



Title	pfaB products determine the molecular species produced in bacterial polyunsaturated fatty acid biosynthesis
Author(s)	Orikasa, Yoshitake; Tanaka, Mika; Sugihara, Shinji; Hori, Ryuji; Nishida, Takanori; Ueno, Akio; Morita, Naoki; Yano, Yutaka; Yamamoto, Kouhei; Shibahara, Akira; Hayashi, Hidenori; Yamada, Yohko; Yamada, Akiko; Yu, Reiko; Watanabe, Kazuo; Okuyama, Hidetoshi
Citation	FEMS Microbiology Letters, 295(2), 170-176 https://doi.org/10.1111/j.1574-6968.2009.01582.x
Issue Date	2009-06
Doc URL	http://hdl.handle.net/2115/43086
Rights	The definitive version is available at www.blackwell-synergy.com
Type	article (author version)
File Information	295-2_p170-176.pdf



[Instructions for use](#)

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

Yoshitake Oriyasa^{1†}, Mika Tanaka^{1†}, Shinji Sugihara¹, Ryuji Hori^{1,2}, Takanori Nishida^{1†}, Akio Ueno^{2†}, Naoki Morita³, Yutaka Yano⁴, Kouhei Yamamoto⁵, Akira Shibahara⁵, Hidenori Hayashi^{6,7}, Yohko Yamada⁸, Akiko Yamada^{9†}, Reiko Yu^{9†}, Kazuo Watanabe⁹, & Hidetoshi Okuyama^{1,2*}

¹ Faculty of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan.

² Faculty of Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan

³ Research Institute of Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology (AIST), Toyohira-ku, Sapporo 062-8517, Japan

⁴ National Research Institute of Fisheries Science, Fisheries Research Agency, Kanazawa-ku, Yokohama 236-8648, Japan

⁵ Department of Clinical Nutrition, Osaka Prefecture University, Habikino, Habikino 583-8555, Japan

⁶ Cell-free Science and Technology Research Center, Ehime University, Matsuyama, 790-8577, Japan

⁷ Satellite Venture Business Laboratory, Ehime University, Matsuyama 790-8577, Japan

⁸ Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Bunkyo-ku, Tokyo 112-8681, Japan

⁹ Sagami Chemical Research Center, Hayakawa, Ayase 252-1193, Japan

Correspondence: Hidetoshi Okuyama, Laboratory of Environmental Molecular Biology, Faculty of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan. Tel.: +81 11 706 4523; fax: +81 11 707 2347; E-mail: hoku@ees.hokudai.ac.jp

†For the present addresses of these authors, contact Hidetoshi Okuyama

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 & Yayanos, 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; Orikasa *et al.*, 2006a). 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 (pEPA Δ 1; see Table 1) and a mutant *pfa* gene construct encoding *pfaA*, *pfaC*, *pfaD*, and *pfaE* (pEPA Δ 6) for EPA biosynthesis.

Materials and methods

Bacterial cells and cultivation

Escherichia coli DH5 α 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 μ L) 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 pEPA Δ 6 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* DH5 α 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'-TGTTGTTTCTACCGCACCTT-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 *Bam*HI and *Sma*I. Treatment of pDHA4 with *Bam*HI and *Sma*I 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 *Bam*HI and *Sma*I. 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 *SfbI* and *EcoRI* and with *BamHI* and *EcoRI*, 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 *SfbI* and *EcoRI* to produce pUC18::*pfaB*(EPA) and with *BamHI* and *EcoRI* to produce pUC18::*pfaD*(EPA). pUC18::*pfaA*(EPA) and pUC18::*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 *pfa* genes for DHA biosynthesis (pDHA4) and its combined expression with individual *pfa* genes for EPA

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

The pDHA4 vector was constructed by linking pDHA3 (pSTV29 carrying *pfaA*, *pfaB*, *pfaC*, and *pfaD* for DHA biosynthesis and five other irrelevant open reading frames) with *pfaE* derived from *M. marina* MP-1 (Fig. 1). The successful construction of pDHA4 was confirmed by the production of DHA

in recombinant *E. coli* DH5 α (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* DH5 α . 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 pEPA Δ 6 produced EPA ($9.2 \pm 0.8\%$) and DHA ($0.2 \pm 0.1\%$) (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. pneumatophori* SCRC-2738. These EPA and DHA contents increased to $13.8 \pm 0.9\%$ and $0.6 \pm 0.1\%$, respectively, in cells grown with cerulenin at $5 \mu\text{g mL}^{-1}$ (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). No

