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MINIREVIEW

Bacterial Genes Responsible for the Biosynthesis of Eicosapentaenoic and Docosahexaenoic Acids and Their Heterologous Expression▽

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Until the 1990s, it was thought that, with the exception of cyanobacteria, bacteria had no polyunsaturated fatty acids (PUFAs). This was probably because the bacterial species whose physiology, biochemistry, and molecular biology had been well studied until that time were mesophilic species such as *Escherichia coli*, which have no PUFAs. It has since been found that *n*-3 long-chain PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are preferentially distributed in psychrophilic bacteria that inhabit relatively unusual environments including low-temperature deep-sea environments and the intestines of sea fish (25, 31, 32). Although several PUFA-producing bacterial strains had been reported until the 1970s (15), the discovery of DHA and EPA in bacteria from deep-sea water and sediments and detailed analysis of them by DeLong and Yayanos in 1986 (2) resulted in the initiation of research into PUFA-producing bacteria.

It is interesting that the PUFAs detected in those bacteria were mostly EPA or DHA and not C_{18} PUFAs such as linoleic and linolenic acids, which are most common in animals, plants, fungi, and cyanobacteria. However, although EPA- and DHA-producing bacteria were discovered in the 1980s, they have not been given much attention. This can easily be imagined, as microorganisms (including eukaryotes such as microalgae) with PUFAs such as EPA and DHA are widespread in marine environments (24, 25, 28). It was also thought that bacterial as well as eukaryotic EPA and DHA were biosynthesized by a combination of elongation and oxygen-dependent desaturation of existing fatty acids. Therefore, the successful cloning of genes involved in the biosynthesis of EPA from *Shewanella* sp. strain SCRC-2738, isolated from marine fish intestines in 1996 (33), was very important and could be regarded as the first stage of research into such genes. This EPA-producing strain is presently identified as *Shewanella pneumatophori* SCRC-2738 (4). It was very surprising that the deduced proteins encoded by the EPA biosynthesis genes (*pfa* genes) (see below) included no conserved sequences from fatty acid desaturase genes (7, 33), although data on fatty acid desaturases were very limited in the early 1990s (11). Five deduced proteins involved in EPA biosynthesis had domains that were conserved in fatty acid synthetase and/or polyketide synthases (PKSs) (7). In addition, evidence has been presented to show that bacterial PUFAs can be synthesized under anaerobic conditions (7).

After the discovery of the EPA biosynthetic *pfa* genes, their homologues were cloned from various marine bacteria. The *pfa* genes of *Moritella marina* MP-1 were the first genes to be cloned from DHA-producing bacteria (27). Recent genome sequencing of various organisms, including bacteria, demonstrated that *pfa* genes are distributed abundantly in members of the bacterial genera *Shewanella* (29) and *Colwellia* (6) and that these strains have been isolated mainly from marine sources. There are some variations in the structures of *pfa* gene clusters, although they have a basic structure that is common among all types of clusters (see below). There is no doubt that all bacterial EPA and DHA are synthesized by the PKS system (7, 16).

Although the recombinant production of EPA in *E. coli* was achieved in 1996 using *pfa* genes from *S. pneumatophori* SCRC-2738 (33), no reports of the heterologous synthesis of DHA were found in the literature. This was because the *pfa* gene cluster in *M. marina* MP-1 lacked a gene corresponding to the *pfaE* gene of *S. pneumatophori* SCRC-2738. Recently, however, the *pfaE* gene was cloned from *M. marina* MP-1 (17), and recombinant DHA was produced (19). This might be called the second stage of research. Here, we review the bacterial genes responsible for the biosynthesis of EPA and DHA, covering such aspects as gene cloning, characterization of the structure of the genes and their domain structures, and recombinant production of EPA and DHA.

