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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

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Abstract

The EntD-like phosphopantetheinyl transferase (PPTase) gene, cloned from the eicosapentaenoic acid-producing bacterium *Photobacterium profundum* strain SS9, has an open reading frame of

690 bp encoding a 230-amino acid protein. When this PPTase gene was expressed in *Escherichia coli* with *pfaA*, *pfaB*, *pfaC* and *pfaD* derived from *Moritella marina* MP-1, which were four of five essential genes for biosynthesis of docosahexaenoic acid (DHA), the DHA production of the recombinant was 2.0% (w/w) of total fatty acids. This is the first report showing that the EntD-like PPTase is involved in producing *n*-3 polyunsaturated fatty acids.

Introduction

Long-chain *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are synthesized *de novo* in bacteria via polyketide biosynthesis (Metz et al. 2001, Orikasa et al. 2006a,b,c). The genes (*pfaA*, *pfaB*, *pfaC*, *pfaD*, and *pfaE*) involved in biosynthesis of EPA or DHA have been cloned from EPA-producing or DHA-producing bacteria, respectively (Allen and Bartlett 2002, Orikasa et al. 2004, Tanaka et al. 1999). The gene structures and domain structures of these five genes essential for biosynthesis of EPA or DHA were recently reviewed (Okuyama et al. 2007).

Photobacterium profundum SS9 is an EPA-producing bacterium that was isolated from deep seawater (Allen and Bartlett 2000). Although *pfaA*, *pfaB*, *pfaC*, and *pfaD* genes were cloned from *P. profundum* SS9 (Allen and Bartlett 2002), the gene encoding phosphopantetheinyl transferase (PPTase) corresponding to *pfaE* was not obtained. According to the *P. profundum* SS9 genome database (Vezzi et al. 2005, <http://getentry.ddbj.nig.ac.jp/top-j.html>), this bacterium has two putative PPTase genes. One (accession number CAG21401) is considered from its size (378 bp) to be an AcpS-type PPTase, and to be involved in the *de novo* biosynthesis of fatty acids, and the other (accession number CAG23685) is a 690-bp putative PPTase, with a primary structure similar to that of Sfp-type PPTases.

According to Orikasa et al. (2006b) Sfp-type PPTases can be divided into two groups. The first group includes PPTases mainly used for *n*-3 PUFA synthesis, while the other group includes PPTases principally involved in polyketide and/or nonribosomal peptide synthesis. The SS9 putative Sfp-type PPTase and EntD of *Escherichia coli*, which is responsible for the synthesis of siderophore enterobactin (Hantash et al. 1997), are included in the latter group (Orikasa et al. 2006b). The SS9 putative Sfp-type PPTase is regarded as EntD (Vezzi et al 2005). The two groups have P2 (GxDxE) and P3 (KExxxKx) domains (where x is a nonconserved amino acid) in common. The PPTases necessary for the biosynthesis of *n*-3 PUFA have highly conserved P0 (L/VRxL/VLS), P1a (KxKP), and P1b (FNxSH) domains at their N-terminal region. However, in PPTases involved in the biosynthesis of polyketides and/or nonribosomal peptides, these domains are replaced with 1A (KRxxEx), P1a' (xRxP), and P1b' (GSIxH) domains, respectively (Orikasa et al. 2006b).

In this study, the putative Sfp-type EntD-like PPTase gene (CAG23685) was cloned from the *P. profundum* SS9 genome and coexpressed with *pfaA–D* genes from DHA-producing *Moritella marina* MP-1.

Materials and Methods

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 at 37 °C for 16 h. A portion of the *E. coli* DH5 α cells carrying pET21::*pfaE* (see below) and pDHA3 (Orikasa et al. 2006a,c) that were precultured at 37 °C was transferred to fresh LB medium and then cultivated at 15 °C for 48 h for DHA production.

