Expression of the eicosapentaenoic acid synthesis gene cluster from *Shewanella* sp. in a transgenic marine cyanobacterium, *Synechococcus* sp.

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The eicosapentaenoic acid (EPA) synthesis gene cluster isolated from a marine bacterium, *Shewanella putrefaciens* strain SCRC-2738, was cloned and expressed in the marine cyanobacterium *Synechococcus* sp. A broad-host-range cosmid vector, pJRD215 (10.2 kb, Sm^r Km^r), was used to clone a 38 kb insert, pEPA, containing the EPA synthesis gene cluster, creating plasmid pJRDEPA (approx. 48 kb). This plasmid was transferred to the cyanobacterial host at a frequency of 2.2×10^{-7} . Cyanobacterial transconjugants grown at 29 °C produced 0.12 mg EPA (g dry weight)⁻¹, whereas those grown at 23 °C produced 0.56 mg EPA (g dry weight)⁻¹. The yield was further improved to 0.64 mg (g dry weight)⁻¹ by incubation for 1 d at 17 °C. This is believed to be the first successful cloning and expression of such a large heterologous gene cluster in a marine cyanobacterium.

Keywords: metabolic engineering, marine cyanobacterium, *Synechococcus* sp., eicosapentaenoic acid (EPA) production, conjugation

INTRODUCTION

Highly unsaturated fatty acids are receiving attention as useful dietary components. In particular, ω -3 fatty acids such as eicosapentaenoic acid (EPA, C20:5) are known to prevent several human diseases (Needleman *et al.*, 1979; Terano *et al.*, 1984; Abbey *et al.*, 1990). At present, the main source of dietary EPA is marine fish. However, the undesirable fishy flavour which remains throughout the extraction process of EPA can become a problem when large amounts of EPA are added to food. Marine microalgae have been considered as new sources of dietary EPA because of their lack of fish flavours. Although there are many marine eukaryotic microalgae that produce EPA (Viso *et al.*, 1993; Cohen *et al.*, 1995), their low growth rate does not suit industrial EPA production. Marine cyanobacteria do not produce EPA, but they can be cultured at high densities and can grow much faster than eukaryotic microalgae. It was demonstrated that up to 10 g dry weight l⁻¹ of a marine cyanobacterium could be cultured using a photo-bioreactor (Takano *et al.*, 1992). Molecular genetic

engineering may be one way to introduce EPA synthesis into cyanobacteria.

Cyanobacterial genetic engineering has been developed mainly with freshwater strains, such as *Synechocystis*, *Synechococcus* and *Anabaena*, to elucidate their photosynthesis mechanisms (Golden, 1994; Xu *et al.*, 1995), nitrogen fixation (Golden *et al.*, 1991; Lyons & Thiel, 1995) and morphological differentiation (Wolk, 1991; Wolk *et al.*, 1993). Many marine cyanobacteria have been screened and some have been found to produce useful materials such as UV-absorbing material (Matsunaga *et al.*, 1993; Wachi *et al.*, 1995a), plant growth regulators (Wake *et al.*, 1992), melanin biosynthesis inhibitors (Wachi *et al.*, 1995b) and antimicrobial compounds (Miura *et al.*, 1993). Genetic manipulation has been considered as a promising technique to enhance production of these substances. However, since the useful strains were not naturally transformable, several gene transfer experiments were carried out as the first step towards genetic engineering of these strains. Transfer by electroporation and conjugation has been established in marine cyanobacteria (Matsunaga *et al.*, 1990; Sode *et al.*, 1992; Matsunaga & Takeyama, 1995; Brahamsha, 1996) and a high-copy-number plasmid was

Abbreviation: EPA, eicosapentaenoic acid.