DHA was detected with the coexpression of pEPA Δ 1 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* DH5 α recombinant carrying a single pEPA Δ 1 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* DH5 α recombinant carrying both pEPA Δ 1 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 ($8.1 \pm 0.2\%$ of total), very low EPA ($0.5 \pm 0.0\%$) at 15 °C, and the contents of both EPA and DHA increased to $1.0 \pm 0.2\%$ and $19.6 \pm 0.8\%$, respectively, in cells grown with $0.5 \mu\text{g mL}^{-1}$ 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* DH5 α containing the pEPA Δ 1,2,3 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.

Acknowledgement

The authors appreciate K. Fujimoto, C. Matsuda, S. Suehiro, and R. Takamura, undergraduate students of the Faculty of Science, Hokkaido University and M. Ootaki, a graduate student of the Graduate School of Environmental Earth Science, Hokkaido University, for their contribution in fatty acid analysis; and A. Mizutani of AIST for her assistance in nucleic acid sequencing. This work was partly supported by the National Institute of Polar Research to H. O. and by Grant-in-Aid for Scientific Research ((C) no. 18608003) from the Ministry of Education, Science, Sports, and Culture of Japan to H. H.

References

- Allen EE, Facciotti D & Bartlett DH (1999) Monounsaturated but not polyunsaturated fatty acids are required for growth of the deep-sea bacterium *Photobacterium profundum* SS9 at high pressure and low temperature. *Appl Environ Microbiol* **65**: 1710–1720.
- Bowman JP, Gosink JJ, McCammon SA, Lewis TE, Nichols DS, Nichols PD, Skerratt JH, Staley JT & McMeekin TA (1998) *Colwellia demingiae* sp. nov., *Colwellia hornerae* sp. nov., *Colwellia rossensis* sp. nov. and *Colwellia psychrotropica* sp. nov.: psychrophilic Antarctic species with the ability to synthesize docosaheptaenoic acid (22:6 omega 3). *Int J Syst Bacteriol* **48**: 1171–1180.
- DeLong EF & Yayanos AA (1986) Biochemical function and ecological significance of novel bacterial lipids in deep-sea procaryotes. *Appl Environ Microbiol* **51**: 730–737.

- Fang J, Chan O, Kato C, Sato T, Peeples T & Niggemeyer K (2003) Phospholipid FA of piezophilic bacteria from the deep sea. *Lipids* **38**: 885–887.
- Hamamoto T, Takada N, Kudo T & Horikoshi K (1995) Characteristic presence of polyunsaturated fatty acids in marine psychrophilic vibrios. *FEMS Microbiol Lett* **129**: 51–56.
- Hirota K, Nodasaka Y, Orikasa Y, Okuyama H & Yumoto I (2005) *Shewanella pneumatophori* sp. nov., eicosapentanoic-acid-producing marine bacterium isolated from pacific mackerel (*Pneumatophorus japonicus*) intestine. *Int J Syst Evol Microbiol* **55**: 2355–2359.
- Jiang H, Zirkle R, Metz JG, Braun L, Richter L, Van Lanen SG & Shen B (2008) The role of tandem acyl carrier protein domains in polyunsaturated fatty acids biosynthesis. *J Am Chem Soc* **130**: 6336–6337.
- Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V & Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* **293**: 290–293.
- Morita N, Nishida N, Tanaka T, Yano Y & Okuyama H (2005) Enhancement of polyunsaturated fatty acid production by cerulenin treatment in polyunsaturated fatty acid-producing bacteria. *Biotechnol Lett* **27**: 389–393.
- Nichols DS, Nichols PD, Russell NJ, Davies NW & McMeekin TA (1997) Polyunsaturated fatty acids in the psychrophilic bacterium *Shewanella gelidimarina* ACAM 456T: molecular species analysis of major phospholipids and biosynthesis of eicosapentaenoic acid. *Biochim Biophys Acta* **1347**: 164–176.
- Nishida T, Orikasa Y, Ito Y, Yu R, Yamada A, Watanabe K & Okuyama H (2006a) *Escherichia coli* engineered to produce eicosapentaenoic acid becomes resistant against oxidative damages. *FEBS Lett* **580**: 2731–2735.

