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A phosphopantetheinyl transferase gene essential for biosynthesis of $n-3$ polyunsaturated fatty acids from *Moritella marina* strain MP-1

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Abstract A phosphopantetheinyl transferase (PPTase) gene (*pfaE*), cloned from the docosahexaenoic acid (DHA)-producing bacterium *Moritella marina* strain MP-1, has an open reading frame of 861 bp encoding a 287-amino acid protein. When the *pfaE* gene was expressed with *pfaA–D*, which are four out of five essential genes for biosynthesis of eicosapentaenoic acid (EPA) derived from *Shewanella pneumatophori* SCRC-2738 in *Escherichia coli*, the recombinant produced 12% EPA of total fatty acids. This suggests that *pfaE* encodes a PPTase required for producing $n-3$ polyunsaturated fatty acids, which is probably involved in the synthesis of DHA in *M. marina* strain MP-1.

Keywords: Docosahexaenoic acid; Eicosapentaenoic acid; $n-3$ Polyunsaturated fatty acid; Phosphopantetheinyl transferase; *pfaE* gene; *Moritella marina* strain MP-1

1. Introduction

Long-chain $n-3$ polyunsaturated fatty acids ($n-3$ PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are synthesized *de novo* via polyketide biosynthesis in bacteria [1]. The genes involved in biosynthesis of EPA have been cloned from some EPA-producing bacteria [2–4] and their homologous from various bacterial and eukaryotic sources have been deposited in databases of DDBJ/GenBank/EMBL. The EPA biosynthesis genes from *Shewanella pneumatophori* SCRC-2738 (formerly *Shewanella* sp. strain SCRC-2738 [5] and hereafter designated SCRC-2738) have been well characterized [1,6,7]. They are clustered (designated as the EPA gene cluster) and the cluster includes five essential open reading frames (ORFs): ORFs 2, 5, 6, 7, and 8 [1,6]. In this study ORFs 5, 6, 7, and 8 of the EPA gene cluster were renamed as *pfaA*, *pfaB*, *pfaC*, and *pfaD*, respectively, according to the recent common designation of related genes [3,4]. The gene *pfaA* (ORF 5) encodes a multifunctional protein including domains of 3-ketoacyl synthase (KS), malonyl-CoA: acyl carrier protein (ACP) acyltransferase (AT), and six repeats of ACP, 3-ketoacyl-ACP reductase (KR). The gene *pfaC* (ORF 7) encodes a protein with domains of KS, chain-length factor, and two 3-hydroxydecanoyl-ACP dehydratases (HD). Genes *pfaB* (ORF 6) and *pfaD* (ORF 8) encode the proteins with a domain of AT and enoyl reductase (ER), respectively [1]. ORF 2, encoding phosphopantetheinyl transferase (PPTase), was designated *pfaE*. Appreciable levels of EPA were produced when *Escherichia coli* was transformed with DNA including one set of those five ORFs [1,6,7].

Genes homologous with *pfaA–D* of the EPA gene cluster have been cloned from the DHA-producing *Moritella marina* strain MP-1 (designated hereafter MP-1) [8]. Those clustered genes are presumably involved in synthesis of DHA, although it has not been proven. A PPTase gene corresponding to *pfaE* of SCRC-2738 has not been recovered from DHA-producing bacteria including MP-1. Tanaka et al. [8] obtained a cosmid clone (p3D5) of 35 kbp including *pfaA–D* from MP-1. However, the clone included no sequence homologous to that of *pfaE*.

PPTase catalyzes the post-translational modification of carrier proteins in multienzyme systems including fatty acid synthases (FASs), polyketide synthetases (PKSs), and nonribosomal polypeptide synthetases (NRPSs) [9,10]. Phosphopantetheinylation occurs by transfer of the 4′-phosphopantetheine prosthetic group from coenzyme A to a conserved serine residue in the carrier proteins, converting the proteins from their inactive "apo" forms to their active "holo" forms. In general, the FAS systems use an AcpS-type PPTase, named after ACP synthase, of approximately 15 kDa, and PKS and NRPS systems use an Sfp-type PPTase, named from Sfp (surfactin phosphopantetheinyl transferase) of approximately 30 kDa [9]. The PPTase for $n-3$ PUFAs is an Sfp-type enzyme from its deduced primary structure (see below). However, it has never attracted much attention, probably because the enzyme has scarcely been characterized. The *pfaE* gene of SCRC-2738 is

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Abbreviations: ACP, acyl carrier protein; ArCP, aryl carrier protein; AT, malonyl-CoA:ACP acyltransferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ER, enoyl reductase; FAME, fatty acid methyl ester; FAS, fatty acid synthases; GC/MS, gas chromatography–mass spectrometry; GLC, gas–liquid chromatography; HD, 3-hydroxydecanoyl-ACP dehydratase; IPTG, isopropyl β-D-thiogalactoside; KR, 3-ketoacyl-ACP reductase; KS, 3-ketoacyl-ACP; LB, Luria Bertani; NRPS, non-ribosomal polypeptide synthetase; ORF, open reading frame; PCP, peptide carrier protein; PKS, polyketide synthetase; PPTase, phosphopantetheinyl transferase; PUFA, polyunsaturated fatty acid

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the sole PPTase gene for $n-3$ PUFAs, of which function has been certified.

