Trace Nutrients Research 29 : 84－91 (2012)

Original Article

Functional analysis of an eicosapentaenoic acid biosynthesis protein Orf2 from a psychrotrophic bacterium, *Shewanella livingstonensis* Ac10

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Summary

A psychrotrophic bacterium, *Shewanella livingstonensis* Ac10, produces eicosapentaenoic acid (EPA) at 4°C, which plays a beneficial role in the cold adaptation of the bacterium. The gene cluster containing *orf2, orf5, orf6, orf7,* and *orf8* is involved in EPA biosynthesis. Here, we investigated the function of *orf2*, which is thought to code for a phosphopantetheinyl transferase (PPTase). The western blot analysis using an anti-Orf2 antibody demonstrates that Orf2 is induced at 4°C and localized to the cytoplasm. PPTase catalyzes the transfer of a phosphopantetheine moiety from CoA to the serine residue of acyl carrier proteins (ACPs). To examine the phosphopantetheinylation of 5 conserved repeated ACP domains of Orf5 by Orf2, we established an *in vitro* phosphopantetheinylation system using the purified recombinant Orf2 as well as ACP domains of Orf5 as its putative substrates. When each of the ACP domains was incubated with Orf2 and CoA at 4°C, the phosphopantetheinylation of individual ACP domains was observed by HPLC and MALDI-TOF/MS analyses. Moreover, the *in vitro* modification did not occur when Ser-less ACP mutants were used, indicating that Orf2 catalyzes the phosphopantetheinylation of the serine residue in ACP domains of Orf5, which may probably be an initial step of EPA biosynthesis in *S. livingstonensis* Ac10.

Introduction

Long-chain ω-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid are beneficial in preventing coronary artery diseases, hypertension, diabetes, arthritis, atherosclerosis, and cancers[1-3, 5, 6]. Much attention has been paid to the production of these fatty acids because of their positive effects on human health[4-6]. Humans generally acquire these fatty acids from fish or other seafoods[7]. Many studies have indicated that EPA is not only good for human health but also essential for bacterial adaptation to low temperatures[18].

Shewanella livingstonensis Ac10, a psychrotrophic Gram-negative bacterium isolated from Antarctic seawater, produces EPA at low temperatures[18]. The function of EPA biosynthesis genes in *S. livingstonensis* Ac10 was previously proposed[10], and these genes can be found in the form of a gene cluster that shares a high sequence similarity with that of *Shewanella pneumatophori* SCRC-2738[21, 25, 29]. The gene cluster consists of 5 genes: *orf2, orf5, orf6, orf7*, and *orf8*. Among them, *orf2* is thought to code for a phosphopantetheinyl transferase (PPTase), whereas *orf5* codes for a multifunctional protein comprising domains of 3-ketoacyl synthase, acyl transferase, 5 repeats of acyl carrier protein (ACP) domains, and 3-ketoacyl reductase[10, 24].

PPTases from *Pseudomonas aeruginosa, Bacillus subtilis, Saccharopolyspora erythraea*, and *Moritella marina* have been characterized[11, 17, 24, 26]. The location of *orf2* in the gene cluster of *S. livingstonensis* Ac10 is different from that of the PPTase gene in *Moritella marina* MP-1 and *Pseudoalteromonas* sp. DS-12[27]. Specifically, *orf2* (*pfaE*) in *Moritella marina* MP-1 is separated from the other genes, whereas *orf2* (*pfaE*) in *Pseudoalteromonas* sp. DS-12 is merged with *pfaC*[27, 30]. In contrast, the location of *orf2* in *S. livingstonensis* Ac10 is similar to that in *S. pneumatophori* SCRC-2738[10]. PPTase encoded by *orf2* in *S. pneumatophori* SCRC-2738 has not been studied in detail.