GENES RESPONSIBLE FOR THE BIOSYNTHESIS OF EPA AND DHA

The genes responsible for the biosynthesis of EPA were first cloned as a cosmid carrying a DNA fragment of approximately 38 kbp (33). This fragment carried at least 18 open reading frames (ORFs), of which a cluster of only five was necessary for the biosynthesis of EPA. These ORFs are now named *pfaA*, *pfaB*, *pfaC*, *pfaD*, and *pfaE* (13, 17) (Fig. 1). The clustered genes were designated the "EPA biosynthesis gene cluster." When various *E. coli* strains were transformed with the EPA biosynthesis gene cluster, they normally produced EPA at 1% to 5% of total fatty acids (20, 33).

Genes homologous to the *pfa* genes have been cloned from

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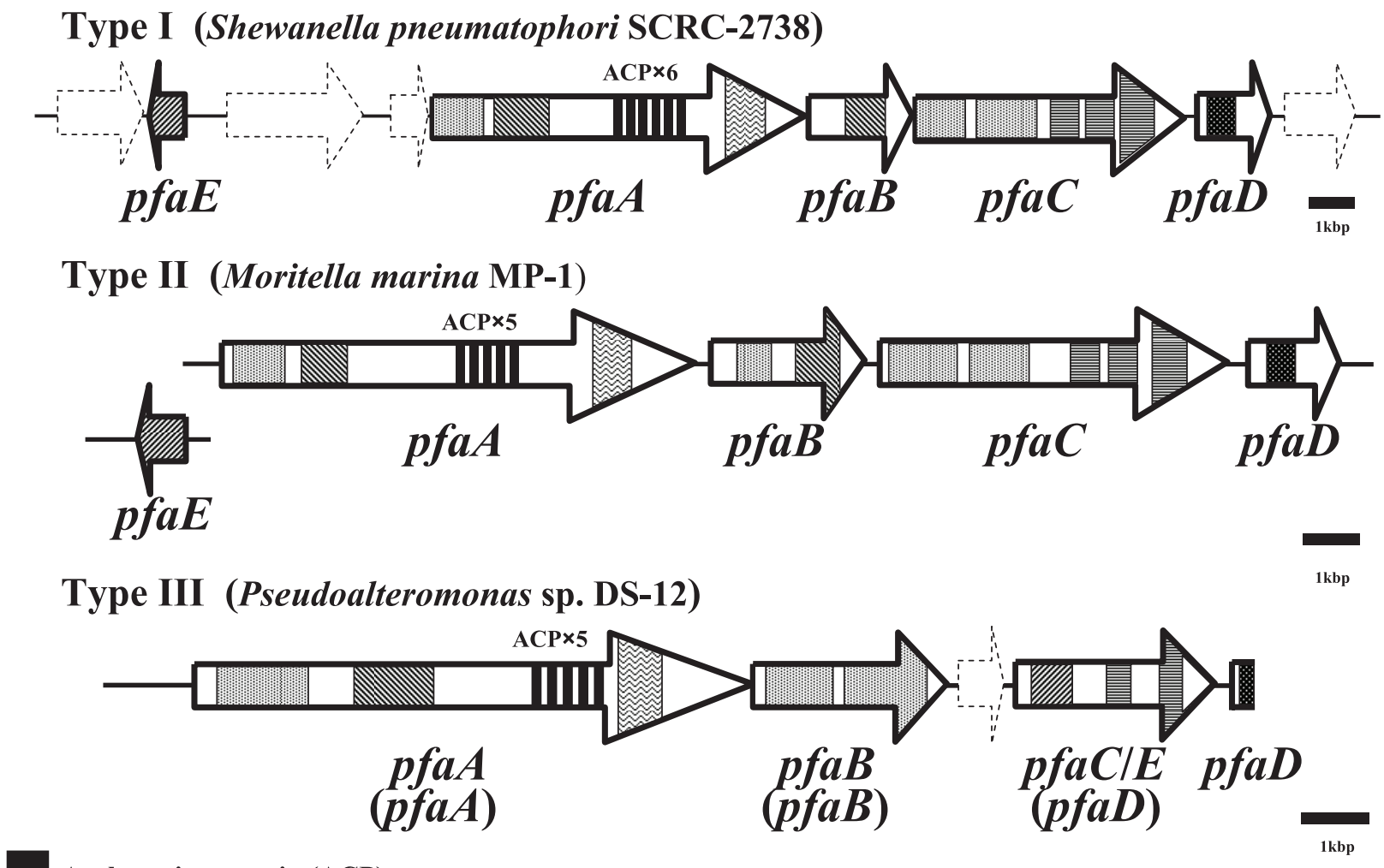