In this study, we attempted to clone the PPTase gene (*pfaE*) required for biosynthesis of DHA from MP-1. The cloned PPTase gene was then examined for its capability to complement the *pfaE*-lacking EPA gene clusters from SCRC-2738.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains and vectors used in this study are listed in Table 1. *E. coli* recombinant cells were cultivated by shaking in Luria Bertani (LB) medium supplemented with indicated antibiotics normally at 37 °C for 16 h. A portion of the 37 °C-grown

Table 1
Strains and vectors used in this study

Strain/plasmid/cosmid	Relevant characteristics	Source
Strain		
E. coli		
DH5α	*deo*R, *end*A1, *gyr*A96, *hsd*R17(rK$^-$ mK$^+$), *rec*A1 *pho*A, *rel*A1, *thi*-1, Δ(*lac* ZYA-*arg*F) U169*ϕ*80d*lac*ZΔM15, F$^-$, *λ*$^-$, *sup*E44	Takara Bio[a]
BL21(DE3)	F$^-$, *dcm*, *omp*T, *hsd*S (rB$^-$ mB$^-$), *gal*, *λ* (DE3)	Takara Bio
M. marina strain MP-1	Wild type	ATCC 15381
Plasmids/cosmids		
pSTV28	Cloning vector	Takara Bio
pET21a	Cloning/expression vector	Takara Bio
pEPAΔ1,2,3	pWE15 carrying an EPA gene cluster that lacks *pfaE* from *S. pneumatophori* SCRC-2738	Ref. [7]
pSTV::*pfaE*	pSTV28 carrying *pfaE* from *S. pneumatophori* SCRC-2738 (formerly designated ORF2/pSTV28)	Ref. [6]
pET21a::*pfaE*	pET21a carrying *pfaE* from *M. marina* strain MP-1	This work
pETSTV::*pfaE*	pSTV28 carrying *pfaE* derived from pET21a::*pfaE*	This work
p3G11	Lorist6 carrying a genomic clone that includes *pfaE* of *M. marina* strain MP-1	Ref. [8]

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PPTase	Domains and their core sequences				
	P0	P1		P2	P3
		P1a	P1b		
for PUFA					
PfaE (SCRC-2738)	6 LRALLS	28 KGKP	45 FNVSH	82 GVDIE	129 KESYIKA
PfaE (SC2A)	80 LRAVLS	102 KGKP	119 FNLSH	164 GVDIE	211 KESYIKA
PfaE (MR-1)	52 LRCVLS	92 KGKP	109 FNLSH	143 GVDIE	191 KESYIKA
for nonribosomal peptide					
Sfp (*B. subtilis*)	51 VRSVIS	73 YGKP	86 FNISH	105 GIDIE	150 KESFIKQ
EntD (*E. coli*)	48 KRKTEH	78 VRQP	89 GSISH	108 GIDIE	154 KESAFKA
for polyketide					
SePptII (*S. erythraea*)	38 KRRREF	69 RGAP	80 GSMTH	103 GIDAE	149 KESVYKA
unspecified					
PcpS (*P. aeruginosa*)	62 KRQAEF	93 DRAP	104 GSITH	127 GLDVE	174 KESLFKA

Fig. 1. Partial core sequence alignment of conserved domains of Sfp-type PPTases involved in the biosynthesis of $n-3$ polyunsaturated fatty acids, nonribosomal peptides, and polyketides. P1, P2, and P3 domains are defined in [9]. P0, P1a and P1b were defined in this study. SCRC-2738, *S. pneumatophori* SCRC-2738; SC2A, *Shewanella* sp. SC2A; MR-1, *Shewanella oneidensis* MR-1. Deduced amino acid sequences for PfaE PPTases, Sfp, EntD, PcpS, and SePptII were retrieved from databases (DDBJ/GenBank/EMBL: http://www.ddbj.nig.ac.jp/Welcome-j.html). Identical amino acid residues are in red and similar amino acid residues are in blue.

precultured *E. coli* DH5α cells carrying pETSTV::*pfaE* (see below) and pEPAΔ1,2,3 [7] was transferred to fresh LB medium and then cultivated at 20 °C for 36–48 h for EPA production. Cells carrying pSTV28 were cultivated in medium containing chloramphenicol at 30 μg ml^{-1}. Cells carrying either a pET21vector or a pCR2.1®–TOPO® vector (Invitrogen, Carlsbad, CA) were grown in the presence of ampicillin at 50 μg ml^{-1}. *M. marina* strain MP-1 (MP-1; ATCC 15381) was cultivated in LB medium containing 3.0% NaCl at 15 °C for 96 h.

