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# Isolation and Characterization of the Eicosapentaenoic Acid Biosynthesis Gene Cluster from *Shewanella* sp. BR-2

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**Forty-four eicosapentaenoic acid (EPA)-producing microbial strains were isolated from the intestines of marine fishes. Among them, one strain showing a maximum level of EPA (4.78% of total fatty acids) was identified as *Shewanella* sp. BR-2 on the basis of its 16S rRNA sequence. The EPA content reached a maximum level during the mid-exponential phase of cell growth, and gradually decreased with further growth of the cells. A cosmid DNA including the EPA biosynthesis gene cluster consisting of *pfaA-E* was isolated from a cosmid library of genomic DNA of *Shewanella* sp. BR-2, named pCosEPA-BR2. An *E. coli* clone harboring pCosEPA-BR2 produced EPA at a maximum level of 7.5% of total fatty acids, confirming the EPA biosynthesis activity of the cloned gene cluster.**

**Keywords:** Eicosapentaenoic acid, *Shewanella* sp. BR-2, PKS-like module, biosynthesis gene cluster

Polyunsaturated fatty acids (PuFAs) such as arachidonic acid (AA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3), and docosahexaenoic acid (DHA, C22:6n-3) are critical components of the glycolipids and phospholipids that compose plasma membranes [9]. They also act as precursors of certain hormones and of signaling molecule such as eicosanoid [1]. Additionally, these fatty acids are commonly known to provide beneficial effects in the prevention and treatment of heart disease, high blood pressure, inflammation, and certain types of cancer [2, 3, 5, 16, 19, 20].

PuFAs are typically formed *via* iterative elongation and desaturation of the saturated fatty acid, palmitic acid (PA, C16:0), generated by fatty acid synthase (FAS) [4, 15, 21]. Metz *et al.* [12] suggested a novel alternative pathway for the production of C20+ PuFAs in the marine microorganism *Shewanella pneumatophori* SCRC-2738. This pathway employs a polyketide synthase (PKS)-like module consisting of the following set of domains: a β-ketoacyl-ACP (acyl carrier protein) synthase (KS) domain, an acyltransferase (AT) domain, multiple ACP domains, a β-ketoacyl-ACP reductase (KR) domain, a chain length factor (CLF) domain, a β-hydroxyacyl-ACP dehydratase/isomerase (DH) domain, and an enoyl reductase (ER) domain. Additionally, a unique phosphopantetheinyl transferase (PPTase) is coupled with the PKS-like modules to activate the active site (serine residue) of the ACP domains. The PKS-like module was assumed to directly condense acetyl units derived from malonyl-CoA to carbon chains of C20+. Each step of the two-carbon extension consists of sequential catalysis by a ketoreductase (KR), a dehyratase (DH), and/or an enoyl reductase (ER), leading to the partial or complete reduction of the keto group. Therefore, in addition to the structural simplicity of the enzymatic complex, the PKS-like pathway has an advantage over the desaturase–elongase pathway in which it reserves the cellular reduction energy (1 NADPH per double bond). In contrast, in the desaturase–elongase pathway, a *cis*-double bond is inserted by aerobic desaturation after the complete reduction of the keto group [12].

Recently, we cloned a PKS-like module from *S. oneidensis* MR-1 that can produce EPA, although the production yield was very low (maximal level of 0.57% of total fatty acids) [10]. Heterologous expression of the gene cluster containing the *S. oneidensis* PKS-like module also led to the production of EPA in *E. coli*, but the production yield was still very low (maximal level of 0.69% of total fatty acids). In contrast, Yazawa [22] reported a very high level of EPA production in *S. pneumatophori* SCRC-2738 and in transgenic *E. coli* expressing the *S. pneumatophori* EPA biosynthesis gene cluster (about 25–40% and 16–22% of total fatty acids, respectively). Our keen interest was in

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knowing the molecular basis for the extreme differences in the production yields of EPA by different microorganisms. It was expected that this information could provide a blueprint for engineering a PKS-like module or a related metabolic pathway in order to increase the production yield of PuFAs. With this goal, we tried to isolate a novel EPA-producing microorganism that showed a high level of EPA production and cloned the PKS-like module from this microorganism. The results of the work presented in this article could lay the groundwork for comparative analysis revealing the molecular basis for differences in EPA production levels.

