Subtle control of carbon chain length in polyunsaturated fatty acid synthases

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ABSTRACT

Some marine bacteria synthesize docosahexaenoic acid (DHA; C_{22}) and eicosapentaenoic acid (EPA; C_{20}) by enzyme complexes composed of four subunits (A to D). We recently revealed that β-ketoacyl synthase (KS_C)/chain length factor (CLF)-like domains in the "C" subunit of DHA synthase catalyzed the last elongation step (C_{20} to C_{22}) even though their amino acid sequences are very similar to those of EPA synthase. To investigate the amino acid residues controlling the product chain length, conserved residues in the KS_C/CLF-like domains in DHA synthase were replaced with corresponding EPA synthase residues. Among 12 mutants, two CLF-like domain-mutated genes completely lost DHA productivity and produced trace amounts of EPA when co-expressed with *dha-ABD* in *Escherichia coli*, whereas when co-expressed with *epa-ABD* they produced the same amounts of EPA as *epa-ABCD*. These results suggested that the product profiles were subtly controlled by several amino acid residues.

Polyunsaturated fatty acids (PUFAs, Figure 1) such as docosahexaenoic acid (DHA; C22:6 ω3), eicosapentaenoic acid (EPA; C20:5 ω3), and arachidonic acid (ARA; C20:4 ω6) are indispensable fatty acids for humans and fishes and fish oils are main supply sources. Because of increasing demand, however, other resources of PUFAs are required and practical fermentative processes have been developed. [1–3]

There are two distinct PUFA biosynthetic pathways. One is the aerobic desaturase/elongase pathway and the other is the anaerobic PUFA synthase pathway. In the aerobic pathway operating in plants, fungi, microalgae, and bacteria, desaturases and elongases catalyze individual desaturation and elongation steps from oleic acid (C18:1 ω9) to synthesize PUFAs. [3] In the anaerobic pathway occurring in eukaryotic microalgae and prokaryotic bacteria, PUFA synthases comprised of large enzyme complexes with various catalytic domains synthesize PUFAs as exemplified by microalgal DHA production (Figure S1). [4] PUFA synthase possesses acyltransferase (AT), multiple tandem acyl carrier protein (ACP), malonyl CoA transacylase (MAT), ketoacyl synthase (KS), ketoacyl reductase (KR), two types of dehydratase (polyketide synthase (PKS)-type DH and FabA-type DH), enoyl reductase (ER), and chain length factor (CLF)-like domains (Figure 1).

We previously revealed that PUFA productivities was dependent on the number of active ACPs. [5] We also revealed that two distinct dehydratases, DH_{PKS} and DH_{FabA}, controlled the formation

of the first *cis* double bonds in ARA and EPA biosynthesis. [6] We also showed that the last elongation step in EPA biosynthesis (C_{18} to C_{20}) was catalyzed by the KS_A domain of the "A" subunit in both EPA and DHA synthases. [7] In contrast, the last elongation step for DHA biosynthesis (C_{20} to C_{22}) was catalyzed by the KS_C/CLF-like domains of the "C" subunit in DHA synthase even though amino acid sequences of the KS_C/CLF-like domains of the "C" subunit in EPA and DHA synthases are very similar (Figure S2).

In this study, to determine the key amino acid residues controlling the product chain length, conserved amino acid residues in DHA synthase, which were suggested to face the catalytic cavities in the KS_C and CLF-like domains by docking simulations, were replaced with corresponding EPA synthase residues and the product profiles were investigated by heterologous expression in *E. coli*. Moreover, the quaternary structure of the subunits was examined with truncated KS_C and CLF-like domains.

RESULTS AND DISCUSSION

The amino acid sequences of the KS_C/CLF-like domains in DHA and EPA synthases are similar. (Figure S2). However, some amino acid residues differ and are specifically conserved in each type of enzyme. Among these, we searched for amino acid residues that might face the cavity with the catalytic Cys residue in the KS_C domain and control the product chain length by docking

67 simulations because we recently succeeded in converting microalgal DHA synthase into EPA
68 synthase by substitutions of three amino acid residues in the KSC/CLF-like domains, all of which
69 faced an estimated catalytic cavity [7]. Moreover, amino acid residues facing the cavity of the CLF-
70 like domain, which has similarity to the KSC domain but has no conserved active residues, were
71 also selected based on an estimated structure.