2.2. Polymerase chain reaction (PCR) and plasmid construction

Chromosomal DNA of MP-1 was isolated as described [8]. For PCR amplification, various types of degenerate oligonucleotide primers were prepared from core sequences of three domains conserved in Sfp-type PPTases from three *Shewanella* species (see Fig. 1). The first round of PCR was carried out using primers of 5′-TAYGGNGMNAARGGNAARCC-3′ and 5′-GCYTTDATRTANSWYTCYTT-3′ designed from the P1a and P3 domains, respectively, and genomic DNA from MP-1 as the template. PCR products purified by electrophoresis were then used as template for the second round of PCR with a primer of 5′-CAYTTYAAYNTIVSNCA-3′ designed from P1b and the primer from P3. The resulting PCR product (fragments of approximately 200 bp) was cloned into the pCR2.1®–TOPO® vector and then sequenced. The oligonucleotide primers (5′-GGCACAAATGATTAAGTTATCGG-3′ and 5′-CTGGACGTTAAAAGAAACCTACA-3′) designed from the sequenced fragment (see Fig. 2) and the genomic library of MP-1 as template [8] were subjected to the third PCR amplification to detect a cosmid clone(s) carrying the gene for PPTase. One positive clone of p3G11 was used to determine the whole sequence of PPTase gene. To obtain the full sequence of the gene for the PPTase of MP-1, flanking DNA sequences of the targeted gene were determined by inverse PCR [11].

To clone the full length of the PPTase gene (*pfaE*) of MP-1, PCR was carried out using one set of oligonucleotide primers: PPTEX_F1 (5′-GTATCCATTCTACATATGTACAG-3′) including an *Nde*I site (underlined) and PPTEX_R1 (5′-AAATAGTCTCGAGCTTCACTC-3′) including an *Xho*I site (underlined) and p3G11 as template. The obtained DNA fragment including the 861 bp ORF was digested with *Nde*I and *Xho*I, cloned into pCR2.1®–TOPO® (pCR2.1–TOPO::*pfaE*) and used for transformation of *E. coli* DH5α. The pCR2.1–TOPO::*pfaE* was treated with *Nde*I and *Xho*I and then the resulting insert DNA was cloned into *Nde*I–*Xho*I-digested pET21a (pET21a::*pfaE*). The *pfaE* gene sequence has been deposited to DDBJ/GenBank/EMBL with the accession number of AB262366.

The plasmid pETSTV::*pfaE* was constructed by ligating *Sal*I–*Bam*HI-digested pSTV28 with a PCR-amplified 2.8 kbp *Sal*I–*Bam*HI fragment that included *pfaE* and a T7 RNA polymerase binding site. For amplification of the 2.8 kbp DNA fragment, PCR was carried out using pET21a::*pfaE* as template and the primers PET_TO_PSTV_F (5′-TCAAGGGCATCGGTCGACATC-3′) including a *Sal*I site (underlined) and PET_TO_PSTV_R (5′-CCGGATATGGATCCTCCTTTC-3′) including a *Bam*HI site (underlined). The plasmid pSTV28 carrying *pfaE* from SCRC-2738 (pSTV::*pfaE*; [6]) was used as reference.

2.3. Nucleotide sequence determination and analysis

The nucleotide sequence of each fragment was determined by the dideoxy-chain termination method. Single-stranded DNA templates were fluorescently labeled with an Amplitaq Dye Primer Cycle Sequencing Kit using a thermal cycler, and then analyzed with an automatic DNA sequencer 3100 (Applied Biosystems, Foster City, CA). Nucleotide sequence analysis and comparative searches were performed using the GENETYX-MAC 9.0 program (Software Development, Tokyo, Japan), and the Swissprot (http://www.swissprot20.org/) and NBRF (http://pir.georgetown.edu/nbrf/) databases, respectively.

