The *Escherichia coli* highly expressed *entD* gene complements the *pfaE* deficiency in a *pfa* gene clone responsible for the biosynthesis of long-chain n-3 polyunsaturated fatty acids

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**Abstract**

The *Escherichia coli* *entD* gene, which encodes an Sfp-type phosphopantetheinyl transferase (PPTase) that is involved in the biosynthesis of siderophore, is available as a high-expression ASKA clone (pCA24N::*entD*) constructed from the *E. coli* K-12 strain AG1. In *E. coli* DH5α, pCA24N::*entD* complemented a *pfaE*-deficient clone that comprised *pfaA*, *pfaB*, *pfaC* and *pfaD*, which are four of the five *pfa* genes that are responsible for the biosynthesis of eicosapentaenoic acid derived from *Shewanella pneumatophori* SCRC-2738. Sfp-type PPTases are classified into the EntD and PfaE groups, based on differences between their N-terminal-domain structures. Here, we showed that all Sfp-type PPTases may have the potential to promote the biosynthesis of long-chain n-3 polyunsaturated fatty acids.

**Introduction**

In some marine bacteria and eukaryotic microorganisms, long-chain n-3 polyunsaturated fatty acids (n-3 PUFAs), such as the eicosapentaenoic acid (EPA) and the docosahexaenoic acid (DHA), are synthesized *de novo* via a polyketide biosynthesis pathway (Metz et al., 2001; Orikasa et al., 2006a, b, c). Five genes (*pfaA*, *pfaB*, *pfaC*, *pfaD* and *pfaE*) that are involved in the biosynthesis of EPA or DHA have been cloned from bacteria (Tanaka et al., 1999; Allen & Bartlett, 2002; Orikasa et al., 2004). Similar eukaryotic genes have been cloned from heterokont algae such as *Schizochytrium* (Metz et al., 2001) and *Ulkenia* (Luy et al., 2009), although their gene structures are different from those of bacteria. The bacterial gene structures and domain structures of all *pfa* genes that are essential for the biosynthesis of EPA and DHA are well conserved (Okuyama et al., 2007).

Among the five *pfa* genes, the *pfaE* gene encodes an Sfp-type phosphopantetheinyl transferase (PPTase) of approximately 30kDa, which catalyses phosphopantetheinylation via transfer of the 4′-phosphopantetheine prosthetic group from coenzyme A to a conserved serine residue in the carrier proteins, thus converting these proteins from their inactive ‘apo’ forms into their active ‘holo’ forms (Gehring et al., 1998). Orikasa et al. (2006b) classified Sfp-type PPTases into two groups: the first includes PPTases that are involved mainly in the biosynthesis of n-3 PUFAs, while the second includes PPTases that are involved principally in polyketide and/or nonribosomal peptide synthesis. The Sfp-type PPTases have three conserved domains: P1, P2 and P3 (Weissman et al., 2004). The P1 and P3 domains are responsible for coenzyme-A binding and domains P2 and P3 are responsible for Mg²⁺ binding (Reuter et al., 1999; Chirgadze et al., 2000). However, the PPTases that are required for the biosynthesis of n-3 PUFAs (i.e., PfaEs) are different from the other Sfp-type PPTases in some aspects: the P1 domain at their N terminus can be separately recognized as P1a and P1b in PfaE and is highly conserved.
among PfaEs. Moreover, PfaEs have an additional conserved P0 domain (L/VRLxL/VLS) (where x is a nonconserved amino acid) upstream of P1a (Orikasa et al., 2006a).

The second representative group of PPTase includes the EntD protein of *Escherichia coli*, which is responsible for the synthesis of the siderophore enterobactin (Hantash et al., 1997). Interestingly, the genome of *Photobacterium profundum* SS9, which is an EPA-producing deep-sea bacterium, includes only one Sfp-type PPTase gene that was categorized into this second group (the EntD type; Sugihara et al., 2008). These findings suggest that this Sfp-type PPTase of *P. profundum* (SS9 PPTase) may be involved in the production of EPA, together with the other pfa genes (*pfaA*, *pfaB*, *pfaC* and *pfaD*) (Allen & Bartlett, 2002) located in the *P. profundum* SS9 genome (Vezzi et al., 2005).

