

# Enhancement of heterologous production of eicosapentaenoic acid in *Escherichia coli* by substitution of promoter sequences within the biosynthesis gene cluster

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**Abstract** To enhance the heterologous production of eicosapentaenoic acid (EPA) in *Escherichia coli*, the EPA biosynthesis gene cluster from *Shewanella oneidensis* MR-1 was cloned under the *lacZ* promoter on a high-copy number plasmid, pBlue-script SK(+). The production of EPA was remarkably enhanced yielding levels of up to 7.5% of the total fatty acid content in the recombinant *E. coli* strain by induction with IPTG, whereas the stimulation of EPA production was abolished by adding glucose into the culture medium, probably due to glucose repression acting on the promoter activity.

**Keywords** Biosynthesis gene cluster · Eicosapentaenoic acid · *Escherichia coli* ·

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Heterologous production · *LacZ* promoter ·  
*Shewanella oneidensis*

## Introduction

Polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3), are critical components of the glycolipids and phospholipids that compose plasma membranes (Lauritzen et al. 2001). They also act as precursors of certain hormones and the signaling eicosanoid molecules (Abbey et al. 1990). 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 (Angerer and Schacky 2000; Braden and Carroll 1986; Fiocchi et al. 1994; Sauer et al. 2001).

The most abundant sources of PUFAs in nature are marine fishes such as herring, mackerel, salmon, and sardines that usually inhabit deep-sea environments. The accumulation of PUFAs in the bodies of marine fishes is actually due to the intake of marine microorganisms that can produce DHA or EPA de novo. Recently, we have cloned and expressed an EPA biosynthesis gene cluster from the microorganism, *Shewanella oneidensis* MR-1, in *Escherichia coli* (Lee et al. 2006). Although production of EPA in the

heterologous host was detected, the yield was extremely low (merely 0.7% of the total fatty acids) despite the presence of multiple copies of the cosmid containing the genes (SuperCos1). In this study, we assessed the effect of substituting promoter sequences involved in directing the expression of the EPA biosynthesis genes with the strong *lacZ* promoter on the heterologous production of EPA.

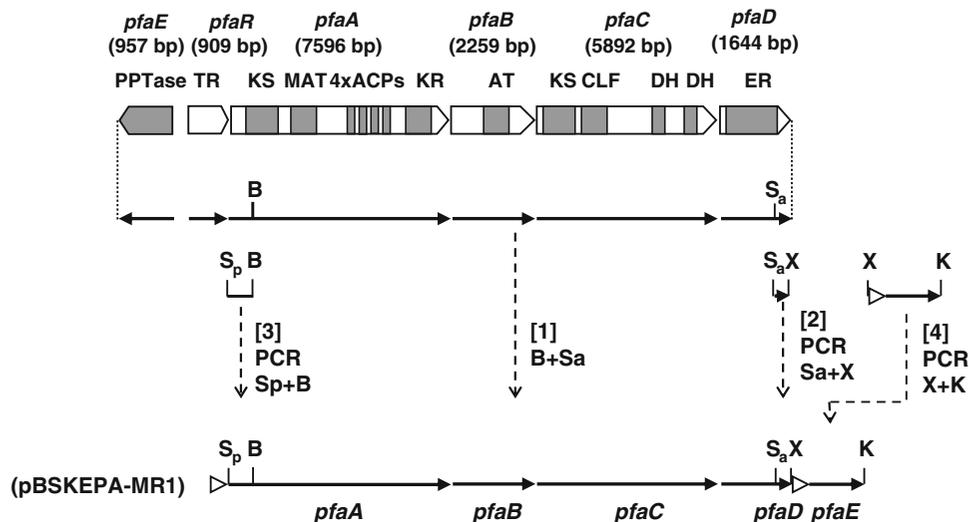
## Materials and methods

### Reconstitution of expression plasmid for the EPA biosynthesis gene cluster

The strategy to reconstitute the expression plasmid for the EPA biosynthesis gene cluster is presented in Fig. 1. Firstly, a 20.2-kb *Bam*HI–*Sal*II fragment from the cosmid, pCosEPA-MR1 (Lee et al. 2006), including the *pfaA* (except for 1.36 kb of *N*-terminal DNA sequence), *pfaB*, *pfaC*, and *pfaD* (except for 129 bp of *C*-terminal DNA sequence) genes was cloned into the corresponding restriction sites of pBluescript SK(+), generating pBSKEPA-1. Secondly, the *C*-terminal 129-bp DNA sequence of the *pfaD* gene was amplified by the method of PCR using the following oligonucleotides: P1 (5′-GTCTGACTTGGCGAAACATTTA-3′;