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1    ATG TAC AGC GGC GTA AAA GAT AAG CTC ACC CTC ACT ACA AAT GAA ATC CAT TTA TGG TCG GTT ACT CCG CAA ACT ATC CAA CAG CCT GAA   90
1    Met Tyr Ser Gly Val Lys Asp Lys Leu Thr Leu Thr Thr Asn Glu Ile His Leu Trp Ser Val Thr Pro Gln Thr Ile Gln Gln Pro Glu   30

91   TTA TTA CAG GCT TAT AGC CAA CTG TTA TCA CCT GCA GAA ACA ATA AAA CAA CAA CGC TTT CGA TTT GAA AAA GAT CGT CAC AAT GCT CTC   180
31   Leu Leu Gln Ala Tyr Ser Gln Leu Leu Ser Pro Ala Glu Thr Ile Lys Gln Gln Arg Phe Arg Phe Glu Lys Asp Arg His Asn Ala Leu   60

181  ATC ACT CGT GCT TTC GTC CGT GAT TTA TTA TCT CAC TAT GCA GAT GTT TTA CCG GCT GAT TGG CAG TTT GTG AAG GGG GAA AAG GAT AAA   270
61   Ile Thr Arg Ala Phe Val Arg Asp Leu Leu Ser His Tyr Ala Asp Val Leu Pro Ala Asp Trp Gln Phe Val Lys Gly Glu Lys Asp Lys   90
                        P0                                                                                    P1a
271  CCA GAG ATA GCG AAT CCC CCA CTC CCA CTG CGC TTT AAT ATT AGT CAT ACC GAT AAC TTA ATC ATT TGT GCC GTC ATG CTC AAT GAT GAT   360
91   Pro Glu Ile Ala Asn Pro Pro Leu Pro Leu Arg Phe Asn Ile Ser His Thr Asp Asn Leu Ile Ile Cys Ala Val Met Leu Asn Asp Asp   120
                                                P1b
361  ATC GGT TGT GAT GTC GAA AAT ACA CTG CGT AGC AGT AAT GTC TTG AGT ATT GCT AAA CAT TCA TTC TCA GAT AGT GAA TTC AAT GAT TTA   450
121  Ile Gly Cys Asp Val Glu Asn Thr Leu Arg Ser Ser Asn Val Leu Ser Ile Ala Lys His Ser Phe Ser Asp Ser Glu Phe Asn Asp Leu   150
     P2
451  CTT ACT CAA CCC ACT GCA CAA CAA ACC AGT CGT TTT TTT GAT TAC TGG ACG TTA AAA GAA TCT TAT ATC AAA GCA TGG GGC TTG GGT TTA   540
151  Leu Thr Gln Pro Thr Ala Gln Gln Thr Ser Arg Phe Phe Asp Tyr Trp Thr Leu Lys Glu Ser Tyr Ile Lys Ala Trp Gly Leu Gly Leu   180
                                                                            P3
541  TCG ATC CCG TTG AAA GAT TTC AGC TTC ACG CTA CCC GAA GGC TTT CAA CAG CAG TAT CAA CAA GAA GAT CAG CAA GAA AAC CAG CAT TGT   630
181  Ser Ile Pro Leu Lys Asp Phe Ser Phe Thr Leu Pro Glu Gly Phe Gln Gln Gln Tyr Gln Gln Glu Asp Gln Gln Glu Asn Gln His Cys   210

631  ATT GAT ACC ATT AAA TTA AGC TTT GCA CCT CAC CGT ATT GAT AAT CCC AAC ATT TGG CGT CAT TGG CTG TTC TAT CCA AAT AAT ACC CAC   720
211  Ile Asp Thr Ile Lys Leu Ser Phe Ala Pro His Arg Ile Asp Asn Pro Asn Ile Trp Arg His Trp Leu Phe Tyr Pro Asn Asn Thr His   240

721  AGA GTT GCA CTG GCT GTG CGC GCG CGA AGT AAT AAT CAG CAG ACT GAA TAT AAA ATG CGA TTT TTT AAT TCG ACA CCA CTG ATT AAT ATC   810
241  Arg Val Ala Leu Ala Val Arg Ala Arg Ser Asn Asn Gln Gln Thr Glu Tyr Lys Met Arg Phe Phe Asn Ser Thr Pro Leu Ile Asn Ile   270

811  ACT GAA ACA CTT ATT TTT AAA CCT GAG ACT AAT TTT AAA CCT GAC GCT AAA 861
271  Thr Glu Thr Leu Ile Phe Lys Pro Glu Thr Asn Phe Lys Pro Asp Ala Lys 287
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Fig. 2. Nucleotide and deduced amino acid sequences of PPTase gene (*pfaE*) from *M. marina* strain MP-1. The deduced amino acid sequence of fragment A is underlined. Amino acid residues corresponding to core sequences of P0, P1a, P1b, P2, and P3 domains are boxed.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