FIG. 1. Organization of genes responsible for bacterial EPA and DHA biosynthesis and domain structures of individual genes. The organization of gene clusters is divided into three types. Type I, which is represented by *Shewanella pneumatophori* SCRC-2738, is a gene cluster including all five *pfa* genes in a similar vicinity. Type II consists of a cluster of the four genes *pfaABCD*, with *pfaE* separate from the other genes. This type of cluster is represented by *Moriella marina* MP-1. The relative direction of *pfaE* has not been determined for this bacterium. In type III, *pfaE* is integrated into *pfaC/E*, and the cluster is considered to consist of four genes. In this review, the remnant sequence downstream of the *pfaC/E* gene was regarded as a partial sequence of *pfaD*. The original nomenclature (Dai and Zhang, unpublished) (GenBank accession no. ABF00130) is shown in parentheses. The third type of cluster has been reported for *Pseudoalteromonas* sp. strain DS-12 only.

TABLE 1. List of known bacterial *pfa* genes[a]

Organism	Product[b]	*pfa* genes in the cluster		No. of ACP repeats in PfaA	Domain(s) in PfaB	HD domains in PfaC or PfaC/E	*pfaE* (PfaE)		Recombinant synthesis of the product	Reference or source
		Type[c]	Cloning				Group[d]	Cloning		
S. pneumatophori SCRC-2738	EPA	I	Yes	6	AT	FabA-FabA-FabA	I	Yes	Yes	20
S. marinintestina IK-1	EPA	I	Yes	6	AT	FabA-FabA-FabA	I	Yes	Yes	Unpublished[e]
Shewanella sp. strain SC2A	EPA	—[f]	No	—	—	—	I	Yes	No	1
S. oneidensis MR-1	EPA	I	No	4	AT	FabA-FabA[g]	I	No	No	29
Shewanella sp. strain GA-22[h]	EPA[i]	—	No	—	—	—	I	—	—	3
P. profundum SS9	EPA	II	Yes	6	AT	FabA-FabA[g]	II	No	No	1, 30
Pseudoalteromonas sp. strain DS-12	(EPA)	III	Yes	5	KS, KS	FabA-FabA[j]	I[k]	Yes	No	GenBank accession no. ABF00130
M. marina MP-1	DHA	II	Yes	5	KS, AT	FabA-FabZ/FabA-FabA	I	Yes	Yes	17, 18
C. psychrerythreae 34H	(DHA)	I	No	6	KS, AT	FabA-FabA[g]	I	No	No	6

[a] Nucleic acid and deduced amino acid sequences were retrieved from databases (DDBJ/GenBank/EMBL) (http://www.ddbj.nig.ac.jp/Welcome-j.html). The name of each domain in individual *pfa* genes is described in the legend of Fig. 1.
[b] In cases of (EPA) and (DHA), the production of EPA or DHA is expected but not confirmed.
[c] Type I, II, and III *pfa* genes are defined in the text.
[d] Group I and II PPTases are defined in the text and reference 18.
[e] Unpublished result by N. Morita, Y. Yano, S. Ohgiya, and H. Okuyama.
[f] —, no information is available.
[g] Unannotated 900- to 1,000-bp sequences are present between the two FabA-like sequences.
[h] Only a 1,624-bp partial *pfaA* sequence has been deposited (GenBank accession no. AJ563807).
[i] Arachidonic acid (20:4*n*-6) and linoleic acid (18:2*n*-6) were also detected.
[j] Unannotated 360- to 450-bp sequences are present between the two FabA-like sequences.
[k] *pfaE* is included in *pfaC/E*.

