The role of tandem acyl carrier protein domains in polyunsaturated fatty acid biosynthesis

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Supporting Information

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Materials and Methods

Strains and cosmids. *Shewanella japonica* PUFA synthase system[1a] and *Moritella marina* PUFA synthase system[1b] were used in this work. A 39,669-bp fragment encoding the *S. japonica* PUFA synthase was cloned into cosmid SuperCos 1 (Stratagene, La Jolla, CA) to construct 3F3.[1a]

Construction of *pfaA-ACP* expression vectors and overproduction of PfaA-ACPs in *E. coli.* Six *pfaA-ACP* genes were amplified by PCR from 3F3 using the following primers: for *ACP1* (forward) 5′-G CGG GAT CCA ACA GCC CTG AGC TCA CAA AA-3′ /(reverse) 5′-CG CGC GGC CGC TTA GCC TGC GGC CGG TAG TTT A-3′; for *ACP2* (forward) 5′-G CGG GAT CCA TCA GGT CTT AGC GCA GAA AC-3′ /(reverse)5′-CG CGC GGC CGC TTA GCC TGC GGC GGG TAG TTT A-3′; for *ACP3* (forward) 5′-G CGG GAT CCA TCT GGC CTT AGC GCT GAA AC-3′ /(reverse)5′-CG CGC GGC CGC TTA ACC AGC AGC GGG TAG CTT A-3′; for *ACP4* (forward) 5′-G CGG GAT CCA TCT GGT CTT AGC GCA GAA AC-3′ /(reverse) 5′-CG CGC GGC CGC TTA GCC TGC GGC GGG TAG TTT A-3′; for *ACP5* (forward) 5′-G CGG GAT CCA TCA GGT TTA AGT GCG GAA CA-3′ /(reverse) 5′-CG CGC GGC CGC TTA GCC TGC GGC GGG TAG TTT A-3′; and for *ACP6* (forward) 5′-G CGG GAT CCA ACA GCC CTG AGC GCT GAG CA-3′ /(reverse) 5′-CG CGC GGC CGC TTA GCC TGC GGC TGG CAG TTT A-3′. The PCR products were cloned as *Bam*HI-*Not*I fragment into pETDuet-1 to obtain expression vectors pETACP1a ~ pETACP6a (Table S1) and sequenced to confirm PCR fidelity. The *pfaA-ACP1* gene was also amplified by PCR from 3F3 using 5′-AGC CAT ATG ACA GCC CTG AGC TCA CAA-3′ as a forward primer and 5′-GGC CTC GAG TTA TGC ATT TGC AGT GTC GCT-3′ as a reverse primer. The resultant PCR product was cloned as *Nde*I-*Xho*I fragment into pET28a to obtain expression vectors pETACP1 (Table S1) and sequenced to confirm PCR fidelity. *E. coli* BL21(DE3) containing the expression vectors were grown at 37 ºC until the OD_{600} reached 0.5. IPTG (1 mM) was then added, and incubation continued at 37 ºC for 4 h. The produced N-His_6 tagged ACP proteins were purified by Ni-NTA agarose (QIAgen, Valencia, CA).

In vitro 4′-phosphopantetheinylation of PfaA-ACPs from apo- into holo-form catalyzed by Svp PPTase. In vitro 4′-phosphopantetheinylation of PfaA-ACPs from apo- into holo-form was performed using Svp PPTase.[2] A typical reaction of 100 μL, containing 100 μM ACP, 500 μM CoA, 5 μM Svp PPTase, 12.5 mM $MgCl_2$, 2.5 mM DTT in 100 mM Tris·HCl, pH 7.5, was incubated at 25 ºC for 30 min. The conversion of apo- to holo-ACP was analyzed by HPLC and ESI-MS analyses as described below.

