pathway involves many different kinds of enzymes, such as fatty acid synthase for the synthesis of long-chain saturated fatty acids (16:0 and 18:0), desaturases, and elongases for introducing various double bonds and for elongating fatty acids with various chain lengths. These enzymes can be localized at very different locations in a cell and use different forms of lipids as the substrates. Due to the complexity in localization and substrate use of these enzymes, VLCPUFA producers using the aerobic pathway for biosynthesis usually accumulate a substantial amount of intermediate fatty acids in addition to major final products, while VLCPUFA producers using PUFA synthase for biosynthesis often produce cleaner final products with fewer and lesser intermediate fatty acids (42, 43), which is considered more desirable for dietary supplementation. In addition, because the anaerobic pathway is a *de novo* system using a single enzyme, it might be more efficient for the production of VLCPUFAs. However, so far, little is known about the molecular mechanism of PUFA synthase for the biosynthesis of VLCPUFAs. PUFA synthase is a megaenzyme encoded by multiple large ORFs with usually high G+C content and repetitive ACP coding sequences. Cloning and expression of the large DNA fragments in heterologous systems for functional characterization are daunting tasks. In addition, the eukaryotic VLCPUFA producers with a PUFA synthase are usually not amenable to genetic manipulation. These make the mechanistic interrogation very challenging. This study attempted dissection of the entire PUFA synthase sequence from *Thraustochytrium* sp. ATCC 26185 into different catalytic domains and then expressed the embedded domains as stand-alone enzymes in *E. coli* for functional characterization. Successful complementation and functional expression of the KS domains in *E. coli* are the first steps toward future studies through *in vitro* assays for elucidating the molecular mechanism underlying the biosynthesis of VLCPUFAs.

MATERIALS AND METHODS

Strains, plasmids, media, and reagents. *Escherichia coli* strains Top10 and BL21 were purchased from Invitrogen Biotechnology Co. (Grand Island, NY, USA) grown in Luria-Bertani medium (1% peptone, 0.5% yeast extract, and 1% sodium chloride). *E. coli fabB* was provided by Charles Rock, Department of Biochemistry, St. Jude Children's Research Hospital, Tennessee, USA (44). The temperature-sensitive *fabB*(Ts) and knockout *fabF* mutants were purchased from National BioResource Project (NBRP *E. coli*, Microbial Genetics Laboratory, National Institute of Genetics, Japan) (45). Expression vectors, such as pET28a(+), pET15b, and pGEM-T, were purchased from Promega Co. (CA, USA). The plasmid extraction, DNA purification, and gel extraction kits were obtained from Bio Basic Inc. (York, Ontario, Canada). HP *Taq* DNA polymerase was purchased from Bio Basic Inc. Q5 polymerase, restriction enzymes, and deoxynucleoside triphosphates (dNTP) were purchased from New England BioLabs (Ipswich, MA, USA). Primers were synthesized through Sigma-Aldrich (St. Louis, MO, USA).

Sequence analysis of the PUFA synthase in *Thraustochytrium*. The sequence analysis of ketoacylsynthase (KS) domains from the polyunsaturated fatty acid (PUFA) synthase in *Thraustochytrium* sp. strain ATCC 26185 (ATCC, Manassas, VA, USA) was performed on the genome sequence (BioProject accession no. [PRJNA368972](https://www.ncbi.nlm.nih.gov/bioproject/26185)). Tools for cataloging fatty acid synthases (FASs)/polyketide synthases (PKSs), including BLAST (NCBI), ClusterMine360 (46), antiSMASH (47), and NRPS-PKS (48) were used to identify putative domains and active sites. The multisequence alignment of homologous sequences from *Aurantiochytrium*, *Schizochytrium*, *Emiliania huxleyi*, and *Shewanella fidelis* by DNAMAN was used to delimit the borderlines of each domain, and on-line homology modeling tools provided by the SWISS-MODEL (49) and Phyre server (50) were used to confirm them.

Cloning and expression of KS domains from the PUFA synthase in *E. coli*. To express the KS domains in *E. coli*, primers were designed for amplifying them from ORF-A (KS-A) and ORF-B (KS-B) with His tags at the N terminus and a stop codon at the C terminus (Table 2). The KS domain from ORF-A was amplified with two primers (F KS-A [F stands for forward] and R KS-A [R stands for reverse]), while the KS domain from ORF-B were amplified with two other primers (F KS-B and R KS-B). Amplicons with the expected size were first cloned into an intermediate pGEM-T vector for sequencing confirmation.