E. coli BL21 (DE3) carrying either pETSTV::*pfaE* or pSTV28 was cultivated at 37 °C for 16 h or at 15 °C for 96 h in LB medium containing chloramphenicol at 30 μg ml^{-1}. Induction of the *pfaE* gene was carried by the addition of isopropyl β-D-thiogalactoside (IPTG) at 0.3 mM. Harvested cells were washed three times with phosphate buffer (pH 7.5) by centrifugation at 3000 × *g* for 15 min and then suspended in 0.2 ml of 60 mM Tris-HCl buffer (pH 9.0) containing 5 M urea, 1 M thiourea, 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% Triton X-100, and Complete-mini (EDTA-free). Cells were disrupted by sonic oscillation using Sonifier Cell Disruptor (model W185; Branson Ultrasonic Corp., Danbury, CT) for 40 s in an ice bath. Cell-free extracts were prepared by centrifuging cell lysates at 20000 × *g* for 60 min. Electrophoresis was performed on slabs of 12.5% polyacrylamide gel containing 0.1% SDS. Gels were stained in a solution containing 0.1% Coomassie blue, 50% methanol, and 10% acetic acid.

2.5. Gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC/MS) analyses of fatty acids

Fatty acids were analyzed as their methyl esters (FAMEs). Cells were directly methanolyzed using 2 M HCl in methanol at 80 °C for 60 min. FAMEs extracted with *n*-hexane were concentrated and then subjected to GLC on a gas chromatograph (model GC-353B; GL Sciences, Tokyo) equipped with a capillary column, BPX70 (25 m long × 0.22 mm I.D., 0.25 μm film thickness, SGE Japan, Yokohama, Japan) and flame ionization detection with nitrogen as the carrier gas. The GLC oven temperature was 80 °C initially and programmed up to 240 °C at a rate of 4.0 °C min^{-1}. The injector temperature was set at 221 °C, and the detector temperature at 221 °C. The data were analyzed using a D-2500 Chromato-integrator (Hitachi, Tokyo).

FAMEs were identified by comparing their retention times with those of authentic standards and their pyrrolidide derivatives prepared as previously [12] were subjected to GC/MS on a Varian system (model CP-3800 gas chromatograph and Saturn 2200 ion trap mass spectrometer, Varian Technologies Japan, Inc., Tokyo) equipped with the same column as described above. Data were analyzed using a Saturn^{TM} Software Workstation Version 5.52. All analyses were carried out with a split ratio of 20:1. Helium was used as the carrier gas at a flow rate of 0.9 ml min^{-1}. The injection temperature was maintained at 250 °C. The MS conditions for electron bombardment ionization–mass spectrometry analysis were set as follows: *m/z* range of 10–500, 1 s scan time, ion trap temperature at 100 °C, 10 μA emission current, and 25000 times automatic gain control setting.

3. Results and discussion

3.1. PCR-based cloning of a PPTase gene fragment from M. marina strain MP-1

To design oligonucleotide primers for PCR the consensus domains of Sfp-type PPTase genes only from *Shewanella* species capable of synthesizing EPA were aligned (Fig. 1). The corresponding deduced amino acid sequences of EntD from *E. coli*, Sfp from *Bacillus subtilis*, PcpS from *Pseudomonas aeruginosa*, and SePptII from *Saccharopolyspora erythraea* are included separately in the figure as representatives of Sfp-type PPTases essential for the biosynthesis of nonribosomal polypeptides (NRPs; for EntD and Sfp) and polyketides (PKs; for SePptII). The attribution of PcpS was not specified, because it is required for biosynthesis of NRPs, PKs, and fatty acids [13]. All the PPTases have three consensus domains of P1, P2, and P3 shown in Fig. 1, and see [9]. However, in this study the P1 domain was recognized separately as two subdomains of P1a and P1b, because four core amino acids (KGKP) of P1a and four (FNxSH, where x is a nonconserved amino acid) of P1b were identical to the sequences of PPTases from the three *Shewanella* species (Fig. 1) and from some other *Shewanella* spp., which have genes homologous with the EPA gene cluster (data not shown). The resulting PCR products (fragments of approximately 200 bp) cloned into a pCR2.1®– pTOPO® vector were sequenced. The deduced amino acid sequence of one DNA fragment (named fragment A) was 50% identical with the corresponding sequence of the PPTase of SCRC-2738. Fragment A was considered a partial sequence of the PPTase gene of MP-1.

Among 260 genomic clones of MP-1 [8] one positive clone of p3G11, which had a sequence of fragment A, was obtained. The determined full sequence of a tentative PPTase gene of an ORF of 861 bp in p3G11 is shown in Fig. 2, where the sequence of fragment A is also indicated.

