pfaB products determine the molecular species produced in bacterial polyunsaturated fatty acid biosynthesis

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Abstract
When pDHA4, a vector carrying all five pfaA–pfaE genes responsible for docosahexaenoic acid (DHA; 22:6) biosynthesis in Moritella marina MP-1, was coexpressed in Escherichia coli with the individual pfaA–pfaD genes for eicosapentaenoic acid (EPA; 20:5) biosynthesis from Shewanella pneumatophori SCRC-2738, both polyunsaturated fatty acids were synthesized only in the recombinant carrying pfaB for EPA synthesis. Escherichia coli coexpressing a deleted construct comprising pfaA, pfaC, pfaD, and pfaE for EPA and pfaB for DHA produced EPA and DHA. Both EPA and DHA were detected in bacteria that inherently contained pfa genes for DHA. These results suggest that PfaB is the key enzyme determining the final product in EPA or DHA biosynthesis.

Running Head: Bacterial polyunsaturated fatty acid molecular species

Keywords
Docosahexaenoic acid; Eicosapentaenoic acid; Moritella marina strain MP-1; Polyunsaturated fatty acid; pfaB gene.
Introduction

Bacteria synthesize polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6), in a mode of polyketide biosynthesis that differs from the combined fatty acid elongation and aerobic desaturation system commonly observed in eukaryotes (Metz et al., 2001). Because there are some benefits, including the need for fewer reducing equivalents such as NADPH and simple fatty acids consisting solely of EPA or DHA, in using this bacterial system to produce PUFA rather than using the aerobic system (Okuyama et al., 2007), the factor(s) that determine the final molecular species in the system must be identified for the practical production of these PUFAs.

The bacterial polyketide synthase-type multienzyme complex responsible for the biosynthesis of EPA and DHA is composed of proteins encoded by five pfa genes: pfaA, pfaB, pfaC, pfaD, and pfaE (Okuyama et al., 2007). Four of the pfa genes of the DHA-producing bacterium Moritella marina MP-1, pfaA, pfaB, pfaC, and pfaD, are clustered on the chromosome (Tanaka et al., 1999), whereas pfaE is located separately (Orikasa et al., 2006a). However, in the EPA-producing bacterium Shewanella pneumatophori SCRC-2738 and some other EPA-producing bacteria, all five genes are clustered in close proximity (see Fig. 1). The number of acyl carrier protein (ACP) repeats in the pfaA gene product is five in M. marina MP-1 and six in S. pneumatophori SCRC-2738. The 3-ketoacyl synthase (KS) domain is present in the pfaB product of M. marina MP-1, but not in that of S. pneumatophori SCRC-2738 (Orikasa et al., 2004).

Although the structures of the pfa genes and their encoded proteins from EPA-producing and DHA-producing bacteria are very similar, the bacterial syntheses of EPA and DHA have been generally considered mutually exclusive; that is, bacteria that produce EPA do not produce DHA and vice versa. Some deep-sea or Antarctic bacteria produce only EPA (Bowman et al., 1998; Fang et al., 2003; Nichols et al., 1997), as do EPA-producing S. pneumatophori SCRC-2738 (Hirota et al., 2005) and S.
marinintestina IK-1 (Satomi et al., 2003). Conversely, some deep-sea bacteria produce only DHA (DeLong & Yamanos, 1986; Hamamoto et al., 1995). However, this criterion is not absolute, because some bacteria from the intestinal contents of marine fish produce both EPA and DHA (Watanabe et al., 1997; Yano et al., 1998; Yano et al., 1994). In most of these cases, DHA is the primary product (approximately 5% or more of total fatty acids) and EPA is a minor component (less than 1% of the total fatty acids). So far, only EPA or DHA has been produced in recombinant E. coli cells carrying the pfa genes responsible for EPA or DHA biosynthesis, respectively (Orikasa et al., 2004; Okuyama et al., 2007). These findings suggest that the molecular species involved in the bacterial biosynthesis of EPA or DHA must be determined by one or more of the pfa gene product(s). However, as demonstrated previously, the pfaE product has been excluded as a candidate determinant because the pfaE genes of S. pneumatophori SCRC-2738 and M. marina MP-1 are compatible with the other pfa genes of both bacterial species (Orikasa et al., 2006a; 2006b). The number of ACP domain repeats in the pfaA product is not involved in determining the molecular species of PUFA (Okuyama et al., 2007). Nonetheless, the details of how these highly similar structures preferentially synthesize either EPA or DHA have not yet been clarified.