PPTase is an ubiquitous enzyme that plays important physiological roles in the synthesis of fatty acids and catalyzes the posttranslational modification of ACPs in multienzyme systems, including fatty acid synthases, polyketide synthetases, and nonribosomal polypeptide synthetases[8, 9, 11, 12, 19]. ACP is an important component in fatty acid biosynthesis. In *S. livingstonensis* Ac10, 5 ACPs are integrated in a multifunctional protein. The phosphopantetheine-binding motifs found in the first and second ACPs are identical to each other, and so are the fourth and fifth ACPs. The number of ACP domains in *S. livingstonensis* Ac10 is different from that in *S. pneumatophori* SCRC-2738, which contains 6 ACP domains, and it is not clear whether any of these 6 ACP domains in *S. pneumatophori* SCRC-2738 is phosphopantetheinylated.

ACP domains are present either in a multifunctional protein in eukaryotic cells or as separate subunits in prokary-

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Table 1 Primers used for construction of overexpression plasmids

Primer name	Primer sequence (5'-3')	Restriction site
5' p-*orf2*	GGAATTCCATATGTCGGTCATGCCTC	NdeI
3' p-*orf2*	GGAATTCGCAGACTCCTTGTATACATTCAG	EcoRI
5' p-ACP1	GGAATTCCATATGGCACCAACAGTCGTATTAACACAGC	NdeI
3' p-ACP1	GGAATTCGCCGATGTTGATCCCGTTGACGCTACC	EcoRI
5' p-ACP3	GGAATTCCATATGGCAGAAAGCGTTCAGCGTACTATGTTAGC	NdeI
3' p-ACP3	GGAATTCGGTGCAGGTGCAGATACAGTTGCTACTGG	EcoRI
5' p-ACP5	GGAATTCCATATGGCTGATACCGTTTTAAGCACCATGATG	NdeI
3' p-ACP5	GGAATTCGCTACCGCCGCAATAGTGGTTGTTGTAGG	EcoRI

otic and plant cells[14, 28]. These domains are modified by enzymes with the conserved motifs, including LGXDSL or ZXGHSXG[12, 13, 20]. In the presence of Mg^{2+}, the phosphopantetheine group of CoA is incorporated into a conserved serine residue of ACP via a PPTase[17], resulting in the conversion of an inactive apo-ACP to an active holo form[15, 22, 23].

In this current study, 5 ACP domains of Orf5, a multifunctional scaffold protein for EPA synthesis, were individually cloned, expressed, and purified. We studied the modification of each ACP domain independently and examined whether these 5 ACPs serve as substrates for Orf2, a putative PPTase expressed and purified from *E. coli*. Our results demonstrate that Orf2 catalyzes the phosphopantetheinylation of conserved ACP domains of Orf5.

Materials and Methods

Strains and culture conditions

Escherichia coli strains were grown in LB media at 30 or 37°C. Ampicillin was used at a final concentration of 100 μg/mL. Transformation and plasmid preparation were carried out following the standard protocols[16]. *S. livingstonensis* Ac10 was cultured in LB media containing 50 μg/mL rifampicin at 4 or 18°C. All cultivations were conducted aerobically.

Intracellular localization of Orf2

Soluble protein extracts and insoluble pellets of *S. livingstonensis* Ac10 grown at 18 and 4°C (OD_{600} = 1.0) were subjected to sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes[33]. Orf2 was detected using a rabbit polyclonal anti-Orf2 antibody raised against the recombinant His-tagged Orf2 protein and the ECL Plus Kit™ (Amersham Pharmacia Biotech).

Construction of expression plasmids

Primers used for the construction of expression plasmids are listed in Table 1. Gene fragments coding for *orf2* (accession number: AB284096.1) and the regions of *orf5* (accession number: AB284098.1) coding for individual ACP domains (the first, third, or fifth ACP) were amplified from *S. livingstonensis* Ac10 genomic DNA using the KOD-plus DNA Polymerase (Toyobo). Purified PCR products digested with NdeI and EcoRI fragments were ligated into the NdeI/EcoRI sites of pET-21a(+) to yield the expression plasmids pOrf2, pACP1, pACP3, and pACP5. To facilitate protein purification, a DNA region coding for a carboxyl (C)-terminal 6×His-tag in these plasmids was utilized. Recombinant Orf2 and ACPs were expressed mostly as soluble proteins. To obtain mutant ACPs, the DpnI-mediated site-directed mutagenesis kit (Stratagene) was used with primers shown in Table 2. Amplified DNA fragments digested with DpnI according to manufacturer's instructions were then introduced into *E. coli* DH5α. The plasmids obtained from the recombinant *E. coli* DH5α were introduced into the expression host, *E. coli* BL21 (DE3).