## Materials and Methods

### Bacterial Strains and Culture Media

Genomic DNA from *Shewanella* sp. BR-2 isolated in this work was used to clone the EPA biosynthesis gene cluster. *E. coli* XL1-Blue MR (Stratagene) was used as the cloning and expression host for the EPA biosynthesis gene cluster. *Shewanella* sp. BR-2 and *E. coli* XL1-Blue MR were cultivated in marine broth medium (Difco) and Luria–Bertani [LB; 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl] medium, respectively. Ampicillin was added at a concentration of 50 mg/ml when necessary.

### Isolation of EPA-Producing Microorganisms

A total of 226 strains were isolated from the intestines of the following 12 species of marine animals: pacific mackerel (*Scomber japonicus*), sardine (*Sardinops melanosticta*), hairtail (*Trichiurus haumela*), sciaenoid fish (*Nibea imbricate*), blue crab (*Portunus trituberculatus*), flatfish (*Paralichthys olivaceus*), prawn (*Penaeus japonicus*), mackerel pike (*Cololabis saira*), cuttlefish (*Sepia esculenta*), hard-shelled mussel (*Mytilus crassitesta*), sea bream (*Acanthopagrus latus*), and matsubara (*Sebastes hubbsi*).

All isolates were further scored for the production of polyunsaturated fatty acids. The microbial cells were cultivated in LB or marine broth medium at 20°C for 12 h, transferred to fresh medium, and further cultivated at 20°C for 24 h. The fatty acid composition of the cultivated cells was analyzed by the method described below. From 226 isolates obtained from fish intestines, 44 microbial strains produced EPA, and *Shewanella* sp. BR-2, showing a maximum level of EPA production, was used for further studies.

### 16S rRNA Sequences Analysis

The 16S rRNA sequence of *Shewanella* sp. BR-2 was determined (GenBank Accession No. EU719603) (SolGent, Korea) and aligned manually with representative 16S rRNA sequences of the genus *Shewanella* and related taxa based on their secondary structures using the ClustalW computer program, version 1.83 [18]. A phylogenetic tree was inferred by the neighbor-joining method with MEGA version 4 [17]. Kimura's two-parameter model was used for calculation of the distance matrix [8] and nucleotide positions containing gaps or missing data were deleted completely. The tree was rooted by using *Alteromonas macleodii* (GenBank Accession No. X82145). A total of 1,260 sites of 16S rRNA were used for the phylogenetic analysis. The tree reliability was evaluated by bootstrap analysis with 1,000 replicates.

### Cloning of the EPA Biosynthesis Gene Cluster

The genomic DNA of *Shewanella* sp. BR-2 was purified using a Wizard Genomic DNA Purification Kit (Promega, U.S.A.) according to the manufacturer's protocol. The purified genomic DNA was partially digested with the restriction enzyme Sau3AI. DNA fragments were ligated with a cosmid, SuperCos1, that had been digested with BamHI and dephosphorylated with alkaline phosphatase. The ligated products were packaged *in vitro* into bacteriophage lambda particles using an *in vitro* packaging kit (Gigapack III Gold Packaging Extract; Stratagene, U.S.A.) and introduced into *E. coli* cells by transfection. In order to isolate cosmid DNA containing the EPA biosynthesis gene cluster, the transformants were screened by the method of PCR amplification using two sets of oligonucleotides, each of which targeted the well-conserved KS and ER domains (Fig. 3). For the KS domain, P1 (5'-CCAAGACCAATATGTCCAC TG-3') and P2 (5'-GCAAAGCCCGCGTCATCATA-3') were used, and for the ER domain, P3 (5'-ACCGGCTCAATCAACCAAGC-3') and P4 (5'-TCTAAATAACTGCCTTTAGCCC-3') were used. The nucleotide sequences of the insert DNA on the selected cosmid were determined using a BigDye Terminator Ready Reaction Kit (Genotech, Korea) and analyzed with the on-line BLAST facility of the National Center for Biotechnology Information (htp://www.ncbi.nlm.nih.gov; NCBI, U.S.A.). The nucleotide sequence was deposited in GenBank under Accession No. EU719604.