72 By homology modelling with the crystal structure of mammalian fatty acid synthase (PDB ID,
73 2VZ8; 25% identity with DHA synthase) [8], we estimated the structures of the KSC domain
74 (Figure S3) and CLF-like domain (Figure S4) in the DHA synthase of *Moritella marina* and
75 selected six amino acid residues in each domain (M229, Q239, N244, N245, H247, and K337 in
76 the KSC domain and E665, Y670, E674, F680, N701, and R705 in the CLF-like domain) (Figures
77 S2–S4). Then, we replaced the selected amino acids with the corresponding residues of the EPA
78 synthase of *Photobacterium profundum*.

79 The mutated *dha-C* genes were co-expressed with *dha-ABD* in *E. coli* and their PUFA profiles
80 were analyzed by GC-MS (Figures 2 and S5). Transformants harboring the mutated *dha-C* genes
81 Q239H, N244H, N245G, or H247S produced almost the same amount of DHA (1.4~2.7 mg L^{-1})
82 and their PUFA profiles were the same as that of the wild type enzyme. In contrast, transformants
83 with M229F, K337L, Y670A, E674D, F680M, or N701Q produced less DHA (0.4~0.8 mg L^{-1})
84 than the wild type enzyme. Moreover, transformants with E665A or R705K completely lost DHA

85 productivity and produced trace amounts of EPA (0.04 mg L^{-1}). These results suggested that some
86 specific amino acid residues in the CLF-like domain mainly control the carbon chain length.
87 We previously showed that transformants harboring *dha-ABD* and *epa-C* produced only EPA.
88 [7] However, the productivity was drastically decreased when compared with that of *dha-ABCD*.
89 This result suggested that a combination of co-expressing genes is important for PUFA productivity.
90 Therefore, we next expressed the abovementioned mutated genes with *epa-ABD*. Among the
91 mutated genes, transformants with Y670A or F680M produced almost the same amounts of DHA,
92 DPA, and EPA (Y670A; 0.7, 0.6, and 1.0 mg L^{-1}, F680M; 0.6, 0.3, and 0.5 mg L^{-1}). Moreover,
93 transformants harboring E665A or R705K produced the same amount of EPA (0.5~1.0 mg L^{-1}) as
94 *epa-ABCD* (0.6 mg L^{-1}) concomitant with DPA (0.4~0.8 mg L^{-1}) (Figure 3). The results showed
95 that the single mutation caused complete change of the function for specifically synthesizing EPA.
96 Human fatty acid synthase, which possesses similar domain structures to those of PUFA
97 synthases, forms a 540 kDa homodimer and the KS domains directly contact each other. [9]
98 Recently, a covalent AcpP–FabB complex, an acyl carrier protein appended to a ketosynthase
99 responsible for fatty acid biosynthesis in *E. coli*, was also predicted to form a dimer structure by
100 X-ray crystallography, NMR, and molecular dynamics simulations. [10] We therefore examined
101 whether the KS_C and CLF-like domains of DHA synthase form a dimer structure. The whole “C”
102 subunit is a large enzyme and is difficult to prepare as a recombinant enzyme. Therefore, we first

103 tried to prepare a truncated recombinant KS_C or CLF-like domain, or KS_C/CLF-like domain of
104 DHA and EPA synthases. We constructed several plasmids so that the truncated recombinant
105 enzymes were expressed with or without an *N*-terminal His-tag. Among these, the non-tagged and
106 His-tagged CLF-like domain, and His-tagged KS_C/CLF-like domain of EPA synthase were
107 expressed as soluble forms, although the His-tagged KS_C domain was mainly expressed as an
108 inclusion body (Figure S6). To examine the interaction between the KS_C and CLF-like domains,
109 the two domains were simultaneously co-expressed in *E. coli* and then purified with a Ni^{2+}-agarose
110 column. As shown in Figure 4, the non-tagged CLF-like domain was co-purified with the *N*-
111 terminal His-tagged KS_C domain in the similar manner as KS and CLF-like domains of type II
112 polyketide synthase for ishigamide biosynthesis [11], suggesting that the two domains interacted
113 with each other. The interaction was also examined by a gel filtration experiment with the purified
114 enzymes. Although no peaks in agreement with the monomer molecular weight of either protein
115 were detected, two peaks corresponding to a hexamer (324 kDa) and a dimer (129 Da) structure of
116 KS_C and CLF-like domains, respectively, were detected (Figure 4). After fractionation of the peaks,
117 they were confirmed to contain KS_C and CLF-like domains by SDS-PAGE (Figure 4). These results
118 showed that the "C" subunit containing KS_C and CLF-like domains perhaps forms a multimer
119 structure. Then, we tried to carry out the same experiments with the purified His-tagged CLF-like
120 domain and His-tagged KS_C/CLF domain (Figure S6). However, these recombinants were very

121 unstable and easily inactivated by aggregation.

