Structure, Activity, and Substrate Selectivity of the Orf6 Thioesterase from *Photobacterium profundum*

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Background: Thioesterases are required for the release of polyunsaturated fatty acids in some bacteria.

Results: The Orf6 protein from *Photobacterium profundum* has been characterized both functionally and structurally.

Conclusion: Orf6 has a substrate preference for long-chain fatty acids.

Significance: This is the first in vitro and structural characterization of a hotdog thioesterase from a deep-sea bacterium.

Thioesterase activity is typically required for the release of products from polyketide synthase enzymes, but no such enzyme has been characterized in deep-sea bacteria associated with the production of polyunsaturated fatty acids. In this work, we have expressed and purified the Orf6 thioesterase from *Photobacterium profundum*. Enzyme assays revealed that Orf6 has a higher specific activity toward long-chain fatty acyl-CoA substrates (palmitoyl-CoA and eicosapentaenoyl-CoA) than toward short-chain or aromatic acyl-CoA substrates. We determined a high resolution (1.05 Å) structure of Orf6 that reveals a hotdog hydrolase fold arranged as a dimer of dimers. The putative active site of this structure is occupied by additional electron density not accounted for by the protein sequence, consistent with the presence of an elongated compound. A second crystal structure (1.40 Å) was obtained from a crystal that was grown in the presence of Mg^2+, which reveals the presence of a binding site for divalent cations at a crystal contact. The Mg^2+-bound structure shows localized conformational changes (root mean square deviation of 1.63 Å), and its active site is unoccupied, suggesting a mechanism to open the active site for substrate entry or product release. These findings reveal a new thioesterase enzyme with a preference for long-chain CoA substrates in a deep-sea bacterium whose potential range of applications includes bioremediation and the production of biofuels.

The biosynthesis of polyunsaturated fatty acids (PUFAs) in deep-sea bacteria takes place anaerobically through the activity of a polyketide synthase system consisting of four essential polypeptides (1–3). The multienzymes PfaA, PfaB, PfaC, and PfaD have been found to be sufficient for the production of PUFAs in *Escherichia coli*, which is normally a non-producer (2, 3). Like other polyketide synthase systems, the PUFA synthases contain many of the required enzyme domains for building long-chain fatty acids, including ketoacyl synthase, acyltransferase, acyl carrier protein (ACP), ketoacyl reductase, dehydratase, and enoyl reductase domains (see Fig. 1A). These enzyme activities are either stand-alone proteins (PfaB and PfaD) or housed within multidomain assemblies (PfaA and PfaC) (1, 2). The order and arrangement of domains in the PUFA synthases have no precedent in land-based biosynthesis machineries, but they are well conserved across marine organisms from different species and geographical locations (4).

One enzyme domain that is typically present in most land-based polyketide synthase systems but absent in the PUFA synthase gene cluster is the thioesterase (TE) domain, which catalyzes the release of free fatty acids and polyketides from ACP after complete product elongation (5, 6). Fatty acid release by TE has been found to be a limiting factor in the microbial production of fatty acids, and the overexpression of TEs has been associated with an increase in the yields of fatty acids and other biofuel precursors in microbial cultures (7–9). Thus, the TE domain is a widely accepted biochemical tool for boosting the production of natural products in industrial fermentations (10).

TE activity has evolved from two structurally distinct protein families: the α/β-hydrolase TE and hotdog TE. The α/β-hydrolase is more commonly associated with the cyclization and release of polyketides and fatty acids from ACP. As its name implies, it consists of an α/β-hydrolase fold with active site residues arranged as a catalytic triad consisting of the amino acids serine, histidine, and aspartic acid. The reaction mechanism is initiated by the nucleophilic attack of the thioester by the active site Ser, followed by hydrolysis of the resulting ester intermediate (6). The hotdog TE is more commonly associated with the cleavage of small products and aberrant intermediates from CoA carriers but not with the release of long-chain fatty acids from ACPs (11, 12).