Table 2 Oligonucleotides used for site-directed mutagenesis

Primer name	Primer sequence (5'-3')	Modified codon
1F1	GGTATCGACGCAATCAAGCGG	Ser44Ala
1R1	CCGCTTGATTGCGTCGATACC	Ser44Ala
1F2	CCAGAACTGGCCCCAGAAGAT	Ser65Ala
1R2	ATCTTCTGGGGCCAGTTCTGG	Ser65Ala
3F	GGTATCGACGCAATTAAACGC	Ser37Ala
3R	GCGTTTAATTGCGTCGATACC	Ser37Ala
5F1	CTTGAACTGGCCATGGACATG	Ser26Ala
5R1	CATGTCCATGGCCAGTTCAAG	Ser26Ala
5F2	GGTATCGACGCAATCAAACGC	Ser37Ala
5R2	GCGTTTGATTGCGTCGATACC	Ser37Ala

Purification of recombinant proteins

Overnight cultures (5 ml) of *E. coli* BL21 (DE3) harboring various expression plasmids grown in LB media with 100 μg/mL ampicillin were inoculated into 500 ml of the same medium. Cultures were grown at 37°C and 180 rpm until OD_{600} reached 0.6. Expression of the target genes was then induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside, and cells were allowed to grow for an additional 5 h at 30°C.

After centrifugation at 5,500×*g* and 4°C, cells were quickly frozen in liquid nitrogen and stored at −80°C. Frozen cells were resuspended in buffer A (0.5 M NaCl, 20 mM Tris-HCl, and 5 mM imidazole, pH 7.9) and disrupted on ice for 15 min in a sonicator (Branson Digital Sonifier®). The resulting crude extracts were centrifuged twice (KUBOTA 7780) at 10,500×*g* for 35 min at 4°C. Supernatants filtered through the 0.45-μm Millipore Cosmonice Filter were applied to Ni-NTA affinity columns equilibrated with buffer A at 4°C. Columns were washed with 25 ml of buffer A at a flow rate of 0.5 ml/min, and recombinant proteins were eluted with a linear gradient from 100% buffer B (0.5 M NaCl, 20 mM Tris-HCl, and 60 mM imidazole, pH 7.9) to 100% buffer C (0.25 M NaCl, 10 mM Tris-HCl, and 500 mM imidazole, pH 7.9). Fractions containing target proteins were detected by SDS-PAGE, collected, and dialyzed against 20 mM Tris-HCl (pH 8.0). After dialysis, purified proteins were further concentrated using the Millipore Amicon membrane filters and stored at −80°C until use.

HPLC analysis of *in vitro*-modified ACPs

In vitro modifications of ACPs by the purified Orf2 were monitored by HPLC as described by Finking et al.[17]. PPTase activity at various pH conditions was determined in the presence of 75 mM MES/NaOAc (pH 4.5−6.5) or Tris-HCl (pH 7.0−9.0). Reaction mixtures (60 μl each) contained apo-ACP (30−80 μM, first ACP; 60−110 μM, third ACP; 30−70 μM, fifth ACP), 75 mM MES/NaOAc (pH 5.5, first ACP; pH 6.0, third ACP; pH 6.5, fifth ACP), 12.5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 500 μM CoA. Reactions were initiated by the addition of Orf2 to a final concentration of 1−4 μM, followed by incubation at 4°C for 5−45 min. Mixtures were subsequently filtered and analyzed using a reversed phase HPLC column (Capcell Pak C_8 SG300Å; 4.6 × 250 mm; Shiseido Co., Japan) equilibrated with 30% solvent B (acetonitrile containing 0.1% trifluoroacetic acid). Absorbance at 280 nm was monitored. As the controls, the mixtures without Orf2 were prepared and analyzed by HPLC immediately after mixing. The apo- and holo-ACPs could be separated from each other as described in Results and Discussion.