various EPA- and DHA-producing bacteria. Allen and Bartlett (1) previously reported the finding of a cluster of EPA biosynthesis genes in *Photobacterium profundum* SS9. However, this cluster did not include *pfaE*, which encodes phosphopantetheinyl transferase (PPTase). A partial sequence corresponding to *pfaA* was cloned from EPA-producing *Shewanella* sp. strain GA-22 (3). Clustered genes homologous to those from *S. pneumatophori* SCRC-2738 were cloned from the DHA-producing deep-sea bacterial species *M. marina* MP-1 (27). This gene cluster also included only *pfaABCD* and lacked *pfaE*. Therefore, attempts at the recombinant production of DHA in *E. coli* or in other host organisms were unsuccessful until quite recently. The *pfa* genes from *M. marina* MP-1 constitute the only DHA biosynthesis gene cluster currently cloned.

Genome sequencing of various bacteria either known or expected to produce EPA or DHA demonstrates the abundance and wide distribution of such genes. Interestingly, bacteria that have genes homologous to *pfa* genes are found mostly in marine sources. In the genome of EPA-producing *Shewanella oneidensis* MR-1 (29), a cluster of *pfaABCDE* genes was found. *Colwellia psychrerythraea* 34H (6), which is expected (but not confirmed) to produce DHA, has a similar cluster of genes. All of the aforementioned *pfa* gene clusters contained *pfaE* as an ORF encoding a single protein either within the cluster or outside the cluster. The former and latter structures of *pfa* gene clusters are designated type I and type II *pfa* genes, respectively, in this review (Fig. 1). However, the structure of the type I *pfa* genes is unlikely to be essentially different from that of the type II *pfa* genes. This is known because even the gene cluster from *S. pneumatophori* SCRC-2738, which contains all five genes, has the sequential arrangement of *pfa* genes broken by the presence of two ORFs unrelated to EPA biosynthesis (Fig. 1). Unlike these structures, *Pseudoalteromonas* sp. strain DS-12 has a unique *pfa* gene cluster consisting of four ORFs (M. Dai and P. Zhang, unpublished data) (GenBank accession no. ABF00130). In this strain, PfaE is integrated into PfaC/E, and *pfaB* and *pfaC/E* genes are separated by one unrelated ORF (Fig. 1). This is designated the type III *pfa* gene. Gene sequences homologous to those of the *pfa* genes have also been found in eukaryotic marine microalgae producing EPA and DHA (7). Table 1 summarizes known bacterial genes that are homologous to the EPA biosynthesis genes *pfaABCDE* of *S. pneumatophori* SCRC-2738.

STRUCTURE OF INDIVIDUAL *pfa* GENES

It is evident that only five *pfaABCDE* genes are generally necessary for the biosynthesis of EPA and DHA. Although the basic structures of all *pfa* genes for EPA and DHA biosynthesis are very similar, the domain structures of some of the individual genes are slightly different (Fig. 1 and Table 1). In the type I and type II *pfa* gene clusters, *pfaA* is tentatively thought to encode a multifunction protein that includes domains for 3-ketoacyl synthase (KS), malonyl coenzyme A:acyl carrier protein (ACP) acyltransferase, normally five or six ACP repeats, and 3-ketoacyl–ACP reductase (KR). The *pfaC* gene encodes a protein with two KS repeats and two or three 3-hydroxydecanoyl–ACP dehydratases (HD). The second KS domain in PfaC of *S. pneumatophori* SCRC-2738, *P. profundum* SS9, and *M. marina* MP-1 was considered to be a chain length factor (1, 17, 19). In this study, however, both domains remained undifferentiated, as information about their precise function was not available. Previously, the number of HD domains in PfaC was recognized to be two (1, 7, 17); however, a very recent database search demonstrated that PfaC includes three sequential domains of HD based on *S. pneumatophori* SCRC-2738, *Shewanella marinintestina* IK-1, and *M. marina* MP-1 only (Fig. 1 and Table 1). Interestingly, in PfaC for EPA, all