Its mechanism involves a single conserved Asp (or Glu) residue that acts either as a nucleophile, generating a covalent anhydride intermediate, or as a general base, activating a water molecule directly (13, 14).
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It has been shown that PUFA producers, such as *Schizochytrium* species, accumulate products as free fatty acids, which are presumably released by the activity of a TE enzyme (15). However, a gene or DNA sequence encoding a dedicated TE enzyme has not been identified near any of the PUFA synthase gene clusters from deep-sea organisms. It has been hypothesized that the enzyme domain exhibiting this necessary TE activity for PUFA release is found somewhere within the known PUFA multienzyme sequence (15). However, this hypothesis is based on the observation that cell-free homogenates from *E. coli* expressing PUFA synthase proteins produce free fatty acids (15). In that work, it was not established whether the TE activity that causes the fatty acid release comes from within the expressed PUFA domains or whether it comes from endogenous TE proteins from the *E. coli* expression host. Thus, the precise location of the TE activity in PUFA synthases has not yet been established.

The PUFA synthase gene cluster in *Photobacterium profundum* SS9 features a gene (*orf6*) that encodes a putative hotdog hydrolase in close proximity to the other four *pfa* genes (*pfaABCD*) (1). In this work, we expressed and purified the Orf6 protein in *E. coli*. The purified protein shows TE activity and a substrate preference for long-chain fatty acyl-CoA substrates, with tolerance for shorter substrates. In addition, two different high resolution crystal structures were obtained for Orf6, which confirm the hotdog fold and active site configuration.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Orf6—orf6 was cloned from fosmid 8E1, which was originally isolated and sequenced by Allen and Bartlett (GenBank™ accession number AF409100.1) (1) and was kindly provided by Dr. Eric Allen (Scripps Institution of Oceanography). PCR amplification using oligonucleotides 5’-CATGCATGggtaccATGAGCAAAATTATCACCCCACTCGGAG-3’ and 5’-CATGCATGGcgggtTTAATTCCTACCACCCAG-3’ yielded a DNA fragment that was digested with BamHI and BamHI (New England Biolabs) and ligated into the same sites in vector pGEX4T-3 (GE Healthcare). The resulting pGEX-Orf6 expression plasmid was used to transform BL21-CodonPlus (DE3)-RIL competent cells. A single colony was used to inoculate 10 ml of LB medium at pH 8.0 supplemented with ampicillin and chloramphenicol (25 μg/ml). A total of four 1-liter culture flasks of *E. coli* were inoculated from the starter culture in a shaker with the same reagents but leaving out the enzyme. The slope data were measured in real time. Control reactions were performed by combining the same reagents but leaving out the substrate to detect any nonspecific conversion of the hydrolysis of acyl-CoA substrates (14). Affinity-purified Orf6 was exchanged into 50 mM K2HEPES (pH 7.5) reaction buffer by gel filtration chromatography on a Superdex 200 10/300 GL column. A total enzyme concentration of 10 μM was preincubated for 1 h with 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) at 25°C, followed by the addition of 1 mM short-chain CoA substrates (acytelyl-, palmitoyl-, arachidonoyl-, or eicosapentaenoyl-CoA) in a total volume of 200 μl. The change in absorbance at 412 nm was monitored on a SpectraMax 190 system (Molecular Devices) in real time. Control reactions were performed by combining the same reagents but leaving out the substrate to detect any nonspecific conversion of 5,5′-dithiobis(2-nitrobenzoic acid) by the protein. The rate, for the uncatalyzed reaction was monitored by combining the same reagents but leaving out the enzyme. The slope data were converted to units of nmol/min/mg of protein using the Beer equation and the extinction coefficient at 412 nm for thionitrobenzoic acid (TNB), 13.6 mM^−1^ cm^−1^.