To the best of our knowledge, pDHA3 is the only vector encoding the bacterial genes responsible for the biosynthesis of DHA, pfaA, pfaB, pfaC, and pfaD of M. marina MP-1 (Okuyama et al., 2007; Orikasa et al., 2006b). However, only DHA was produced in recombinant E. coli cells coexpressing the genes encoded by pDHA3 and pfaE from M. marina MP-1 (Orikasa et al., 2006b) or pfaE from other bacteria (Orikasa et al., 2006a; Sugihara et al., 2008). Various constructs are available that contain the pfa genes of S. pneumatophori SCRC-2738. In addition to cosmid and plasmid vectors that carry all five pfa genes, deletion constructs lacking one of the five pfa genes and clones containing individual pfa genes have been prepared (Orikasa et al., 2004; Orikasa, 2007; Yazawa et al., 1998). These clones have been used to increase the heterologous production of EPA (Orikasa et al., 2004;
Orikasa et al., 2007) and to determine the physiological functions of EPA in E. coli recombinant systems (Nishida et al., 2006a; 2006b; Okuyama et al., 2008).

In this study, a vector that carries all five pfa genes derived from M. marina MP-1 was constructed (pDHA4; Fig. 1). To identify the factor(s) determining the final product in the polyketide mode of PUFA biosynthesis, the pfa genes on pDHA4 were coexpressed with individual pfa genes derived from S. pneumatophori SCRC-2738. The pfaB gene was also cloned from pDHA4 and expressed with a construct encoding all five pfa genes (pEPAΔ1; see Table 1) and a mutant pfa gene construct encoding pfaA, pfaC, pfaD, and pfaE (pEPAΔ6) for EPA biosynthesis.

**Materials and methods**

**Bacterial cells and cultivation**

*Escherichia coli* DH5α was the host bacterium for the recombinant pfa genes. It was grown in LB medium supplemented with the indicated antibiotics at 37°C for 16 h. Part (40 μL) of the culture was transferred to 2 mL of fresh LB medium and was cultivated with shaking at 180 rpm at 15°C for 96 h to stimulate DHA and/or EPA production. Bacterial strains and vectors used in this study are summarized in Table 1.

EPA-producing *S. pneumatophori* SCRC-2738 from the intestines of Pacific mackerel (Hirota et al., 2005) and *Shewanella marinintestina* IK-1 from squid body (Satomi et al., 2003) were cultivated in 3 mL of LB medium containing 3.0% NaCl at 20°C for 48 h. DHA-producing *M. marina* MP-1 from deep-sea waters (DeLong & Yayanos, 1986) was cultivated in the same medium at 15°C for 72 h. *Moritella marina* MP-1 and *E. coli* recombinant carrying pEPAΔ6 and pSTV28::pfaB(DHA) were cultivated in the presence and in the absence of cerulenin at indicated concentrations.
DNA procedures