In vivo 4′-phosphopantetheinylation of PfaA-ACPs from apo- into holo-form by co-expression of *pfaA-ACPs* with *pfaE*. The *pfaE* gene was amplified by PCR from 3F3 using the following primers: (forward) 5′-CAA GCG CAT ATG TCT TAT TGC TAT TAT AAA-3′ /(reverse) 5′-AGA CTC GAG TCA GTT GGT TTT TAT GAA CAT TT-3′. The PCR product was cloned as *Nde*I-*Xho*I fragment into the same sites of pETACP1a ~ pETACP6a to obtain co-expression vectors pETACP1a+E ~ pETACP6a+E (Table S1). Expression of the ACP genes and purification of the ACPs were performed identical to as above.

HPLC and ESI-MS analyses of apo- and holo-forms of PfaA-ACPs. HPLC analysis of purified ACP proteins was carried out on a Jupiter C-18 column (5 μm, 300 Å, 250 × 4.6 mm, Phenomenex, Belmont, CA), eluted with a gradient from 15% to 90% CH_3CN in 0.1% TFA-H_2O in 30 min at a flow rate of 1.0 mL/min, and UV detection was at 220 nm. Individual protein peaks were collected and lyophilized for ESI-MS analyses. The latter was performed on an Agilent (Palo Alto, CA) 1000 HPLC-MSD SL instrument.

Construction of *S. japonica* PUFA synthases expression constructs and production of EPA/DPA in *E. coli.* The genes responsible for EPA and DPA biosynthesis from *S. japonica* have been previously cloned, sequenced and analyzed.[1a] *S. japonica* genes *pfaB* and *pfaD* were amplified by PCR and cloned as *Nco*I-*Not*I and *Nde*I-*Xho*I fragments respectively into multiple cloning site 1 (MCS1) and multiple cloning site 2 (MCS2) of pACYCDuet-1 to create expression plasmid pREZ65. The 5′ and 3′ ends of *pfaC* were PCR amplified and cloned with central portion of *pfaC* as an *Avr*II fragment and the entire *pfaC* gene cloned as an *Nde*I-*Xho*I fragment into pCOLADuet-1 to create the expression plasmid pREZ71. The *pfaE* gene was amplified by PCR and cloned as an *Nde*I-*Xho*I fragment into MCS2 of pETDuet-1. The *pfaA* gene was subsequently cloned into the construct containing *pfaE* by PCR amplification the 5′ and 3′ ends of *pfaA* and cloning the central portion of the gene as an *Eco*RV-*Pfl*MI fragment. The entire *pfaA* was then cloned as a *Bbs*I (*Nco*I)-*Not*I fragment into MCS1 to generate the expression plasmid pREZ67. *E. coli* strain BLR(DE3) was co-transformed with the three expression plasmids pREZ65, pREZ71 and pREZ67 containing *pfaB* and *pfaD*; *pfaC*; and *pfaE* and *pfaA*, respectively. Cultures were grown in 25 mL of LB at 20 ºC until the OD_{600} reached 0.5. IPTG (1 mM) was then added, and incubation continued at 20 ºC for 12 h. The cells were then collected by centrifugation for PUFA analysis.

GC-MS analysis of PUFA production. PUFA production was monitored by GC-FID-MS analysis of corresponding fatty acyl methyl esters (FAMEs). To the cells, 2 mL of 1.5 M anhydrous HCl in methanol and 1 mL toluene were added. The resulting sample was then stirred at 100 °C for 2 h. After cooling to room temperature, the organic layer was washed with 1 mL of saturated brine, and removed by vacuum. The resulting FAMEs were identified and quantified by comparing the GC-FID-MS peaks with authentic FAME standards.

