

## Characterization of cyanobacterial cells synthesizing 10-methyl stearic acid

著者別名	鈴木 石根
journal or publication title	Photosynthesis Research
year	2018-06-25
権利	This is a post-peer-review, pre-copyedit version of an article published in Photosynthesis Research. The final authenticated version is available online at: <a href="https://doi.org/10.1007/s11120-018-0537-5">https://doi.org/10.1007/s11120-018-0537-5</a> ” .
URL	<a href="http://hdl.handle.net/2241/00152135">http://hdl.handle.net/2241/00152135</a>

doi: 10.1007/s11120-018-0537-5

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1 **Characterization of cyanobacterial cells synthesizing 10-methyl**  
2 **stearic acid**

3  
4 **Authors**

5 Shuntaro Machida<sup>a, b</sup> and Iwane Suzuki<sup>c,\*</sup>

6  
7 **Affiliations**

8 *<sup>a</sup>Graduate School of Life and Environmental Sciences, University of Tsukuba, Tennoudai 1-1-1,*  
9 *Tsukuba, Ibaraki 305-8572, Japan*

10 *<sup>b</sup>Food Research Institute, National Agriculture and Food Research Organization, Kannondai*  
11 *2-1-12, Tsukuba, Ibaraki 305-8642, Japan*

12 *<sup>c</sup>Faculty of Life and Environmental Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba,*  
13 *Ibaraki 305-8572, Japan*

14  
15 **\*Corresponding author**

16 Faculty of Life and Environmental Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba,  
17 Ibaraki 305-8572, Japan. Tel: +81-29-853-4908, Fax: +81-29-853-6614

18 E-mail address: iwanes6803@biol.tsukuba.ac.jp

19 **Abstract**

20

21 Recently, microalgae have attracted attention as sources of biomass energy. However, fatty  
22 acids from the microalgae are mainly unsaturated and show low stability in oxygenated  
23 environments, due to oxidation of the double bonds. The branched-chain fatty acid, 10-methyl  
24 stearic acid, is synthesized from oleic acid in certain bacteria; the fatty acid is saturated, but  
25 melting point is low. Thus, it is stable in the presence of oxygen and is highly fluid. We  
26 previously demonstrated that BfaA and BfaB in *Mycobacterium chlorophenolicum* are involved  
27 in the synthesis of 10-methyl stearic acid from oleic acid. In this study, as a consequence of the  
28 introduction of *bfaA* and *bfaB* into the cyanobacterium, *Synechocystis* sp. PCC 6803, we  
29 succeeded in producing 10-methyl stearic acid, with yields up to 4.1% of the total fatty acid  
30 content. The synthesis of 10-methyl stearic acid in *Synechocystis* cells did not show a significant  
31 effect on photosynthetic activity, but the growth of the cells was retarded at 34°C. We observed  
32 that the synthesis of 10-methylene stearic acid, a precursor of 10-methyl stearic acid, had an  
33 inhibitory effect on the growth of the transformants, which was mitigated under microoxic  
34 conditions. Eventually, the amount of 10-methyl stearic acid present in the  
35 sulfoquinovosyldiacylglycerol and phosphatidylglycerol of the transformants was remarkably  
36 higher than that in the monogalactosyldiacylglycerol and digalactosyldiacylglycerol. Overall,

37 we successfully synthesized 10-methyl stearic acid in the phototroph, *Synechocystis*,  
38 demonstrating that it is possible to synthesize unique modified fatty acids via photosynthesis  
39 that are not naturally produced in photosynthetic organisms.

40

#### 41 **Keywords**

42

43 10-methyl octadecanoic acid, *bfaAB*, microalgae, mid-chain methyl-branched fatty acid,  
44 *Synechocystis* sp. PCC 6803, tuberculostearic acid.

45

#### 46 **Abbreviations**

47

48 MGDG; monogalactosyldiacylglycerol, DGDG; digalactosyldiacylglycerol, SQDG;  
49 sulfoquinovosyldiacylglycerol, PG; phosphatidylglycerol, *cobfaAB*; codon optimized *bfaA* and  
50 *bfaB*, GC; gas chromatography, FAME; fatty acid methyl ester, 16:0; palmitic acid, 16:1 $\Delta$ 9;  
51 palmitoleic acid, 18:0; stearic acid, 18:1 $\Delta$ 9; oleic acid, 18:2 $\Delta$ 9,12; linoleic acid, 18:3 $\Delta$ 6,9,12;  
52  $\gamma$ -linolenic acid, 18:3 $\Delta$ 9,12,15;  $\alpha$ -linolenic acid, 18:4 $\Delta$ 6,9,12,15; stearidonic acid, 19:0Me10;  
53 10-methyl stearic acid, 19:1 $\Delta$ Me10; 10-methylene stearic acid.

54

55 **Introduction**

56

57 Contemporary society is dependent on the consumption of enormous quantities of fossil fuels.

58 The fossil fuels are used not only as resources for transportation and generation of electricity at

59 the large-scale but also as raw materials for the production of various chemicals. However, the

60 use of fossil fuels is thought to accelerate global warming and increase environmental pollution.