Previously, we provided evidence that the SS9 PPTase gene complemented a pfaE-deficient *pha* gene clone, pDH4A3, which carried only *pfaA*, *pfaB*, *pfaC* and *pfaD* derived from the DHA-producing *Moritella marina* MP-1 (Sugihara et al., 2008). However, there is no evidence that *pfaE* is replaced with the *E. coli* entD gene. In the past, *E. coli* entD was considered as not being responsible for the biosynthesis of n-3 PUFAs, as neither EPA nor DHA was detected in any *E. coli* recombinant cells that carried vectors harbouring pfaE-deficient *pha* genes prepared from *Shewanella pneumatophori* SCRC-2738 (Orikasa et al., 2004), *M. marina* MP-1 (Tanaka et al., 1999; Orikasa et al., 2006a,b) and *P. profundum* SS9 (Allen & Bartlett, 2002).

To elucidate whether *pfaE* is replaced with *entD*, we used the ASKA clone pCA24N::*entD*, which is a plasmid that expresses *entD* at high levels. This clone was obtained from the cloning vector collection of the *E. coli* Strain National BioResource Project (http://www.shigen.nig.ac.jp/ecoli/ strain/top/top.jsp). In this study, pCA24N::*entD* was coexpressed with pEPA1,2,3, which was a pWE15 cosmid clone carrying an EPA biosynthesis gene cluster that lacked *pfaE* from *S. pneumatophori* SCRC-2738 (Orikasa et al., 2004).

### Materials and methods

#### Bacterial strains and culture conditions

The bacterial strains and vectors used in this study are listed in Table 1. *Escherichia coli* DH5α recombinant cells were precultivated in Luria–Bertani (LB) medium supplemented with the indicated antibiotics at 37°C for 16 h under shaking at 160 r.p.m. Portions of the culture were then transferred to the same medium and grown at 20°C for 72 h, for EPA production.

#### Plasmid preparation and transformation

The ASKA library is a comprehensive *E. coli* K-12 ORF plasmid library in which one gene was cloned into each *E. coli* strain via gene cloning at the Nara Institute of Science and Technology (Kitagawa et al., 2005). The *E. coli* strain K-12 carrying pCA24N::*entD* was obtained from the National BioResource Project. The ASKA clone library is based on the *E. coli* K-12 strain AG1 and individual genes were cloned into the pCA24N vector (see Table 1).

*Escherichia coli* K-12 cells carrying pCA24N::*entD* were grown at 30°C for 16 h in LB medium. pCA24N::*entD* was isolated using the mini-prep method and was used to transform *E. coli* DH5α cells carrying pEPA1,2,3 using the heat-shock method. The transformed *E. coli* DH5α cells were grown in LB medium containing ampicillin at 50 μg mL<sup>-1</sup> and chloramphenicol at 30 μg mL<sup>-1</sup> at 20°C for 72 h with shaking.

#### Fatty-acid analysis and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Transformed *E. coli* DH5α cells were collected by centrifugation. The precipitated cells were washed and were then directly subjected to methanolysis using 10% v/v acetyl chloride in methanol at 100°C for 1 h. The resulting fatty-acid methyl esters were analysed by gas-liquid chromatography.

### Table 1. Strains and vectors used in this study

| Strain/plasmid/cosmid | Relevant characteristics | Source |
|-----------------------|--------------------------|--------|
| *Escherichia coli* DH5α | deoR, endA1, gyrA96, rpsL17(:Km<sup>+</sup>), recA1, phaA, relA1, thi-1, Δ(lacZΔM15), F<sup>−</sup>, supE44 | Takara Bio* |
| *E. coli* K-12 strain AG1 | recA1, endA1, gyrA96, thi-1, rpsL17(:Km<sup>+</sup>), supE44, relA; provided as a host of pCA24N::*entD* | Kitagawa et al. (2005) |
| Plasmid/cosmid | pWE15 carrying an EPA gene cluster that lacks *pfaE* from *S. pneumatophori* SCRC-2738 | Orikasa et al. (2004) |
| pCA24N::*entD* | pCA24N carrying *entD* from *E. coli* K-12 strain AG1 | Kitagawa et al. (2005) |

* Takara Bio Inc. (Tokyo, Japan).
and GC/MS using the mode of electron impact, as described by Orikasa et al. (2006a).

The proteins produced by the recombinant cells were analysed by SDS-PAGE 7 h after treatment with or without 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG), as described previously (Orikasa et al., 2006a, c). The concentration of the proteins was estimated using the method of Bradford (1976).