MALDI-TOF/MS analysis of *in vitro*-modified ACPs

For the phosphopantetheinylation of ACPs, reaction mixtures contained 75 mM MES/NaOAc (pH 6.5), 15 mM $MgCl_2$, 30 or 200 μM ACP, and 1 mM CoA. Reactions were initiated by the addition of 20 μM Orf2 and then incubated at 4°C overnight. A reaction mixture incubated at 4°C without Orf2 was also prepared as a control. All mixtures were subjected to SDS-PAGE, and the gel was stained with the Gel Negative Stain Kit (Nacalai Tesque, Inc.). For MALDI-TOF/MS analysis, bands corresponding to ACPs were excised, treated with DTT and iodoacetamide, and digested with trypsin (Promega, Madison, WI) overnight at 37°C[31, 32]. Digested peptides were concentrated, desalted using the C_{18} ZipTip (Millipore Corporation), and analyzed using the Bruker Microflex MALDI-TOF/MS.

Results and Discussion

Intracellular localization of Orf2

S. livingstonensis Ac10, an EPA-producing bacterium, has an EPA biosynthetic gene cluster that contains 5 genes: *orf2, orf5, orf6, orf7,* and *orf8*. Among them, *orf2* is an essential gene for EPA biosynthesis and shares a sequence similarity with a PPTase, which is considered to catalyze the phosphopantetheinylation of ACP domains of a multifunctional scaffold protein, Orf5. We previously reported that the gene-disrupted mutants of each of these 5 genes did not produce EPA, and the EPA-less mutants showed growth retardation at low temperatures[18]. Moreover, the addition of a synthetic phosphatidylethanolamine containing EPA at the *sn*-2 position complemented the growth defect, indicating that EPA plays an important role in the cold adaptation of *S. livingstonensis* Ac10[18]. In this study, we cloned *orf2* that presumably codes for a PPTase and generated a rabbit polyclonal antibody against the recombinant Orf2 protein. The western blot analysis using the anti-Orf2 antibody showed that a 40-kDa band was detected only in soluble fractions and that the band intensity at 4°C was higher than that at 18°C (Fig. 1), indicating that *S. living-*

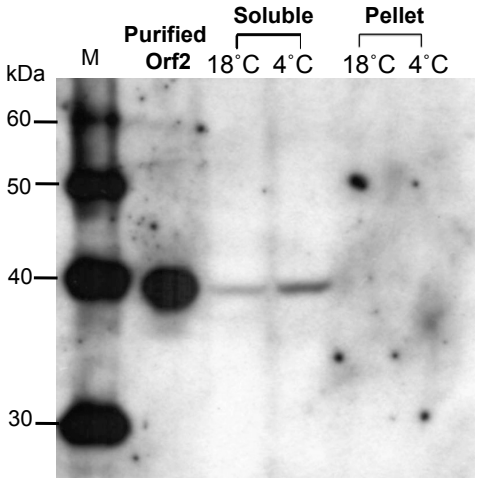

Fig. 1 Intracellular localization of Orf2
The soluble fractions and insoluble pellet of *S. livingstonensis* Ac10 grown at 18 or 4°C were subjected to SDS-PAGE. Equal amounts of proteins (8 μg) were applied to each lane, and the western blot analysis was performed using an anti-Orf2 antibody. As a positive control, 0.2 μg of purified recombinant Orf2 was also loaded.

stonensis Ac10 produces Orf2 in a soluble form at low temperatures (4°C).