122 In conclusion, we identified several key amino acid residues in the CLF-like domain of DHA
123 synthase for chain length control of the product. In particular, mutant genes carrying E665A or
124 R705K, which were constructed by replacing the amino acid residue with the corresponding residue
125 of EPA synthase, lost DHA productivity when they were heterologously expressed with *dha-ABD*
126 in *E. coli*. In contrast, transformants harboring the mutated genes and *epa-ABD* produced the same
127 amount of EPA as those carrying *epa-ABCD*. Moreover, truncated enzymes containing CLF-like
128 and KS_C domains were shown to form a hetero multimer. Together, these results suggest that the
129 product profiles are subtly controlled by some amino acid residues in the CLF-like domains.

130

131 **METHODS**

132 **General**. All chemicals, primers, and enzymes were obtained from the same suppliers as described
133 previously. [6]. PUFA production was analyzed by GC/MS as the same methods. [6]

134

135 **Bacterial strains, media, and plasmids.** *Escherichia coli* XL1-Blue (Nippon Gene Co. Ltd.,
136 Tokyo, Japan) was used for construction of plasmids (Supporting Information). An *E. coli* mutant
137 defective in β-oxidation, BLR(DE3)Δ*fadE*, [5] was utilized for the heterologous PUFA production.
138 *E. coli* BL21(DE3) (Nippon Gene Co. Ltd.) was used to prepare recombinant enzymes. LB broth

139 medium (Sigma-Aldrich Japan) and terrific broth (TB) medium (Becton, Dickinson and Company,
140 NJ, USA) were used for cultivation. If necessary, 1.5% agar was added into the media. Ampicillin
141 (Ap), chloramphenicol (Cm), kanamycin (Km), and streptomycin (Sm) were added to the media at
142 concentrations of 100, 30, 25, and 20 μg ml^{-1}, respectively, when needed.
143
144 **Preparation of recombinant enzymes and gel filtration**. To prepare truncated recombinant
145 enzymes, *Escherichia coli* BL21(DE3) harboring the recombinant expression plasmid pET-*epa*-
146 *C*_KS_C (His-tagged KS_C domain), pET-*epa*-*C*_CLF (His-tagged CLF-like domain), pCDF-*epa*-
147 *C*_CLF (non-tagged CLF-like domain), or pET-*epa*-*C*_KS_C-CLF (His-tagged KS_C/CLF-like
148 domain) [7] was used. For analysis of interaction between the KS_C domain and CLF-like domain,
149 *E. coli* was transformed with pET-*epa*-*C*_KS_C and pCDF-*epa*-*C*_CLF. The transformant was
150 cultivated at 37 °C in LB broth medium including Km for 16 h and was inoculated into 100 ml LB
151 medium containing Km. Recombinant enzymes were prepared as described previously. [6] Enzyme
152 purities and concentration were analyzed by SDS-PAGE on 8–12% gels and determined by the
153 Bradford method using bovine serum albumin as a standard. The purified enzymes were subjected
154 to gel filtration with a HiLoad 16/600 Superdex 200 pg column (GE Healthcare UK Ltd., Little
155 Chalfont, Buckinghamshire, England) connected to an ÄKTAexplorer 10S system (GE Healthcare).
156 The column had been equilibrated with phosphate buffer (50 mM sodium phosphate, 150 mM

NaCl; pH 7.2) and the proteins were eluted at flow rate of 1 mL min^{-1}. Ferritin (440,000 Da), aldolase (158,000 Da), conalbumin (75,000 Da), and ovalbumin (44,000 Da) were used as standards for calculation of apparent molecular mass.

Structure modelling. The 3D structures of the KS_C and CLF-like domains in subunit "C" were obtained by homology modelling using the Phyre2 server with the structure of mammalian fatty acid synthase (PDB ID, 2VZ8).

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SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website at DOI: xxx.

Detailed experimental procedures, Supporting Information Figures S1–S6, Supporting Information Table S1 (PDF)