three HD domains were homologous to FabA, with a high degree of similarity, while PfaC for DHA had two HD domains similar to that of FabA and one domain, in the center, similar to that of FabZ/FabA (Table 1). Genes *pfaB* and *pfaD* encode proteins with KS and acyltransferase domains and an enoyl reductase domain, respectively (7, 20). A KS domain is included only in PfaB for DHA derived from *M. marina* MP-1 (27) and *C. psychrerythraea* 34H (6). However, the KS domain of PfaB in *M. marina* MP-1 lacked an active-site sequence (1). The domain structure of the *pfa* genes of *Pseudoalteromonas* sp. strain DS-12 was quite different from that of the same genes in the other bacterial strains. PfaB was found to have two KS domains, and PfaC/E (which was registered as a product of the *pfaD* gene in the database) (Dai and Zhang, unpublished) has one PPTase domain and two HD domains (Fig. 1). At the downstream end of the *pfaC/E* gene was a 483-bp remnant DNA region (this was regarded as the *pfaD* gene in this review), which included a partial sequence similar to that of enoyl reductase.

Compared with PfaABCD, the domain structures of PfaE are well characterized. *pfaE* (PPTase gene) for the biosynthesis of EPA or DHA can be regarded as a member of a large gene family of Sfp-type PPTases based on the sizes of the deduced protein molecules and their domain structures (17). Based on their domain structures, Orikasa et al. (17, 18) divided all Sfp-type PPTases into two groups: PPTases responsible mainly for the biosynthesis of EPA or DHA (group I), characterized by P0, P1a, and P1b domains, and those responsible mainly for the synthesis of polyketides and nonribosomal peptides (group II), characterized by the domains 1A, P1a′, and P1b′, which correspond to the P0, P1a, and P1b domains of group I (18). Although the P2 and P3 domains are commonly conserved in the two groups, there is a higher degree of similarity in these domains within groups than between groups (18). The *pfaE* gene complementing the *pfaABCD* genes of *P. profundum* SS9 has not been cloned. However, genome sequencing of this bacterium (30) provided a candidate sequence (GenBank accession no. CAG23685) from the group II Sfp-type PPTase (PfaE). On the other hand, the Sfp-type PPTase of *Bacillus subtilis*, Sfp itself, which is involved in the biosynthesis of surfactin (a nonribosomal peptide) (12), belongs to group I (18).

FUNCTIONAL COMPATIBILITY OF INDIVIDUAL *pfa* GENES

The compatibility of *pfa* genes involved in the biosynthesis of PUFAs has been investigated mostly using the *pfa* genes from the EPA-producing *S. pneumatophori* strain SCRC-2738 and DHA-producing *M. marina* strain MP-1. The *pfaE* gene (pETSTV::*pfaE*) from *M. marina* MP-1 complemented the pDHA3 vector carrying *pfaABCD* genes from DHA-producing *M. marina* MP-1 (19) as well as the pEPAΔ1,2,3 vector carrying *pfaABCD* genes from EPA-producing *S. pneumatophori* SCRC-2738 (17). To examine the compatibility of *pfaE* from EPA-producing *S. pneumatophori* SCRC-2738 with pDHA3 from *M. marina* MP-1, an *E. coli* DH5α transformant that utilized pEPAΔ5 carrying *pfaBCDE* genes from *S. pneumatophori* SCRC-2738 and pDHA3 was produced (18). Both EPA and DHA were produced in this combination, suggesting that the *pfaE* gene in pEPAΔ 5 is involved in producing DHA (18); that is, the PPTase (PfaE) from *S. pneumatophori* SCRC-2738 was able to recognize the ACP repeats (substrate) integrated into the *pfaA* gene product of *M. marina* MP-1, and the *pfaA* product of the DHA biosynthesis gene cluster played a role in EPA biosynthesis. Orikasa et al. (18, 19) obtained pDHA2 in the course of preparing pDHA3. Although pDHA2 carried *pfaABCD* genes from *M. marina* MP-1, the combined expression of pDHA2 and *pfaE* produced neither EPA nor DHA, as the *pfaA* in pDHA2 had one fatal base pair replacement, inactivating the gene cluster. However, when pDHA2 was coexpressed with any of the three types of deletion clones of the pEPA clusters from *S. pneumatophori* SCRC-2738 (that is, the clones carrying *pfaA*, *pfaC*, *pfaD*, and *pfaE*; *pfaA*, *pfaB*, *pfaD*, and *pfaE*; or *pfaA*, *pfaB*, *pfaC*, and *pfaE*) in *E. coli*, all transformants produced both EPA and DHA. In contrast, neither EPA nor DHA was produced by a combination of pDHA2 and a deletion clone carrying *pfaB*, *pfaC*, *pfaD*, and *pfaE* (Y. Orikasa, A. Yamada, A. Yu, K. Watanabe, and H. Okuyama, unpublished data). All these results suggest that not only *pfaE* and *pfaA* but also *pfaB*, *pfaC*, and *pfaD* are functionally compatible in the biosynthesis of EPA and DHA. The recombinant production of EPA and trace levels of DHA in *E. coli* was confirmed by the use of *pfa* genes (pIK814) of *S. marinintestina* IK-1 and by a combination of these genes with pDHA2 from *M. marina* MP-1 (N. Morita, Y. Yano, S. Ohgiya, and H. Okuyama, unpublished data).