Several desaturase genes have been isolated from freshwater cyanobacteria (Wada *et al.*, 1990; Sakamoto *et al.*, 1994a, b). The *desA* gene (Δ^{12} desaturase gene) was isolated from *Synechocystis* PCC 6803 and expressed in *Synechococcus* sp. PCC 7942; it resulted in a change of

fatty acid composition and enhancement of chilling tolerance of the latter strain (Wada *et al.*, 1990). On the other hand, elongation genes for fatty acid synthesis have not been isolated. Yazawa *et al.* (1988) isolated a marine bacterium, *Shewanella* sp. SCRC-2738, from fish intestines that produced EPA. The EPA synthesis gene cluster (approx. 38 kb) was isolated from this strain (Yazawa, 1996); it comprised eight open reading frames (ORFs). Three of the ORFs showed similarities with genes encoding enzymes which related to *de novo* synthesis of fatty acids or to carbon chain elongation. Since EPA was produced in *Escherichia coli* into which the EPA synthesis gene cluster was cloned, it was speculated that desaturase genes were included in the remaining ORFs (Yazawa, 1996).

In this paper, we describe metabolic engineering of a marine cyanobacterium by expressing a large gene cluster from *Shewanella* sp. SCRC-2738 in *Synechococcus* sp., and demonstrate the effect of temperature conditions on EPA synthesis in marine cyanobacterial cells.

METHODS

Strains and plasmids. A marine cyanobacterium, *Synechococcus* sp. NKBG042902, isolated from a Japanese coastal area was used as a host strain (Takano *et al.*, 1992). It was cultured in BG 11 medium (ATCC catalogue, medium no. 617) supplemented with 3% NaCl, under aerobic conditions at 29 or 23 °C with a light intensity of 50 $\mu\text{E m}^{-2} \text{s}^{-1}$. *Escherichia coli* S17-1 (Simon *et al.*, 1983) and NM554 (Raleigh *et al.*, 1988) were cultured under aerobic conditions using LB medium (bact-tryptone 10 g l⁻¹, bact-yeast extract 5 g l⁻¹ and NaCl 3 g l⁻¹; pH 7.4) at 37 or 20 °C. The broad-host-range vector plasmids pJRD215 (10.2 kb, Km^r Sm^r) (Davison *et al.*, 1987) and pKT230 (10.2 kb, Km^r Sm^r) (Bagdasarian *et al.*, 1981) were used. pEPA (Yazawa, 1996) consisted of pWE15 and the EPA synthesis gene cluster (GenBank accession number U73935) isolated from *Shewanella putrefaciens* SCRC-2738.

Construction of plasmid for expression of EPA synthesis genes. Fig. 1 shows the construction of a plasmid containing the EPA synthesis gene cluster in the vector pJRD215. The EPA synthesis gene cluster (approx. 38 kb) was excised from pEPA by *Sau3AI* digestion. pJRD215 was digested with *SalI* or *XhoI*, and the linear vector DNAs were cleaved with *BamHI*. The two vector fragments containing *cos* sequence were isolated and ligated to the EPA synthesis gene cluster generated by digestion with *Sau3AI*. This concatemer was packaged *in vitro* to bacteriophage lambda particles using an *in vitro* packaging kit (Gigapack II Packaging Extracts, Stratagene) and used to infect *E. coli* NM554. Infected cells were plated onto LB medium plates containing 50 μg kanamycin ml⁻¹.

Conjugal gene transfer of cyanobacterium. Cyanobacterial cells in mid-exponential phase were centrifuged, washed with BG11 marine medium, and resuspended in fresh medium. Freshly transformed *E. coli* S17-1 with pJRD215, pKT230 or cosmids isolated from transfected *E. coli* NM554 were collected from LB plates and resuspended in BG11 marine medium. Suspensions of cyanobacterial cells and transformed *E. coli* cells were mixed at a cell number ratio of 1:10 (cyanobacterium:*E. coli*) and spotted onto dried BG11 marine

medium agar plates. After 48 h incubation under light, the collected cell mixtures were subjected to selection of cyanobacterial transconjugants on the BG11 marine agar plates containing 25 μg kanamycin ml⁻¹.

