RESEARCH LETTER

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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Keywords
ASKA clone; EntD; Eicosapentaenoic acid; *pfaE*; Phosphopantetheinyl transferase; Polyunsaturated fatty acid.

Running title
*entD* complements *pfaE*

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Abstract

The *Escherichia coli* entD gene, which encodes an Sfp-type phosphopantetheiny 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 (n-3 PUFAs).

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 and c). Five genes (pfaA, pfaB, pfaC, pfaD and pfaE) that are involved in the biosynthesis of EPA or DHA have been cloned from bacteria (Allen & Bartlett, 2002; Orikasa et al., 2004; Tanaka et al., 1999). Similar eukaryotic genes have been cloned from heterocont 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 30 kDa, 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 to their active “holo” forms. 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 non-ribosomal 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 Mg^{2+} binding (Chirgadze et al., 2000; Reuter et al., 1999). 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/VRxL/VLS) (where x is a non-conserved 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 and 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 *pfa* gene clone, pDHA3, 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 PUFA s, as neither EPA nor DHA was detected in any E. coli recombinant cells that carried vectors harbouring pfaE-deficient pfa genes prepared from S. pneumatophori SCRC-2738 (Orikasa et al., 2004), M. marina MP-1 (Orikasa et al., 2006a, b; Tanaka et al., 1999) 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, pCN24N::entD was co-expressed with pEPAΔ1,2,3, which was a pWE15cosmid 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. E. coli DH5α recombinant cells were pre-cultivated 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 open reading frame 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).
**E. 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 pEPAΔ1,2,3 using the heat-shock method. The transformed **E. coli** DH5α cells were grown in LB medium containing ampicillin at 50 µg mL⁻¹ and chloramphenicol at 30 µg mL⁻¹ 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 and gas chromatography–mass spectrometry (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 proteins was estimated using the method of Bradford (1976).

**Results and discussion**

**Co-expression 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 polyunsaturated fatty acids, and analysis of the fragmentation profile using a program from the National Institute of Standard and Technology databases (http://www.nist.gov/srd/nist1a/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 total fatty acids from cells grown at 20 °C for 72 h. Polyunsaturated fatty acids 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 band of 26 kDa was detected only in recombinant cells carrying pEPAΔ1,2,3 plus pCA24N::entD in the presence and absence of IPTG (lanes 3 and 4; indicated by arrow). 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 whether or not ASKA library plasmids are expressed at low temperature without 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(+), was highly expressed in the absence of IPTG in *E. coli* (Lee et al. 2008), which was assessed by the high content of produced EPA at 20 °C. Thus, the inducer (IPTG)-independent leaky expression of the ASKA library plasmid would be due to low temperature effects on this plasmid.

The entD gene sequence encodes a predicted protein of 23,579 Da. The size detected for the
induced band (EntD; 26 kDa) corresponds to the protein with a His tag and four and five spacer amino-acid sequences at its N and C termini, respectively (Kitagawa et al., 2005). Native EntD was not detected in cells carrying only pEPAΔ1,2,3 (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 over-expressed 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 pEPAΔ1,2,3 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.
Acknowledgement

This work was partly supported by the National Institute of Polar Research. pEPAΔ1,2,3 was kindly provided by Sagami Chemical Research Center, Ayase 252-1193, Japan.

References


*Escherichia coli.* Biotechnol Lett **28:** 1841–1847.


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*Chembiochem* **5:** 116–125.
Table 1. Strains and vectors used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid/cosmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tr>
<td><strong>Strain</strong></td>
<td></td>
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<tr>
<td><em>Escherichia coli</em></td>
<td><em>deoR, endA1, gyrA96, hsdR17 (rK⁻/mK⁺)</em>, Takara Bio&lt;sup&gt;a)&lt;/sup&gt;</td>
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<tr>
<td>DH5α</td>
<td><em>recA1, phoA, relA1, thi-1, Δ(lac ZYA-argF)</em>, U169φ80dlacZΔM15, F⁻, λ⁻, supE44</td>
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<tr>
<td><em>E. coli K-12 strain</em></td>
<td><em>recA1, endA1 gyrA96 thi-1, hsdR17 (rK⁻/mK⁺)</em></td>
<td>Kitagawa et al., 2005</td>
</tr>
<tr>
<td>AG1</td>
<td><em>supE44, relA1; provided as a host of pCA24N::entD</em></td>
<td></td>
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<tr>
<td><strong>Plasmid/cosmid</strong></td>
<td></td>
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<tr>
<td>pEPAΔ1,2,3</td>
<td><em>pWE15 carrying an EPA gene cluster that lacks pfaE from S. pneumatophori SCRC-2738</em></td>
<td>Orikasa et al., 2004</td>
</tr>
<tr>
<td>pCA24N::entD</td>
<td><em>pCA24N carrying entD from <em>E. coli</em> K-12 strain AG1</em></td>
<td>Kitagawa et al., 2005</td>
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<sup>a</sup>Takara Bio Inc., Tokyo, Japan
Figure legends

**Fig. 1.** Gas chromatograms of total fatty-acid methyl esters prepared from *E. coli* DH5α recombinants, and electron-impact mass spectrometry of the unknown fatty-acid peak. *E. coli* carrying pEPAΔ123 plus pCA24N::entD (A) and *E. coli* carrying pEPAΔ1,2,3 (B). Mass spectrum of the unknown peak with a retention time of 30.2 min detected in A (C). Peaks a and b are those of cis-vaccenic and 3-hydroxyl tetradecanoic acids, respectively. Heneicosanoic acid (21:0) was used as an internal standard. *E. coli* DH5α cells carrying pEPAΔ1,2,3 and cells carrying pEPAΔ1,2,3 plus pCA24N::entD were cultivated at 20 °C for 72 h in LB medium containing ampicillin at 50 µg mL⁻¹ and ampicillin at 50 µg mL⁻¹ and chloramphenicol at 30 µg mL⁻¹, respectively.

**Fig. 2.** SDS–PAGE analysis of EntD at high levels in *E. coli* DH5α cells carrying pEPAΔ1,2,3 plus pCA24N::entD. *E. coli* DH5α cells carrying pEPAΔ1,2,3 and not treated with IPTG (lane 1); *E. coli* DH5α cells carrying pEPAΔ1,2,3 and treated with IPTG (lane 2); *E. coli* DH5α cells carrying pEPAΔ1,2,3 plus pCA24N::entD and not treated with IPTG (lane 3); and *E. coli* DH5α cells carrying pEPAΔ1,2,3 plus pCA24N::entD and treated with IPTG (lane 4). 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 was loaded onto each lane. The dense band detected below EntD corresponds to chloramphenicol acetyltransferase derived from the pCA24N vector.
Fig. 2