- Nishida T, Orikasa Y, Watanabe K & Okuyama H (2006b) The cell membrane-shielding function of eicosapentaenoic acid for *Escherichia coli* against exogenously added hydrogen peroxide. *FEBS Lett* **580**: 6690–6694.
- Okuyama H, Orikasa Y, Nishida T & Morita N (2007) Bacterial genes responsible for the biosynthesis of eicosapentaenoic and docosahexaenoic acids and their heterologous expression. *Appl Environ Microbiol* **73**: 665–670.
- Okuyama H, Orikasa Y & Nishida N (2008) Significance of antioxidative function of eicosapentaenoic and docosahexaenoic acids in marine microorganisms. *Appl Environ Microbiol* **74**: 570–574.
- Omura S (1976) The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. *Bacteriol Rev* **40**: 681–697.
- Ootaki M, Morita N, Nishida T, Tanaka M, Hase H, Yano Y, Yamada A, Yu R, Watanabe K & Okuyama H (2003) Genes and pathways involved in biosynthesis of eicosapentaenoic and docosahexaenoic acids in bacteria. *Advanced Researches of Plant Lipids* (Murata N, Nishida I, Yamada M, Sekiya J, Okuyama H, & Wada H, eds), pp. 49–52. Kluwer Academic Publishers, Tokyo.
- Orikasa Y (2007) Molecular biological and biochemical studies on bacterial genes responsible for the biosynthesis of long chain n-3 polyunsaturated fatty acids. PhD Thesis, Hokkaido University, Sapporo, Japan.
- Orikasa Y, Ito Y, Nishida T, Watanabe K, Morita N, Ohwada T, Yumoto I & Okuyama H (2007) Enhanced heterologous production of eicosapentaenoic acid in *Escherichia coli* cells that coexpress eicosapentaenoic acid biosynthesis *pfa* genes and foreign DNA fragments including a high-performance catalase gene, *vktA*. *Biotechnol Lett* **29**: 803–809.
- Orikasa Y, Nishida T, Hase A, Watanabe K, Morita N & Okuyama H (2006a) A phosphopantetheinyl transferase gene essential for biosynthesis of n-3 polyunsaturated fatty acids from *Moritella marina* strain MP-1. *FEBS Lett* **580**: 4423–4429.

- Orikasa Y, Nishida T, Yamada A, Yu R, Watanabe K, Hase A, Morita N & Okuyama H (2006b) Recombinant production of docosahexaenoic acid in a polyketide biosynthesis mode in *Escherichia coli*. *Biotechnol Lett* **28**: 1841–1847.
- Orikasa Y, Yamada A, Yu R, Ito Y, Nishida T, Yumoto I, Watanabe K & Okuyama H (2004) Characterization of the eicosapentaenoic acid biosynthesis gene cluster from *Shewanella* sp. strain SCRC-2738. *Cell Mol Biol* **50**: 625–630.
- Satomi M, Oikawa H & Yano Y (2003) *Shewanella marinintestina* sp. nov., *Shewanella schlegeliana* sp. nov. and *Shewanella sairae* sp. nov., novel eicosapentaenoic-acid-producing marine bacteria isolated from sea-animal intestines. *Int J Syst Evol Microbiol* **53**: 491–499.
- Sugihara S, Orikasa Y & Okuyama H (2008) 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. *Biotechnol Lett* **30**: 411–414.
- Tanaka M, Ueno A, Kawasaki K, Yumoto I, Ohgiya S, Hoshino T, Ishizaki K, Okuyama H & Morita N (1999) Isolation of clustered genes that are notably homologous to the eicosapentaenoic acid biosynthesis gene cluster from the docosahexaenoic acid-producing bacterium *Vibrio marinus* strain MP-1. *Biotechnol Lett* **21**: 939–945.
- Watanabe K, Ishikawa C, Ohtsuka I, Kamata M, Tomita M, Yazawa K & Muramatsu H (1997) Lipid and fatty acid compositions of a novel docosahexaenoic acid-producing marine bacterium. *Lipids* **32**: 975–978.
- White SW, Zheng J, Zhang Y-M & Rock CO (2005) The structural biology of type II fatty acid synthesis. *Annu Rev Biochem* **74**: 791–831.
- Yano Y, Nakayama A, Ishihara K & Saito H (1998) Adaptive changes in membrane lipids of barophilic bacteria in response to changes in growth pressure. *Appl Environ Microbiol* **64**: 479–485.