61 Moreover, the demand for fossil fuels is rising due to global industrial expansion, while the

62 availability of these fuels is gradually diminishing. Therefore, exploitation of alternative

63 sources of liquid fuels is required to meet the needs of the society. In recent years, microalgae

64 have attracted attention as next-generation sources of biomass energy because of their high

65 productivity and because they do not compete directly with the production of land crops, which

66 are the primary sources of foods (Chisti 2007; Parmar et al. 2011). Methyl esters of fatty acids

67 from microalgae are primarily expected to serve as biodiesel. However, most of the fatty acids

68 in microalgae are C16–22 saturated and unsaturated fatty acids. The saturated fatty acids are

69 stable against atmospheric oxidation, but they solidify at ambient temperatures due to their

70 melting points being high. In contrast, the melting points of the polyunsaturated fatty acids are

71 relatively low, and they are fluid at ambient temperatures. However, carbon-carbon double

72 bonds in the carbon skeleton of polyunsaturated fatty acids are unstable due to susceptibility to

73 oxidation, making long-term storage difficult. These characteristics of fatty acids from the  
74 microalgae limit their application as liquid fuels. Previously, we developed *Synechocystis* cells  
75 which produce cyclopropane fatty acids, *cis*-9,10-methylene hexadecanoic and octadecanoic  
76 acids, by the introduction of *cfa* for cyclopropane fatty acid synthase from *Escherichia coli*  
77 (Machida et al. 2016). Finally, the ratio of the cyclopropane fatty acids in the total fatty acid  
78 content in the cells comprises more than 30%. To obtain the more stable fatty acid than the  
79 cyclopropane fatty acids, we attempted to develop cells producing branched-chain fatty acid.

80           In living organisms, modified fatty acids are essential for the functioning of the cellular  
81 membranes and storage of lipids, where the fatty acids are esterified (Kniazeva et al. 2004).  
82 Certain bacteria produce methylated fatty acids, such as cyclopropane fatty acids, branched-  
83 chain fatty acids, and mycolic acids (Akamatsu and Law 1970; Cronan et al. 1974; Takayama  
84 et al. 2005). As a branched-chain fatty acid, 10-methyl stearic acid (19:0Me10), also called  
85 tuberculostearic acid or 10-methyl octadecanoic acid, is primarily known as a significant  
86 component of the lipids of tubercle bacilli (Lennarz et al. 1962). The melting-point of 19:0Me10  
87 is low (13.2°C), and the fatty acid is resistant to oxidation because it is saturated and branched.  
88 It had been hypothesized that 19:0Me10 is produced by a two-step biosynthetic pathway  
89 (Akamatsu and Law 1970; Jaureguiberry et al. 1965). The first step of biosynthesis is the  
90 methylenation of oleic acid (18:1Δ9) with *S*-adenosyl-*L*-methionine as the methyl donor. The

91 10-methylene stearic acid (19:1 $\Delta$ Me10) formed has been identified in cells of *Corynebacterium*  
92 *urealyticum* (Couderc et al. 1991). The second step is the reduction of 19:1 $\Delta$ Me10 to 19:0Me10,  
93 with NADPH as the reducing agent (Akamatsu and Law 1970). Our previous study revealed  
94 that BfaB and BfaA from *Mycobacterium chlorophenicum* catalyze these two steps,  
95 respectively (Machida et al. 2017). By heterologous expression of *bfaA* and *bfaB* in *Escherichia*  
96 *coli*, 19:0Me10 is synthesized *in vivo* from 18:1 $\Delta$ 9, which was supplemented in the media; cells  
97 expressing only *bfaB* produce 19:1 $\Delta$ Me10.

98 Fatty acids are the main constituents of cell membranes in all living organisms. The  
99 unsaturated fatty acids are essential to maintaining membrane fluidity, which is critical for  
100 membrane function. Acyl-lipid desaturases of cyanobacteria introduce double bonds at specific  
101 positions in the fatty acids that are esterified with the glycerol backbone of the membrane lipids  
102 (Murata et al. 1992). The genes *desA*, *desB*, *desC*, and *desD* of *Synechocystis* sp. PCC 6803  
103 encode the acyl-lipid desaturases that introduce double bonds at the positions  $\Delta$ 12,  $\Delta$ 15,  $\Delta$ 9,  
104 and  $\Delta$ 6, respectively, of the C18 fatty acids bound at the *sn*-1 position of the lipids. In  
105 *Synechocystis*, the expression of *desA*, *desB*, and *desD* is induced under low-temperature  
106 conditions (Los et al. 1997). The membrane lipids of *Synechocystis* cells are composed of  
107 glycolipids, such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol  
108 (DGDG), and sulfoquinovosyldiacylglycerol (SQDG) and a single phospholipid,

109 phosphatidylglycerol (PG). Oxygenic photosynthetic organisms producing the unusual  
110 modified fatty acids, such as branched-chain fatty acids, were not found in nature until now.  
111 The laboratory-based synthesis of 19:0Me10 in *Synechocystis* is the first attempt.

112           In this study, we synthesized the fatty acid 19:0Me10 which is saturated and has a low  
113 melting point in *Synechocystis* by expressing the *M. chlorophenolicum* genes, *bfaA* and *bfaB*.  
114 To increase the yield of 19:0Me10 *in vivo*, we also examined the effects of mutations in *desA*  
115 and *desD* in *Synechocystis*, which lead to accumulation of 18:1 $\Delta$ 9, such that 18:1 $\Delta$ 9 comprises  
116 more than 40% of the total fatty acid content (Tasaka et al. 1996). We analyzed changes in fatty  
117 acid composition, growth, and respiratory and photosynthetic activities in the *Synechocystis*  
118 transformants.

119

120

## 121 **Materials and Methods**

122

### 123 **Organisms and culture conditions**

124

125 A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (Williams 1988) was used as the wild-  
126 type strain in this study. The *Synechocystis* cells were grown in BG11 medium (Stanier et al.



127 1971) buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–  
128 NaOH (pH 7.5) at 34°C or 24°C under continuous illumination at 70  $\mu\text{mole photons m}^{-2} \text{ s}^{-1}$  by  
129 white fluorescent lamps and aerated with 1% (v/v) CO<sub>2</sub>-enriched air (Wada and Murata 1989).  
130 For screening of transformants and maintaining of *Synechocystis* cells, we used BG11 medium  
131 solidified with 1.5% (w/v) Bacto-agar (BD Biosciences Japan, Tokyo, Japan) including the 25  
132  $\mu\text{g/mL}$  kanamycin sulfate, 25  $\mu\text{g/mL}$  spectinomycin dihydrochloride pentahydrate, or 25  $\mu\text{g/mL}$   
133 chloramphenicol (Wako Pure Chemicals, Osaka, Japan), depending on the selection markers.  
134 For growth under microoxic conditions, liquid cultures were bubbled with 1% (v/v) CO<sub>2</sub>-mixed  
135 nitrogen gas (Japan Fine Products, Kanagawa, Japan).