Construction of PfaA-ACP point-mutants. First, a pair of pET-linker and a pair of pUC-linker were inserted into the *Bgl*II-*Xho*I fragment of pET28a and *Eco*RI-*Hin*dIII fragment of pUC18, generating pETm and pUCm, respectively (Figure S3). The MCSs of pETm and pUCm were modified to contain all needed restriction endonuclease sites. Second, each ACP gene was cloned from pREZ67 to pETm or pUCm via several cycles of cloning steps. Each ACP point-mutant gene was obtained by mutagenesis PCR using primers listed in Table S5. Third, each ACP-point-mutant gene was cloned into pREZ67 via several cycles of reverse cloning steps to obtain 16 pREZ67 derivate expression plasmids (Table S1).

For pREZ67-1m: The 2.3-kb *Kpn*I-*Bgl*II fragment of pREZ67 was cloned into the identical sites of pETm to generate plasmid pACP1-5. The 1.1-kb *Kpn*I-*Eag*I fragment of pACP1-5 was cloned into the identical sites of pETm to generate plasmid pACP1. Reaction was performed using pACP1 as template with the ACP1mu5 and ACP1mu3 as primers. Successful reaction mixtures consisted of 20 ng of template plasmid, 300 nM each primer, 500 μM dNTPs, 2.5 units of

platinum *pfx* DNA polymerase (Invitrogen, Carlsbad, CA), 1 mM $MgSO_4$, and 1 × pfx amplification buffer (Invitrogen, Carlsbad, CA). The PCR program was as follows: initial denaturing at 94 °C for 2 min, followed by 18 cycles at 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 7 min, and completed by additional 10 min at 68 °C. Upon completion, 10 units of *Dpn*I was added directly to the PCR mixture and digested at 37 °C for 1 h. The 10 μL of mixture was directly transformed into DH5α. The resulting mutant construct was confirmed by sequencing to give plasmid pACP1m. The 1.1-kb *Kpn*I-*Eag*I fragment of pACP1m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-1m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-1m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-1m.

For pREZ67-2m: The 1.2-kb *Eag*I-*Bgl*II fragment of pACP1-5 was cloned into the identical sites of pETm to generate plasmid pACP2-5. The 1.0-kb *Eag*I-*Hin*dIII fragment of pACP2-5 was cloned into the identical sites of pUCm to generate plasmid pUCACP2-4. The 0.3-kb *Eag*I-*Dra*III fragment of pUCACP2-4 was cloned into the identical sites of pUCm to generate plasmid pUCACP2. Reaction was performed using pUCACP2 as template with the ACP1mu5 and ACP1mu3 as primers. The mutagenesis PCR, *Dpn*I digestion, and transformation of DH5α were performed identical to as above. The resulting mutant constructed was confirmed by sequencing to give plasmid pUCACP2m. The 0.3-kb *Eag*I-*Dra*III fragment of pUCACP2m was cloned into the identical sites of pUCACP2-4 to generate plasmid pUCACP2-4-2m. The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-2m was cloned into the identical sites of pACP2-5 to generate plasmid pACP2-5-2m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-2m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-2m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-2m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-2m.

For pREZ67-3m: The 0.7-kb *Dra*III-*Hin*dIII fragment of pUCACP2-4 was cloned into the identical sites of pUCm to generate plasmid pUCACP3-4. Reaction was performed using pUCACP3-4 as template with the ACP1mu5 and ACP1mu3 as primers. The mutagenesis PCR, *Dpn*I digestion, and transformation of DH5α were performed identical to as above. The resulting mutants constructed were selected by sequencing to give plasmid pUCACP3-4-34m. The 0.3-kb *Dra*III-*Blp*I fragment of pUCACP3-4-34m was cloned into the identical sites of pUCACP3-4 to generate plasmid pUCACP3-4-3m. The 0.7-kb *Dra*III-*Hin*dIII fragment of pUCACP3-4-3m was cloned into the identical sites of pUCACP2-4 to generate plasmid pUCACP2-4-3m. The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-3m was cloned into the identical sites of pACP2-5 to generate plasmid pACP2-5-3m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-3m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-3m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-3m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-3m.