Crystallography and Data Collection—Crystallization trials were done by the hanging drop vapor diffusion technique at 25°C using commercially available sparse matrix crystallization screens (Hampton Research). To crystallize Orf6 without magnesium, 2.0 μl of purified Orf6 protein (4.5 mg/ml) using 0.1 mM sodium citrate tribasic dihydrate (pH 6.6), and 30% (w/v) PEG 4000) on a siliconized glass coverslip and sealed above a reservoir of 700 μl of precipitant solution in a VDX format crystallization tray. Crystallization in the presence of magnesium was identical except that the
Table 1

X-ray diffraction data collection and model refinement statistics

| Orf6 | Orf6 + Mg²⁺ |
|---|---|
| (PDB 3R87) | (PDB 4I45) |
| **Data collection** | | |
| Space group | P6₁₂₂ | 1222 |
| Cell dimensions (Å) | 103.37 | 51.29 |
| a | 103.37 | 54.36 |
| b | 64.71 | 88.36 |
| c | | |
| Beamline | APS 23-ID | ALS 4.2.2 |
| Wavelength (Å) | 1.031 | 1.000 |
| Resolution range (Å) | 52.44–1.05 | 28.50–1.40 |
| Total reflections | 1,165,318 | 106,202 |
| Unique reflections | 90,851 | 23,821 |
| Completeness (%) | 95.9 (69.3)* | 96.3 (76.8) |
| I/σ | 13.7 (2.0) | 15.2 (3.6) |
| Rsym (%) | 5.4 (73.6) | 4.5 (22.1) |

* The numbers in parentheses are for the highest resolution shell.

| **Refinement** | | |
| Rwork/Rfree (%) | 15.0/17.0 | 14.8/19.6 |
| Resolution range (Å) | 52.44–1.05 | 28.5–1.40 |
| No. of atoms (B-factor) | | |
| Protein | 115.0 (16.3) | 1090 (20.3) |
| Water | 111 (28.2) | 171 (34.7) |
| Other | 1 (10.5) | | |
| r.m.s.d. values | | |
| Bond lengths (Å) | 0.015 | 0.015 |
| Bond angles | 1.55⁰ | 1.61⁰ |
| Ramachandran (%) | | |
| Favored/disallowed | 96.9/0.8 | 100/0 |
| MolProbity Clashscore (%) | 6.59 (58) | 8.27 (67) |
| MolProbity score (%) | 1.55 (63) | 1.77 (57) |

RESULTS

The anaerobic biosynthesis of PUFAs in several marine organisms requires the presence of a TE enzyme (15). It has been shown that PUFAs are released as free fatty acids, and it has been postulated that such activity may reside within the known enzyme domains of the multienzyme cluster (15). Thus far, no dedicated TE has been characterized as a plausible candidate to carry out this reaction. Interestingly, the gene for Orf6 (GenBankTM accession number AF409100.1) is located directly upstream of the pfaA gene of *P. profundum*, which encodes the first multienzyme of the PUFA synthase complex. The proximity of the *orf6* gene to the PUFA gene cluster suggested a potential involvement of Orf6 in PUFA release. A database search for Orf6 homologs using BLAST resulted in the identification of a single unique orthologous protein in a number of organisms, many of which are known producers of PUFAs. The most similar orthologs were found in *Moritella marina* (86% identical, 0 gaps), *Vibrio splendidus* (85% identical, 0 gaps), and *Colwellia psychrerythraea* (84% identical, 0 gaps), followed by the orthologs from a number of species of the *Shewanella* genus (~60% identical, 0 gaps) (Fig. 1B). The genomic location of these orthologs with respect to the PUFA synthase genes tends to vary between organisms. In some organisms, such as *P. profundum, M. marina*, and *V. splendidus*, the *orf6* ortholog is located within 1000 bases of the start for the *pfaA* gene, suggesting a possible involvement in PUFA biosynthesis. In other organisms, such as *Psychromonas* sp. and most bacteria of the *Shewanella* genus, the *orf6* ortholog is located >100,000 bases away.