136 *E. coli* strain JM109 (Yanisch-Perron et al. 1985) was grown in 1.8 mL of LB medium  
137 (Bertani 1951) at 37°C with shaking at 200 rpm. All transformants of *E. coli* were maintained  
138 on LB medium solidified with 1.5% (w/v) Bacto-agar in the presence of 50  $\mu\text{g/mL}$  sodium  
139 ampicillin or 50  $\mu\text{g/mL}$  spectinomycin dihydrochloride pentahydrate (Wako Pure Chemicals),  
140 depending on the selection markers.

141

## 142 **Plasmid construction and transformation**

143

144 To express the heterologous target genes in *Synechocystis*, we constructed five plasmids,

145 pTHT2031-bfaA-S, pTHT2031-bfaB-S, pTHT2031-bfaAB-S, pTHT2031-cobfaAB-S, and  
146 pTC2031-cobfaAB-S (Table S1), which were derived from the expression vector,  
147 pTCHT2031v (Ishizuka et al. 2006). Fig. S1 shows the processes for the construction of these  
148 plasmids. The plasmid pTCHT2031v contains five DNA fragments in the following order: the  
149 upstream sequence of *slr2031* (*slr2031up*), a chloramphenicol resistance gene cassette ( $\text{Cm}^r$ ),  
150 the *trc* promoter sequence ( $\text{Ptrc}$ ), the downstream sequence of *slr2031* (*slr2031dn*), and the  
151 plasmid backbone of the pUC vector (Ishizuka et al. 2006). First, to replace the selection marker  
152  $\text{Cm}^r$  with the spectinomycin resistance gene cassette ( $\text{Sp}^r$ ), we constructed pTHT2031, a  
153 plasmid lacking  $\text{Cm}^r$ , from pTCHT2031v by polymerase chain reaction (PCR) amplification of  
154 the entire sequence of pTCHT2031v, except the  $\text{Cm}^r$  sequence, using the primer set,  
155 pTCHT\_Cm\_remove\_InF\_F and pTCHT\_Cm\_remove\_InF\_R (Table S2). The resulting  
156 fragment was circularized with In-Fusion<sup>®</sup> HD cloning kit (Takara Bio, Ōtsu, Japan). The  
157 genomic fragments corresponding to only *bfaB*, and to both *bfaA* and *bfaB*, were amplified by  
158 PCR using *M. chlorophenolicum* JCM 7439 chromosomal DNA as the template and primer sets,  
159 *bfaB*\_Nde\_F and *bfaB*\_Bam\_R and *bfaA*\_Nde\_F and *bfaB*\_Bam\_R, respectively. The  
160 amplified DNA fragments were subcloned into a T-vector pMD19 simple vector (Takara Bio)  
161 to obtain the plasmids pMD-bfaB and pMD-bfaA-4-bfaB. The DNA sequences of the inserts  
162 were confirmed by using BigDye<sup>®</sup> Terminator v.3.1 (Life Technologies, Foster City, CA, USA)

163 and ABI 3130 Genetic Analyzer (Life Technologies). We then performed PCR to amplify a  
164 DNA fragment containing the Sp<sup>r</sup> cassette using pAM1146 (Tsinoremas et al. 1994) as the  
165 template and the primer set, Sp\_Bgl\_F and Sp\_Bam\_R. The Sp<sup>r</sup> fragment was digested with  
166 *Bgl*III and *Bam*HI, inserted into *Bam*HI-cleaved pMD-bfaB and pMD-bfaA-4-bfaB, to obtain  
167 pMD-bfaB-S and pMD-bfaA-4-bfaB-S, respectively. We selected plasmids in which the Sp<sup>r</sup>  
168 fragments were transcribed in the same orientation as the genes of interest that were to be  
169 expressed. The fragments containing bfaB-Sp<sup>r</sup> and bfaA-4-bfaB-Sp<sup>r</sup> were excised from these  
170 plasmids by *Nde*I and *Bam*HI digestion, and inserted into pTHT2031 digested with the same  
171 restriction enzymes, to obtain pTHT2031-bfaB-S and pTHT2031-bfaA-4-bfaB-S, respectively.  
172 The native sequence of *bfaA* and *bfaB* shows a 4-bp overlap between the genes. We previously  
173 observed that the Shine–Dalgarno (SD) sequence is essential for the stable translation of the  
174 BfaB protein in *E. coli* cells (Machida et al. 2017). To insert the canonical SD sequence (5'-  
175 AGGAGGAATAAACC-3'), which is also present in the *trc* promoter region of the original  
176 pTCHT2031v (Ishizuka et al. 2006) between the two open reading frames of *bfaA* and *bfaB*,  
177 pTHT2031-bfaA-4-bfaB-S was amplified using the primer set, SD\_add\_I\_F and SD\_add\_I\_R,  
178 and circularized using In-Fusion<sup>®</sup> HD cloning kit to obtain pTHT2031-bfaAB-S. To construct  
179 pTHT2031-bfaA-S, pTHT2031-bfaA-4-bfaB-S was linearized by PCR using the primer set,  
180 Sp\_up\_F and bfaA\_dn\_inf\_R, and circularized using the In-Fusion<sup>®</sup> HD cloning kit.

181 Both *bfaA* and *bfaB* were artificially synthesized (Life Technologies Japan, Tokyo),  
182 and optimized for codon usage by *Synechocystis* (*cobfaAB*) (Fig. S2). The *cobfaAB* fragment  
183 was amplified from pEX-*cobfaAB* using the primer set, *coBfaAB\_trc\_inf\_F* and  
184 *coBfaAB\_Sp\_inf\_R*. After linearization of pTHT2031-*bfaAB-S*, using the primer set, *Sp\_up\_F*  
185 and *pTHT\_trcdn\_R*, it was ligated with the *cobfaAB* fragment using In-Fusion<sup>®</sup> HD cloning  
186 kit, resulting in the formation of pTHT2031-*coBfaAB-S*.