Yano Y, Nakayama A, Saito H & Ishihara K (1994) Production of docosahexaenoic acid by marine bacteria isolated from deep sea fish. *Lipids* **29**: 527–528.

Yazawa K, Yamada A, Kondo K, & Kato S (1998) Process for producing icosapentaeoic acid by genetic recombination. European patent application. EP 0 913 473.

Figure legends

Fig. 1. Organization of the five *pfa* 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 *pfa* genes based on analysis by Okuyama *et al.* (2007) is shown in different colors. The relative direction of *pfaE* 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 *pfa* 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 1. Strains and vectors used in this study

Strain/plasmid/cosmid	Relevant characteristics	Source or reference
Strain		
<i>E. 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
<i>M. marina</i> MP-1	Wild type	ATTC 15381
<i>S. pneumatophori</i> SCRC-2738	Wild type	Hirota <i>et al.</i> , 2005
<i>S. marinintestina</i> IK-1	Wild type	Satomi <i>et al.</i> , 2003
Plasmids/Cosmids		
pET21a:: <i>pfaE</i>	pET21a carrying <i>pfaE</i> for DHA	Orikasa <i>et al.</i> , 2006a
pSTV29	Low-copy-number cloning vector, Cm ^r	Takara Bio
pDHA3	pSTV29 carrying <i>pfaA,B,C,D</i> for DHA	Orikasa <i>et al.</i> , 2006a
pDHA4	pSTV29 carrying <i>pfaA,B,C,D,E</i> for DHA	This study
pSTV28	Low-copy-number cloning vector, Cm ^r	Takara Bio
pSTV28:: <i>pfaB</i> (DHA)	pSTV28 carrying <i>pfaB</i> for DHA	This study
pSTV28:: <i>pfaB</i> (EPA)	pSTV28 carrying <i>pfaB</i> for EPA	Yazawa <i>et al.</i> , 1998
pSTV28:: <i>pfaD</i> (EPA)	pSTV28 carrying <i>pfaD</i> for EPA	This study
pUC18	High-copy-number expression vector, Amp ^r	Takara Bio
pUC18:: <i>pfaA</i> (EPA)	pUC18 carrying <i>pfaA</i> for EPA	Yazawa <i>et al.</i> , 1998
pUC18:: <i>pfaB</i> (EPA)	pUC18 carrying <i>pfaB</i> for EPA	This study

pUC18:: <i>pfaC</i> (EPA)	pUC18 carrying <i>pfaC</i> for EPA	Yazawa <i>et al.</i> , 1998
pUC18:: <i>pfaD</i> (EPA)	pUC18 carrying <i>pfaD</i> for EPA	This study
pWE15	Cosmid expression vector, Km ^r , Amp ^r	Stratagene ^c
pEPAΔ1	pWE15 carrying <i>pfaA,B,C,D,E</i> for EPA	Orikasa <i>et al.</i> , 2004
pEPAΔ6	pWE15 carrying <i>pfaA,C,D,E</i> for EPA	Orikasa <i>et al.</i> , 2006a; Yazawa <i>et al.</i> , 1998

^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* DH5 α

Combination ^a		EPA or DHA produced (% of total fatty acids)		
Vector carrying a <i>pfa</i> gene(s) from SCRC-2738 ^b	Vector carrying a <i>pfa</i> gene(s) from MP-1 ^c	EPA	DHA	(n =)
None	pDHA4 ^d	ND ^e	3.7 \pm 0.1	(5)
pUC18:: <i>pfaA</i> (EPA)	pDHA4	ND	2.1 \pm 0.5	(3)
pUC18:: <i>pfaB</i> (EPA)	pDHA4	3.4 \pm 0.8	0.8 \pm 0.2	(5)
pUC18:: <i>pfaC</i> (EPA)	pDHA4	ND	5.0 \pm 1.5	(3)
pUC18:: <i>pfaD</i> (EPA)	pDHA4	ND	3.5 \pm 0.4	(3)
pEPA Δ 1	pSTV28:: <i>pfaB</i> (DHA)	12.8 \pm 0.2	ND	(3)
pEPA Δ 6	pSTV28:: <i>pfaB</i> (DHA)	9.2 \pm 0.8	0.2 \pm 0.1	(6)
pEPA Δ 6	pSTV28:: <i>pfaB</i> (DHA)	13.8 \pm 0.9 ^f	0.6 \pm 0.1 ^f	(3)