Fatty acid analysis. Cyanobacteria or *E. coli* cells in late-exponential phase were centrifuged at 6000 g and 4 °C, washed with Tris/phosphate buffer (pH 7.5), and the cell pellet was lyophilized. The lyophilized cells (30 mg) were treated with 2 ml 5% methanolic hydrochloride acid (Kokusan Co.) including pentadecanoic acid (C15:0) as a internal standard, and heated at 90 °C for 40 min. After the samples had cooled down, they were diluted with 1 ml H₂O and extracted with 1 ml n-hexane. The collected hexane layer, in which methyl esters of fatty acids were partitioned, was dried and re-dissolved in n-hexane. Fatty acid methyl esters were analysed by gas chromatography (GC-14B, Shimadzu) with a capillary column (HR-Thermon 300B, 0.25 mm $\times$ 25 m, Shinwa Chemical Industries). The injection temperature was 250 °C. The column temperature was programmed to increase from 180 to 220 °C (180 °C for 1 min, then increasing by 1 °C min⁻¹ to 190 °C, 190 °C for 2 min, then increasing by 5 °C min⁻¹ to 210 °C, 210 °C for 15 min, then increasing by 15 °C min⁻¹ to 220 °C, 220 °C for 5 min). The quantities of fatty acids were estimated from the peak areas on the chromatogram using the internal standard by C-RA6A Chromatopac (Shimadzu).

Southern hybridization analysis. Southern hybridization analysis of plasmids was carried out using as a probe a 3.2 kb fragment including ORF8 that was labelled with the DIG chemiluminescence kit (Boehringer Mannheim). Plasmids were extracted from *E. coli* by a standard method (Sambrook *et al.*, 1989).

Evaluation of plasmid stability in cyanobacterial cells. The stability of plasmids was evaluated as the ratio of cells harbouring the plasmid to total cells. Cell samples were collected at several times during the growth of cyanobacterial transconjugants in BG11 marine medium containing kanamycin (25 μg ml⁻¹), and were plated out on BG11 marine medium with or without kanamycin. The ratio of cells harbouring the plasmid was calculated as the number of colonies on plates with kanamycin divided by the number of colonies on plates without kanamycin.

GC-MS analysis of gene product. Fatty acid methyl esters extracted from transformed cells were separated on TLC silica gel impregnated with silver nitrate in n-hexane/ether (80:20) into several fatty acid groups. Groups of unsaturated fatty acid methyl esters with more than three double bonds were identified by comparison with standards. They were extracted from TLC plates and subjected to GC-MS analysis using a Hitachi GC-mass spectrometer (M-80A) with a capillary column (DB-WAX, 0.32 mm $\times$ 30 m; J & W Scientific). The injection and column temperature were 220 °C and 250 °C, respectively. The temperature was programmed to increase from 80 to 240 °C (80 °C for 1 min, then increasing by 20 °C min⁻¹ to 180 °C, 180 °C for 5 min, then increasing by 5 °C min⁻¹ to 190 °C, 190 °C for 5 min, then increasing by 5 °C min⁻¹ to 240 °C, 240 °C for 20 min).

RESULTS

Construction of a plasmid containing an EPA synthesis gene cluster and EPA production in *E. coli*

Since a broad-host-range cosmid vector for Gram-negative bacteria, pJRD215, was confirmed to replicate in the marine cyanobacterium *Synechococcus* sp.

Fig. 2. Southern hybridization analysis of a plasmid harboured by exconjugant E2 using a 3.2 kb fragment (ORF8) as a probe. Lane 1, 1 kb ladder; lane 2, pEPA digested with *Xba*I and *Spe*I; lane 3, the constructed plasmid from *E. coli* E2 digested with *Xba*I and *Spe*I; lane 4, pJRD215.

NKBG042902 during preliminary experiments, this vector was used to clone an EPA synthesis gene cluster into the cyanobacterial host. To select the transconjugants of *E. coli* NM554 having the EPA synthesis gene cluster, plasmids extracted from the *E. coli* transconjugants were digested with *Xba*I and *Spe*I. Among 35 transconjugants resistant to kanamycin, the plasmids of three

transconjugants, E1, E2 and E3, showed the same restriction pattern as that of pEPA. As EPA production was affected by growth temperature, EPA production by these three transconjugants grown at 20 °C was measured according to the previous report (Yazawa, 1996). Transconjugants E1 and E2 produced larger amounts of EPA [3.2 ± 0.7 and 3.3 ± 0.7 mg (g dry weight)⁻¹] than transconjugant E3 [0.3 ± 0.1 mg (g dry weight)⁻¹]. E1 and E2 may have a plasmid harbouring the intact EPA synthesis gene cluster. Fig. 2 shows the restriction pattern of the plasmid from E2 and pEPA restricted with *Xba*I and *Spe*I, and the result of Southern hybridization analysis. pEPA and the constructed plasmid appeared to share four fragments identical to those from the EPA synthesis gene cluster. The fragment including ORF8 hybridized to the 3.2 kb fragment from both plasmids. The plasmid in *E. coli* E2 was designated pJRDEPA and was used for further experiments.

