Production of Eicosapentaenoic Acid by a Recombinant Marine Cyanobacterium, *Synechococcus* sp.

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ABSTRACT: The eicosapentaenoic acid (EPA) synthesis gene cluster from an EPA-producing bacterium, *Shewanella* sp. SCRC-2738, was cloned into a broad-host range vector, pJRD215, and then introduced into a marine cyanobacterium, *Synechococcus* sp. NKBG15041c, by conjugation. The transconjugant cyanobacteria produced 3.7 ± 0.2% (2.24 ± 0.13 mg/L) EPA (n-3) and 2.5 ± 0.2% (1.49 ± 0.06 mg/L) eicosatetraenoic acid (n-3) of the total fatty acids when the cells were cultured at 23°C at a light intensity of 1,000–1,500 Lux. The EPA and eico-satetraenoic acid contents of the cells were increased to 4.6 ± 0.6% (3.86 ± 1.11 mg/L) and 4.7 ± 0.3% (3.86 ± 0.82 mg/L), and 7.5 ± 0.3% (1.76 ± 0.10 mg/L) and 5.1 ± 0.2% (1.19 ± 0.06 mg/L) when they were cultured at low temperature (18°C) and at lower light intensity (40 Lux), respectively.

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The n-3 group of polyunsaturated fatty acids (PUFA), in particular eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3) have received much attention for their importance in keeping the human circulation and nervous systems in a healthy condition (1,2). Moreover, these compounds are also essential nutrients to larval marine fish (3). At present, the main sources of dietary EPA are marine fish and algae such as *Porphyridium* and *Nannochloropsis* (4,5). Marine cyanobacteria like *Synechococcus* are widely distributed in the sea and have important roles as primary producers in the marine food chain. Utilization of these cyanobacteria for purposes of fish culture is convenient, because they are bioavailable as whole cells without any extraction. However, marine cyanobacteria do not produce EPA. Therefore, by introducing an EPA synthesis gene cluster to marine cyanobacteria, it is expected that they might be a valuable source of n-3 fatty acids for fish culture.

The EPA synthesis gene cluster [approximately 38 kb, composed of nine open reading frames (ORF) (Yamada, A., Yu, R., Watanabe, K., Yazawa, K., and Konda, K., unpublished data)] was isolated from a marine bacterium, *Shewanella* sp. SCRC-2738 (6,7). In previous work, we confirmed that a marine cyanobacterium, *Synechococcus* sp. NKBG042902, produced EPA after the introduction of an EPA synthesis gene cluster (8). However, plasmid pJRDEPA, including all nine ORF of this gene cluster, was unstable in the cells, and the total amount of EPA produced was low. In this paper, we demonstrate EPA production by a transgenic marine cyanobacterium, *Synechococcus* sp. NKBG15041c (9), carrying a plasmid containing the essential ORF for EPA synthesis and its enhancement under different growth conditions.

EXPERIMENTAL PROCEDURES

Strains and plasmids. A marine cyanobacterium, *Synechococcus* sp. NKBG15041c, isolated from a Japanese coastal area, was used as a host strain. It was cultured in BG11 medium (American Type Culture Collection catalog, medium no. 617) supplemented with 3% (wt/vol) NaCl (BG11-M) under aerobic conditions with continuous light at 23°C unless otherwise stated. *Escherichia coli* JM109 and S17-1 (10) were cultured under aerobic conditions in LB medium [10 g/L Bacto-tryptone 5 g/L Bacto-yeast extract, both from Difco (Detroit, MI) and 10 g/L NaCl] at 37 or 25°C. Cloning vector pBSIIKS(+) (Stratagene, La Jolla, CA) and broad-host range vector pJRD215 (10.2 kb, containing Km^r and Sm^r genes; 11) were used in this work. As the source of the EPA synthesis gene cluster, pEPA (46.6 kb; 7,8) was used. pEPA is a plasmid that is carrying an EPA synthesis gene cluster (GenBank accession number, U73935) isolated from *Shewanella* sp. SCRC-2738.