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213

214 **CONFLICT OF INTEREST**

215 The authors declare no conflict of interest.

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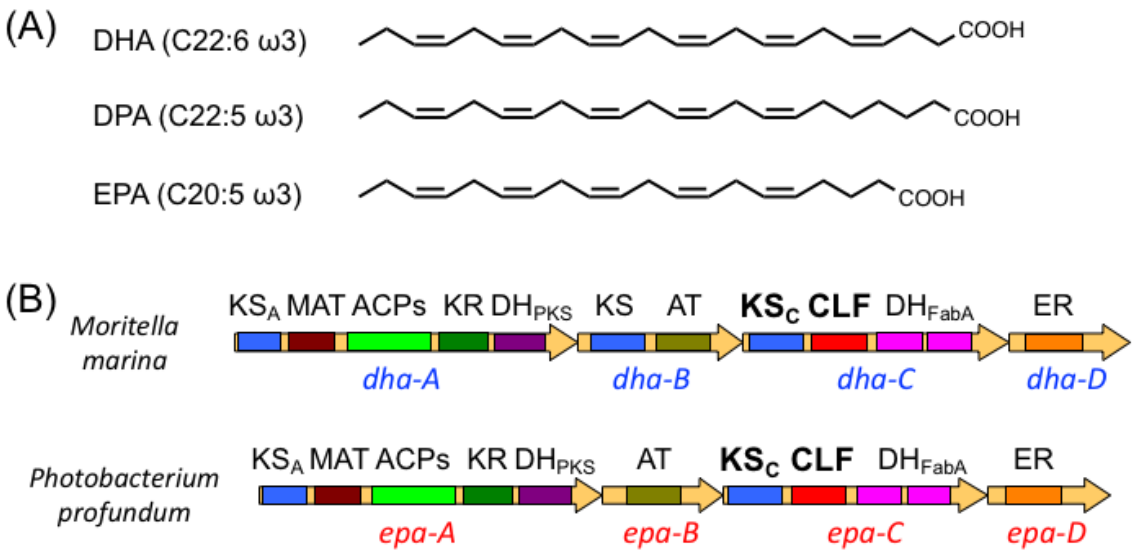


Figure 1. (A) Chemical structures of DHA, DPA, and EPA. (B) Domain organizations of bacterial PUFA synthases. KS: β-ketoacyl synthase, MAT: malonyl CoA transacylase, ACP: acyl carrier protein, KR: β-ketoacyl reductase, DH_{PKS}: polyketide synthase (PKS)-type dehydratase, AT: acyltrasferase, CLF: chain length factor-like domain, DH_{FabA}: FabA-type dehydratase, ER: enoyl reductase.

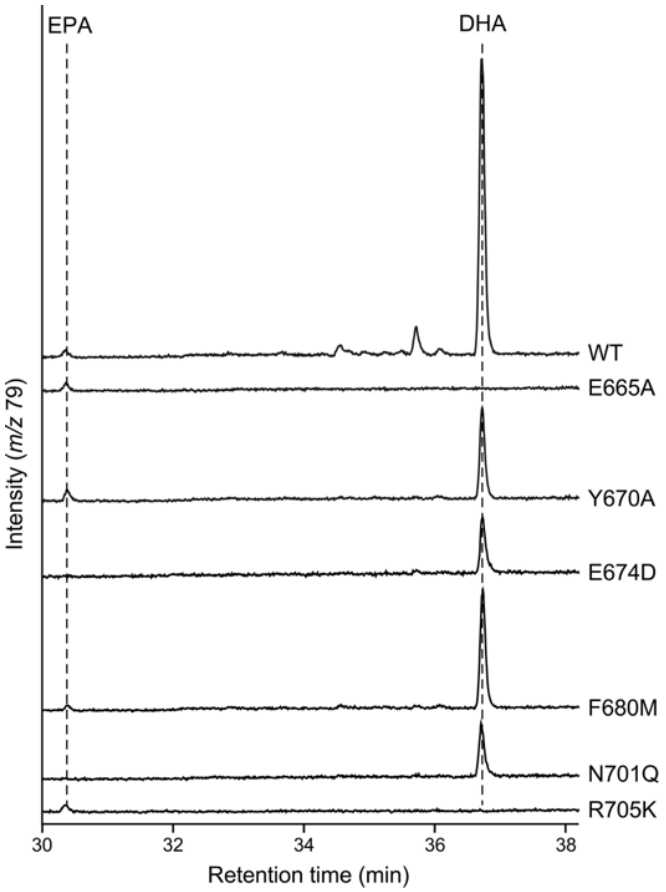


Figure 2. GC-MS analysis (traced at *m/z* 79) of products produced by *E. coli* expressing Dha-ABD and CLF-like domain mutants of Dha-C. The traces show the wild-type (WT) and E665A, Y670A, E674D, F680M, N701Q, and R705K mutants (from top to bottom).