^a *E. coli* DH5 α cells carrying pDHA4 and any pUC18 vector were cultivated in LB medium containing chloramphenicol at 30 $\mu\text{g mL}^{-1}$ and ampicillin at 50 $\mu\text{g mL}^{-1}$ at 15°C for 72 h. Cells carrying the cosmid vector (pEPA Δ 6 or pEPA Δ 1) and pSTV28::*pfaB*(DHA) were cultivated in LB medium containing ampicillin at 50 $\mu\text{g mL}^{-1}$ and chloramphenicol at 50 $\mu\text{g mL}^{-1}$ at 15°C for 96 h. In the combination of pEPA Δ 6 and pSTV28::*pfaB*(DHA), recombinants were cultivated in the presence and in the absence of cerulenin at 5 $\mu\text{g mL}^{-1}$.

^b *S. pneumatophori* SCRC-2738

^c *M. marina* MP-1

^d Details for vectors, see Table 1.

^e ND, not detected.

^f These values are those from the recombinant cells cultivated in the presence of cerulenin.

Fig. 1

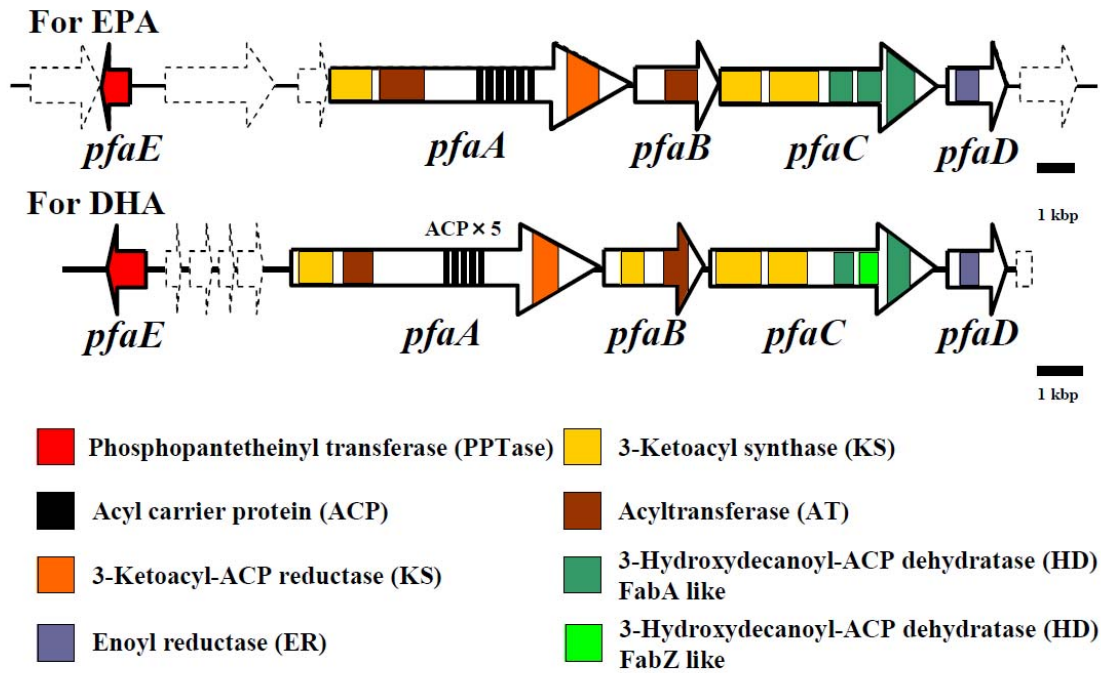


Fig. 2.