Expression and purification of Orf2 and ACPs

Orf2 shares a sequence similarity with the PPTase superfamily proteins, and it is considered to transfer the 4′-phosphopantetheine moiety from CoA to the invariant serine residue of ACPs to initiate EPA synthesis in *S. livingstonensis* Ac10. We also found that Orf5, another essential gene for EPA synthesis, has 5 repeated phosphopantetheine-binding motifs. The first and second ACPs are identical to each other, and so are the fourth and fifth. To examine whether the protein encoded by *orf2* has PPTase activity and modifies the conserved ACP domains of Orf5 at low temperatures as well as which ACP domain is involved in EPA synthesis, we expressed and purified Orf2 along with its putative substrates (the first, third, and fifth ACPs of Orf5). All proteins were produced with a C-terminal His-tag and purified using the Ni-NTA affinity column. Proteins obtained were found to be homogeneous by SDS-PAGE (data not shown).

Determination of *in vitro* phosphopantetheinylation of Apo-ACPs by purified recombinant Orf2

Based on the sequence analysis, *orf2* was assumed to code for a PPTase that modifies ACPs in EPA synthesis. The modification of ACPs was examined by monitoring the incorporation of the 4′-phosphopantetheine group *in vitro*. We created constructs expressing individual ACP domains of Orf5, which is a general approach successfully used to elucidate domain functions for large multidomain proteins involved in polyketide and fatty acid synthesis[34, 35]. Three versions of Orf5 ACPs (first, third, and fifth ACPs) were heterologously produced in *E. coli* and purified. After ACPs were incubated with Orf2 and CoA at 4°C, the reaction mixtures were subjected to HPLC analysis (Fig. 2). In the absence of Orf2, a peak corresponding to the apo form of ACP with a retention time of about 17 min was detected (Fig. 2A, C, and E). In the presence of Orf2, the peak corresponding to apo-ACP decreased, and a new peak with a retention time of about 15 min appeared (Fig. 2B, D, and F). The area of the new peak was increased in a time-dependent manner (data not shown). These results demonstrate that Orf2, in the presence of CoA and Mg^{2+}, modifies individual ACPs *in vitro*[14]. Optimal pH values for *in vitro* modification of the first, third, and fifth ACPs were determined to be 5.5, 6.0, and 6.5, respectively, at 4°C.