PCR and plasmid construction

Chromosomal DNA of SS9 (Allen and Bartlett 2002) was kindly provided by Dr. D. H. Bartlett (Center for Marine Biotechnology and Biomedicine, Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093-0202). To clone the full length of the PPTase gene (designated *pfaE*) of SS9, PCR was carried out using one set of oligonucleotide primers: forward (5'-GAAACCTCAATAATACATATGAA-3') including an *NdeI* site (underlined) and reverse (5'-TCATTATGAATTCATTCAAC-3') including an *EcoRI* site (underlined) and SS9 genome DNA as template. The resulting DNA fragment including the 690 bp ORF was digested with *NdeI* and *EcoRI*, cloned into pCR2.1[®]-TOPO[®] (pCR2.1-TOPO::*pfaE*) and used for transformation of *E. coli* DH5 α . The pCR2.1-TOPO::*pfaE* was treated with *NdeI* and *EcoRI* and then the resulting insert DNA was cloned into *NdeI*–*EcoRI*-digested pET21a (pET21a::*pfaE*). The *pfaE* gene sequence was determined by the dideoxy-chain termination method as described previously (Orikasa et al. 2006a).

Table 1

SDS-PAGE of proteins and GC and GC/MS analysis of fatty acids

E. coli BL21 (DE3) carrying either pET21a::*pfaE* or pET21a was cultivated at 37 °C for 16 h or at 15 °C for 96 h in LB medium containing ampicillin at 50 $\mu\text{g ml}^{-1}$. Expression of the *pfaE* gene was induced by the addition of isopropyl β -D-thiogalactoside (IPTG) at 0.3 mM. The proteins produced by the recombinant bacteria were analyzed by SDS-PAGE, as described previously (Orikasa et al. 2006a,c).

Cells were directly methanolized and the resulting fatty acid methyl esters were analyzed by gas-liquid chromatography (GC) and gas chromatography/mass spectrometry (GC/MS), as described previously (Orikasa et al. 2006a,c).

Results and Discussion

Cloning and characterization of *pfaE* from *P. profundum* SS9

The PPTase gene of 690 pb was cloned as pCR2.1-TOPO::*pfaE*, and then integrated into pET21a (pET21a::*pfaE*). The *pfaE* of SS9 encodes a deduced protein consisting of 230 amino acids. Its calculated molecular mass of 25.5 kDa is slightly less than that of other Sfp-type PPTases involved in biosynthesis of *n*-3 PUFA (Orikasa et al. 2006a) and close to that (23.6 kDa) of EntD of *E. coli*.

Expression of *pfaE* in *E. coli*

In SDS-PAGE analysis, the recombinant *E. coli* BL21(DE3) harboring pET21a::*pfaE*, which was grown at 37 °C and then treated with IPTG at 0.3 mM, showed an intense band of 25 kDa (not shown). No band corresponding to a PPTase was observed from recombinant cells that were not treated with IPTG or from cells that carried an empty vector (not shown, see Orikasa et al. 2006c).

Figure 1

pET21a::*pfaE* was able to complement pDHA3, a vector carrying an insert DNA derived from the genome of MP-1 that included *pfaA–D* but no PPTase gene (*pfaE*) (Orikasa et al. 2006a,c). GC-based analysis of the total fatty acid methyl esters of the recombinant cells carrying pDHA3 and pET21a::*pfaE* grown at 15 °C showed an unknown peak with a retention time of 31.0 min (Fig. 1A), which was the same as that of known DHA (data not shown). The GC/MS profile of this unknown component was the same as that of known DHA (not shown) and the $[M - H]^+$ and base peak ions at m/z 341 and at m/z 79, respectively, were evident (Fig. 1B). Analysis of the fragmentation profile with a program from the National Institute of Standard and Technology databases (<http://www.nist.gov/srd/nist1a/htm>) indicated that the profile of the unknown component was closest to that of DHA. From these results, we conclude that this component is DHA, making up 2.0% (w/w) of total fatty acids, and that this *pfaE* is a PPTase gene involved in the biosynthesis of DHA. It is predicted that this PPTase is involved in the *in situ* biosynthesis of EPA in SS9 cells.