For pREZ67-4m: The 0.4-kb *Blp*I-*Hin*dIII fragment of pUCACP3-4-34m was cloned into the identical sites of pUCACP3-4 to generate plasmid pUCACP3-4-4m. The 0.7-kb *Dra*III-*Hin*dIII fragment of pUCACP3-4-4m was cloned into the identical sites of pUCACP2-4 to generate plasmid pUCACP2-4-4m. The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-4m was cloned into the identical sites of pACP2-5 to generate plasmid pACP2-5-4m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-4m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-4m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-4m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-4m.

For pREZ67-5m: The 0.2-kb *Hin*dIII-*Bgl*II fragment of pACP2-5 was cloned into the identical sites of pETm to generate plasmid pACP5. Reaction was performed using pACP5 as template with the ACP5mu5 and ACP5mu3 as primers. The mutagenesis PCR, *Dpn*I digestion, and transformation of DH5α were performed identical to as above. The resulting mutant constructed was confirmed by sequencing to give plasmid pACP5m. The 0.2-kb *Hin*dIII-*Bgl*II fragment of pACP5m was cloned into the identical sites of pACP2-5 to generate plasmid pACP2-5-5m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-5m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-5m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-5m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-6m.

For pREZ67-6m: The 0.5-kb *Bgl*II-*Bse*RI fragment of pREZ67 was cloned into the identical sites of pETm to generate plasmid pACP6. Reaction was performed using pACP6 as template with ACP6mu5 and ACP6mu3 as primers. The mutagenesis PCR, *Dpn*I digestion, and transformation of DH5α were performed identical to as above. The resulting mutant constructed was confirmed by sequencing to give plasmid pACP6m. The 0.5-kb *Bgl*II-*Bse*RI fragment of pACP6m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-6m.

For pREZ67-34m: The 0.7-kb *Dra*III-*Hin*dIII fragment of pUCACP3-4-34m was cloned into the identical sites of pUCACP2-4 to generate plasmid pUCACP2-4-34m. The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-34m was cloned into the identical sites of pACP2-5 to generate plasmid pACP2-5-34m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-34m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-34m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-34m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-34m.

For pREZ67-123m: The 0.7-kb *Dra*III-*Hin*dIII fragment of pUCACP3-4-3m was cloned into the identical sites of pUCACP2-4-2m to generate plasmid pUCACP2-4-23m. The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-23m was cloned into the identical sites of pACP2-5 to generate plasmid pACP2-5-23m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-23m was cloned into the identical sites of pACP1-5-1m to generate plasmid pACP1-5-123m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-123m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-123m.

For pREZ67-345m: The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-34m was cloned into the identical sites of pACP2-5-5m to generate plasmid pACP2-5-345m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-345m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-345m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-345m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-345m.

For pREZ67-456m: The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-4m was cloned into the identical sites of pACP2-5-5m to generate plasmid pACP2-5-45m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-45m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-45m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-45m was cloned into the identical sites of pREZ67-6m to generate plasmid pREZ67-456m.

For pREZ67-1234m: The 0.7-kb *Dra*III-*Hin*dIII fragment of pUCACP3-4-34m was cloned into the identical sites of pUCACP2-4-2m to generate plasmid pUCACP2-4-234m. The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-234m was cloned into the identical sites of pACP2-5 to generate plasmid pACP2-5-234m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-234m was cloned into the identical sites of pACP1-5-1m to generate plasmid pACP1-5-1234m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-1234m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-1234m.

For pREZ67-3456m: The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-34m was cloned into the identical sites of pACP2-5-5m to generate plasmid pACP2-5-345m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-345m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-345m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-345m was cloned into the identical sites of pREZ67-6m to generate plasmid pREZ67-3456m.

For pREZ67-2345m: The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-234m was cloned into the identical sites of pACP2-5-5m to generate plasmid pACP2-5-2345m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-2345m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-2345m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-345m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-2345m.