the underlined letters indicate a *Sal*II site and the italicized letters correspond to the Leu-510 codon of *pfaD*) and P2 (5′-CTCGAGTTAGCAGCGTTGCAG-3′; the underlined letters indicate a *Xho*I site and the italicized letters indicate the stop codon of *pfaD*). The PCR product was inserted into the restriction sites, *Sal*II and *Xho*I, on pBSKEPA-1, generating pBSKEPA-2. Next, the *N*-terminal 1.36-kb DNA sequence of the *pfaA* gene was amplified by the method of PCR using the following set of oligonucleotides: P3 (5′-ACTAGTATGAGCCATACCCCT-3′; the underlined letters indicate a *Spe*I site and the italicized letters indicate the start codon of *pfaA*) and P4 (5′-GGATCCAAGGGCGCGCCT-3′; the underlined letters indicate a *Bam*HI site and the italicized letters correspond to the Gln-454 codon of *pfaA*). The PCR product was inserted into the restriction sites, *Spe*I and *Bam*HI, on pBSKEPA-2, generating pBSKEPA-3, where the expression of the *pfaA*–*D* genes was driven by the *lacZ* promoter on pBluescript SK(+).

To insert the *lacZ* promoter upstream of the *pfaE* gene encoding phosphopantetheine transferase (PPTase), the 129-bp *lacZ* promoter and the 957-bp DNA sequence for the *pfaE* gene were amplified by the method of PCR using the following oligonucleotides: P5 (5′-CTCGAGAGCGGGCAGTGA-3′; the underlined letters indicate a *Xho*I site) and P6



**Fig. 1** Strategy for substituting the promoter sequences of the EPA biosynthesis gene cluster. The procedure used to construct pBSKEPA-MR1 was explained in the materials and methods section. KS,  $\beta$ -ketoacyl-ACP (acyl carrier protein) synthase; AT, acyltransferase; ACPs, multiple ACPs; KR,  $\beta$ -ketoacyl-

ACP reductase; CLF, chain length factor; DH,  $\beta$ -hydroxyacyl-ACP dehydratase/isomerase; ER, enoyl reductase. B, *Bam*HI; K, *Kpn*I; Sa, *Sal*I; Sp, *Spe*I; X, *Xho*I. Arrow heads indicate *lacZ* promoter sequences

(5'-GGATCCTCTAGAAGCTGTTTCCTGTG-3'; the underlined and the italicized letters indicate a *Bam*HI and a *Xba*I site, respectively) for the *lacZ* promoter and P7 (5'-TCTAGA*AATGAAGATTGAGCTT*-3'; the underlined letters indicate a *Xba*I site and the italicized letters indicate the start codon) and P8 (5'-GGATCCGGTACCTT*AGTCAGCCAA*-3'; the underlined and the italicized letters indicate a *Bam*HI and a *Kpn*I site, respectively, and the bold letters indicate the stop codon) for the *pfaE* gene. The DNA fragments were combined and cloned into the pGEM TEasy vector and the resulting 1.08-kb *Xho*I–*Kpn*I fragment was excised and inserted into the corresponding restriction sites downstream of the *pfaA-D* genes on pBSKEPA-3, generating pBSKEPA-MR1.

#### Analysis of EPA production in *E. coli*

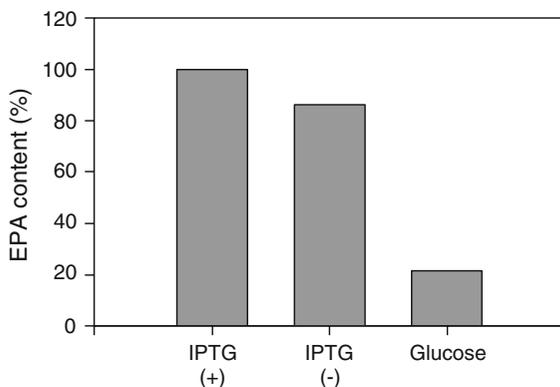
*Escherichia coli* clones harboring pBSKEPA-MR1 were cultivated in LB medium supplemented with ampicillin (50 µg/ml) at 25°C for 24 h. The cells were inoculated into 1 l of fresh medium and grown at 20°C to mid-growth stage (OD<sub>600</sub> of approx 0.5) with shaking at 180 rpm and then expression of the EPA biosynthesis genes was induced by addition of 0.1 mM IPTG followed by incubation for 36 h. To assess the effect of glucose on the gene expression, glucose was added directly to the medium (1%, w/v). The cultivated cells were harvested from 500 ml of culture broth by centrifugation at 5,000g for 15 min at 4°C. The cells were washed twice with 1 M sorbitol and dried for 3 h at 60°C. The lipid of cells was extracted by the chloroform–methanol (3:1, v/v). The total lipid extract was dried and then suspended in 3 ml 5% (v/v) 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 hexane and then applied to a GC equipped with a flame-ionized detector and a HP-5 (30 m × 0.25 mm, 0.25 µm, Agilent Technologies, USA) capillary column. The column temperature was raised from 150°C (2 min) to 270°C (2 min) at 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).

