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

Keywords
Docosahexaenoic acid, Eicosapentaenoic acid, \textit{Moritella marina} strain MP-1, n-3 Polyunsaturated fatty acid, Phosphopantetheinyl transferase, \textit{pfaE} gene.

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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 synthase; 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
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 homologies 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 *E. coli* was transformed with DNA including one set of those five ORFs [1,6,7].

Genes homologous with *pfas* A–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 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. *Escherichia 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 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 30μg ml⁻¹. Cells carrying either a pET21 vector or a pCR.1®-TOPO® vector (Invitrogen, Carlsbad, CA) were grown in the presence of ampicillin at 50 μg ml⁻¹. *Moritella 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 Figure 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 Figure 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′-TCAAGGCGATCGGTCGACATC-3′) including a...
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**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.

**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 3,000 × 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% 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 20,000 × 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™ 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 25,000 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 PepS
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 Figure 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 open
reading frame (ORF) of 861 bp in p3G11 is shown in Figure 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 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 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 psychroerythraea* 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 \textit{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:\textit{pfaE} (formerly ORF2/pSTV28 \cite{6}) from SCRC-2738, in \textit{E. coli} DH5α, the production of EPA was 12.3\% of total fatty acids. The combination of a cosmid clone of p3D5 including \textit{pfaA–D} from MP-1 \cite{8} with either \textit{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) \cite{9,10}. Sfp of \textit{B. subtilis} phosphopantetheinylates not only peptide carrier protein (PCP) for NRPs but also ACPs for polyketides and fatty acids \cite{17}. A unique Sfp-type PcpS, which is the sole enzyme responsible for phosphopantetheinylation of carrier proteins of \textit{P. aeruginosa}, utilizes ACP, the aryl carrier protein (ArCP), and PCP \cite{13} as substrate. AngD of \textit{V. anguillarum} utilizes EntF (PCP) of \textit{E. coli} \cite{18}. Compared with these Sfp-type PPTases, \textit{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 \textit{E. coli} by combining \textit{pfaA–D} from \textit{P. profundum} SS9 and \textit{pfaE} from \textit{Shewanella} sp. SC2A—both of which are EPA-producing bacteria—was unsuccessful \cite{3}. However, the \textit{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 \textit{P. profundum} SS9) or six (for SCRC-2738), which are integrated in the large multifunctional \textit{pfaA} product of approximately 250 kDa as substrates \cite{3,6,8}. Carrier proteins with such unique structures have never been reported for any proteins other than \textit{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 \cite{6,8}. The \textit{B. subtilis} Sfp uses a 130 kDa TycA protein containing substrate recognition and adenylation, PCP, and racemization domains in that order \cite{10}. Tandemly, two or three repeated ACP domains are found in the polyketide
antibiotic mupirocin biosynthesis gene (Mmp) cluster from *Pseudomonas fluorescens* [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].

References


**Figure legends**

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.

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.

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.

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 pyrrolidine derivative of an unknown peak with a retention time of 27.8 min is shown in (A).
Table 1. Strains and vectors used in this study.

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\(^a\) Takara Bio Inc., Tokyo, Japan
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**Fig. 1** Domains and their core sequences
Fig. 2
Fig. 4