Previously, all *pfaE* genes involved in the biosynthesis of *n*-3 PUFA were considered Sfp-type PPTases with P0, P1a, and P1b domains at their N-terminal region (see Orikasa et al. 2006b;

Okuyama et al. 2007). However, the present study shows that the SS9 EntD-like PPTase (*pfaE*), which is included in the group of Sfp-type PPTases possessing 1A, P1a', and P1b' domains, can also be involved in the biosynthesis of *n*-3 PUFA. This is, to our knowledge, is the first report showing that an EntD-like PPTase is responsible for the biosynthesis of *n*-3 PUFA.

Although *E. coli* DH5 α should have had its own EntD gene in its genome, the recombinant cells transformed with pDHA3 that carried only *pfaA*–*pfaD* from *M. marina* MP-1 or those with pEPA Δ 2 carrying only *pfaA*–*pfaD* from EPA-producing *Shewanella pneumatophori* SCRC-2738 produced neither DHA nor EPA (Orikasa et al. 2006a,c). Considering that any *pfaE* cloned to date was coexpressed in high copy number plasmids (Orikasa et al. 2004, Orikasa et al. 2006a,c), relatively high amounts of *E. coli* EntD protein (PPTase) compared with that of PfaA–D proteins may be needed for recombinant production of EPA or DHA in *E. coli*.

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Table 1. Strains, genome DNA, and vectors used in this study.

Strain/genome/plasmid	Relevant characteristics	Source
Strain		
<i>Escherichia coli</i>		
DH5 α	<i>deoR</i> , <i>endA1</i> , <i>gyrA96</i> , <i>hsdR17</i> (rK ⁻ mK ⁺), <i>recA1</i> <i>phoA</i> , <i>relA1</i> , <i>thi-1</i> , Δ (<i>lac ZYA-argF</i>) U169 ϕ 80 <i>dlacZ</i> Δ M15, F ⁻ , λ ⁻ , <i>supE44</i>	Takara Bio ^a
BL21(DE3)	F ⁻ , <i>dcm</i> , <i>ompT</i> , <i>hsdS</i> (rB ⁻ mB ⁻), <i>gal</i> , λ (DE3)	Takara Bio ^a
Genome DNA of		Allen and Bartlett (2002)
<i>P. profundum</i> SS9		
Plasmids		
pCR2.1 [®] -TOPO [®]	Cloning vector	Invitrogen ^b
pCR2.1-TOPO:: <i>pfaE</i>	pCR2.1 [®] -TOPO [®] carrying <i>pfaE</i> from SS9	This work
pET21a	Cloning/expression vector	Takara Bio
pET21a:: <i>pfaE</i>	pET21a carrying <i>pfaE</i> from SS9	This work
pDHA3	pSTV29 carrying <i>pfaA-pfaD</i> from <i>Moritella marina</i> MP-1	Orikasa et al. (2006b)

^a Takara Bio Inc., Tokyo, Japan

^b Invitrogen Corp., Carlsbad, CA, USA

Figure legend

Figure 1. (A) Gas chromatogram of total fatty acid methyl esters prepared from *E. coli* DH5 α carrying pET21a::pfaE and pDHA3. Peaks a and b are those of *cis*-vaccenic acid and 3-hydroxy tetradecanoic acid, respectively. Heneicosanoic acid (peak c) was used as internal standard. (B) The mass spectrum of the unknown peak with a retention time of 31.0 min shown in (A). *E. coli* DH5 α cells carrying pET21a::pfaE and pDHA3 were cultivated at 15 °C for 48 h in LB medium containing ampicillin and chloramphenicol at 50 $\mu\text{g ml}^{-1}$ and 30 $\mu\text{g ml}^{-1}$, respectively.