3.2. Cloning and characterization of the full length of PPTase gene (pfaE)

The full length PPTase gene (*pfaE*) of MP-1 was amplified by PCR using p3G11 as template. The resulting DNA fragment including one 861 bp ORF was cloned as pCR2.1–TOPO::*pfaE*, and then integrated into pET21a (pET21a::*pfaE*), from which pETSTV::*pfaE* was constructed. The *pfaE* of MP-1 encodes a deduced protein consisting of 287 amino acids. Its calculated molecular mass of 33.46 kDa is almost the same as that (31.69 kDa) of PPTase from SCRC-2738

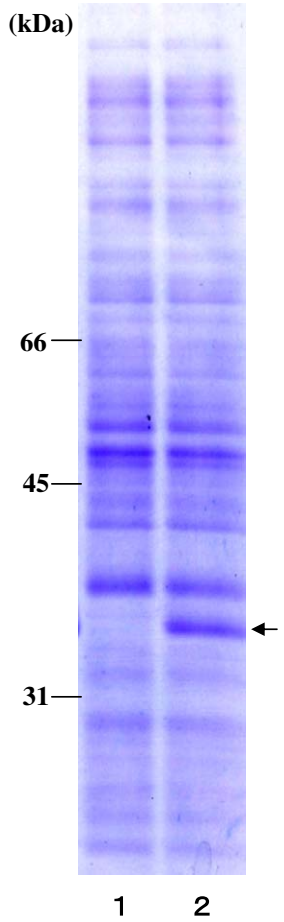

Fig. 3. SDS-PAGE analysis of *M. marina* strain MP-1 PPTase. pETSTV::*pfaE* was expressed in *E. coli* BL21(DE3). The recombinant cells were grown at 15 °C for 96 h. No treatment of cells with IPTG was performed (lane 2). Control cells were transformed with pSTV28 (lane 1). The arrow indicates the position of a 33 kDa band.

and with that of other Sfp-type PPTases. The predicted isoelectric point of this protein is 6.31.

The deduced PPTase of MP-1 has P2 and P3 domains conserved in Sfp-type PPTases for NRPs and PKs [9] and those for EPA (Figs. 1 and 2). P1a and P1b domains also existed and only the replacement of G with D and V/L with I was found in P1a and P1b domains, respectively, in MP-1 (Fig. 2). Our cloning of *pfaE* from MP-1 succeeded because