Enzyme Activity—Purified Orf6 was found to have hydrolase activity against a number of commercially available thioesters of CoA. The enzyme activity was recorded as the increase in the production of TNB by the CoA thiol, which results from thioester hydrolisis. The values for the specific activity of Orf6 toward CoA thioester substrates ranged from 0.8 to 16 nmol of TNB/min/mg of pure enzyme (Table 2). The uncatalyzed reaction, which was monitored as a control, took place at a rate of 0.01 nmol of TNB/min. The substrate preference of Orf6 was explored through the use of different CoA thioester substrates (Table 2). Orf6 showed a preference for long-chain acyl-CoA thioesters, which is somewhat of a rarity among hotdog TEs, which have been associated more with the hydrolysis of aromatic thioesters. In particular, the highest specific activities measured were against palmitoyl-CoA (16 milliunits/mg), eicosapentaenoic-CoA (15 milliunits/mg), and arachidonoyl-CoA (12 milliunits/mg). Orf6 was also shown to be active against benzoyl-CoA and β-hydroxybutyryl-CoA. The D17A variant of Orf6, with the putative active site carboxylate removed, showed no activity above background levels with palmitoyl-CoA or eicosapentaenoic-CoA.

Overall Structure—Orf6 crystallized as a dimer of dimers with two putative active sites per dimer (Figs. 2 and 3A), as had been originally described for the hotdog hydrolases (24). The monomer unit has a structure that is typical of the double hotdog hydrolase/dehydratase family, featuring a central helix surrounded by a five-strand antiparallel β-sheet. The double hotdog dimer is formed by the interaction of two β-sheets from neighboring subunits to form one extended structure serving as
an extra wide “bun,” which can accommodate two antiparallel Hα2-helical “sausages” (Fig. 2A). A tetramer with 222 symmetry (Fig. 3A) is formed via dimerization of Orf6 dimers, primarily through polar interactions between the helical portions of each molecule. The tetrameric structure is very similar to that of the well studied *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase (Protein Data Bank code 1BVQ) (25), despite these enzymes sharing only 22% sequence identity. Purified Orf6 was analyzed on a size-exclusion chromatography column, and its mass relative to standards was estimated to be 43.5 kDa. Because the nominal monomeric molecular mass is 15.5 kDa, we hypothesize that Orf6 exists in solution in rapid equilibrium between dimeric and tetrameric forms, which would result in a single peak with an apparent size that is intermediate between the dimeric and tetrameric forms (Fig. 3B). The alternative explanation of a trimeric form in solution is unlikely given the 222 symmetry of the tetramer observed in the crystal structure (Fig. 3A).

**Active Site**—One of the defining features of hotdog TEs is the single acidic residue invariably occupying position 17. As expected, Orf6 contains a conserved Asp-17 residue (Fig. 1B) located within a loop between strand β1 and helix α1 (Fig. 2). The Asp-17 residue, the replacement of which with alanine abolished TE activity, seems to be held in place by hydrogen bonding with Ser-19, as it points inward toward the active site,
TABLE 2
Specific activities of Orf6 towards different substrates

|                      | Uncatalyzed | Orf6-catalyzed |
|----------------------|-------------|----------------|
|                      | nmol TNB/   | nmol TNB/      |
|                      | min/mg      | min/mg         |
| **Short-chain fatty acids** |            |                |
| Acetyl-CoA           | 0.08 ± 0.02 | 1.2 ± 0.1      |
| Benzyol-CoA          | 0.06 ± 0.01 | 9.7 ± 0.1      |
| β-Hydroxybutyryl-CoA | -0.01 ± 0.02| 4.6 ± 0.1      |
| Crotonyl-CoA         | 0.09 ± 0.01 | 0.85 ± 0.03    |
| **Long-chain fatty acids** |          |                |
| Palmitoyl-CoA        | 0.2 ± 0.2   | 15.9 ± 0.1     |
| Stearoyl-CoA         | 0.1 ± 0.1   | 9.7 ± 0.2      |
| Arachidoyl-CoA       | 0.05 ± 0.01 | 5.2 ± 0.1      |
| Arachidonoyl-CoA     | 0.2 ± 0.02  | 11.8 ± 0.2     |
| Eicosapentaenoyl-CoA | 0.07 ± 0.01 | 14.4 ± 0.1     |