For further analysis of this *in vitro* modification of

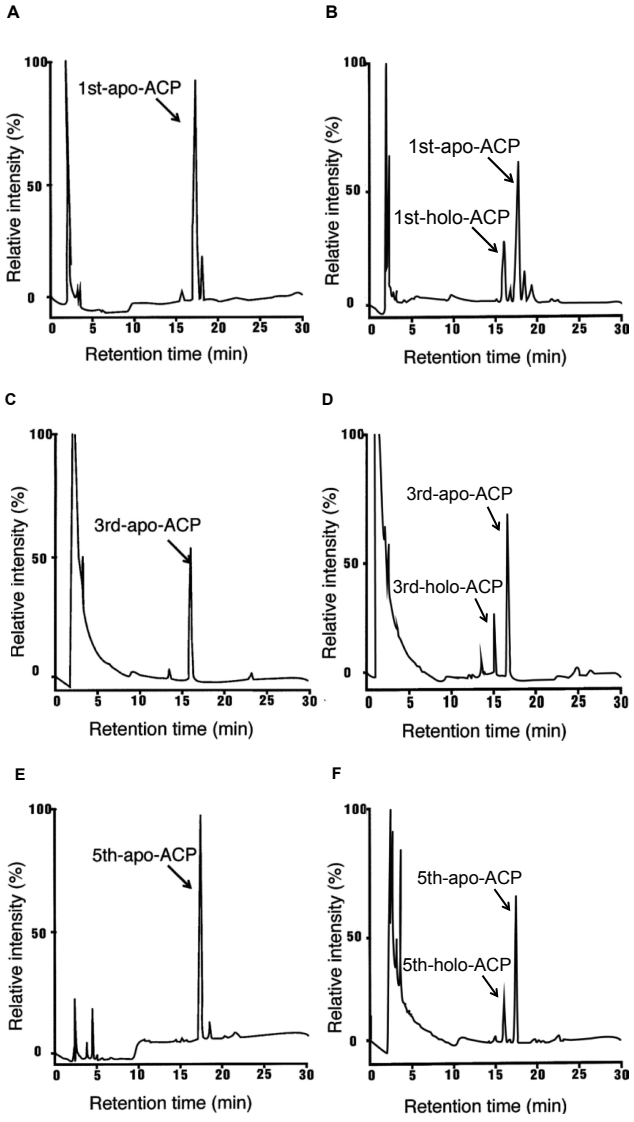

Fig. 2 *In vitro* modification of ACPs by Orf2

The reaction mixtures (pH 5.5–6.5) containing 2 mM DTT, 12.5 mM $MgCl_2$, 50–60 μM ACPs, 1–4 μM Orf2, and 500 μM CoA were incubated at 4°C. The first, third, and fifth ACPs were incubated with Orf2 for 15, 5, and 45 min, respectively, and subjected to HPLC analysis (B, D, and F). As negative controls, the mixtures not containing Orf2 were also analyzed by HPLC (A, C, and E).

ACPs by Orf2, we performed the MALDI-TOF/MS analysis of tryptic digests of each ACP that was incubated with or without Orf2. A digested peptide of the first ACP containing the putative phosphopantetheine-binding motif (T_{23}GYPTEMLELGMDMEADLGIDSIKR$_{47}$) was detected at *m/z* 2783.97 (Fig. 3A). In the presence of Orf2, a new peak was detected at *m/z* 3198.9, which corresponds to the peptide containing the Ser-phosphopantetheine group with chloride ions ($[M+340+H+2Cl]^-$, Fig. 3B). As for the third and fifth ACPs, the peaks corresponding to the peptides modified with phosphopantetheine, $[M+340-H]^-$ and $[M+340+H+2Cl]^-$, were detected (Figs. 4 and 5). In contrast, when the serine residue of the phosphopantetheine-binding motif of ACPs was replaced with alanine, the modified peaks were not observed in the

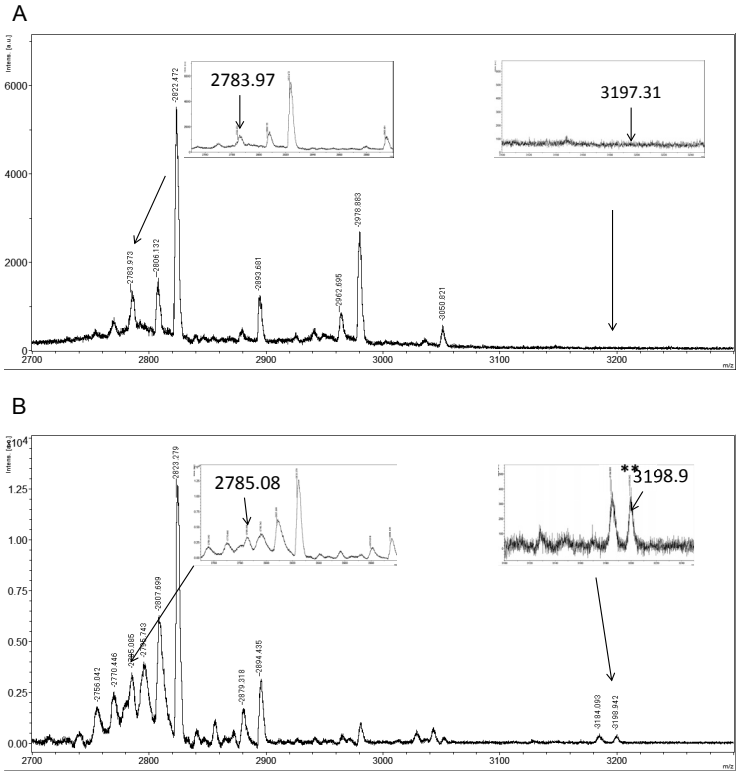

Fig. 3 MALDI-TOF/MS spectra of the phosphopantetheine-binding peptide of the first ACP