Construction of pJRDEPA-S. DNA constructs were produced using standard methods. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Takara Shuzo (Kyoto, Japan). At first, the subcloning of essential ORF (2,5,6,7, and 8) for EPA production from pEPA was carried out. A 8,398 bp *Xba*I-*Spe*I fragment (23,045–31,443 bp, containing the 3′ end of ORF6, ORF7, and the 5′ end of ORF8), a 10,731 bp *Xba*I-*Xba*I fragment (12,314–23,045 bp, containing the 3′ end of ORF3, ORF4, 5 and the 5′ of ORF6), and a 1,071 bp *Spe*I-*Nhe*I fragment (31,443– 32,514 bp, containing the 3′ end

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Abbreviations: EPA, eicosapentaenoic acid; GLC, gas–liquid chromatography; GC–MS, gas chromatography–mass spectrometry; ORF, open reading frame; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography.

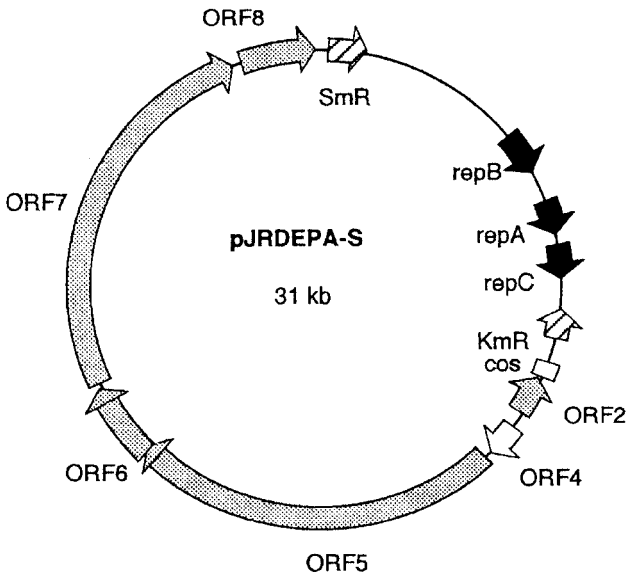
of ORF8) of the EPA synthesis gene cluster were cloned in order into the *Xba*I-*Spe*I, *Xba*I site, and *Spe*I sites of pBSIIKS(+), respectively. The resultant plasmid was named ΔX4XbNh/pBS. After digestion with *Not*I, ΔX4XbNh/pBS was treated with T4 DNA polymerase (Takara Shuzo) to make blunt ends, and then cut with *Xho*I to obtain fragment A. For ORF2, a fragment cut out from R/pSTV28 [a plasmid containing ORF2, 7,951–9,129 bp, in pSTV28 (Takara Shuzo, Kyoto, Japan)] with *Eco*RI and *Pst*I, was cloned into the *Eco*RI-*Pst*I site of pBSIIKS(+) to obtain R/pBS. After treatment with *Pst*I and T4 DNA polymerase, a *Xho*I linker was inserted into R/pBS. Then, fragment B was obtained from R/pBS by XhoI digestion. Fragments A and B were cloned into the *Xho*I-*Stu*I and *Xho*I sites of pJRD215 by *in vitro* packaging (PackageneR Lambda DNA Packaging System; Promega, Madison, WI) and by using a DNA ligation kit (Takara Shuzo), respectively. The final plasmid constructed was designated as pJRDEPA-S (Scheme 1).

Conjugal gene transfer to the cyanobacterium. Conjugal gene transfer to the cyanobacterium was carried out as previously reported (8) with some modifications. Cyanobacterial cells at the mid-growth phase were centrifuged, washed with BG11-M medium, and then resuspended in fresh medium. Freshly transformed *E. coli* S17-1 cells harboring pJRDEPA-S were collected from Luria broth plates containing 50 μg kanamycin/mL and 50 μg streptomycin/mL, and then suspended in BG11-M medium. Suspensions of cyanobacterial cells and *E. coli* cells were mixed in the cell number ratio of 1:10 (cyanobacterium/*E. coli*) and then spotted onto dried BG11 plates supplemented with 15 mM NaCl. After 48 h of incubation under light, the cells were collected from the plates with BG11-M medium, and then streaked on BG11-M plates containing 75 μg kanamycin/ mL to select cyanobacterial transconjugants.