187 Two fragments, which were amplified from pTHT2031 and *Synechocystis*  
188 chromosomal DNA using the primer sets, *pTHT\_cpc\_inf\_F* and *pTHT\_cpc\_inf\_R* and  
189 *cpc560\_F* and *cpc560\_R*, respectively, were ligated using the In-Fusion<sup>®</sup> HD cloning kit to  
190 produce pTC2031. We then performed PCR to amplify a DNA fragment containing the *Sp<sup>f</sup>*  
191 cassette using pAM1146 as the template and the primer set, *Sp\_Bgl\_F* and *Sp\_Bam\_R*. After  
192 digestion of pTC2031 and the amplified fragment containing *Sp<sup>f</sup>* with *NdeI* and *BglIII*, we  
193 constructed pTC2031-S by ligation. To obtain plasmid pTC2031-*cobfaAB-S*, pTC2031-S was  
194 linearized by PCR using the primer set, *Sp\_up\_F* and *cpc560\_R*, and ligated with the *cobfaAB*  
195 fragment using primer set *coBfaAB\_cpc\_inf\_F* and *coBfaAB\_Sp\_inf\_R*.

196

## 197 **Fatty acid analysis**

198

199 The fatty acid profiles of the *Synechocystis* transformants were examined using the methods  
200 described in our previous studies (Kotajima et al. 2014; Machida et al. 2016; Machida et al.  
201 2017). The cells were precipitated by centrifugation, re-suspended in 2 mL of methanol, and  
202 transferred to glass test tubes. After thoroughly drying by a concentrating centrifuge (CC-105,  
203 Tomy Seiko, Tokyo, Japan), the residue was re-suspended in 0.1 M hydrochloric acid  
204 methanolic solution (Wako Pure Chemicals). The tubes were tightly capped and incubated at  
205 100°C for 1 h to allow for methyl esterification of the acyl groups in the lipids and conversion  
206 into fatty acid methyl esters (FAMES). The resulting FAMES were recovered using *n*-hexane.  
207 The recovered hexane phases were evaporated, and the residues containing the FAMES were  
208 dissolved in 200 µL of *n*-hexane. To identify the FAMES of palmitic acid (16:0), palmitoleic  
209 (16:1Δ9), stearic acid (18:0), 18:1Δ9, linoleic acid (18:2Δ9,12), γ-linolenic acid (18:3Δ6,9,12),  
210 α-linolenic acid (18:3Δ9,12,15), stearidonic acid (18:4Δ6,9,12,15), 19:0Me10, and 19:1ΔMe10,  
211 we performed gas chromatography (GC) using a GC-2010 gas chromatograph equipped with a  
212 QP-2010 mass spectrometer (Shimadzu, Kyoto, Japan). Helium was used as a carrier gas at a  
213 constant flow rate of 1.25 mL/min in splitless mode. A CP-Sil5 CB column (Agilent  
214 Technologies, Santa Clara, CA, United States) was used at the following temperatures: 60°C  
215 for 1.5 min, followed by 130°C at a temperature increase rate of 20°C/min, and then a further  
216 increase to 230°C at an increase rate of 4°C/min. We confirmed the retention times and mass

217 spectra using commercial FAME standards (Nu-Chek Prep, Elysian, MN, >99%) and  
218 methylesterified standard of 19:0Me10 (Larodan Fine Chemicals, Malmö, Sweden,  $\geq 97\%$ ). To  
219 quantify the FAMES, we applied 1  $\mu\text{L}$  of the hexane solution to a GC-2014 gas chromatograph  
220 equipped with a flame ionization detector (Shimadzu). The conditions of GC were identical to  
221 those used for FAME identification.

222

### 223 **Separation of lipid classes**

224

225 In order to analyze the fatty acid composition attached to the lipids, the harvested cells were  
226 stored at  $-80^\circ\text{C}$ , and freeze-dried using FDU-1100 (Tokyo Rikakikai, Tokyo, Japan). The lipids  
227 were extracted with chloroform/methanol solution (2:1, v/v). The cell debris was precipitated  
228 by centrifugation, and the resulting supernatant was transferred into new tubes and evaporated.  
229 The samples were then re-suspended in 300  $\mu\text{l}$  chloroform/methanol solution (2:1, v/v), and  
230 applied to a silica gel plate (Silicagel 70 FM Plate, Wako Pure Chemicals). The lipids were  
231 separated by thin-layer chromatography using acetone/toluene/water (91:30:7, v/v/v) as an  
232 eluent. The spots of each lipid were detected by staining with primulin, and then the silica gel  
233 corresponding to the lipid spots was scraped off. The fatty acids in the lipids obtained from  
234 silica gel were saponified and analyzed as described above.

235

236 **Photosynthetic and respiratory activities**

237

238 Photosynthetic and respiratory activities were measured as evolution and absorption of oxygen,  
239 respectively, using an oxygen electrode (Oxytherm System, Hansatech, Norfolk, UK). The  
240 liquid culture of *Synechocystis* cells were grown at 34°C or 24°C for 1 d and assayed at the  
241 same temperature. Photosynthetic activity in the samples was measured at a light intensity of  
242 600  $\mu\text{mole photons m}^{-2} \text{ s}^{-1}$ , which represented saturated light conditions. Sodium bicarbonate  
243 (2.5 mM) was added to the cell suspensions as the carbon source. Respiratory activity was  
244 measured under dark conditions.