To construct a vector carrying all five pfa genes responsible for the biosynthesis of DHA, pDHA3 harboring pfaA, pfaB, pfaC, and pfaD from M. marina MP-1 and a fragment of pET21a::pfaE harboring pfaE from the same bacterium were combined (Table 1). Polymerase chain reaction (PCR) amplified a DNA fragment that included pfaE and a T7 RNA polymerase binding site using pET21a::pfaE as a template, with the forward primer (5’-TCAAGGGCATCGGTCGACATC-3’) and reverse primer (5’-CCGGATATAGGTCGACCTTTC-3’), in which SalI sites were introduced by base modification and are underlined. The PCR-amplified 2.8 kbp DNA fragment containing pfaE was digested with SalI and then purified by agarose gel electrophoresis. It was ligated into pDHA3, which had been digested with SalI, and the recombinant DNA was introduced into E. coli DH5α cells by heat shock. Colony direct PCR was used to confirm the presence of the insert by using primers designed against the inner sequence of the pfaE gene (Orikasa et al., 2006a): 5’-TGTTGTTCCTACCGCACCTT-3’ for the forward primer and 5’-GTTGGCCGATTCATTAATGC-3’ for the reverse primer. A positive clone was cultured to prepare the plasmid. Plasmid digested with SalI gave two bands of 25 kbp and 2.8 kbp on agarose gels. The plasmid was designated pDHA4.

This study used individual pfa genes for the biosynthesis of EPA or DHA. pfaB(DHA) gene of 2.5 kbp was excised from pDHA4 using restriction enzymes BamHI and SmaI. Treatment of pDHA4 with BamHI and SmaI provided four major DNA bands of 3.2 kbp, 5.6 kbp, 8.3 kbp, and 10.9 kbp on agarose gels. The band of 3.2 kbp, which was expected to contain the pfaB gene of 2.5 kbp, was extracted and purified, and then ligated into the pSTV28 plasmid, which had been digested with BamHI and SmaI. The plasmid was designated pSTV28::pfaB(DHA).

pUC18::pfaB(EPA) and pUC18::pfaD(EPA) were prepared from the pSTV28::pfaB(EPA) and pSTV28::pfaD(EPA) vectors, respectively. Plasmids pSTV28::pfaB(EPA) and pSTV28::pfaD(EPA)
(Orikasa, 2007; Yazawa et al., 1998) were digested with \textit{S}f\textit{b}I and \textit{E}coRI and with \textit{B}amHI and \textit{E}coRI, respectively. The required DNA fragments were separated on agarose gels, purified and ligated into the pUC18 plasmid vector (Takara Bio), which had been digested with \textit{S}f\textit{b}I and \textit{E}coRI to produce pUC18::\textit{pfaB}(EPA) and with \textit{B}amHI and \textit{E}coRI to produce pUC18::\textit{pfaD}(EPA). pUC18::\textit{pfaA}(EPA) and pUC18::\textit{pfaC}(EPA) have been previously described (Orikasa, 2007; Yazawa et al., 1998).

**Analysis of fatty acids**

Fatty acids were converted to methyl esters and then analyzed as described (Orikasa et al., 2006a). Identification of DHA and EPA was performed by comparing their retention time on gas–liquid chromatography with standards and by gas chromatography–mass spectrometry as described previously (Orikasa et al., 2006a; Orikasa, 2007).

**Results and discussion**

**Construction of a vector carrying the five \textit{pfa} genes for DHA biosynthesis (pDHA4) and its combined expression with individual \textit{pfa} genes for EPA**

The five \textit{pfa} genes—\textit{pfaA}, \textit{pfaB}, \textit{pfaC}, \textit{pfaD}, and \textit{pfaE}—derived from DHA-producing \textit{M. marina} MP-1 were designated \textit{pfaA}(DHA), \textit{pfaB}(DHA), \textit{pfaC}(DHA), \textit{pfaD}(DHA), and \textit{pfaE}(DHA), respectively, to differentiate them from the \textit{pfa} genes from EPA-producing \textit{S. pneumatophori} SCRC-2738: \textit{pfaA}(EPA), \textit{pfaB}(EPA), \textit{pfaC}(EPA), \textit{pfaD}(EPA), and \textit{pfaE}(EPA) (Table 1).