The first ACP was incubated either without (A) or with (B) Orf2 at 4°C. Reaction mixtures were subjected to SDS-PAGE, and the band corresponding to the first ACP was excised and digested with trypsin. Tryptic digests were analyzed by MALDI-TOF/MS. (A) Tryptic peptides of the first ACP incubated without Orf2. Arrows indicate the peak corresponding to the peptide containing the phosphopantetheine-binding motif. Inset shows the enlarged spectra. (B) Tryptic peptides of the first ACP incubated with Orf2. The mass peak with an m/z value of 2783.97 (A) corresponds to the $[M-H]^-$ ion of the apo-peptide without modification (calculated m/z value = 2784.31), and that with an m/z value of 3198.9 (indicated by asterisks in B) corresponds to the $[M+340+H+2Cl]^-$ ion of the holo-peptide with modification (calculated m/z value = 3197.31). The apo-peptide was not modified completely. The spectra contain impurities derived from the reaction mixtures, which did not correspond to any tryptic digests of Orf2.

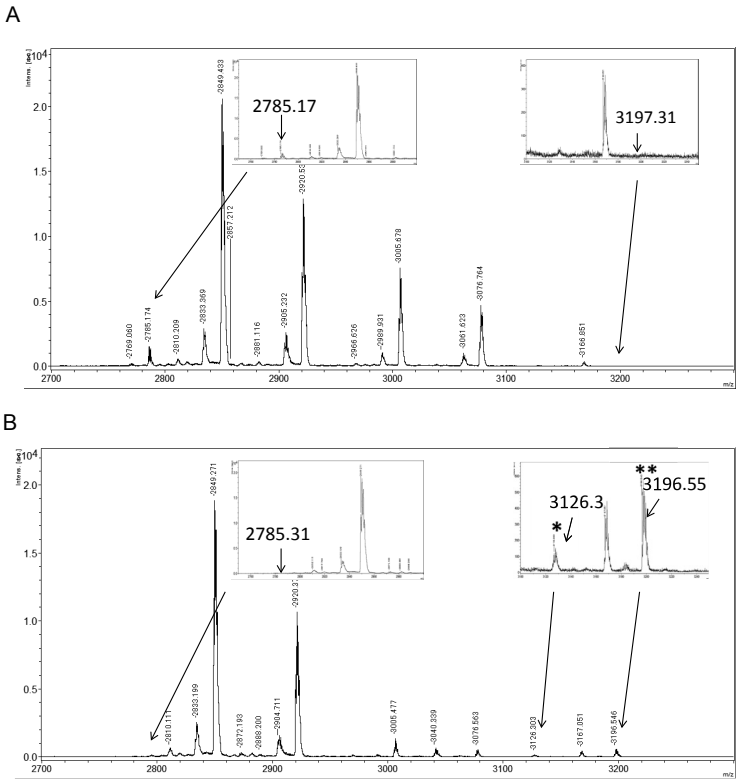

Fig. 4 MALDI-TOF/MS spectra of the phosphopantetheine-binding peptide of the third ACP

The MALDI-TOF/MS spectra of tryptic digests of the third ACP incubated without (A) or with (B) Orf2 at 4°C. Peptides containing the phosphopantetheine-binding motif are shown. The mass peak with an m/z value of 2785.17 (A) corresponds to the $[M-H]^-$ ion of the apo-peptide without modification (calculated m/z value = 2784.31), and those with m/z values of 3126.3 and 3196.55 (indicated by asterisks in B) correspond to the $[M+340-H]^-$ and $[M+340+H+2Cl]^-$ ions of the modified peptide (calculated m/z values = 3124.31 and 3197.31).