Gas–liquid chromatography (GLC). Fatty acid methyl esters were prepared from the lyophilized cyanobacterial cells or *E. coli* cells by treatment with 5% methanolic hydrochloric acid (12). The fatty acid methyl esters, which were purified by using thin-layer chromatography (TLC), were analyzed by GLC. The analytical conditions were as previously reported (8), with minor modifications.

Fatty acid methyl esters prepared from *Shewanella* sp. SCRC-2738 that had been confirmed to contain EPA and 20:4n-3 (12) and EPA methyl ester (Sigma Chemical Co., St.Louis, MO) were used as standard samples. Heneicosanoic acid (21:0) was also used as an internal standard.

GC–mass spectrometry (GC–MS). PUFA methyl ester fractions were prepared from the fatty acid methyl esters by $AgNO_3$-TLC (12), and then subjected to GC–MS. The analytical conditions were previously reported (8) with minor modifications.



SCHEME 1

RESULTS AND DISCUSSION

Construction of a plasmid containing an EPA synthesis gene cluster and EPA production in E. coli. Functional analysis of each ORF in the EPA synthesis gene cluster isolated from SCRC-2738 indicated that five (ORF2, 5, 6, 7, and 8) of the nine ORF were required for EPA synthesis in *E. coli* (Yamada, A., Yu, R., Watanabe, K., Yazawa, K., and Kondo, K., unpublished data). In this experiment, after excluding noncoding regions present both upstream and downstream of the ORF, five essential ORF and one nonessential ORF (ORF4) for EPA synthesis were ligated into pJRD215. With this procedure, plasmid pJRDEPA-S (Scheme 1) is approximately 17 kb shorter than pJRDEPA. To determine the EPA productivity of pJRDEPA-S, it was transferred into *E. coli* JM109. The fatty acid methyl esters of the JM109 were analyzed with GLC and GC–MS. The EPA content in the total fatty acids reached 5.7%, i.e., more than that of pEPA (less than 4.5%; 7), although the copy number was lower in *E. coli* cells (data not shown). This suggested reduction in the size of the EPA synthesis gene cluster enhanced the EPA productivity.

EPA production by cyanobacterial cells. Fatty acid methyl esters prepared from the total wild type cyanobacterial cells and transconjugant cells harboring pJRDEPA-S were analyzed by GLC (Table 1). For the transconjugant, there were two novel peaks that were never detected in wild-type, corresponding to authentic 20:4n-3 and EPA (20:5n-3), respectively. The methyl esters of total fatty acids and PUFA were analyzed by GC–MS (Table 1). For the two GLC peaks mentioned above, ion peaks were detected at *m/z* 318 and 316, consistent with the molecular weights of 20:4 and 20:5, respectively, and an ion peak at *m/z* 79, typical of polyunsaturated fatty acids was also seen (data not shown). Therefore, it was confirmed that the transgenic cyanobacterium was able to produce EPA and 20:4n-3 on the introduction of pJRDEPA-S.

In comparing with the wild-type cyanobacteria, percentages of 18:1n-9 and 18:2n-6 to total fatty acids were lower in the transconjugant (Table 1). The decrease might be correlated to production of EPA and 20:4n-3.

EPA production in cyanobacterial cells under different culture conditions. The cyanobacterial transconjugant harboring

TABLE 1
Fatty Acid Composition (% of total fatty acid) of Wild-Type *Synechococcus* sp. NKBG15041c and a Transconjugant Harboring pJRDEPA-S Under Different Growth Conditions[a]