For pREZ67-12345m: The 1.0-kb *Eag*I-*Hin*dIII fragment of pUCACP2-4-234m was cloned into the identical sites of pACP2-5-5m to generate plasmid pACP2-5-2345m. The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-2345m was cloned into the identical sites of pACP1-5-1m to generate plasmid pACP1-5-12345m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-12345m was cloned into the identical sites of pREZ67 to generate plasmid pREZ67-12345m.

For pREZ67-23456m: The 1.2-kb *Eag*I-*Bgl*II fragment of pACP2-5-2345m was cloned into the identical sites of pACP1-5 to generate plasmid pACP1-5-2345m. The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-2345m was cloned into the identical sites of pREZ67-6m to generate plasmid pREZ67-23456m.

For pREZ67-123456m: The 2.3-kb *Kpn*I-*Bgl*II fragment of pACP1-5-12345m was cloned into the identical sites of pREZ67-6m to generate plasmid pREZ67-123456m.

Production of EPA/DPA by these mutants in *E. coli*. Each pREZ67 derivate expression plasmid was co-transformed in *E. coli* BLR(DE3) with pREZ71 and pREZ65. Fermentation of these mutants and analysis of fatty acids production were performed as same as above.

Production of PUFAs by a hybrid PUFA synthase. The *pfaA* homolog, *orf8* was amplified by PCR from *M. marina* genomic DNA and cloned as an *Nco*I-*Sal*I fragment into MCS1 of pETDuet-1, which contained a previously cloned fragment of *S. japonica pfaE* cloned into MCS2 to generate the final expression construct pLR5 (Table S1). This expression plasmid was co-transformed in *E. coli* BLR(DE3) with pREZ71 and pREZ65. Fermentation of this hybrid and analysis of fatty acids production were performed as same as above.

References

S1 (a) Weaver, G. A.; Zirkle, R.; Metz, J. G. U.S. Patent 7,217,856 B2, **2007**; (b) Morita, N.; Tanaka, M.; Okuyama, H. *Biochem. Soc. Trans.* **2000,** *28*, 943-945.

S2 Sanchez, C.; Du, L.; Edwards, D. J.; Toney, M. D.; Shen, B. *Chem. Biol.* **2001,** *8*, 725-738.