### Production of EPA in Transgenic *E. coli*

To examine the production of EPA in the *E. coli* clone containing the EPA biosynthesis gene cluster from *Shewanella* sp. BR-2, the cells were cultivated in LB medium supplemented with ampicillin (50 μg/ml) at 25°C for 24 h, transferred to fresh medium, and further cultivated at 20°C for 36 h. The fatty acid composition of the cultivated *E. coli* clone was analyzed by the method described below.

### Analysis of Fatty Acid Composition

The cultivated cells were harvested from 50 ml of culture broth by centrifugation at 4,800 rpm for 15 min at 4°C. The cells were washed twice with 1 M sorbitol and dried for 3 h at 60°C. The dried cells were resuspended in 3 ml of 5% methanolic sulfuric acid and heated at 90°C for 1 h in sealed tubes. Fatty acid methyl esters (FAMEs) were extracted from the cells with 0.6 ml of hexane and then applied to a gas chromatograph (GC, Hewlett Packard 6890N) equipped with a flame-ionized detector (FID) and a HP-5 (30 m×0.32 mm, 0.25 mm; Agilent Technologies, U.S.A.) capillary column. The column temperature was raised from 150°C (2 min) to 270°C (2 min) at a rate of 7°C per min. All the results were confirmed by at least three independent experiments. The FAMEs were subjected to GC–MS analysis using a Hitachi GC–mass spectrometer (M-80A) with a capillary column (DB-WAX, 0.32 mm×30 m; J & W Scientific). Eicosapentaenoic acid methyl ester (Sigma) was used as a standard.

## Results and Discussion

### Screening of EPA-Producing Marine Microorganisms

The most abundant sources of polyunsaturated fatty acids (PuFAs), typically DHA and EPA, in nature are marine animals such as herring, mackerel, salmon, and sardines that usually inhabit deep-sea environments. The accumulation

of PuFAs in the body of marine animals is actually due to the intake of marine microorganisms that can produce DHA or EPA *de novo*. This means that the intestines of marine animals can be a rich source of microorganisms capable of producing high levels of PuFAs. A total of 226 microbial strains were isolated from the intestines of various marine animals. The fatty acid composition of the isolates was examined by GC analysis. Among them, 44 strains contained EPA at levels that ranged from 0.35 to 4.78% of total fatty acids at 20°C (data not shown). Although the cellular levels of EPA were lower than those of *Shewanella pneumatophori* SCRC-2738 (25–40% at 20°C), they were higher than those of other EPA-producing microorganisms such as *Shewanella* sp. GA-22 (5% and 2% at 4°C and 20°C, respectively), *S. baltica* MAC1 (3.5% and 1.5% at 4°C and 10°C, respectively), and *S. oneidensis* MR-1 (0.57% and 0.18% at 4°C and 15°C, respectively).

**Table 1.** Effect of growth temperature on fatty acid composition of *Shewanella* sp. BR-2.

| Fatty acid | % of total fatty acid | | | |
|---|---|---|---|---|
| | 4°C | 15°C | 20°C | 30°C |
| 12:0 | 3.3 | 2.3 | 3.0 | 3.0 |
| 13:0 | 6.0 | 5.9 | 5.6 | 7.0 |
| 14:0 | 3.4 | 2.2 | 3.0 | 2.8 |
| 15:0 | 13.1 | 14.8 | 15.5 | 21.6 |
| 16:0 | 11.0 | 10.3 | 14.0 | 15.8 |
| 16:1 | 39.9 | 31.3 | 34.2 | 29.4 |
| 17:0 | 0.5 | 1.3 | 0.9 | 1.5 |
| 18:1 | 6.5 | 9.5 | 8.6 | 7.4 |
| 20:5 (EPA) | 7.7 | 5.4 | 4.6 | 2.6 |
| Others | 8.6 | 17 | 10.6 | 8.9 |

## Isolation and Identification of *Shewanella* sp. BR-2

Strain BR-2, showing the highest level of EPA (4.78% at 20°C), was selected and analyzed further. A peak corresponding to EPA was detected in gas chromatography analysis of the fatty acid composition of strain BR-2, and it was confirmed to be EPA by GC–MS analysis (data not shown).