Transjugation of *Synechococcus* sp.

Plasmids pJRDEPA (approx. 48 kb) and pJRD215 (10.2 kb) were transferred to *Synechococcus* sp. NKBG042902 by conjugation. For comparison, another broad-host-range vector plasmid, pKT230 (11.9 kb), was also used in a gene transfer experiment. The transjugation efficiencies of pKT230, pJRD215 and pJRDEPA in cyanobacterial cells were 2.3×10^{-6} , 3.3×10^{-6} and 2.2×10^{-7} transconjugants per recipient cell, respectively.

Table 1. Fatty acid composition of wild-type *Synechococcus* sp. NKBG042902 and a transconjugant harbouring pJRDEPA, grown at different temperatures

Fatty acid composition	Fatty acid content (weight percentage of total fatty acids)					
	Wild-type			Transconjugant		
	29 °C	23 °C	23 °C to 17 °C*	29 °C	23 °C	23 °C to 17 °C*
less than 15:0	14.0 ± 0.4	14.2 ± 0.4	14.1 ± 0.8	14.1 ± 0.3	14.3 ± 0.7	10.4 ± 1.1
16:0	37.5 ± 0.3	32.4 ± 0.7	32.3 ± 1.7	32.9 ± 0.9	31.4 ± 0.8	34.8 ± 1.4
16:1	7.5 ± 0.1	8.8 ± 0.4	11.7 ± 0.5	9.6 ± 0.6	9.1 ± 0.2	10.7 ± 0.7
17:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
17:1	1.1 ± 0.0	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.0	1.3 ± 0.1	1.3 ± 0.1
18:0	1.4 ± 0.0	1.3 ± 0.1	1.0 ± 0.1	1.32 ± 0.1	1.4 ± 0.0	1.5 ± 0.3
18:1	20.7 ± 0.3	26.0 ± 2.1	20.6 ± 0.6	25.1 ± 0.8	23.7 ± 1.9	21.5 ± 5.3
18:2	7.8 ± 0.1	5.3 ± 0.9	7.2 ± 0.8	5.7 ± 0.2	5.4 ± 0.4	5.4 ± 0.2
18:3 γ	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1
18:3 α	4.4 ± 0.1	4.4 ± 0.4	6.4 ± 0.2	5.0 ± 0.2	4.4 ± 0.2	4.5 ± 0.7
20:0	trace	trace	trace	trace	trace	trace
20:2	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	4.0 ± 0.1	0.6 ± 0.1	0.6 ± 0.2
20:3	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.3	0.7 ± 0.1
20:4	—	—	—	trace	trace	trace
20:5(EPA)	—	—	—	≤ 0.1	0.4 ± 0.3	0.5 ± 0.2
Others	4.4 ± 0.8	4.7 ± 1.7	4.0 ± 0.5	3.3 ± 0.7	9.3 ± 1.2	10.7 ± 3.4

trace, < 0.05; —, not detected.

*Cells grown at 23 °C were incubated for 1 d at 17 °C under light and aerobic conditions.

Fig. 3. Ratio of cells harbouring plasmid pJRDEPA (Km^r) to total cells (■) and growth curve of the cyanobacterial transconjugant (●).