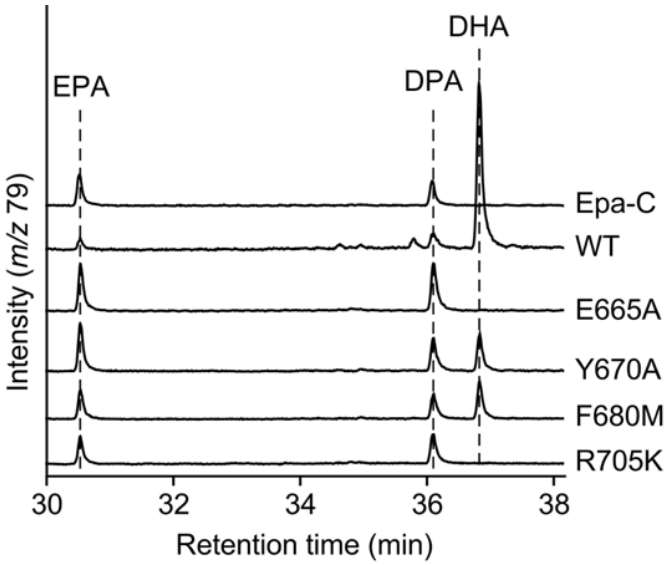
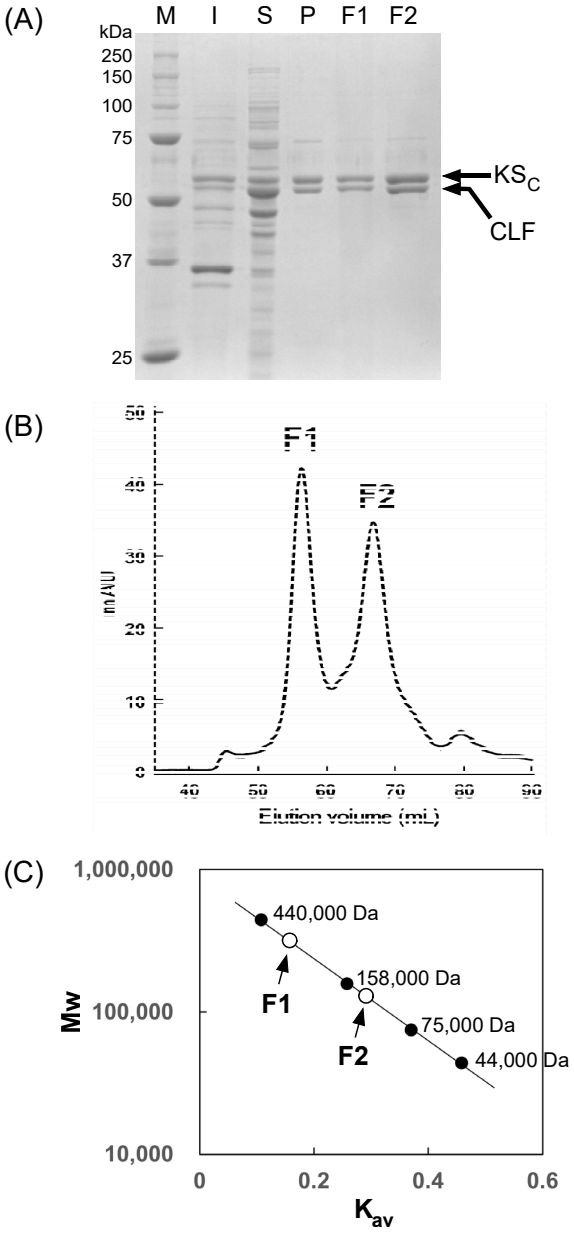


Figure 3. GC-MS analysis (traced at *m/z* 79) of products produced by *E. coli* expressing Epa-ABD and Epa-C or CLF-like domain mutants of Dha-C. The traces show Epa-C, Dha-C wild-type (WT), E665A, Y670A, F680M, and R705K mutants of Dha-C (from top to bottom).



240

241

242 **Figure 4.** Analysis of interaction between truncated KS_C and CLF-like domains of EPA synthase.

243 (A) SDS-PAGE analysis of co-expressed His-tagged KS_C and non-tagged CLF-like domains (58.6

244 and 53.6 kDa) in *E. coli*. I, insoluble fraction; S, soluble fraction; P, purified fraction obtained by

245 Ni^{2+}-agarose chromatography; F1 and F2, F1 and F2 fractions shown in (B). (B) Gel filtration

246 chromatography (traced at 280 nm) of the co-purified His-tagged KS_C and non-tagged CLF-like
247 domains. The two major fractions are shown as F1 (K_{av} = 0.155) and F2 (K_{av} = 0.295). (C)
248 Calculation of molecular weights of proteins involved in the F1 and F2 fractions (open circles)
249 with standards; ferritin (440,000 Da), aldolase (158,000 Da), conalbumin (75,000 Da), and
250 ovalbumin (44,000 Da) (closed circles).