The EPA content reached a maximum level during the mid-exponential phase of cell growth and then gradually

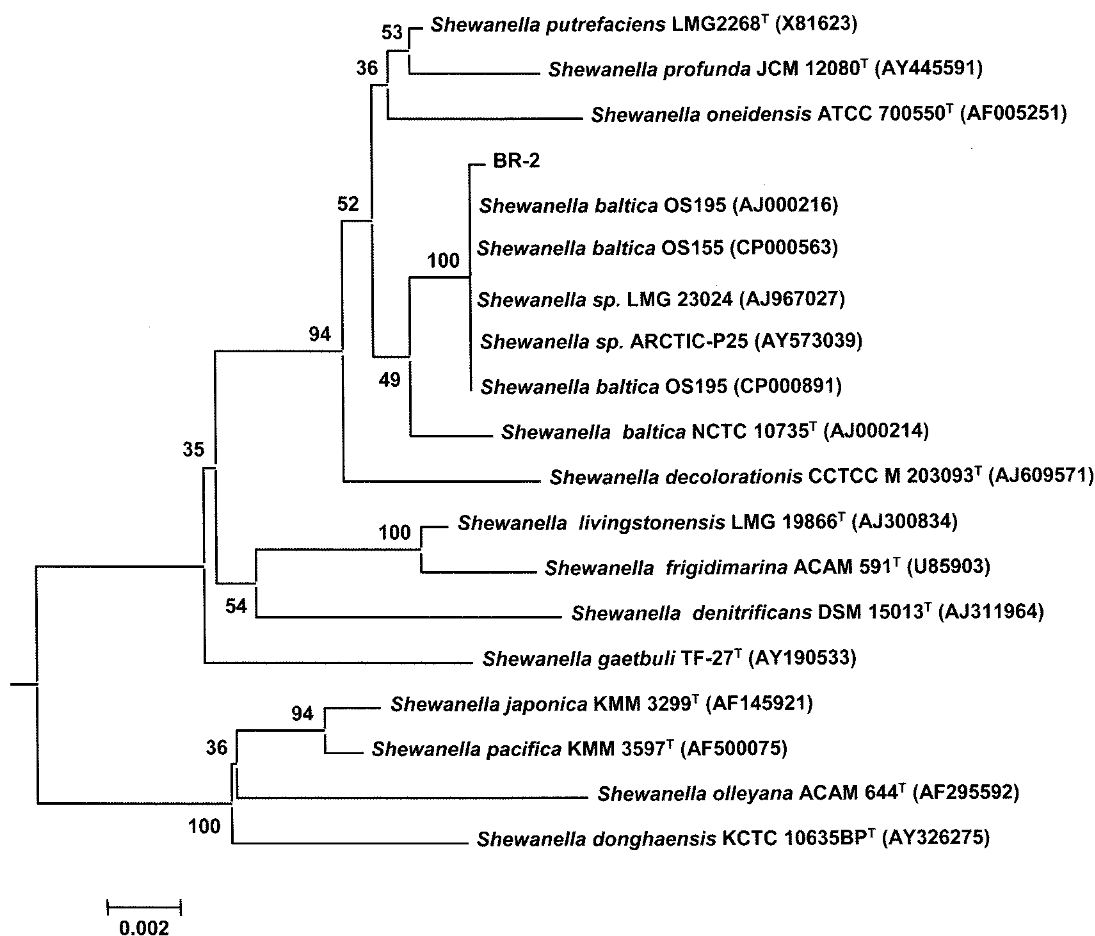


**Fig. 1.** Phylogenetic tree estimated from 16S rRNA sequences in order to position the BR-2 isolate.
The 16S rRNA sequence of *Shewanella* sp. BR-2 was deposited in the GenBank nucleotide sequence database under Accession No. EU719603.

decreased with further growth of the cells (data not shown). A similar trend was observed for *S. oneidensis* MR-1 [4].