245

246

247 **Results**

248

249 **Fatty acid analysis of *Synechocystis* cells transformed with *bfaA* and *bfaB***

250

251 Mycobacteria, including the members of the genera *Mycobacterium*, *Nocardia*, *Rhodococcus*,  
252 and *Corynebacterium*, synthesize the branched-chain fatty acid 19:0Me10. In our previous

253 study (Machida et al. 2017), we demonstrated that BfaA and BfaB from *M. chlorophenolicum*  
254 are involved in the biosynthesis of 19:0Me10 using 18:1 $\Delta$ 9 as a substrate, which is one of the  
255 main components of fatty acids in *Synechocystis*. In the present study, we examined whether  
256 19:0Me10 was synthesized from 18:1 $\Delta$ 9 and incorporated into membrane lipids *in vivo* by the  
257 heterologous expression of *bfaA* and *bfaB* in wild-type *Synechocystis*.

258 In wild-type *Synechocystis* cells, 18:1 $\Delta$ 9, which is believed to be the substrate for BfaB,  
259 comprised  $10.3 \pm 0.8\%$  of the total fatty acid content (Table 1). In the strain containing *bfaA*  
260 and *bfaB* (*bfaAB*<sup>+</sup>), 19:0Me10 comprised  $1.7 \pm 0.4\%$  of the total fatty acid content.  
261 Simultaneously, the 18:1 $\Delta$ 9 content was slightly decreased to  $9.3 \pm 2.0\%$ . The relative amount  
262 of 18:2 $\Delta$ 9,12 in the *bfaAB*<sup>+</sup> strain was  $16.5 \pm 1.3\%$ , which was also lower than that in the wild-  
263 type cells ( $24.0 \pm 0.4\%$ ). These results indicated that BfaA and BfaB were functional in the  
264 *Synechocystis* cells and that 18:1 $\Delta$ 9 was used as the substrate.

265 To increase the amount of 19:0Me10 in the *Synechocystis* cells, we attempted to  
266 introduce *bfaA* and *bfaB* into the *desAD*<sup>-</sup> strain, in which *desA* and *desD*, encoding for  $\Delta$ 12 and  
267  $\Delta$ 6 desaturases, respectively, are disrupted and does not produce C18 polyunsaturated fatty  
268 acids (Tasaka et al. 1996). 18:1 $\Delta$ 9 constituted  $43.1 \pm 0.3\%$  of the total fatty acid content of the  
269 *desAD*<sup>-</sup> strain. Although we attempted to increase the amount of 19:0Me10 in the *desAD*<sup>-</sup>  
270 */bfaAB*<sup>+</sup> strain, it unexpectedly constituted only  $2.8 \pm 0.5\%$  of the total fatty acid content.



271 Moreover, the fatty acid composition of the *Synechocystis* cells grown at 24°C was analyzed.  
272 As a result, *bfaAB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup> strains comprised 0.8 ± 0.1% and 4.0 ± 0.4% of  
273 19:0Me10 to total fatty acid (Table 2).

274

### 275 **Analysis of growth and photosynthetic activity in *Synechocystis* transformants**

276

277 In *Synechocystis* cells, 19:0Me10 is an unnatural fatty acid. Therefore, we examined the effects  
278 of synthesis of this fatty acid on growth and photosynthetic activity of the *Synechocystis* cells.  
279 The photosynthetic and respiratory activities of *bfaAB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup> cells at 24°C and  
280 34°C showed no significant difference compared to those in each parental strain (Fig. 1),  
281 respectively, whereas the growth of *bfaAB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup> cells, at the 34°C, was reduced  
282 compared to those of the wild-type and *desAD*<sup>-</sup> cells, respectively (Fig. 2A and B). These results  
283 suggested that 19:0Me10 biosynthesis, or the expression of BfaA and BfaB, may disturb cell  
284 growth, but not the function of photosynthesis.

285         The cells of *bfaAB*<sup>+</sup> cultured at 24°C showed lower growth rate than the wild-type cells  
286 as well as the both types of cells cultured in 34°C (Fig. 2C). The *desAD*<sup>-</sup> strain scarcely grew at  
287 24°C, whereas the growth of the *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup> strain was slightly recovered (Fig. 2D) and  
288 was similar to the growth of the *bfaAB*<sup>+</sup> strain (Fig. 2C). It seems that the synthesis of 19:0Me10

289 in the *desAD*<sup>-</sup> strain which cannot synthesize any polyunsaturated fatty acids was helpful for  
290 maintenance of the membrane fluidity.

291

## 292 **Heterologous expression of *bfaA* or *bfaB* in *Synechocystis* cells**

293

294 Because the cells transformed with *bfaA* and *bfaB* showed a decline in growth, we hypothesized  
295 that the production or incorporation of 19:0Me10 into the membrane lipids, and the expression  
296 of BfaA and/or BfaB, were harmful to the cells. To investigate this, cells expressing either BfaA  
297 or BfaB were generated and assayed.

298 In our previous study, *E. coli* cells expressing the only *bfaB* accumulated 19:1ΔMe10,  
299 which is the precursor of 19:0Me10 (Machida et al. 2017). Similarly, *Synechocystis* cells of  
300 strains *bfaB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaB*<sup>+</sup>, which were transformed with only the *bfaB* gene, produced  
301  $1.2 \pm 0.1\%$  and  $1.8 \pm 0.2\%$ , respectively, of 19:1ΔMe10 relative to the total fatty acid content  
302 (Table 1). The cells of *bfaA*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaA*<sup>+</sup> expressing the only *bfaA*, which is essential  
303 for the reduction of 19:1ΔMe10 to 19:0Me10, did not show a significant difference in fatty  
304 acid composition compared with the respective parental strains. The growth of *bfaA*<sup>+</sup> and  
305 *desAD*<sup>-</sup>/*bfaA*<sup>+</sup> strains was also almost the same as that of the wild-type and *desAD*<sup>-</sup> cells,  
306 respectively (Fig. 2A and B). In contrast, *bfaB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaB*<sup>+</sup> strains showed significant

307 decreases in growth compared with their respective parental strains. Moreover, the growth of  
308 cells expressing both *bfaA* and *bfaB* (*bfaAB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup>) was slightly better than that  
309 of the cells expressing only *bfaB* (*bfaB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaB*<sup>+</sup>). These results indicated that  
310 19:1ΔMe10, which is a precursor of 19:0Me10, may be toxic to the cells, and its reduction by  
311 BfaA decreased the toxicity.