FIGURE 2. A, stereo view of the Orf6 double hotdog dimer. Orf6 is displayed as a colored rainbow (blue (N terminus) → red (C terminus)) for the left monomer with secondary structure elements labeled; the right monomer is colored white. The structure of the second crystal form (colored black) is superimposed on the right monomer to illustrate the conformational changes around helices α2 and α3. The bound Mg$^{2+}$ ion in the second crystal form is shown as a purple sphere. The active site residue Asp-17 (D17) is shown as sticks and labeled. The extra electron density ($F_o - F_c$ map, contoured at +2.5σ) in the Orf6 binding pocket in the first crystal form is shown as a magenta surface. B, stereo view of the active site. Coloring is the same as in A. Electron density is displayed as mesh instead of surface, and two chains of 13 atoms each (light and dark gray) are modeled into the extra continuous density for reference.

where the acyl-CoA substrate is expected to bind (Fig. 2B), based on other hotdog TE structures in complex with substrate analogs (26, 27). The putative active site is located approximately at the dimerization interface (Fig. 2) and comprises residues from each monomer. An unexpected finding within the Orf6 active site is the presence of additional electron density that cannot be accounted for by the sequence of the protein. This additional electron density (magenta surface in Fig. 2A) is consistent with the presence of a long tubular flexible compound in the active site (Fig. 2B). Interestingly, one end of this additional electron density is within hydrogen bonding distance of the active site residue Asp-17 (Fig. 2B), suggesting that the compound occupies the active site of Orf6 much in the same way we expect the natural substrate would.

To identify the compound bound in the active site, we tried an organic extraction with ethyl acetate from 2 mg of pure Orf6 protein, intending to make a crude structural assignment of the extracted compound by gas chromatography coupled with mass spectrometry. However, the organic extracts did not contain enough of the compound to detect any distinguishable components relative to the controls (data not shown). Additionally, in an effort to generate Orf6 with an unoccupied and more available active site, we tried unfolding and refolding Orf6 in a dilute solution using guanidinium hydrochloride, but the protein precipitated during refolding.

**Conformational Changes**—Many (although not all) of the conditions resulting in crystallization of Orf6 contained divalent cations, which occasionally promoted the simultaneous growth of two different crystal forms within the same drop. We therefore solved the structure of Orf6 from a second crystal grown in the presence of MgCl$_2$. The resulting structure is largely similar to the one described previously, but it reveals significant conformational changes in the vicinity of Mg$^{2+}$ with a root mean square deviation of 1.63Å relative to the structure obtained without divalent cations when comparing all Cα atoms of each structure. The new structure features an Mg$^{2+}$-binding site at a crystal contact between two dimers that are not involved in a tetramerization interface. The Mg$^{2+}$ ion is coordinated by His-51 and Asp-125 from one Orf6 molecule and Asp-66 (bidentate) from another molecule. Additionally, two ordered water molecules are coordinated at axial positions, producing a distorted octahedral coordination geometry. In the Mg$^{2+}$-bound structure, there is a shift in helix α2 by up to 6 Å at its C-terminal end, giving rise to a more open angle between helices α1 and α2. Because helix α2 is part of the active site of the neighboring subunit, this conformational change produces a more open active site, which could explain why the active

FIGURE 3. A, three orthogonal views (down each 2-fold rotation axis) of the Orf6 tetramer observed in the crystal structures. The structure is displayed as a schematic with cylindrical helices, colored by molecule. B, size exclusion chromatogram of purified Orf6. The inset shows the standard curve (log molecular mass versus retention volume) used to estimate the size and oligomeric state of Orf6. The estimated solution size (43.5 kDa), together with the tetramer observed in the crystal structure, suggests a rapid dimer-tetramer equilibrium in solution.
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site in this structure is empty, with all electron density accounted for by the protein sequence and ordered water molecules. To determine whether binding of Mg\(^{2+}\) or Ca\(^{2+}\) to Orf6 affected its enzyme activity, we performed enzyme activity assays in the presence of both divalent cations (1 mM), but there was no change in the activity (data not shown).