Results and discussion

Coexpression of pCA24N::entD with pEPAΔ1,2,3 in E. coli DH5α cells

pCA24N::entD was used to transform E. coli DH5α cells carrying pEPAΔ1,2,3. GC/MS analysis of fatty-acid methyl esters prepared from E. coli DH5α cells that carried pCA24N::entD plus pEPAΔ1,2,3 revealed the presence of an unknown peak with a retention time of 30.2 min (Fig. 1a), which was not detected in E. coli DH5α host cells carrying only pEPAΔ1,2,3 (Fig. 1b). The retention time of the unknown peak was the same as that of the methyl ester of authentic EPA (data not shown). The GC/MS profile of the unknown peak shown in Fig. 1c was typical of methylene-interrupted PUFAs, and analysis of the fragmentation profile using a program from the National Institute of Standard and Technology Databases (http://www.nist.gov/srd/htm) indicated that the profile of this unknown component was closest to that of EPA. Based on these results, this compound was identified as EPA methyl ester. The content of EPA was 9.2 ± 0.2% of the total fatty acids from cells grown at 20 °C for 72 h. PUFAs other than EPA were not detected.

Expression of the EntD protein in E. coli DH5α cells

Figure 2 shows the SDS-PAGE profiles of E. coli DH5α cells carrying either pEPAΔ1,2,3 or pEPAΔ1,2,3 plus pCA24N::entD, in the presence or absence of IPTG. A significantly denser peak was detected in lanes 3 and 4 compared to lanes 1 and 2, respectively. Recombinant cells were grown at 37 °C for 7 h. The arrow indicates the band corresponding to EntD, at 26.1 kDa. Fifty micrograms of protein were loaded onto each lane. The dense band detected below EntD corresponds to chloramphenicol acetyltransferase derived from the pCA24N vector.
band of 26 kDa was detected only in recombinant cells carrying pEPAA1,2,3 plus pCA24N::entD in the presence and absence of IPTG (lanes 3 and 4, indicated by arrows). Although the intensity of this band was slightly stronger in cells treated with IPTG than that observed in cells not treated with IPTG, it is evident that pCA24N::entD can be highly expressed without induction by IPTG. There is no information regarding whether or not ASKA library plasmids are expressed at a low temperature without an inducer (see Kitagawa et al., 2005). However, it is interesting to note that the EPA biosynthesis gene cluster from Shewanella oneidensis MR-1 cloned under the lacZ promoter on a high copy number plasmid, pBluescript SK(+) (lanes 1 and 2 of Fig. 2). According to Armstrong et al. (1989), no native band of EntD was detected in E. coli strains by SDS-PAGE, unless it was overexpressed in the T7 promoter-directed high-expression system. The present results suggest that PfaE can be replaced by significantly higher levels of EntD. An undetectable level of expression of the native entD gene product of host E. coli DH5α cells (lanes 1 and 2 of Fig. 2) was insufficient to complement pEPAA1,2,3 lacking pfaE. The difference in the N-terminal domain structure between EntD and PfaE, and the addition of a His tag and of spacer amino-acid sequences to EntD would affect its affinity for its substrates, i.e. coenzyme A and/or a conserved serine residue in carrier proteins (such as acyl carrier proteins). This would be the most relevant reason for the partial replacement of PfaE with high levels of EntD. However, we have no idea how the addition of a His tag and of spacer amino-acid sequences to native EntD affects the structure and the catalytic activity of the Pfa enzyme complex. The pfaE from the EPA biosynthesis genes is compatible with that from the DHA biosynthesis genes (Orikasa et al., 2006a, c). It should be noted that the Sfp-type PPTases responsible for the biosynthesis of siderophores (and probably other polyketide compounds) and those responsible for the biosynthesis of n-3 PUFAs from terrestrial and marine bacteria, respectively, are partially compatible.

The PPTase involved in the production of EPA in P. profundum SS9 is an EntD-type enzyme (see above and Sugihara et al., 2008). This suggests that the PfaA–D proteins of this bacterium do not need high levels of EntD to synthesize EPA in an E. coli recombinant. Considering that the entD gene is expressed under low-iron conditions (Armstrong et al., 1989), no production of EPA in the E. coli recombinant grown in nutrient broth (2216 Marine Medium, Difco; Allen & Bartlett, 2002) might be caused by lack of the EntD protein.

The dense band detected below EntD corresponded to chloramphenicol acetyltransferase derived from the pCA24N vector, as assessed from its amino-acid sequencing.