The pDHA4 vector was constructed by linking pDHA3 (pSTV29 carrying \textit{pfaA}, \textit{pfaB}, \textit{pfaC}, \textit{pfaD} for DHA biosynthesis and five other irrelevant open reading frames) with \textit{pfaE} derived from \textit{M. marina} MP-1 (Fig. 1). The successful construction of pDHA4 was confirmed by the production of DHA
in recombinant *E. coli* DH5α (Table 2). No EPA was detected. The level of DHA was approximately 4% of the total fatty acid content when the cells were grown at 15 °C for 96 h. Table 2 shows the results of the combined expression of pDHA4 and either *pfaA*(EPA), *pfaB*(EPA), *pfaC*(EPA), or *pfaD*(EPA) in *E. coli* DH5α. In any combination, DHA was produced. However, EPA was detected in the cells coexpressing pDHA4 and *pfaB*(EPA). These results suggest that EPA was synthesized by an enzyme complex encoded by *pfaA*(DHA), *pfaC*(DHA), *pfaD*(DHA), and *pfaE*(DHA) and *pfaB*(EPA), and that the final EPA production was determined by the *pfaB*(EPA) product in this enzyme complex. The reason for the relatively low level of DHA (less than 1% of total fatty acids) in only the pDHA4 and *pfaB*(EPA) combination (Table 2) is unknown. It is considered that, in addition to the Pfa enzyme complex containing PfaB(EPA), these recombinant cell should have the complex of PfaA, PfaB, PfaC, PfaD, and PfaE for DHA synthesis derived only from pDHA4. When the two types of *pfaB* gene products in the enzyme complexes are compared, PfaB(EPA) may be more efficacious in producing EPA, even in the Pfa enzyme complex comprising PfaA, PfaC, PfaD, and PfaE for DHA and PfaB(EPA), than PfaB(DHA) is in producing DHA in the Pfa enzyme complex for DHA. Another possibility is that there is competition for the precursors of EPA and DHA biosynthesis between the two Pfa enzyme complexes.

**Cloning of *pfaB*(DHA) and its combined expression with clustered *pfa* genes for EPA**

The *pfaB*(DHA) gene was excised from pDHA4 and integrated into pSTV28 (designated pSTV28::*pfaB*(DHA); Table 1). The *E. coli* recombinant cells carrying pSTV28::*pfaB*(DHA) and pEPAΔ6 produced EPA (9.2 ± 0.8%) and DHA (0.2 ± 0.1%) (Table 2), indicating that *pfaB*(DHA) has the potential to synthesize both DHA and EPA in combination with *pfaA*, *pfaC*, *pfaD*, and *pfaE* from *S. pneumoniae* SCRC-2738. These EPA and DHA contents increased to 13.8 ± 0.9% and 0.6 ± 0.1%, respectively, in cells grown with cerulenin at 5 μg mL⁻¹ (Table 2), which inhibits the de novo synthesis of fatty acids (Omura, 1976), but not that of EPA (Allen *et al.*, 1999) or DHA (Morita *et al.*, 2005).
DHA was detected with the coexpression of pEPAΔ1 and pSTV28::pfaB(DHA) and this vector combination produced EPA at approximately 13% of total fatty acids (Table 2). This content of EPA is almost the same as that of E. coli DH5α recombinant carrying a single pEPAΔ1 vector (Orikasa et al., 2004), suggesting that the expression of pSTV28::pfaB(DHA) has no effect of the formation of the Pfa enzyme complex for EPA in the E. coli DH5α recombinant carrying both pEPAΔ1 and pSTV28::pfaB(DHA).

**Detailed analysis of fatty acids in EPA-producing and DHA-producing bacteria**

*Shewanella pneumatophori* SCRC-2738 (Hirota et al., 2005) and *S. marinintestina* IK-1 (Satomi et al., 2003) have been reported to synthesize EPA (10%–20% of total fatty acids) but not DHA. In this study, too, only EPA was detected in these two species. Conversely, it has been reported that *M. marina* MP-1 produces DHA but no EPA (DeLong & Yayanos, 1986; Morita et al., 2005). However, reanalysis of its fatty acids showed that this bacterium has, in addition to DHA (8.1 ± 0.2% of total), very low EPA (0.5 ± 0.0%) at 15 °C, and the contents of both EPA and DHA increased to 1.0 ± 0.2% and 19.6 ± 0.8%, respectively, in cells grown with 0.5 μg mL⁻¹ cerulenin. These results suggest that pfaB(DHA) functions in the production of both DHA and EPA in bacteria that have inherent pfa genes for DHA synthesis. EPA was not detected in recombinant *E. coli* carrying either pDHA4 (Table 2) or pDHA3 and pfaE (Orikasa et al., 2006b), which would be caused by the formation of undetectable levels of EPA in these systems.