**DISCUSSION**

We have structurally and functionally characterized Orf6, a thioesterase from *P. profundum* whose genomic location is directly upstream of the *pfu* gene cluster encoding the production of PUFAs (1). We were able to identify an Orf6 ortholog in a variety of other species known to produce PUFAs. In some organisms, however, the genomic location of the *orf6* orthologs is >100,000 bases away from the PUFA gene cluster, suggesting that they may have a different function in those organisms or that their function may not be related to PUFA biosynthesis in their natural context.

Among the substrates that we tested, the highest measured specific activity of Orf6 was toward palmitoyl-CoA (C16:0; 16 milliunits/mg), eicosapentaenoyl-CoA (C20:5; 15 milliunits/mg), and arachidonoyl-CoA (C20:4; 12 milliunits/mg). Thus, it is clear that although the rate enhancement of Orf6 is modest (100–200-fold), Orf6 has a preference for long-chain acyl-CoA substrates over shorter substrates and that the presence of double bonds is not a criterion for substrate discrimination in Orf6 because palmitoyl-CoA does not contain double bonds, yet it is hydrolyzed with a relatively high efficiency. However, other CoA thioesters, such as benzoyl-CoA and \(\beta\)-hydroxybutyryl-CoA, were hydrolyzed with a lower efficiency, suggesting that Orf6 has significant tolerance for other substrates.

An alternative explanation for the modest rate enhancement displayed by Orf6 is the possibility that it normally acts on ACP-linked substrates instead of CoA-linked substrates. However, this seems unlikely because most hotdog TEs are specific for the hydrolysis of CoA thioesters (14, 28). For instance, in vitro assays showed that the human hotdog fold TE (hTHEM2) is unable to hydrolyze ACP-loaded thioesters, although it has a broad tolerance for many CoA thioesters (14). Similarly, the EntH hotdog TE from the enterobactin biosynthetic pathway of *E. coli* shows no higher selectivity for substrate analogs loaded onto its cognate ACP than for substrates loaded on CoA, indicating that the EntH TE recognizes the pantetheinyl portion of the substrates with few or no contact with the ACP (28).

Other hotdog TEs show a similar tolerance for a variety of CoA thioesters and display a wide range of specific activities. EntH, the hotdog TE involved in the final release of the *E. coli* natural product enterobactin, is considerably more active than Orf6. Its specific activity ranges from 17 milliunits/mg against acetyl-CoA up to 8400 milliunits/mg against 3-hydroxyphenylacetyl-CoA: an ~500-fold preference for aromatic CoA thioesters over acetyl-CoA (28). Similarly, the phenyl acetate-specific TE Paa from *Azorarcus evansii* has an ~1600-fold preference for 3-hydroxyphenylacetyl-CoA over the short-chain crotonyl-CoA with a range from 17–30,000 milliunits/mg (29). In other hotdog TEs, the substrate preference is much less pronounced. For instance, hTHEM2, also slightly more active than Orf6, shows a 40-fold substrate preference for \(\beta\)-hydroxybutyryl-CoA over palmitoyl-CoA (C16:0) with specific activities that range between 16 and 674 milliunits/mg (14). Orf6 has much lower activity and shows a 20-fold substrate preference (0.85–15.9 milliunits/mg), suggesting that this level of activity and specificity is sufficient for endogenous PUFA production, that the assay conditions are significantly different from conditions in vivo, or that none of the substrates in this report resemble the bona fide natural substrate for this enzyme.

The additional electron density in the Orf6 active site, although somewhat branched and not unambiguously assignable as a specific molecule, is best fit by a chain at least 10 atoms in length (Fig. 2B). Given the partially hydrophilic nature of the protein cavity, it is possible that the density represents a partially ordered portion of a PEG polymer molecule from the crystallization precipitant. The large size of the delineated active site binding pocket is consistent with the observed preference for longer chain substrates.

When comparing our Orf6 crystal structures, we observed substantial localized conformational changes that alter the size and shape of the binding pocket. These two structural snapshots likely represent two states of the conformational ensemble that exists in solution that allow recognition and accommodation of a large substrate. There are previous reports of conformational changes in hotdog TEs that correlate with effects on enzyme activity (30, 31). However, given that we observed no difference in enzyme activity in the presence of Mg\(^{2+}\) or Ca\(^{2+}\) ions, we suspect that the Mg\(^{2+}\)-binding site observed in our structure is a consequence of the crystal packing arrangement and not physiologically relevant.