TABLE 2 Primers used for KS domain amplifications

Primer	Sequence (5'–3')	Sequence annotation
F KS-A	<u>GAATTC</u> ATGGCGGCCCGCG	EcoRI site underlined
R KS-A	AAGCTTCTACGCGTCTCCGGCTC	HindIII site underlined
F KS-B	<u>GAATTC</u> ATGGCGCGCGCAAC	EcoRI site underlined
R KS-B	AAGCTTCTAGTGCTCCTCGAAGACGGCG	HindIII site underlined

To construct plasmids expressing KS domains, the KS sequences from the pGEM-T vector and a *fabB* gene from the pET15b vector were subcloned into a His-tagged vector, pET28a(+). The purified KS fragment from pGEM-T and pET28a(+) vector digested with EcoRI and HindIII were ligated according to the standard procedure. Similarly, purified *fabB* fragment from pET15b and pET28a(+) digested with XbaI and BamHI were ligated. The digested DNAs were run on an agarose gel with wide-toothed comb wells. After electrophoresis, target bands were cut from the gel and purified using BioBasic's miniprep kit (2015). Purified KS and *fabB* fragments and corresponding digested plasmids were ligated as follows: 2 μ l of 5 $\times$ ligation buffer, 1.5 μ l of pET28a(+) linearized vector, 4.5 μ l of KS domain fragments and 0.5 μ l of Invitrogen T4 DNA ligase at 4°C overnight. The ligates, KS/pET28a and *fabB*/pET28a, were transformed into competent *E. coli* Top10 cells. The transformation was carried out using a chemical transformation approach. The sample was incubated at 37°C for 45 min, shaken vigorously (250 rpm), and placed on LB agar plates containing 50 μ g/ml kanamycin for selection. The selection plates were incubated at 37°C overnight, and putative transformants were selected, subcultured, and screened by PCR using a universal primer (T7 promoter) and a gene-specific primer. Positive transformants were inoculated into 5 ml of liquid LB medium plus 50 μ g/ml kanamycin and incubated at 37°C overnight. The plasmids were extracted using BioBasic's miniprep kit (2015) and digested with restriction enzymes to confirm the recombinant plasmids.

The confirmed plasmids pET28a-KS-A, pET28a-KS-B, and pET28a-*fabB* were transformed into competent *E. coli* BL21(DE3) cells for expression. A single colony from the host *E. coli* BL21 harboring an expression plasmid was grown overnight in 10 ml of LB medium with 50 μ g/ml kanamycin at 37°C. A 200- μ l aliquot of the resulting culture was inoculated into 20 ml of fresh LB medium containing kanamycin and incubated aerobically at 37°C until an A_{600} of 0.4 to 0.6 was reached. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) was added to the final concentration of 1 mM to induce protein expression. After 12-h induction, the cells were harvested using 6,000 rpm centrifugation (Eppendorf centrifuge 5804 R) at 4°C for 10 min and resuspended in 5 volumes of ice-cold buffer (20 mM Tris-HCl [pH 7.0]). The cells were disrupted with a beadbeater (Mini-BeadBeater 8; Biospec Products), and the mixture was centrifuged at 15,000 rpm at 4°C. The proteins in cell pellets and supernatant in 200 μ l of SDS-PAGE loading buffer (60 mM Tris-HCl [pH 6.8], 2% SDS, 0.1% bromophenol blue, 25% glycerol, and 14.4 mM β -mercaptoethanol) were separated by electrophoresis. To improve protein expression, the transformants were cultivated under different temperatures (20°C and 37°C) with different concentrations of IPTG (1 mM, 0.5 mM, and 0.01 mM).

Complementation of *E. coli* mutants defective in ketoacyl-ACP synthase activity with KS domains. The complementation tests were conducted in two *E. coli* mutants defective in ketoacyl-ACP synthase I and II activity, respectively. The *fabB*(Ts) temperature-sensitive mutant was derived from lysogenic *E. coli* strain K-12, mutagenized by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). This mutant could not grow at 42°C (but could survive at 30°C). The temperature-sensitive phenotype could be complemented with infection of a Kohara phage carrying a wild-type *fabB* gene. The other mutant is a knockout mutant from the Keio collection where the *fabF* gene was deleted (51).