EPA levels usually exhibit an inverse relation to cultivation temperature. This was also observed in the case of BR-2. A maximum level of EPA was achieved at 4°C, and EPA levels gradually decreased with increasing temperature (Table 1). This is quite reasonable as the EPA-producing *Shewanella* species are the inhabitants of cold, deep-sea environments.

The 16S rRNA gene sequence of strain BR-2 was determined, and phylogenetic analysis showed that it is closely related to and clusters with *S. baltica* strains with high sequence homology (99%) and bootstrap value (100%) (Fig. 1), designating the strain as *Shewanella* sp. BR-2.

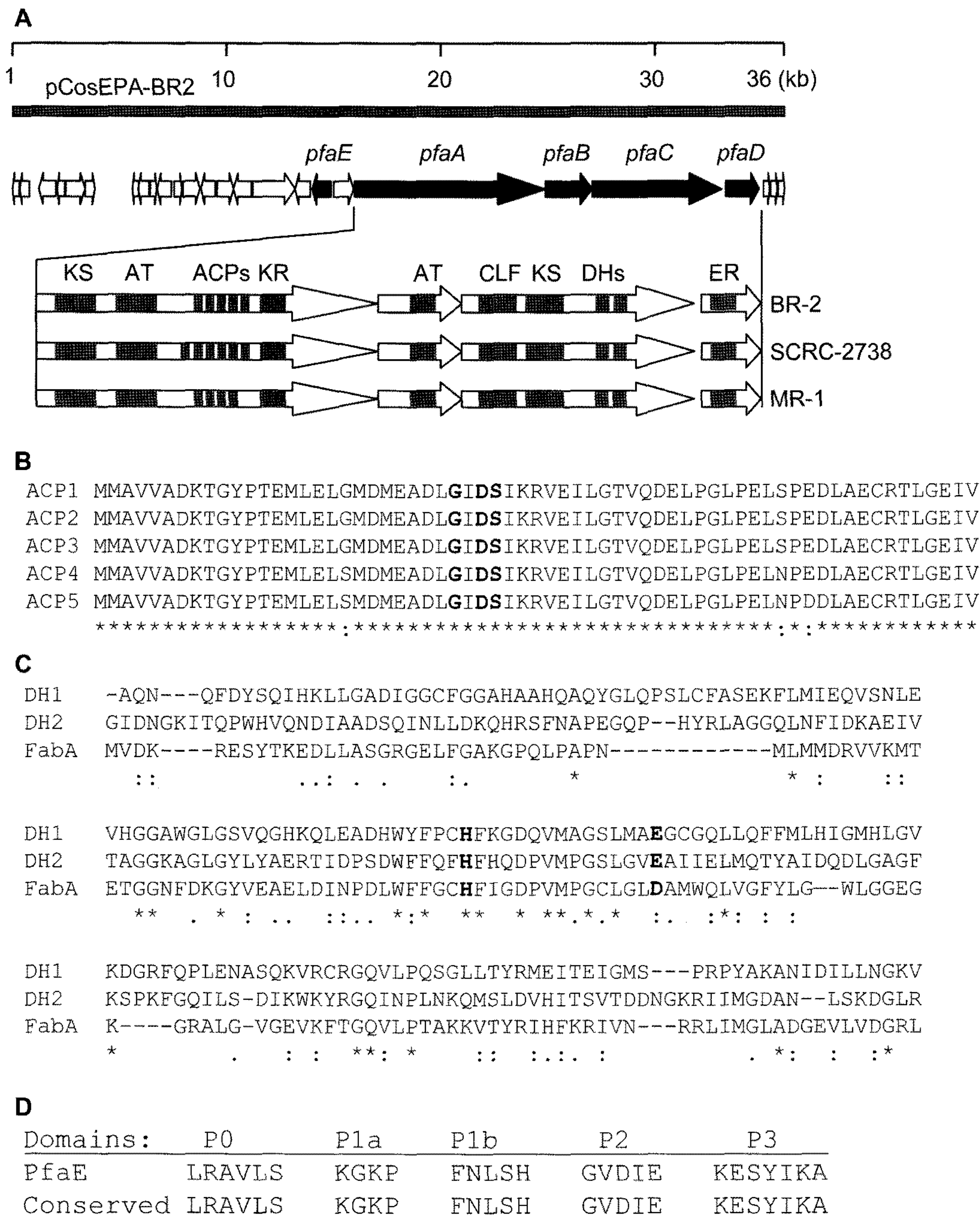


**Fig. 2.** Genomic organization of the EPA biosynthesis gene cluster and the domain structure of the multifunctional proteins (GenBank Accession No. EU719604).

Twenty-four complete and two truncated ORFs within an approximately 36-kb DNA insert of pCosEPA-BR2 are indicated as arrows. KS, β-ketoacyl-ACP (acyl carrier protein) synthase; AT, acyltransferase; ACPs, multiple ACPs; KR, β-ketoacyl-ACP reductase; CLF, chain length factor; DH, β-hydroxyacyl-ACP dehydratase/isomerase; ER, enoyl reductase.

**Cloning of the EPA Biosynthesis Gene Cluster from *Shewanella* sp. BR-2**

The bacterial EPA biosynthesis genes that have been identified to date were recently summarized in a review [13]. The genetic organization of the gene cluster is well conserved. It consists of four genes encoding multifunction enzymes, generally termed *pfaA–D*, which collectively constitute a PKS-like module consisting of KS, AT, multiple ACP, and KR domains on PfaA; an AT domain on PfaB; KS, CLF, and DH domains on PfaC; and an ER domain on PfaD. In order to clone the EPA biosynthesis gene cluster from *Shewanella* sp. BR-2, we