It has been shown that hotdog TEs can catalyze hydrolysis by two distinct chemistries: covalent catalysis and general base catalysis (13, 14). The mechanism of covalent catalysis involves the formation of an anhydride intermediate resulting from the nucleophilic attack of the active site Asp (or Glu) residue, which causes the immediate release of the CoA thiol. This step is followed by the hydrolysis of the anhydride intermediate, which causes the release of the free acid. The main evidence in support of this mechanism is the burst-phase kinetics observed for the enzyme-catalyzed reaction, which are consistent with a fast nucleophilic attack, followed by a rate-limiting hydrolytic step (13). In terms of enzyme structure, the covalent mechanism is validated by the absence of water molecules in the active site when intact substrate is bound (13, 27). By contrast, the general base catalytic mechanism involves direct activation of a water molecule, which acts as the nucleophile (14). The kinetics of Orf6-catalyzed hydrolysis observed in this study show perfectly linear steady-state kinetics with no evidence of multiple steps taking place. This means either that the reaction mechanism does not involve a covalent intermediate or that the second step of hydrolysis is not rate-limiting. In terms of the Orf6 crystal structures, although the Mg\(^{2+}\)-free structure (with the occupied active site) does not have any water molecules in the vicinity of Asp-17, the Mg\(^{2+}\)-bound structure (with the unoccupied active site) does contain two water molecules within 2.5 Å of Asp-17. This last observation may suggest that the presence of the substrate in the active site excludes water, consistent with covalent catalysis. However, it is not possible to draw any mechanistic conclusions from the Mg\(^{2+}\)-free structure because the nature of the substance occupying the active site is not known.
To our knowledge, this is the first report of a hotdog TE with a preference for long-chain acyl-CoA substrates. Most other bacterial TEs of the hotdog type display selectivity toward aromatic acyl-CoA compounds. Both *Arthrobacter* and *Pseudomonas* species have dedicated hotdog TEs for the hydrolysis of 4-hydroxybenzoyl-CoA (26, 32). EntH, a hotdog TE from *E. coli* that catalyzes the release of the natural product enterobactin, has a 10-fold preference for its cognate benzoyl-CoA substrates over the extended palmitoyl-CoA substrate (28). Even hTHEM2 has a clear *in vitro* preference for aromatic CoA thioesters, even though benzoyl-CoA and phenylacetly-CoA are not known human metabolites (14). One notable exception is CalE7, a hotdog TE that catalyzes the release of the elongated 10-carbon backbone for calicheamicin (33). However, the active site of CalE7 does not contain the conserved acidic residue in position 17 and uses a different mechanism that involves a subsequent decarboxylation step following thioester hydrolysis, which results in the release of a terminal methyl ketone (33). In this work, we have shown that Orf6 has a 15-fold preference for the long-chain thioesters palmitoyl-CoA and eicosapentaenoyl-CoA over the shorter acetyl-CoA and crotonyl-CoA and a 3-fold preference over benzoyl-CoA and β-hydroxybutyryl-CoA. However, the rate enhancement of the catalyzed reaction versus the uncatalyzed reaction (100–200-fold) is low enough to raise doubts as to whether Orf6 is naturally dedicated to the release of PUFA in *P. profundum*.

CONCLUSIONS

The biosynthesis of PUFAs in many marine species requires TE activity to catalyze the release of the products. In this work, we have characterized, both structurally and functionally, the first TE from this class of marine organisms. Even though the enzyme shows a preference for long-chain fatty acyl thioesters, including the eicosapentaenoyl thioesters, it also displays tolerance for shorter fatty acid thioesters. Future work will focus on determining whether Orf6 is indeed necessary for physiological PUFA production in marine bacteria and on engineering the PUFA biosynthesis pathways for production of biofuels.

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