Complementation test in a *fabB*(Ts) mutant. The competent cells of a *fabB*(Ts) mutant were prepared by the method of Chung et al. (52). The recombinant plasmids pET28a-KS-A, pET28a-KS-B, and pET28a-*fabB* and empty vector pET28a were transformed into competent *fabB*(Ts) mutant cells as described above, and the transformants were spread on the plates containing 50 μ g/ml kanamycin and grown at 30°C overnight for selection. The putative transformant colonies were selected and screened by PCR with a universal primer (T7 promoter) and a gene-specific primer. Positive colonies were inoculated into 5 ml of liquid LB medium plus 50 μ g/ml kanamycin and incubated at 30°C overnight. For the complement plate assay, 2- μ l cell cultures were dropped in duplicate on LB agar plates with 50 μ g/ml kanamycin and 1 mM IPTG. One of the duplicate plates was incubated at 30°C, and the other duplicate plate was incubated at 42°C.

The fatty acid profiles of the cell cultures were obtained through gas chromatography (GC) analysis. A single colony from transformants harboring the plasmid with the KS domain was grown overnight in 10 ml of LB medium with 50 μ g/ml kanamycin at 30°C. A 200- μ l aliquot of the resulting culture was inoculated into 20 ml of fresh LB medium containing kanamycin and grown aerobically at 30°C until an A_{600} of 0.4 to 0.6 reached. IPTG was added to the final concentration at 0.5 mM for inducing expression. After 12-h expression, the cells were harvested by centrifugation at 6,000 rpm and 4°C for 10 min and washed three times with 5 volumes of phosphate-buffered saline (PBS) buffer (20 mM PBS [pH 7.0]). Western blot analysis showed that KS domain under the control of the T7 promoter was expressed in the mutant strain lacking T7 RNA polymerase (see Fig. S1 in the supplemental material). The cell densities in samples were measured using a NanoDrop 2000c spectrophotometer and adjusted to an optical density at 600 nm (OD_{600}) of 1.0 with PBS buffer. An equal amount of cells (10 ml) was used for fatty acid analysis with heptadecanoic acid (17:0) as an internal standard (IS). For the analysis, 2 ml of 1% sulfuric methanol was added to cell pellets, and the mixture was incubated at 80°C for 1 h. After cooling down on ice, the

TABLE 3 Primers used for site-directed mutagenesis of KS domains

Primer	Sequence (5'–3') ^a
F KS-M-A	GCCGCCAAAGCCTCGTCGCTCATCG
R KS-M-A	CGAGGCTTTGGCGGCGTCGACGAC
F KS-M-B	GCCGCTAAAGCGACCGCGCTCTAC
R KS-M-B	GGTCGCTTTAGCGGCGTCGACCGA

^aMutation sites are shown underlined.

sample was added with 2 ml hexane and 1 ml of 0.9% NaCl. The mixture was centrifuged for 10 min at $2,200 \times g$. The upper hexane phase was transferred to a new glass tube and dried with N_2 gas. After drying, 200 μ l of hexane was added to resolubilize the lipid, and the solution was transferred into insert-containing vials for the GC analysis using an Agilent 7890A system with an autoinjector G4513A.

Complementation test in a *fabF* deletion mutant. For preparing the expression plasmid for a complementation test in a *fabF* deletion mutant, *KS-A* and *KS-B* domain genes from pET28a were subcloned into the expression vector pET15b. The confirmed recombinant plasmid was then transformed into the competent *fabF* mutant, and the transformant was selected on the plates containing 50 μ g/ml kanamycin and 100 μ g/ml ampicillin. For screening the positive colonies, a universal forward T7 promoter primer and a gene-specific reverse primer were used for PCR amplification. The positive colonies were inoculated into 5 ml of liquid LB medium plus 50 μ g/ml kanamycin and 100 μ g/ml ampicillin and grown at 37°C overnight. For the fatty acid analysis, the mutant transformants harboring the expression plasmid were grown overnight in 10 ml of LB medium with 50 μ g/ml kanamycin and 100 μ g/ml ampicillin at 37°C. A 200- μ l aliquot of the overnight culture was inoculated into 20 ml of fresh LB medium containing kanamycin and ampicillin and grown aerobically at 37°C until an A_{600} of 0.4 was reached. IPTG was added to the final concentration at 0.5 mM for inducing expression of the proteins. Fatty acid analysis of the *fabF* deletion mutant carrying the recombinant plasmids was conducted as described above.