According to Allen and Bartlett (1), the *pfaABCD* gene cluster in *P. profundum* SS9 did not complement PPTase genes from *Shewanella* sp. strain SC2A and *B. subtilis*, both of which are classed into group I, in the production of EPA. No sequences similar to those PPTases, other than the deduced sequence reported under GenBank accession no. CAG23685, have been found in the genome (i.e., in either chromosome 1 or 2) of *P. profundum* SS9 (30). It would be interesting to examine the compatibility of the PPTase gene from *P. profundum* SS9 with the *pfaABCD* genes from this strain and those from other EPA- or DHA-producing bacteria such as *S. pneumatophori* SCRC-2738 and *M. marina* MP-1. The functional compatibility of each *pfa* gene is summarized in Table 2.

FUTURE PERSPECTIVES

The EPA and DHA biosynthesis gene clusters were initially cloned with the aim of expressing them in various host organisms such as cyanobacteria, yeast, and plants (27, 33) for the purpose of producing commercially important materials. However, only low levels of EPA were produced by the recombination of the genes in cyanobacteria (26, 34). The recombinant production of DHA in *E. coli* has been reported quite recently (18). Although fish oils are the most important source of EPA and DHA, the contamination of fish due to pollution, as well as unstable fish catches, has created a need for alternative ways to provide those PUFAs (21). The use of the PKS system to produce EPA or DHA in heterologous host organisms has some benefits, such as the need for lesser amounts of reducing equivalents such as NADPH (19, 23) and the simplicity that the PUFAs have in consisting solely of EPA or DHA. Since bacterial PKS systems involved in the production of EPA and DHA are generally less active at moderate temperatures (19,

TABLE 2. Recombinant production of EPA and/or DHA using *pfa* genes from *Shewanella pneumatophori* SCRC-2738, *Moritella marina* MP-1, and *Photobacterium profundum* SS9[a]

Source and combination of *pfa* genes of *S. pneumatophori* SCRC-2738	Source and combination of *pfa* genes		Production of EPA and/or DHA	Reference
	M. marina MP-1	*P. profundum* SS9		
pfaABCDE[b]			EPA	20
pfaABCD[c] plus *pfaE*[d]			EPA	20
pfaABCD[c]	*pfaE*[e]		EPA	17
	pfaABCD[f] plus *pfaE*[g]		DHA	19
pfaBCDE[h]	*pfaABCD*[f]		EPA and DHA	18
pfaE[d]	*pfaA*BCD*[i]		None	17
	*pfaA*BCD*[i] plus *pfaE*[g]		None	17
		pfaABCD[j] plus *pfaE*[k]	None	1

[a] This table is modified from data reported previously in reference 18.
[b] *pfa* genes are harbored in pEPAΔ1.
[c] *pfa* genes are harbored in pEPAΔ1,2,3.
[d] The *pfaE* gene is harbored in pSTV::*pfaE*.
[e] The *pfaE* gene is harbored in pETSTV::*pfaE*.
[f] *pfa* genes are harbored in pDHA3.
[g] The *pfaE* gene is harbored in pET21::*pfaE*.
[h] *pfa* genes are harbored pEPAΔ5.
[i] *pfa* genes are harbored in pDHA2, where *pfaA** is inactive.
[j] *pfa* genes are harbored in pFOS8E1.
[k] PPTase genes from *Bacillus subtilis* and *Shewanella* sp. strain SC2A were used.