In the polyketide biosynthesis mode, a double bond is anaerobically introduced into the elongating fatty acid (Metz et al., 2001). The process requires three steps: condensation of the elongating fatty acid and the C2 donor (malonyl–ACP), introduction of a double bond by dehydration, and the subsequent geometric or positional isomerization of the double bond, although no direct experimental evidence of this has ever been reported. Ootaki et al. (2003) speculated that
3,6,9,12,15-octadecapentaenoic acid (18:5) is one of the intermediate fatty acids, at which point the pathway branches into EPA or DHA biosynthesis (Fig. 2). One cycle of C2 elongation of 18:5 without the introduction of further double bonds would allow the production of EPA, whereas two cycles of C2 elongation of 18:5 with the introduction of a single double bond would produce DHA. The KS domain of PfaB(DHA) may be specifically involved in the condensation step from C18 to C20 or C20 to C22 in DHA biosynthesis. The subsequent introduction of double bonds and the isomerization steps could be catalyzed by both PfaC(DHA) and PfaC(EPA) (Fig. 2). As indicated previously (Okuyama et al., 2007), a slight difference in the domain structures of PfaC(DHA) and PfaC(EPA) may alter their association with the KS domain of PfaB(DHA). In PfaC(EPA), all three dehydratase domains (HDs) are homologous to FabA, a 3-hydroxydecanoyl–ACP dehydratase, whereas PfaC(DHA) has two HDs similar to FabA and one domain similar to FabZ/FabA. FabZ is a 3-hydroxyacyl–ACP dehydratase (White et al., 2005). If the association between the KS domain of PfaB(DHA) and the FabZ/FabA domain of PfaC(DHA) predominates over its association with the corresponding FabA domain of PfaC(EPA), DHA would be preferentially produced as in the combination of pDHA4 and pUC18::pfaC(EPA). The KS domain structure of PfaB is conserved in Colwellia psychrerythraea 34H, which is expected to produce DHA, but not in any of the known PfaBs of EPA-producing bacteria (Okuyama et al., 2007). The quantities of DHA are limited in native DHA-producing bacteria (DeLong & Yayanos, 1986; Hamamoto et al., 1995; Watanabe et al., 1997) and recombinants carrying their genes, compared with the quantities of EPA produced by native EPA-producing bacteria and recombinants carrying their genes (Table 2) (Orikasa et al., 2004; Satomi et al., 2003; Orikasa et al., 2007), and this must be controlled by pfa gene products other than PfaB and PfaE. In the case of PfaE, pfaE genes from EPA-producing S. pneumatophori SCRC-2738 and DHA-producing M. marina MP-1 resulted in almost the same levels of EPA (10%–12% of total fatty acids) in combined expression in E. coli DH5α containing the pEPAΔ1,2,3 vector which included four pfaA–pfaD genes derived from S. pneumatophori.
SCRC-2738 (Nishida et al., 2006a; Orikasa et al., 2006a). Recently Jiang et al., (2008) showed that the total number of ACPs in PfaA controls the levels of polyunsaturated fatty acids.

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References


Figure legends

**Fig. 1.** Organization of the five *pfα* genes for biosynthesis of EPA of *S. pneumatophori* SCRC-2738 and pDHA4 for biosynthesis of DHA of *M. marina* MP-1. The functional domain structure of individual *pfα* genes based on analysis by Okuyama *et al.* (2007) is shown in different colors. The relative direction of *pfαE* in pDHA4 has not been determined. Blank arrows are open reading frames unnecessary for EPA or DHA biosynthesis. The structure of the vector is omitted.