312

### 313 **Cultivation of cells under microoxic conditions**

314

315 A methylene group (C=CH<sub>2</sub>) is present in the middle of the acyl chain of 19:1ΔMe10. Because  
316 this functional group may be somewhat reactive, it is predicted that it may produce radicals  
317 under an oxygenic environment, resulting in oxidative stress in the cells. In the studies  
318 mentioned above, the *Synechocystis* cells were cultured under conditions bubbling of air  
319 containing 1% (v/v) CO<sub>2</sub> through the media. In the present study, to diminish the oxidative  
320 stress to cells, which is anticipated to be caused by the synthesis of 19:1ΔMe10, the cells were  
321 cultured under microoxic conditions with 1% (v/v) CO<sub>2</sub> mixed in nitrogen gas.

322 Fig. 2E shows the cell growth in microoxic conditions. The decreased growth of *bfaB*<sup>+</sup>  
323 and *bfaAB*<sup>+</sup> strains shown in Fig. 2A was alleviated by the microoxic conditions. However, the  
324 growth of the transformants was still slightly lower than that of the wild-type strain. Even a

325 small amount of oxygen synthesized during photosynthesis may trigger oxidative stress in the  
326 cells producing 19:1 $\Delta$ Me10. The growth of the wild-type cells in microoxic conditions was  
327 slightly slower than that in aerobic conditions. It is speculated that the cells cultivated in the  
328 microoxic conditions may have interfered with respiration, which subsequently retarded the  
329 growth rate. Moreover, analysis of the fatty acid composition of the cells cultured under  
330 microoxic conditions revealed no significant difference compared with that of cells cultured  
331 under aerobic conditions (Table S3).

332

### 333 **Transfection of codon-optimized *bfaA* and *bfaB* and the *cpc* promoter**

334

335 In the *bfaAB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup> strains, 19:0Me10 constituted  $1.7 \pm 0.4\%$  and  $2.8 \pm 0.5\%$  of  
336 the total fatty acid content, respectively (Table 1). To improve the production of 19:0Me10 in  
337 *Synechocystis* cells, we introduced codon optimized (co) *bfaA* and *bfaB* (Fig. S2), and the *cpc*  
338 promoter instead of the *trc* promoter. The *cpc* promoter is involved in the expression of the  
339 genes for the cyanobacterial antenna protein phycocyanin, which is one of the most abundantly  
340 synthesized proteins in the cells, and has been reported to be one of the most robust promoters  
341 inducing higher expression in *Synechocystis* cells of exogenous genes than the *trc* promoter (Ng  
342 et al. 2015; Zhou et al. 2014).

343 Plasmids pTHT2031-cobfaAB-S, in which the codon-optimized *bfaA* and *bfaB* genes  
344 are driven by *trc* promoter, and pTC2031-cobfaAB-S, in which these genes driven by *cpc*  
345 promoter, were transformed into wild-type *Synechocystis* and *desAD*<sup>-</sup> cells. As a result, in the  
346 wild-type cells expressing both *cobfaA* and *cobfaB* under the regulation of *trc* and *cpc*  
347 promoters, 19:0Me10 constituted  $2.3 \pm 0.2\%$  and  $1.3 \pm 0.1\%$  of the total fatty acid content,  
348 respectively (Table 3). In contrast, in the *desAD*<sup>-</sup> cells expressing the *cobfaA* and *cobfaB* under  
349 the regulation of *trc* and *cpc* promoters, 19:0Me10 consisted of  $4.1 \pm 0.6\%$  and  $2.7 \pm 0.1\%$  of  
350 the total fatty acid content, respectively. The *desAD*<sup>-</sup> strain expressing both *cobfaA* and *cobfaB*  
351 under the regulation of *trc* promoter showed the highest production of 19:0Me10 in the study,  
352 which was approximately two times higher than that in the wild-type cells expressing both *bfaA*  
353 and *bfaB* under regulation of *trc* promoter at  $1.7 \pm 0.4\%$ . While the production of 19:0Me10 in  
354 the cells expressing *cobfaA* and *cobfaB* under the control of *cpc* promoter was lower than that  
355 in the cells expressing *bfaA* and *bfaB* under the control of *trc* promoter. These results indicated  
356 that optimization of codon usage improved the efficiency of translation of BfaA and BfaB, and  
357 contributed to increased production of 19:0Me10.

358

### 359 **Fatty acid composition of each lipid class in *Synechocystis* cells**

360

361 The highest production of 19:0Me10 reported in this study was  $4.1 \pm 0.6\%$  of the total fatty acid  
362 content (Table 3). The source of *bfaA* and *bfaB* is *M. chlorophenolicum*, in which 19:0Me10  
363 constitutes 14% of the total fatty acid content (Hagglblom et al. 1994). Although the target  
364 genes were overexpressed in *Synechocystis* cells, along with a significant amount of 18:1 $\Delta$ 9 as  
365 the substrate for 19:0Me10, the production of 19:0Me10 in *Synechocystis* transformants was  
366 lower than that in *M. chlorophenolicum*. As a reason why the productivity of 19:0Me10 was  
367 kept at the low level, we considered the differences in the lipid classes in *Synechocystis* and *M.*  
368 *chlorophenolicum*. The lipids in *M. chlorophenolicum* are phospholipids, including  
369 phosphatidylethanolamine, PG, diphosphatidylglycerol, phosphatidylinositol, and  
370 phosphatidylinositol mannosides (Hagglblom et al. 1994). In contrast, the lipids in  
371 *Synechocystis* are primarily glycolipids, including 50% of MGDG 10% to 20% of DGDG,  
372 SQDG, and PG (Wada and Murata 1990; Wada et al. 1994). Based on this information, we  
373 speculated that BfaA and BfaB would preferentially modify 18:1 $\Delta$ 9 bound to the phospholipid,  
374 as the substrate. We fractionated the lipids extracted from *Synechocystis* transformants using  
375 thin-layer chromatography and analyzed the fatty acid composition in each lipid class.