Table S1. Plasmids used in the paper

plasmids	Descriptions
pREZ71	*pfaC* cloned as *Nde*I-*Xho*I fragment into pCOLADuet-1
pREZ65	*pfaB* cloned as *Bbs*I-*Not*I fragment into *Nco*I-*Not*I of MCS1 of pACYCDuet-1 *pfaD* cloned as *Nde*I-*Xho*I fragment into MCS2 of pACYCDuet-1
pREZ67	*pfaA* cloned as *Bbs*I-*Not*I fragment into *Nco*I-*Not*I of MCS1 of pETDuet-1 *pfaE* cloned as *Nde*I-*Xho*I fragment into MCS2 of pETDuet-1
pLR5	*orf8* cloned as *Nco*I-*Sal*I fragment into MCS1 of pETDuet-1 *pfaE* cloned as *Nde*I-*Xho*I fragment into MCS2 of pETDuet-1
pREZ67-1m	Derived from pREZ67. The active Ser residue of ACP1 was mutated into Ala.
pREZ67-2m	Derived from pREZ67. The active Ser residue of ACP2 was mutated into Ala.
pREZ67-3m	Derived from pREZ67. The active Ser residue of ACP3 was mutated into Ala.
pREZ67-4m	Derived from pREZ67. The active Ser residue of ACP4 was mutated into Ala.
pREZ67-5m	Derived from pREZ67. The active Ser residue of ACP5 was mutated into Ala.
pREZ67-6m	Derived from pREZ67. The active Ser residue of ACP6 was mutated into Ala.
pREZ67-34m	Derived from pREZ67. The active Ser residues of ACP3 and ACP4 were mutated into Ala.
pREZ67-123m	Derived from pREZ67. The active Ser residues of ACP1, ACP2, and ACP3 were mutated into Ala.
pREZ67-345m	Derived from pREZ67. The active Ser residues of ACP3, ACP4, and ACP5 were mutated into Ala.
pREZ67-456m	Derived from pREZ67. The active Ser residues of ACP4, ACP5, and ACP6 were mutated into Ala.
pREZ67-1234m	Derived from pREZ67. The active Ser residues of ACP1, ACP2, ACP3, and ACP4 were mutated into Ala.
pREZ67-3456m	Derived from pREZ67. The active Ser residues of ACP3, ACP4, ACP5, and ACP6 were mutated into Ala.
pREZ67-2345m	Derived from pREZ67. The active Ser residues of ACP2, ACP3, ACP4, and ACP5 were mutated into Ala.
pREZ67-12345m	Derived from pREZ67. The active Ser residues of ACP1, ACP2, ACP3, ACP4, and ACP5 were mutated into Ala.
pREZ67-23456m	Derived from pREZ67. The active Ser residues of ACP2, ACP3, ACP4, ACP5, and ACP6 were mutated into Ala.
pREZ67-123456m	Derived from pREZ67. The active Ser residues of all six ACPs were mutated into Ala.
pETACP1	*pfaA-ACP1* gene cloned as *Nde*I-*Xho*I fragment into pET28a (N-His_6 tagged ACP1, 116aa)
pETACP1a	*pfaA-ACP1* gene cloned as *Bam*HI-*Not*I fragment into pETDuet-1 (N-His_6 tagged ACP1, 100aa)
pETACP2a	*pfaA-ACP2* gene cloned as *Bam*HI-*Not*I fragment into pETDuet-1 (N-His_6 tagged ACP2, 100aa)
pETACP3a	*pfaA-ACP3* gene cloned as *Bam*HI-*Not*I fragment into pETDuet-1 (N-His_6 tagged ACP3, 100aa)
pETACP4a	*pfaA-ACP4* gene cloned as *Bam*HI-*Not*I fragment into pETDuet-1 (N-His_6 tagged ACP4, 100aa)
pETACP5a	*pfaA-ACP5* gene cloned as *Bam*HI-*Not*I fragment into pETDuet-1 (N-His_6 tagged ACP5, 100aa)

Table S1. Plasmids used in the paper (continued)

plasmids	Descriptions
pETACP6a	*pfaA-ACP6* gene cloned as *Bam*HI-*Not*I fragment into pETDuet-1 (N-His_6 tagged ACP6, 100aa)
pETACP1a+E	*pfaA-ACP1* gene cloned as *Bam*HI-*Not*I fragment into MCS1 of pETDuet-1 *pfaE* cloned as *Nde*I-*Xho*I fragment into MCS2 of pETDuet-1 (N-His_6 tagged ACP1, 100aa)
pETACP2a+E	*pfaA-ACP2* gene cloned as *Bam*HI-*Not*I fragment into MCS1 of pETDuet-1 *pfaE* cloned as *Nde*I-*Xho*I fragment into MCS2 of pETDuet-1 (N-His_6 tagged ACP2, 100aa)
pETACP3a+E	*pfaA-ACP3* gene cloned as *Bam*HI-*Not*I fragment into MCS1 of pETDuet-1 *pfaE* cloned as *Nde*I-*Xho*I fragment into MCS2 of pETDuet-1 (N-His_6 tagged ACP3, 100aa)
pETACP4a+E	*pfaA-ACP4* gene cloned as *Bam*HI-*Not*I fragment into MCS1 of pETDuet-1 *pfaE* cloned as *Nde*I-*Xho*I fragment into MCS2 of pETDuet-1 (N-His_6 tagged ACP4, 100aa)
pETACP5a+E	*pfaA-ACP5* gene cloned as *Bam*HI-*Not*I fragment into MCS1 of pETDuet-1 *pfaE* cloned as *Nde*I-*Xho*I fragment into MCS2 of pETDuet-1 (N-His_6 tagged ACP5, 100aa)
pETACP6a+E	*pfaA-ACP6* gene cloned as *Bam*HI-*Not*I fragment into MCS1 of pETDuet-1 *pfaE* cloned as *Nde*I-*Xho*I fragment into MCS2 of pETDuet-1 (N-His_6 tagged ACP6, 100aa)