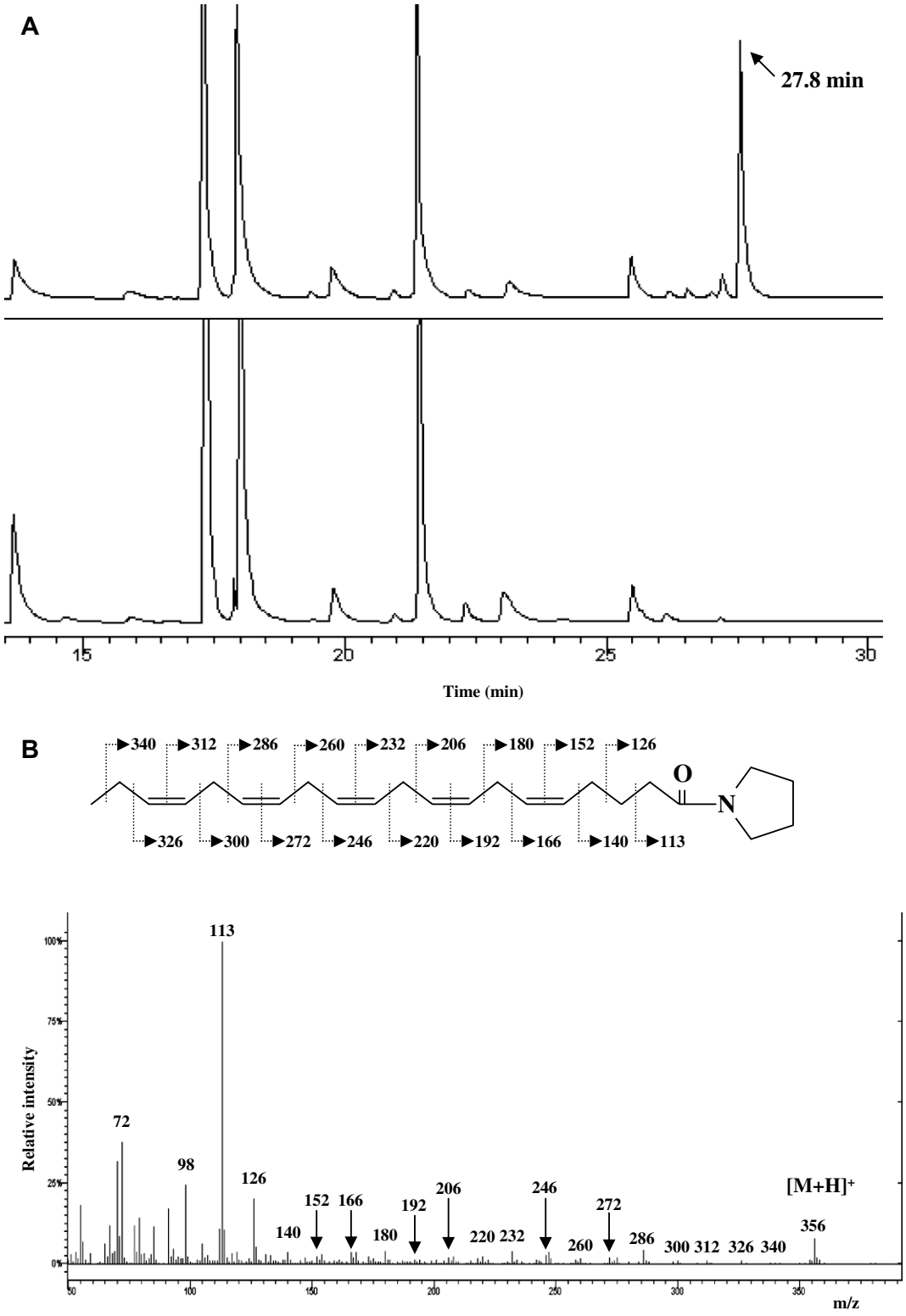

Fig. 4. Gas chromatogram (A) of total fatty acid methyl esters prepared from *E. coli* DH5α carrying pETSTV::*pfaE* and pEPAΔ1,2,3 (upper panel) and from *E. coli* DH5α carrying no vector (lower panel). The mass spectrum (B) of the pyrrolidide derivative of an unknown peak with a retention time of 27.8 min shown in (A).

regions including the P1a and P1b core sequences were selected to make degenerate oligonucleotide primers for the first and second rounds of PCR, respectively. Also, the targeted sequences were, as expected, shared by the PPTase gene of the species belonging to the genus of *Moritella*. Interestingly, *pfaE* for EPA and DHA had another conserved sequence of L/VRxL/VLS (P0), where x is a nonconserved amino acid (see Figs. 1 and 2), which lay 10–20 amino acids upstream of P1a. Among the amino acid residues conserved in P0, only R (and S for Sfp) was commonly found in Sfp-type PPTases for PKs or NRPs, implying that the P0 domain would not be present in these PPTases. This unique P0 sequence, and the P1a and P1b sequences, could be used to design generate primers to amplify PPTase genes for $n-3$ PUFAs of other bacterial genera such as *Photobacterium* [3], *Vibrio* [14], and *Colwellia* [15]. Actually, core sequences of the P0 (IRDLLS), P1a (KGKP), and P1b (FNISH) domains of the tentative PPTase of a psychrophilic bacterium *Colwellia psychrerythraea* 34H, of which the genome sequence has been determined [16], are nearly identical to those of MP-1 and EPA-producing bacteria (see Figs. 1 and 2).

3.3. Expression of pfaE in E. coli

In SDS-PAGE analysis the recombinant *E. coli* BL21(DE3) harboring pETSTV::*pfaE*, which had been grown at 37 °C and then treated with IPTG at 0.3 mM, showed an intense band of 33 kDa (data not shown). No band corresponding to a PPTase was observed from recombinant cells that had not been treated with IPTG, or from cells that carried an empty vector. In recombinant cells grown at 15 °C the 33 kDa band was detected even without IPTG treatment (Fig. 3).