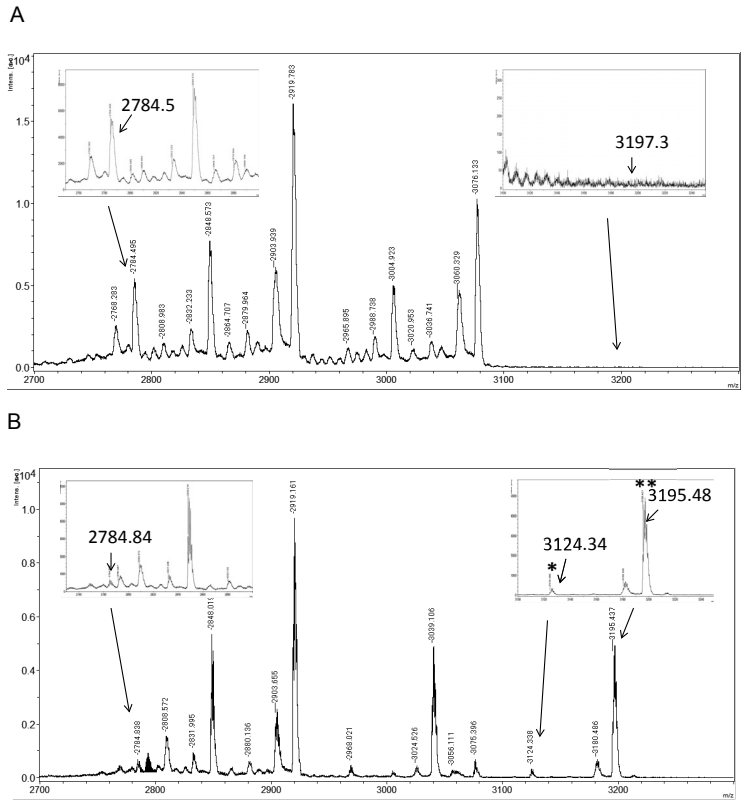

Fig. 5 MALDI-TOF/MS spectra of the phosphopantetheine-binding peptide of the fifth ACP

The MALDI-TOF/MS spectra of tryptic digests of the fifth ACP incubated without (A) or with (B) Orf2 at 4°C. Peptides containing the phosphopantetheine-binding motif are shown. The mass peak with an *m/z* value of 2784.5 (A) corresponds to the $[M-H]^-$ ion of the apo-peptide without modification (calculated *m/z* value = 2784.31), and those with *m/z* values of 3124.34 and 3195.48 (indicated by asterisks in B) correspond to the $[M+340-H]^-$ and $[M+340+H+2Cl]^-$ ions of the holo-peptide with modification (calculated *m/z* values = 3124.31 and 3197.31).

presence of Orf2 (data not shown). These results suggest that Orf2 catalyzes the transfer of the phosphopantetheine moiety to the serine residue of the putative phosphopantetheine-binding motif of all ACP domains in Orf5 as well as that the modification by Orf2 may be involved in EPA synthesis in *S. livingstonensis* Ac10.

Conclusion

This current study focuses on the EPA synthesis mechanism of a psychrotrophic bacterium, *S. livingstonensis* Ac10, which produces EPA at low temperatures. It was shown that EPA contributes to cold adaptation of the bacterium[18]. Five genes essential for EPA synthesis, *orf2, orf5, orf6, orf7, and orf8*, were previously reported to form a cluster and share sequence similarities with genes involved in polyketide synthesis[18, 36]. Among these EPA synthesis genes, *orf2* is predicted to code for a PPTase and catalyze the modification of ACP domains of a multifunctional scaffold protein, Orf5, to promote EPA synthesis at low temperatures. However, their functions have not been determined experimentally. Here, we demonstrate that *orf2* codes for a PPTase that catalyzes the phosphopantetheinylation of ACP domains of Orf5 *in vitro*. The phosphopantetheine moiety of CoA was incorporated into the conserved serine residue in each ACP domain of Orf5 via Orf2 PPTase activity, suggesting that all these domains may be involved in EPA synthesis.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research (B) from JSPS (20360372, 22404021, and 24380047 to T.K.), a Grant-in-Aid for Challenging Exploratory Research 22658028 (to T.K.), a Grant from the Institute for Fermentation, Osaka (to T.K.), a Grant from the Japan Foundation for Applied Enzymology (to T.K.), and a Grant for Research for Promoting Technological Seeds 10-041 from JST (to J.K.).

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