**Fig. 2.** A possible pathway for the bacterial biosynthesis of EPA and DHA. All five *pfα* gene products for EPA [Pfa(EPA)] and those for DHA [Pfa(DHA)] are involved in the biosynthesis of EPA (closed arrow and box of solid line). The KS domain of PfaB(DHA) is not necessary for the production of EPA, but is specifically required for the biosynthesis of DHA (open arrow and box of broken line). Unsaturated fatty acids tentatively considered to be intermediates are abbreviated as 4,7,10,13–16:4 (hexadecatetraenoic acid), where the number before the colon is the number of carbon atoms and the number after the colon is the number of double bonds in the fatty acid molecule. Intermediates before 4,7,10,13–16:4 were omitted. This pathway is slightly modified from that presented previously (Ootaki *et al.*, 2003).
<table>
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<tr>
<th>Strain/plasmid/cosmid</th>
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<th>Source or reference</th>
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\(^{a}\) Takara Bio Inc., Tokyo, Japan.

\(^{b}\) Abbreviations of antibiotics: Km, kanamycin; Amp, ampicillin; and Cm, chloramphenicol.

\(^{c}\) Stratagene, La Jolla, CA, USA.
Table 2. Combined expression of pfa genes from *S. pneumatophori* SCRC-2738 and from *M. marina* MP-1 in *E. coli* DH5α

<table>
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<th>Vector carrying a pfa gene(s) from MP-1(^c)</th>
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<td>ND</td>
<td>2.1 ± 0.5</td>
<td>(3)</td>
</tr>
<tr>
<td>pUC18::pfaB(EPA)</td>
<td>pDHA4</td>
<td>ND</td>
<td>3.4 ± 0.8</td>
<td>(5)</td>
</tr>
<tr>
<td>pUC18::pfaC(EPA)</td>
<td>pDHA4</td>
<td>ND</td>
<td>5.0 ± 1.5</td>
<td>(3)</td>
</tr>
<tr>
<td>pUC18::pfaD(EPA)</td>
<td>pDHA4</td>
<td>ND</td>
<td>3.5 ± 0.4</td>
<td>(3)</td>
</tr>
<tr>
<td>pEPAΔ1</td>
<td>pSTV28::pfaB(DHA)</td>
<td>12.8 ± 0.2</td>
<td>ND(^f)</td>
<td>(3)</td>
</tr>
<tr>
<td>pEPAΔ6</td>
<td>pSTV28::pfaB(DHA)</td>
<td>9.2 ± 0.8</td>
<td>0.2 ± 0.1</td>
<td>(6)</td>
</tr>
<tr>
<td>pEPAΔ6</td>
<td>pSTV28::pfaB(DHA)</td>
<td>13.8 ± 0.9(^f)</td>
<td>0.6 ± 0.1(^f)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

\(^a\) *E. coli* DH5α cells carrying pDHA4 and any pUC18 vector were cultivated in LB medium containing chloramphenicol at 30 μg mL\(^{-1}\) and ampicillin at 50 μg mL\(^{-1}\) at 15°C for 72 h. Cells carrying the cosmid vector (pEPAΔ6 or pEPAΔ1) and pSTV28::pfaB(DHA) were cultivated in LB medium containing ampicillin at 50 μg mL\(^{-1}\) and chloramphenicol at 50 μg mL\(^{-1}\) at 15°C for 96 h. In the combination of pEPAΔ6 and pSTV28::pfaB(DHA), recombinants were cultivated in the presence and in the absence of cerulenin at 5 μg mL\(^{-1}\).

\(^b\) *S. pneumatophori* SCRC-2738

\(^c\) *M. marina* MP-1

\(^d\) Details for vectors, see Table 1.
ND, not detected.

These values are those from the recombinant cells cultivated in the presence of cerulenin.
Fig. 2.