## Results and discussion

We have previously demonstrated the heterologous production of EPA in *E. coli* harboring a cosmid, pCosEPA-MR1, that includes an EPA biosynthesis gene cluster from a Gram-negative bacterium, *S. oneidensis* MR1 (see Fig. 1) (Lee et al. 2006). Although the production level of EPA was slightly increased in the recombinant host as compared to the native host probably because of the presence of multiple copies of the biosynthesis genes in the heterologous host, the production level was still extremely low (merely 0.7% of the total fatty acids). The low production yield could be explained by the expression level of the biosynthesis gene cluster or by the enzyme activity levels of the biosynthetic machinery. Here, we assessed the effect of the expression level of the biosynthesis gene cluster on the production yield. For this purpose, we replaced the native promoter sequences of the biosynthesis gene cluster with the strong *lacZ* promoter. Two putative promoter sequences for the biosynthesis gene cluster were found from extensive analysis of its genetic organization, one in a 667-bp DNA region between divergent genes encoding a transcriptional regulator and the PPTase upstream of the *pfaA-C* genes and another in a 172-bp DNA region upstream of the *pfaD* gene. The activity levels of the promoter sequences might be under the control of the upstream transcriptional regulator. Likewise, Allen and Bartlett (2002) suggested that the EPA biosynthesis gene cluster in *Photobacterium profundum* consisted of two transcriptional units, an mRNA containing the *pfaA-C* genes and an mRNA containing the *pfaD* gene. As a strategy to increase the expression of the biosynthesis gene cluster, the *pfaA-D* genes were introduced into the pBluescript SK(+) vector under the control of the strong *lacZ* promoter (see “Materials and Methods”). To remove any effect of the upstream transcriptional regulator on the expression levels, this gene was omitted in the pBluescript SK(+) clone. In addition, PPTase is known to be essential for the activity of the EPA biosynthesis machinery (Orikasa et al. 2004). This is due to its activation of catalytic sites (serine residues) within the machinery via phosphopantethinylation. To maximize the function of PPTase, *pfaE* was also placed under the control of the *lacZ* promoter downstream of the *pfaA-D* genes (Fig. 1). As a result,

**Table 1** Fatty acid composition of *E. coli* harboring pBSKEPA-MR1

Fatty acid	Percentage of total fatty acid	
	<i>E. coli</i> pBluescript SK(+)	<i>E. coli</i> pBSKEPA-MR1
12:0	2.2	2.8
14:0	5.2	5.0
16:0	34.9	34.4
16:1	27.4	18.9
18:1	17.8	15.6
20:5 (EPA)	–	7.5
Others	12.5	15.8

**Fig. 2** Effect of glucose on the production level of EPA in *E. coli* harboring pBSKEPA-MR1. EPA production was presented as a relative value. IPTG and glucose were added into LB medium at a concentration of 1 mM and 1% (w/v), respectively

a new expression plasmid for the EPA biosynthesis gene cluster was constructed and designated pBSKEPA-MR1.

The production level of EPA in *E. coli* strains harboring pBSKEPA-MR1 was examined (Table 1). New peaks appeared in the GC analysis of fatty acids from *E. coli* DH5 $\alpha$  harboring pBSKEPA-MR1 at 13.1, 15.3, and 15.5 min at the level of 5.4%, 7.5%, and 2.0% of the total fatty acid, respectively (Supplementary Fig. 1), without significant change in the total lipid content of approximately 0.04 g/g dry cell weight. The major peak at 15.3 min was identified as EPA-methyl ester (Supplementary Fig. 2), for which the production level was dramatically increased by

approximately 11-fold as compared to that of the native promoter sequence on pCosEPA-MR1 (Lee et al. 2006). The characterization of chemical structure of peaks at 13.1 and 15.5 min is in process.

The production level of EPA in *E. coli* harboring pBSKEPA-MR1 was slightly reduced by omitting IPTG induction (Fig. 2). Moreover, when glucose was added to the culture medium, the production level of EPA returned to the similar level as shown in *E. coli* harboring pCosEPA-MR1, probably due to glucose repression acting on the promoter activity. These results indicate that the enhanced production of EPA was due to the stimulated expression of biosynthesis gene cluster directed by the strong *lacZ* promoter.

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