	Transconjugant (pJRDEPA-S)			Wild-type
Fatty acid	Condition A	Condition B	Condition C	Condition A
Shorter than 16:0	1.8 ± 0.8	3.5 ± 1.0	10.3 ± 0.2	7.4 ± 0.1
16:0	37.2 ± 0.8	38.3 ± 0.7	38.7 ± 1.4	37.6 ± 1.6
16:1n-7	12.0 ± 0.5	10.2 ± 0.3	12.2 ± 0.4	13.4 ± 1.0
18:0	1.0 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.0 ± 0.2
18:1n-9	13.4 ± 0.2	9.4 ± 1.6	9.9 ± 0.4	19.5 ± 1.2
18:2n-6	13.4 ± 0.5	8.6 ± 0.8	7.6 ± 0.1	15.5 ± 0.7
18:3n-3	7.0 ± 0.0	2.7 ± 0.1	6.7 ± 0.7	6.4 ± 0.5
20:4n-3	2.5 ± 0.2	5.1 ± 0.2	4.7 ± 0.3	ND
20:5n-3, EPA	3.7 ± 0.2	7.5 ± 0.3	4.6 ± 0.6	ND
Others	8.6 ± 0.9	15.0 ± 1.4	9.3 ± 2.4	3.1 ± 0.6

[a]Condition A: 23°C, 1,000–1,500 Lux, 14 d; Condition B: 23°C, *ca.* 40 Lux, 48 d; Condition C: 18°C, 800–1,000 Lux, 22 d (with shaking at 100 rpm). For conditions A–C $n = 3$. Abbreviation, ND, not detected.

pJRDEPA-S was cultured at different growth temperatures and light intensities. Table 1 shows the major fatty acid compositions of the transconjugant and wild type grown at 18 and 23°C, and at high and low light intensities. In the case of culture at 18°C, flasks were shaken at 100 rpm to compensate for biased illumination. The transconjugant grown at 18°C exhibited higher EPA content and higher absolute yield (4.6 ± 0.6%; 3.86 ± 1.11 mg/L) than when grown at 23°C (3.7 ± 0.2%; 2.24 ± 0.13 mg/L). The increase of EPA content associated with a decrease of culture temperature was also observed in the experiments using *E. coli* JM109 carrying pEPA at 20 and 25°C (7). It seems that these tendencies might be related to chilling tolerance by accumulation of PUFA into cell membranes (13,14). For the transconjugant grown at 18°C, the increase of EPA productivity might also be affected by aeration. In a marine cyanobacterium, *Synechococcus* sp. NKBG042902, harboring pJRDEPA, the EPA content in the total fatty acids was 0.7 ± 0.2% at 17°C (8). With a different cyanobacterial host and a shortened plasmid, the EPA content increased to eightfold more than in the previous work.

When the light intensity was lowered to 40 Lux, the transconjugant exhibited the highest content of EPA (7.5 ± 0.3%). Its absolute yield, however, was lower than that of the transconjugant cultured at 23°C (1.76 ± 0.10 mg/L), even though given a longer culture period (Table 1). The cause of this phenomenon is not clear. The cells might find a more suitable condition to synthesize EPA under weak light intensity because the light-induction of photosynthetic systems does not fully function (15,16).

In SCRC-2738 or *E. coli* with the EPA synthesis gene cluster introduced, as high content of 20:4n-3 as that of the cells in this study was not observed (12; Yamada, A., Yu, R., Watanabe, K., Yazawa, K., and Kondo, K., unpublished data). This tendency may depend on the fatty acid composition intrinsic to the particular organisms. According to the hypothetical pathway for the EPA synthesis in SCRC-2738 (17), 18:3n-3, which is present in considerable amounts in cyanobacteria (Table 1; 18), is a likely precursor of 20:4n-3 or EPA.

In the previous work, pJRDEPA was shown to be unstable in *Synechococcus* sp. NKBG042902 (8). In contrast, pJRDEPA-S was maintained well in NKBG15041c. This stability may be attributed to the size of plasmid and host characteristics of NKBG15041c, carrying no native plasmids to compete against introduced plasmids (19).

In this paper, we demonstrated that a transgenic marine cyanobacteria produced EPA and 20:4n-3, and the productivity was changed related to culture conditions. We need more precise optimization of culture condition to make them useful on a large scale. These transgenic cyanobacteria may also be useful in elucidating the synthetic pathway of EPA in bacteria, because they make precursors to EPA such as 20:4n-3 (17), and undergo regulation by factors such as temperature, osmotic pressure and light intensity (13,14,16).

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