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Statement
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References
Allen EE & Bartlett DH (2002) Structure and regulation of the omega-3 polyunsaturated fatty acid synthase genes from the deep-sea bacterium Photobacterium profundum strain SS9. Microbiology 148: 1903–1913.
Armstrong SK, Pettis GS, Forrester LJ & McIntosh MA (1989) The Escherichia coli enterobactin biosynthesis gene, entD: nucleotide sequence and membrane localization of its protein product. Mol Microbiol 3: 757–766.
Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.
Chirgadze NY, Briggs SL, McAllister KA, Fischl AS & Zhao G (2000) Crystal structure of Streptococcus pneumoniae acyl carrier protein synthase: an essential enzyme in bacterial fatty acid biosynthesis. EMBO J 19: 5281–5287.
Gehring AM, Mori I & Walsh CT (1998) Reconstitution and characterization of the Escherichia coli enterobactin synthetase from EntB, EntE, and EntF. Biochemistry 37: 2648–2659.
Hantash FM, Ammerlaan M & Earhart CF (1997) Enterobactin synthase polypeptides of Escherichia coli are present in an osmotic-shock-sensitive cytoplasmic locality. Microbiology 143: 147–156.
Kitagawa M, Ara T, Arafuzzaman M, Joka-Nakamichi T, Inamoto E, Toyonaga H & Mori H (2005) Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive); unique resources for biological research. DNA Res 12: 291–299.
Lee S-J, Kim CH & Seo P-S (2008) Enhancement of heterologous production of eicosapentaenoic acid in *Escherichia coli* by substitution of promoter sequences within the biosynthesis gene cluster. *Biotechnol Lett* 30: 2139–2142.

Luy M, Rusing M & Kiy T (2009) PUFA-PKS genes from *Ulkenia*. US Patent Application Publication No. US 20090093033 A1.

Metz JG, Roessler P, Facciotti D et al. (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* 293: 290–293.

Okuyama H, Orikasa Y, Nishida T & Morita N (2007) Bacterial genes responsible for the biosynthesis of eicosapentaenoic and docosahexaenoic acids and their heterologous expression. *Appl Environ Microb* 73: 665–670.

Orikasa Y, Yamada A, Yu R, Ito Y, Nishida T, Yumoto I, Watanabe K & Okuyama H (2004) Characterization of the eicosapentaenoic acid biosynthesis gene cluster from *Shewanella* sp. strain SCRC-2738. *Cell Mol Biol* 50: 625–630.

Orikasa Y, Nishida T, Hase A, Watanabe K, Morita N & Okuyama H (2006a) A phosphopantetheinyl transferase gene responsible for biosynthesis of n-3 polyunsaturated fatty acids from *Moritella marina* strain MP-1. *FEBS Lett* 580: 4423–4429.

Orikasa Y, Nishida T, Watanabe K, Morita N & Okuyama H (2006b) Phosphopantetheinyl transferase genes essential for biosynthesis of polyunsaturated fatty acids and their domain structures and compatibility. *Current Advances in the Biochemistry and Cell Biology of Plant Lipids: Proceedings of the 17th International Symposium on Plant Lipids, East Lansing, Michigan, July 2006* (Benning C & Ohlrogge J, eds), pp. 169–173. Aardvark Global Publishing Company, LLC, Salt Lake City, UT.

Orikasa Y, Nishida N, Yamada A, Yu R, Watanabe K, Hase A, Morita N & Okuyama H (2006c) Recombinant production of docosahexaenoic acid in a mode of polyketide biosynthesis in *Escherichia coli*. *Biotechnol Lett* 28: 1841–1847.

Reuter K, Mofid MR, Marahiel MA & Ficner R (1999) Crystal structure of the surfactin synthetase-activating enzyme Sfp: a prototype of the 4′-phosphopantetheinyl transferase superfamily. *EMBO J* 18: 6823–6831.

Sugihara S, Orikasa Y & Okuyama H (2008) An EntD-like phosphopantetheinyl transferase gene from *Photobacterium profundum* SS9 complements pfa genes of *Moritella marina* strain MP-1 involved in biosynthesis of docosahexaenoic acid. *Biotechnol Lett* 30: 411–414.

Tanaka M, Ueno A, Kawasaki K, Yumoto I, Ohgiya S, Hoshino T, Ishizaki K, Okuyama H & Morita N (1999) Isolation of clustered genes that are notably homologous to the eicosapentaenoic acid biosynthesis gene cluster from the docosahexaenoic acid-producing bacterium *Vibrio marinus* strain MP-1. *Biotechnol Lett* 21: 939–945.

Vezzi A, Campanaro S, D’Angelo M et al. (2005) Life at depth: *Photobacterium profundum* genome sequence and expression analysis. *Science* 307: 1459–1461.

Weissman KJ, Hong H, Oliynyk M, Siskos AP & Leadlay PF (2004) Identification of a phosphopantetheinyl transferase for erythromycin biosynthesis in *Saccharopolyspora erythraea*. *Chembiochem* 5: 116–125.