20), their genetic modification and selection in host organisms should be considered.

The PKS systems would provide a useful tool for investigating the physiological roles of EPA and DHA and their biosynthetic mechanisms. EPA levels in recombinants can be changed at random using various vectors carrying the *pfa* gene(s) from *S. pneumatophori* SCRC-2738 (20). The antioxidative function of EPA was first observed using such a recombinant in *E. coli* (13, 14, 15). Similar systems could be constructed for DHA by isolating individual *pfa* genes involved in DHA production. A biosynthetic mechanism has been proposed for EPA (and probably DHA) production, which is similar to the anaerobic pathway of unsaturated fatty acid biosynthesis (7, 16). However, no direct evidence is available to support this proposed mechanism. To detect intermediates in the biosynthesis of EPA or DHA, PKS recombinant systems could be used. The heterologous production of DHA in *E. coli* that had been transformed with *pfa* genes from the marine *M. marina* MP-1 was more active at lowered growth temperatures (19). The finding coincided with the finding that this bacterium is psychrophilic and that it inherently formed more DHA at low temperatures (2). However, the effects of salinity of culture media and hydrostatic conditions on the recombinant production of PUFAs have not been elucidated. The transcriptional regulation of *pfaABCD* genes has been studied by targeting the *pfaABCD* genes of *P. profundum* SS9 (1). More information would be produced by using recombinant systems carrying various combinations of *pfa* genes. Normal concentrations (10 to 100 μM) of cerulenin, an inhibitor of de novo biosynthesis of fatty acids, enhanced the synthesis of EPA and DHA in *P. profundum* SS9, *S. marinintestina* IK-1, and *M. marina* MP-1 (1, 8). It has been demonstrated that *M. marina* MP-1 has a fatty acid biosynthetic (*fab*) gene cluster that takes part in the de novo synthesis of fatty acids with moderate chain lengths up to C_{18} (9, 10). The relationship between the PKS system and de novo fatty acid biosynthesis could be investigated using the PKS recombinant systems.

Menzella et al. (5) previously proposed combinatorial polyketide biosynthesis by the design and rearrangement of modular PKS genes. Type I PKS genes of 3 to 6 kbp are similar to the *pfa* genes. Some *pfa* genes, such as *pfaA* and *pfaC*, that are structurally similar to PKS genes might be used for the production of novel and commercially beneficial polyketides. If five or six repeats of ACP domains operate as a cluster to enhance the biosynthesis of PUFAs, as is the case in the biosynthesis of the polyketide antibiotic mupirocin in *Pseudomonas fluorescens*, in which two tandem repeats of carrier proteins are involved (22), the corresponding DNA region would become a useful tool for the enhanced production of various types of polyketides.

We were unable to identify the factor(s) that determines the final product in PKS systems that produce PUFAs. It is speculated that some cooperative interactions between domains of different Pfa proteins, rather than the activity of any single Pfa protein, might be involved in directing the final product in the system. To find an answer to this question would be the third stage in this research.

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ADDENDUM IN PROOF

A recent study (A. Hauvermale, J. Kuner, B. Rosenzweig, D. Guerra, S. Diltz, and J. M. Metz, Lipids **41:**739–347, 2006) reports that PPTase genes from *B. subtilis* and *Nostoc* sp. strain PCC7120 complemented *pfa* genes from *Schizochytrium* sp. in the *E. coli* recombinant system. In this review, those two PPTases can be categorized to group I Sfp-type PPTase, which is involved in the biosynthesis of EPA or DHA.

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