screened a cosmid library containing genomic DNA from the bacterial strain by PCR amplification targeting two conserved KS and ER domains that define the boundaries of the gene cluster (Fig. 2). For this purpose, we first amplified and cloned these two domains from *Shewanella* sp. BR-2 genomic DNA. Nucleotide sequences of the cloned fragments corresponded to the KS and ER domains. Next, we confidently screened a cosmid library of genomic *Shewanella* sp. BR-2 DNA by the same PCR method. As a result, we isolated a cosmid that yielded both PCR products with expected sizes corresponding to the KS and ER domains, which were further confirmed by nucleotide sequencing. The cosmid was shown to contain an approximately 36-kb insert and it was named pCosEPA-BR2.

**Genetic Organization of the *Shewanella* sp. BR-2 EPA Biosynthesis Gene Cluster**

Twenty-four complete and two truncated ORFs were found in pCosEPA-BR2. Analysis and multiple alignments of the deduced amino acid sequences were carried out and the results are summarized in Table 2. A typical EPA biosynthesis gene cluster consisting of *pfaA*, *pfaB*, *pfaC*, and *pfaD* was found in the nucleotide sequence (GenBank Accession No. EU719604). The domain structure of the multifunction proteins was well conserved with respect to that of other EPA biosynthesis gene clusters (Fig. 2A), especially that of *S. pneumatophori* SCRC-2738 [14] and *S. oneidensis* MR-1 [4] that were previously reported to synthesize EPA in heterologous hosts (KS, AT, multiple ACP, and KR domains in PfaA; AT domain in PfaB; CLF, KS, and multiple DH domains in PfaC; ER domain in PfaD). Recently, Okuyama *et al.* [13] divided the PKS-like polyunsaturated fatty acid biosynthesis gene cluster into three types based on the genetic organization. According to