pETSTV::*pfaE* complemented pEPAΔ1, 2, 3 [7], which was a vector carrying an insert DNA that included *pfaA–D* but no PPTase gene (*pfaE*) derived from genome of SCRC-2738. GC-based analysis of the total FAMEs of the 20 °C-grown recombinant *E. coli* DH5α cells carrying pEPAΔ1, 2, 3 and pETSTV::*pfaE* showed an unknown peak with a retention time of 27.8 min (Fig. 4A), which was the same as that of authentic EPA (data not shown). In GC/MS analysis of the pyrrolidide derivative of this unknown component the $[M+H]^+$ ion at *m/z* 356 and a series of ions at *m/z* 113, 126, 140, 152, 166, 180, 192, 206, 220, 232, 246, 260, 272, 286, 300, 312, 326, and 340 were detected (Fig. 4B), suggesting that this fatty acid is indeed EPA [12]. Analysis of the fragmentation profile with a program of the National Institute of Standard and Technology databases (http://www.nist.gov./srd/nist1a/htm) indicated that it was closest to that of EPA. From these results, we conclude that this component is EPA, making up 11.6% (w/w) of total fatty acids. Thus, it is evident that *pfaE* is a PPTase gene involved in the biosynthesis of $n-3$ PUFA in MP-1. When pEPAΔ1, 2, 3 was coexpressed at the same temperature with pSTV::*pfaE* (formerly ORF2/pSTV28 [6]) from SCRC-2738, in *E. coli* DH5α, the production of EPA was 12.3% of total fatty acids. The combination of a cosmid clone of p3D5 including *pfaA–D* from MP-1 [8] with either *pfaE* from the same bacterium or that from SCRC-2738 produced no DHA or EPA (unpublished).

The Sfp-type PPTases have a relatively broad specificity for substrates (carrier proteins) [9,10]. Sfp of *B. subtilis* phosphopantetheinylates not only peptide carrier protein (PCP) for NRPs but also ACPs for polyketides and fatty acids [17]. A unique Sfp-type PcpS, which is the sole enzyme responsible for phosphopantetheinylation of carrier proteins of *P. aeruginosa*, utilizes ACP, aryl carrier protein (ArCP), and PCP [13] as substrate. AngD of *V. anguillarum* utilizes EntF (PCP) of *E. coli* [18]. Compared with these Sfp-type PPTases, *pfaE* products have been considered to have a very strict specificity even for their cognate substrates. This is because the recombinant production of EPA in *E. coli* by combining *pfaA–D* from *P. profundum* SS9 and *pfaE* from *Shewanella* sp. SC2A—both of which are EPA-producing bacteria—was unsuccessful [3]. However, the *pfaE* genes of MP-1 and SCRC-2738 were completely compatible, implying that the PPTase for $n-3$ PUFAs would have a rather broad specificity, at least for cognate substrates. However, it could not utilize carrier proteins such as PCP, ArCP, and ACP for PKs as substrates.

PPTases for $n-3$ PUFAs (PfaE) are required specifically to recognize an uncommon structure of substrates, that is, five repeated ACP domains (for MP-1 or *P. profundum* SS9) or six (for SCRC-2738), which are integrated in the large multifunctional *pfaA* product of approximately 250 kDa as substrates [3,6,8]. Carrier proteins with such unique structures have never been reported for any proteins other than *pfaA* gene products, which are deduced proteins having multiple functional domains of KS, AT, six or five repeats of ACP, and KR in that order [6,8]. The *B. subtilis* Sfp uses a 130 kDa TycA protein containing substrate recognition and adenylation, PCP, and racemization domains in that order [10]. Tandemly, two or three repeated ACP domains are found in the polyketide antibiotic mupirocin biosynthesis gene (*Mmp*) cluster from *Pseudomonas fluorescence* [19]. The deduced MmpB (MmpII) protein of 222 kDa contains domains of KS, HD, KR, triplicated ACPs, and thioesterase. All these proteins had a domain(s) of carrier protein with common core sequences [10]. However, considering the notable difference in number, order, and expected function of each domain and in the size of the whole protein, *pfaA*-encoded proteins are probably the sole substrates of PPTases for $n-3$ PUFA. The presence of highly conserved P1a, P1b, and P0 domains of *pfaE* products is thought to reflect the presence of the unique tertiary structure of the cognate substrate proteins encoded by *pfaA* in EPA- or DHA-producing bacteria. P2 and P3 are domains participating in Mg^{2+} binding and P1 (both P1a and P1b) and P3 are involved in substrate (coenzyme A) binding and catalysis [20,21]. The P0 domain and P1a and P1b domains might be associated with recognition of the specific tertiary structure of the substrates carrying repeated ACP domains of the *pfaA* product.

In this study, we have cloned the PPTase gene (*pfaE*) from MP-1 responsible for the synthesis of EPA in recombinant *E. coli* (Fig. 4). Although we do not have direct evidence, the *pfaE* probably operates as a PPTase gene for DHA synthesis in MP-1. First, this is because the protein structure deduced from the *pfaE* gene is similar to that of PPTases from various types of EPA-producing bacteria. In addition to P2 and P3 domains, it has two subdomains (P1a and P1b) and one domain (P0) highly conserved only in PPTases responsible for the biosynthesis of $n-3$ PUFAs. Second, the protein encoded by *pfaE* could completely replace PPTase of SCRC-2738, and third, EPA is scarcely produced in MP-1 [22].

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