376 The composition of MGDG, DGDG, SQDG, and PG was approximately 49%, 18%,  
377 30%, and 3% of the total lipid extracted from the *bfaAB*<sup>+</sup> strain, respectively. These percentages  
378 were not significantly different from those previously reported for the wild-type cells (Wada

379 and Murata 1990; Wada et al. 1994), or from values obtained in this study (data not shown),  
380 suggesting that the expression of *bfaA* and *bfaB* did not affect the lipid composition of the cells.  
381 As a result, the amount of 19:0Me10 esterified to MGDG was  $0.3 \pm 0.1\%$  of the total fatty acid  
382 content and it esterified to DGDG was trace amount in the *bfaAB*<sup>+</sup> strain, whereas for SQDG  
383 and PG, it was  $3.4 \pm 0.4$  and  $6.4 \pm 1.6\%$  (Table 4). Moreover, in the *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup> strain,  
384 MGDG was included only  $0.6 \pm 0.1\%$  of 19:0Me10, while SQDG and PG were included  $5.7 \pm$   
385  $1.6\%$  and  $8.8 \pm 2.2\%$ . In both the *bfaAB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup> strains, the amount of 19:0Me10  
386 bound to the SQDG and PG was remarkably higher than that bound to MGDG and DGDG.  
387 Especially, the ratios of 19:0Me10 to total fatty acid comprised in PG extracted from the *bfaAB*<sup>+</sup>  
388 and *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup> strains were approximately 4 and 3 times higher than those in total lipid  
389 contents extracted from both strains, respectively. On the other hand, the ratios of 18:1 $\Delta$ 9 to the  
390 total fatty acid comprised in SQDG and PG was not significantly altered in that in MGDG, and  
391 DGDG in the *bfaAB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup> strains. These results indicated that 18:1 $\Delta$ 9 which  
392 binds to SQDG and PG is likely to be specifically converted into 19:0Me10 by the action of  
393 BfaA and BfaB.

394

395

396 **Discussion**

397

398 **Synthesis of branched-chain fatty acid in *Synechocystis* and substrate specificity of BfaA**  
399 **and BfaB**

400

401 We succeeded in synthesizing 19:0Me10 *in vivo* by the introduction of *bfaA* and *bfaB* into  
402 *Synechocystis*, with 18:1Δ9 accumulation in the cells being slightly decreased (Table 1). To  
403 increase the total relative amount of 19:0Me10 in the cells, we introduced *bfaA* and *bfaB* into  
404 the *desAD*<sup>-</sup> strain, which accumulates 18:1Δ9 to a much higher level than do the wild-type cells.  
405 However, the amount of 19:0Me10 in *bfaA*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaA*<sup>+</sup> strains showed no significant  
406 difference. Additionally, we attempted to employ codon optimized *bfaA* and *bfaB*, and the *cpc*  
407 promoter, but the amount of 19:0Me10 in the transformants remained at less than 5% of the  
408 total fatty acid content (Table 3). We predicted that differences in the lipid composition between  
409 *Synechocystis* and *M. chlorophenolicum*, the source of *bfaA* and *bfaB*, might be problematic.  
410 Fatty acid analysis of each lipid type revealed the amount of 19:0Me10 incorporated in SQDG  
411 and PG was remarkably higher than that in MGDG and DGDG of the *Synechocystis*  
412 transformants (Table 4). These results suggest that BfaA and BfaB may specifically modify the  
413 18:1Δ9 that is bound to SQDG and PG, and the production of 19:0Me10 may be limited in  
414 *Synechocystis* since the amount of SQDG and PG in *Synechocystis* is only about 20%–40% of



415 the total lipid (Wada and Murata 1990; Wada et al. 1994). The head groups of both SQDG and  
416 PG are negatively charged by sulfate- and phosphate-groups, respectively. BfaA and BfaB  
417 might have high affinity to the negatively charged lipids, rather than galactolipids. However,  
418 acyl groups in *Synechocystis* cells exist as free fatty acid, bound to acyl carrier protein, and as  
419 lipids. In particular, fatty-acid desaturases, which modify acyl groups like those of BfaA and  
420 BfaB, have the following characteristics. There are three types of fatty acid desaturase, acyl-  
421 lipid, acyl-CoA, and acyl-ACP desaturase (Murata and Wada 1995). The acyl-lipid desaturases  
422 introduce unsaturated double bond into fatty acids bound to lipids (Murata et al. 1992; Murata  
423 and Wada 1995) and possess a transmembrane domain (e.g., DesA, DesB, DesC, and DesD).  
424 In contrast, the acyl-CoA and acyl-ACP desaturases recognize acyl groups bound to coenzyme  
425 A or acyl-carrier protein, respectively, as substrates (Murata et al. 1992; Murata and Wada 1995).  
426 In particular, one of the acyl-ACP desaturases, stearyl-ACP desaturase from *Arabidopsis*  
427 *thaliana* (e.g., AC002333), does not possess a transmembrane domain. From these features of  
428 fatty acid desaturases, it is predicted that the enzymes that modify fatty acids bound to lipids  
429 are insoluble, while enzymes that modify fatty acids bound to ACP are soluble. Both, BfaA and  
430 BfaB, do not possess any transmembrane domains. As mentioned above, if BfaA and BfaB  
431 specifically modify oleic acid bound to SQDG and PG, it is uncommon that BfaA and BfaB do  
432 not possess any transmembrane domains. However, the cyclopropane fatty acid synthase (Cfa)

433 from *E. coli*, which can methylate oleic acid and convert it to cyclopropane fatty acid, does not  
434 contain a transmembrane domain (Wang et al. 1992). Moreover, in a previous study, it was  
435 reported that Cfa can modify oleic acid that is bound to phospholipid (Grogan and Cronan 1997).  
436 These phenomena are consistent with our hypothesis regarding substrate specificity of BfaA  
437 and BfaB.