**Table 2.** Deduced ORFs surrounding the EPA biosynthesis gene cluster.

| ORF | Start | Stop | NA/AA | Putative function |
|---|---|---|---|---|
| 1 | 276 | 1 | 276/92 | PTS glucose-like IIB subunit (truncated) |
| 2 | 692 | 231 | 462/154 | Flavodoxin/nitric oxide synthase |
| 3 | 2,083 | 1,223 | 861/287 | Pseudouridine synthase |
| 4 | 241 | 2,073 | 339/113 | Unknown function |
| 5 | 2,553 | 3,563 | 1,011/337 | Conserved hypothetical |
| 6 | 3,563 | 3,946 | 384/128 | Conserved hypothetical |
| 7 | 5,970 | 5,668 | 303/101 | Conserved hypothetical |
| 8 | 6,601 | 5,957 | 645/215 | GCN5-related *N*-acetyltransferase |
| 9 | 6,748 | 6,981 | 234/78 | Conserved hypothetical |
| 10 | 7,402 | 6,998 | 405/135 | Conserved hypothetical |
| 11 | 7,567 | 7,947 | 381/127 | Conserved hypothetical |
| 12 | 7,950 | 8,720 | 771/257 | Conserved hypothetical |
| 13 | 9,501 | 8,854 | 648/216 | Syd family protein |
| 14 | 9,528 | 10,415 | 888/296 | GTP cyclohydrolase I |
| 15 | 11,453 | 10,515 | 939/313 | Transcriptional regulator |
| 16 | 11,577 | 13,562 | 1,986/662 | β-Lactamase domain protein |
| 17 | 14,256 | 13,708 | 549/183 | Lipoprotein |
| 18 | 15,342 | 14,452 | 891/297 | 4'-Phosphopantetheinyl transferase (PfaE) |
| 19 | 15,994 | 16,860 | 867/289 | Transcriptional regulator |
| 20 | 16,860 | 24,935 | 8,076/2,692 | EPA synthase (PfaA) |
| 21 | 25,040 | 27,199 | 2,160/720 | EPA synthase (PfaB) |
| 22 | 27,199 | 33,177 | 5,979/1,993 | EPA synthase (PfaC) |
| 23 | 33,300 | 34,943 | 1,644/548 | EPA synthase (PfaD) |
| 24 | 35,360 | 35,533 | 174/58 | Unknown function |
| 25 | 35,560 | 35,751 | 192/64 | Conserved hypothetical |
| 26 | 35,922 | 36,260 | 339/113 | Diacylglycerol kinase (truncated) |

ORF, open reading frame; NA, nucleic acids; AA, amino acids.

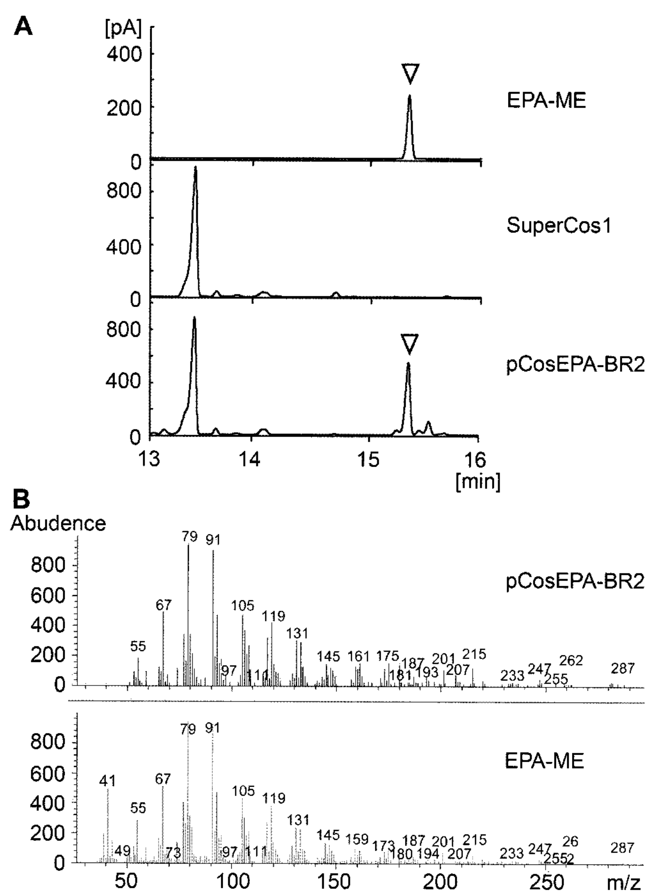


**Fig. 3.** Gas chromatography of fatty acid methyl esters (FAMEs) of *E. coli* harboring SuperCos1 (center) and pCosEPA-BR2 (bottom). EPA-methyl ester was analyzed as a standard (top). The triangle indicate the EPA-methyl ester.