Site-directed mutagenesis of KS domains. Site-directed mutagenesis was performed by overlapping PCR by the method of Ho et al. (53). Two pairs of internal primers with complementary ends and desired mutation sites were designed for PCR amplification to create mutant versions of the *KS* domains (Table 3). The recombinant plasmids pET28a-*KS-A* and pET28a-*KS-B* carrying wild-type *KS-A* and *KS-B* fragments were used as the templates for mutagenesis. For each mutagenesis, the first round of two PCRs was performed with two sets of one flanking primer and one internal primer using the expression plasmid as the template, and the second round of one PCR was conducted with two flanking primers using a mixture of the two amplified fragments as the template. For mutagenizing *KS-A*, two DNA fragments were amplified by overlapping PCRs with two sets of primers (one set is the T7 promoter primer and R KS-M-A primer, and one set is the F KS-M-A primer and T7 terminator primer) using pET28a-*KS-A* as a template. For mutagenizing *KS-B*, DNA fragments were amplified by overlapping PCRs with two sets of primers (one set is the T7 promoter primer and R KS-M-B primer, and one set is the F KS-M-B primer and T7 terminator primer) using pET28a-*KS-B* as the template. After PCR amplifications, the mutagenized *KS* domains (named *KS-M-A* and *KS-M-B*) were subcloned into pET28a or pET15b and transformed into *E. coli* Top10 cells for propagation of recombinant plasmids. The plasmids pET28a-*KS-M-A* and pET28a-*KS-M-B* were confirmed by restriction digestion and DNA sequencing. The confirmed plasmids were transformed into *E. coli* strains for functional analysis.

Lipid extraction and GC analysis. A single colony from *E. coli* transformants harboring the expression plasmid was grown overnight in 10 ml of LB medium with 50 μ g/ml kanamycin or with 50 μ g/ml kanamycin and 100 μ g/ml ampicillin at 30°C. A 200- μ l aliquot of the overnight culture was inoculated into 20 ml of fresh LB medium containing kanamycin or kanamycin and ampicillin and incubated aerobically at 30°C until an A_{600} of 0.4 was reached. IPTG was added to a final concentration of 0.5 mM for inducing expression of the proteins. Fatty acid profiles of transformants carrying the original or mutagenized *KS* domains were analyzed by GC as described above.

Overexpression of the *KS* domains in wild-type *E. coli*. Overexpression of the *KS* domains was performed in the wild-type strain *E. coli* BL21 star (DE3). The expression plasmids pET28a-*KS-A*, pET28a-*KS-B*, and pET28a-*FabB* and control vector pET28a were transformed into the competent cells. The transformants were then spread on the plates containing 50 μ g/ml kanamycin. A single colony of the positive transformant harboring the expression plasmid was grown overnight in 10 ml of LB medium with 50 μ g/ml kanamycin at 37°C. A 500- μ l aliquot of the overnight culture was inoculated into 50 ml of fresh LB medium containing kanamycin and incubated aerobically at 37°C until an A_{600} of 0.4 was reached. After IPTG was added, two sets of aliquots were prepared. One set of three aliquots was incubated at 20°C, while the other set of three aliquots was incubated at 37°C, for inducing expression of the domains. Fatty acid profiles of *E. coli* BL21 star transformants expressing different *KS* domains at different temperatures were analyzed by GC as described above.

Accession number(s). Data for *Thraustochytrium* sp. strain ATCC 26185 may be found in the NCBI BioProject database under accession number [PRJNA368972](https://doi.org/10.1128/AEM.03133-16).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.03133-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

We thank Charles O. Rock, Department of Biochemistry, St. Jude Children's Research Hospital, Tennessee, USA for providing the plasmid with the *E. coli fabB* gene and the National BioResource Project (NBRP *E. coli*, Microbial Genetics Laboratory, National Institute of Genetics, Japan) for providing *E. coli* mutant strains.

This research was supported by the Natural Sciences and Engineering Research Council of Canada.

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