EPA production in cyanobacterial cells

The cyanobacterial transconjugant harbouring pJRDEPA was cultured at several growth temperatures. Table 1 shows the fatty acid compositions of the transconjugant and wild-type *Synechococcus* sp. NKBG042902 grown at 29 and 23 °C, and at a culture temperature of 23 °C but transferred to 17 °C for 1 d before fatty acids were analysed. There was no apparent difference in the fatty acid composition between the wild-type and transconjugant. EPA production was not detected in the wild-type, whereas cyanobacterial transconjugants harbouring pJRDEPA produced EPA. The

transconjugant grown at 23 °C appeared to have a higher EPA content [$0.4 \pm 0.3\%$; $0.56 \pm 0.37 \text{ mg (g dry weight)}^{-1}$] than that grown at 29 °C ($\leq 0.1\%$; $\leq 0.12 \text{ mg (g dry weight)}^{-1}$). The highest amount of EPA was produced when the temperature was lowered to 17 °C for 1 d under light and aeration [$0.5 \pm 0.2\%$; $0.64 \pm 0.27 \text{ mg (g dry weight)}^{-1}$].

Stability of plasmid in cyanobacterial cells

Fig. 3 shows the growth curve of the cyanobacterial transconjugant harbouring pJRDEPA at 23 °C and the loss of this plasmid during growth. The ratio of cells harbouring pJRDEPA to total cells decreased from an initial value of almost 100% to 58% in the stationary phase ($4.8 \times 10^6 \text{ cells ml}^{-1}$).

GC-MS analysis of EPA produced by *Synechococcus* sp. harbouring pJRDEPA

EPA produced by *E. coli* and the cyanobacterial transconjugant that was identified by gas chromatography analysis was further characterized by mass spectral analysis (Fig. 4). Both organisms produced a substance detected at m/z 316 that corresponds to the molecular mass of EPA. An ion peak typically detected in highly unsaturated fatty acid at m/z 79 was also present. These results indicate that the product was EPA.

DISCUSSION

Transconjugation using a cosmid vector with an origin of replication from a *Nostoc* sp. plasmid was reported in the freshwater filamentous cyanobacterium *Anabaena* sp. PCC 7120 and in *Synechococcus* sp. PCC 7920 (Wolk *et al.*, 1988; Vega-Palas *et al.*, 1990; Buikema &

Fig. 4. Mass chromatograms of EPA produced by *E. coli* (a) and the cyanobacterial transconjugant (b).

Haselkorn, 1991). In these studies, however, only small fragments were cloned and successfully transferred to the host. Furthermore, these experiments were carried out for the purpose of gene isolation. In the present study, a very large gene cluster encoding proteins required in EPA synthesis was introduced into and expressed in the marine cyanobacterium *Synechococcus* sp., which normally produced unsaturated fatty acids only up to C18:3. This result should lead to further investigations on the cloning and expression of genes encoding enzymes comprising multiple subunits. Since deletion of any ORF in the EPA synthesis gene cluster cloned into *E. coli* resulted in lack of EPA production as well as absence of intermediate products (Yazawa, 1996), it is not clear which fatty acid was used for EPA synthesis. Accordingly, few differences were observed in the fatty acid composition of the cyanobacterial trans-conjugant and the wild-type. Yazawa (1996) showed that EPA production in *E. coli* was higher at a lower temperature than the optimum growth temperature; this finding is corroborated by our results with trans-conjugants of *Synechococcus* sp.

Plasmid pEPA possesses T3 and T7 promoters upstream of the EPA synthesis gene cluster, but pJRDEPA does not. Nevertheless, EPA production in *E. coli* harbouring pEPA was not higher than that in *E. coli* harbouring pJRDEPA. These results strongly suggest that the EPA synthesis genes contain their own promoters which work in *E. coli* and in cyanobacterial cells. However, the amount of EPA produced by the cyanobacterial trans-conjugant was much lower than that of *E. coli*. One reason may be a lower copy number of the broad-host-range cosmid vector in the cyanobacterium as compared to *E. coli*. The copy number of pJRD215 was estimated to be less than five per cyanobacterial cell (data not shown), and that of the 48 kb pJRDEPA must be even lower. Furthermore, plasmid replication of very large cosmids may not follow cell division, and cells without plasmids may already have been enriched when cells were extracted for EPA analysis. Introduction of a cyanobacterial replication region to a cosmid vector might enhance the stability of the transferred plasmid, consequently increasing EPA production.

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