Table S2. ESI-MS analysis of purified ACPs

	apo-form		holo-form		
	Calcd	Found	Calcd	Found (in vitro assay)[a,c]	Found (in vivo assay)[b,c]
ACP1 (116aa) (pET28a construct)	12454	12450	12794	12790	ND
ACP1 (100aa)	10896	10894	11236	ND	11232
ACP2 (100aa)	10781	10778	11121	ND	11117
ACP3 (100aa)	10882	10879	11222	ND	11218
ACP4 (100aa)	10869	10872	11207	ND	11205
ACP5 (100aa)	10799	10802	11139	ND	11134
ACP6 (100aa) (pETDuet-1 constructs)	10797	10795	11137	ND	11133

[a] Phosphopantetheinylation of PfaA-ACP1 catalyzed by Svp PPTase in vitro.
[b] Phosphopantetheinylation of PfaA-ACPs by coexpression of *pfaA-ACPs* with *pfaE* in vivo.
[c] Not determined.

Table S3. FAME results from EPA expression system.

		% EPA[a]	Rel % EPA	% DPA[a]	EPA/DPA
Control	Wild-type	8.9		1.4	6.4
		9.2		1.4	6.4
		8.6		1.4	6.3
	Average	8.9 ± 0.3	100	1.4 ± 0.1	
Single mutation	ACP1m	7.4		1.5	4.9
		7.7		1.5	5.1
	ACP2m	7.6		1.5	5.1
		7.9		1.5	5.4
	ACP3m	7.1		1.2	5.9
		7.7		1.5	5.2
	ACP4m	8.5		1.4	6.1
		7.1		1.2	5.8
	ACP5m	6.7		1.1	6.3
		6.7		1.0	6.5
	ACP6m	9.2		1.4	6.7
		8.9		1.4	6.4
	Average	7.7 ± 0.8	87	1.3 ± 0.2	
Double mutation	ACP34m	8.0		1.2	6.7
		8.5		1.2	6.9
	Average	8.2 ± 0.3	92	1.2 ± 0.1	
Triple mutation	ACP123m	6.9		0.70	9.8
	ACP345m	6.239		0.65	9.6
	ACP456m	6.7		0.67	10.0
	Average	6.6 ± 0.3	74	0.7 ± 0.1	
Quadruple mutation	ACP1234m	5.8		0.70	8.2
	ACP3456m	5.4		0.45	12.1
	ACP2345m	5.2		0.61	8.5
		5.4		0.67	8.0
	Average	5.4 ± 0.2	61	0.6 ± 0.1	
Quintuple mutation	ACP12345m	2.7		0.39	6.9
		3.1		0.33	9.4
	ACP23456m	3.7		0.33	11.3
		3.8		0.33	11.4
	Average	3.3 ± 0.5	37	0.3 ± 0.1	
Hextuple mutation	ACP123456m	0.0		0.0	
		0.0		0.0	
	Average	0.0	0.0	0.0	

[a] %EPA and %DPA: percentage of EPA and DPA of total fatty acid, respectively.

Table S4. EPA and DPA production by the *S. japonica-M. marina* hybrid PUFA synthase Orf8/PfaB/PfaC/PfaD/PfaE

	%EPA[a]	%DPA[a]	%DHA[a]	EPA/DPA
Pfa PUFA synthase (PfaA/PfaB/PfaC/PfaD/PfaE)	8.9	1.4	0.0	6.4
	9.2	1.4	0.0	6.5
	8.6	1.4	0.0	6.3
	8.9 ± 0.3	1.4 ± 0.1	0.0	
Hybrid PUFA synthase (Orf8/PfaB/PfaC/PfaD/PfaE)	8.7	3.3	0.0	2.6
	8.6	3.2	0.0	2.7
	9.2	3.4	0.0	2.7
	8.9 ± 0.3	3.3 ± 0.1	0.0	

[a]%EPA, %DPA, and %DHA: percentage of EPA, DPA, and DHA of total fatty acid, respectively.