their classification, the EPA biosynthesis gene cluster from *Shewanella* sp. BR-2 belongs to the type I group, in which a gene (*pfaE*) encoding phosphopantetheinyl transferase (PPTase) is present in close proximity to the *pfaA–D* genes (Fig. 2A).

Five ACPs were contained in the PfaA protein from *Shewanella* sp. BR-2 (Fig. 2B), whereas four and six ACPs were found in the PfaA proteins of *S. oneidensis* MR-1 and *S. pneumatophori* SCRC-2738, respectively [4, 13, 14]. Interestingly, the number of ACP domains is likely to correlate with the production level of EPA in these microorganisms. It has been reported that EPA production reaches 24–40% of the total fatty acid content in *S. pneumatophori* SCRC-2738, in which the biosynthesis machinery includes six ACP domains [22]. In contrast, slightly lower and much lower levels of EPA were shown to be produced in *Shewanella* sp. BR-2 (4.78%) and *S. oneidensis* MR-1 (0.57%), where the clusters are composed of five and four copies of the ACP domain, respectively [10]. Jiang *et al.* [6] have demonstrated the role of tandem ACP domains in the synthesis of EPA by serial deletion of the domain. This suggests that the ACP domain is an attractive subject for engineering the PKS-like system to increase the yield of EPA.

Two DH domains were present in PfaC, which showed high homology to FabA from *E. coli* that is a β-hydroxyacyl-ACP dehydrate (Fig. 2C). FabA is known to be involved in the synthesis of unsaturated fatty acid by catalyzing dehydration on β-hydroxy-decenoylacyl-ACP and subsequent isomerization of *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP [7]. The active residues of enzyme were conserved in the DH domains.

Orikasa *et al.* [14] have classified phosphopantetheinyl transferases (PPTases) into two groups; one is involved in the biosynthesis of polyunsaturated fatty acids (group I) and another is involved in polyketides and nonribosomal peptides (group II), which shows a unique domain structure. As shown in Fig. 2D, PfaE conserved well the consensus sequences of signature domains in PPTases belonging to the group I.

**Heterologous Production of EPA in *E. coli***

To confirm whether the cloned PKS-like module can catalyze the production of EPA in a heterologous host, *E. coli*, the fatty acid composition of the clone harboring pCosEPA-BR2, was analyzed using GC. Novel fatty acid peaks appeared in *E. coli* owing to the introduction of the PKS-like module (Fig. 3A). The major fatty acid with a level of 7.5% of the total fatty acids was identified as EPA-methyl ester by GC–MS analysis (Fig. 3B). The level of EPA in the transgenic *E. coli* was slightly higher than in the native *Shewanella* sp. BR-2 strain. This might be due to the high copy number of the biosynthesis gene cluster on the episomal cosmid DNA.

Fish oils are the current major source of polyunsaturated fatty acids for industrial applications, wherein the two main unsaturated fatty acids, DHA and EPA, are always found in fish oils at the level of approximately 18% and 12%, respectively [7]. The close similarity in the chemical structure usually requires very complicated processes to prepare highly pure DHA or EPA [11], which can be a critical obstacle for the application of the valuable materials to higher value-added products such as pharmaceuticals. However, as shown in this study, microorganisms employing the PKS-like modules produce just one of the polyunsaturated fatty acids, DHA or EPA [10, 13, 14, 22]. We expect that further genetic and metabolic engineering of the PKS-like modules and microbial cells containing the modules can enhance the production yields of polyunsaturated fatty acids, which can be beneficial commercially.

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