Table S5. Primers used for PCR mutagenesis

Primer	Sequence
ACP1mu5[a]	GCC GAT TTA GGC ATC GAT gCA ATT AAA CGc GTT GAA ATA TTA GGT ACT
ACP1mu3[a]	AGT ACC TAA TAT TTC AAC gCG TTT AAT TGc ATC GAT GCC TAA ATC GGC
ACP5mu5	GCG GAT TTA GGC ATC GAT gCA ATT AAA CGc GTT GAG ATC TAC TGA CTT
ACP5mu3	AAG TCA GTA GAT CTC AAC gCG TTT AAT TGc ATC GAT GCC TAA ATC CGC
ACP6mu5	GCG GAT TTA GGC ATC GAT gCA ATT AAA CGc GTT GAA ATT TTA GGG ACG
ACP6mu3	CGT CCC TAA AAT TTC AAC gCG TTT AAT TGc ATC GAT GCC TAA ATC CGC

[a] These pair primers were used for PCR mutagenesis of ACP1, ACP2, ACP3 and ACP4. The codons designed for the Ser-to-Ala mutation are underlined.

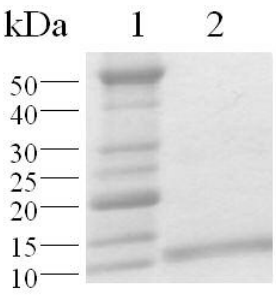

Figure S1. Purified PfaA-ACP1 on 15% SDS-PAGE. Lane 1, protein markers; lane 2, PfaA-ACP1 (100aa, calculated molecular weight of 10896).

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pfaA-ACP4       SGLSAETVLNTMLEVVAEKTGYPTDMLELSMDMEADLGIDSIKRVEILGTVQDELPTLPE
pfaA-ACP3       SGLSAETVLNTMLEVVAEKTGYPTEMLELSMDMEADLGIDSIKRVEILGTVQDELPTLPE
pfaA-ACP2       SGLSAETVLNTMLEVVAEKTGYPTEMLELSMDMEADLGIDSIKRVEILGTVQDELPTPPE
pfaA-ACP1       TALSSQKVLDTMLEVVAEKTGYPTEMLELSMDMEADLGIDSIKRVEILGTVQDELPTLPE
pfaA-ACP6       TALSAEQVQSTMMTVVAEKTGYPTEMLELSMDMEADLGIDSIKRVEILGTVQDELPGLPE
pfaA-ACP5       SGLSAEQVQSTMMTVVAEKTGYPTEMLELSMDMEADLGIDSIKRVEILGTVQDELPTLPE
                :.**:: * .**: **********:******************************  **

pfaA-ACP4       LSPEDLAECRTLGEIVDYMGSKLPAA
pfaA-ACP3       LSPEDLAECRTLGEIVDYMNSKLPAA
pfaA-ACP2       LSPEDLAECRTLGEIVSYMGSKLPAA
pfaA-ACP1       LSPEDLAECRTLGEIVDYMGSKLPAA
pfaA-ACP6       LNPEDLAECRTLGEIVSYMGAKLPAA
pfaA-ACP5       LNPEDLAECRTLGEIVSYMGGKLPAA
                *.**************.**..*****
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Figure S2. Sequence alignment of six PfaA-ACPs from *S. japonica*. The active Ser residue in each ACP is shown in red.

Figure S3. DNA sequences of pET-linker (a) and pUC-linker (b).