438           Small amounts of 19:0Me10 were detected not only from SQDG and PG, but also from  
439 the MGDG. The fatty acids in the cells are ACP bound, lipid bound, and are also present as free  
440 fatty acids; these are synthesized by acyl-ACP synthase, acyltransferase, and hydrolase,  
441 respectively. However, Gao et al. (2012) demonstrated that the amount of free-oleic acid is  
442 much lower than that of the other free fatty acids, including 16:0; 18:0; 18:2  $\Delta$  9,12; and 18:3  
443  $\Delta$  6,9,12 accumulated in the *Synechocystis* cells with a disrupted *slr1609* (encodes acyl-ACP  
444 synthase) (Gao et al 2012). This result indicates that oleic acid may tend to be present in the  
445 lipid-bound. In our study, if 19:0Me10 synthesized on the SQDG and PG was rapidly  
446 transferred to other lipids without any deflection, there should have been no significant  
447 difference in the amount of 19:0Me10 for each lipid class; however, a difference was observed.  
448 19:0Me10 may not be easily released into the free-fatty acid pool, like oleic acid. Besides,  
449 19:0Me10 might be difficult to catalyze by an acyl-ACP synthase or acyltransferases because  
450 the fatty acid is non-native in *Synechocystis*.

451

452 **Effect of synthesizing 10-methylene stearic acid (19:1 $\Delta$ Me10) on cell growth**

453

454 Although the growth of the cells transformed with *bfaA* and *bfaB* was lower than that of the  
455 parental strain expressing neither *bfaA* nor *bfaB*, the cells transformed with the only *bfaA*  
456 exhibited no significant difference in growth compared with the parental strain at 34°C (Fig.  
457 2A, B). The cells expressing only *bfaB* accumulated 19:1 $\Delta$ Me10 (Table 1), and showed a lower  
458 growth rate than the cells transformed with both *bfaA* and *bfaB* at 34°C. These results indicate  
459 that 19:1 $\Delta$ Me10 produced by BfaB may be toxic to the cells, and may, thereby, inhibit cell  
460 growth. In contrast, the growth of the cells expressing both *bfaA* and *bfaB* was slightly better  
461 than that of the cells expressing only *bfaB* at 34°C. From this result, it is hypothesized that the  
462 toxicity of 19:1 $\Delta$ Me10 was reduced by the activity of BfaA. Because the photosynthetic and  
463 respiratory activities of four strains, wild-type, *bfaAB*<sup>+</sup>, *desAD*<sup>-</sup>, and *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup>, did not  
464 show any significant differences (Fig. 1), the toxicity of 19:1 $\Delta$ Me10 may have a negative  
465 influence on a physiological response other than photosynthesis. Moreover, because the  
466 reduction in the growth of cells transfected with *bfaB* was mitigated by microoxic conditions,  
467 it is assumed that 19:1 $\Delta$ Me10 induced oxidative stress in the cells. To produce 19:0Me10  
468 efficiently, the expression level of *bfaA* and *bfaB* should be regulated, and the toxicity of

469 19:1ΔMe10 must be avoided.

470 At present, only *C. urealyticum* is found to be a natural producer of 19:1ΔMe10  
471 (Couderc et al. 1991). In contrast, the production of 19:0Me10 has been reported in many  
472 mycobacteria and related species, including *Mycobacterium tuberculosis* (Khuller et al. 1982),  
473 *Corynebacterium tuberculostearicum* (Brown et al. 1984), *Rhodococcus ruber* (Hwang et al.  
474 2015), and *Nocardia donostiensis* (Ercibengoa et al. 2016). This information may suggest that  
475 a system was adopted in nature to avoid the toxicity of 19:1ΔMe10 by reducing it to 19:0Me10.  
476 Moreover, the accumulation of 19:1ΔMe10 in *C. urealyticum* suggests that this organism uses  
477 another strategy for detoxifying the oxidants of fatty acids.

478

479

## 480 **Conclusions**

481

482 We succeeded in the synthesis of 19:0Me10, which is saturated and has a low melting point in  
483 *Synechocystis* by the introduction of *bfaA* and *bfaB* from *M. chlorophenolicum*. The production  
484 of 19:0Me10 in *Synechocystis* was 4.1% of the total fatty acid content due to the use of cells  
485 with disruptions in genes *desA* and *desD* and the optimization of codon usage for *bfaA* and *bfaB*  
486 in *Synechocystis*. For *Synechocystis* cells synthesizing 19:1ΔMe10, the precursor of 19:0Me10,

487 growth was decreased, but the decline was mitigated by microoxic conditions. This indicates  
488 that 19:1 $\Delta$ Me10 caused oxidative stress on the cells. The amount of 19:0Me10 present in SQDG  
489 and PG in the *Synechocystis* transformants was remarkably higher than that in MGDG and  
490 DGDG. This suggests that the substrate specificity of BfaA and BfaB is for oleic acid bound to  
491 SQDG and PG. Based on our findings, it is expected that the efficient production of 19:0Me10  
492 in microalgae can be achieved through the regulation of *bfaA* and *bfaB* expression levels, and  
493 the modification of the substrate recognition site for BfaA and BfaB. Finally, our study indicates  
494 that by genetic manipulations via the action of photosynthesis, photosynthetic organisms can  
495 produce unconventional modified fatty acids that are not found naturally in the cells. This  
496 technique will be key in the production of useful compounds related to the fatty acids in the  
497 microalgae.

498

499

## 500 **Acknowledgments**

501

502 The genomic DNA of *M. chlorophenolicum* JCM 7439<sup>T</sup> was obtained from RIKEN BRC, which  
503 is a participant in the National BioResources Project of the MEXT, Japan.

504 **Tables**505 **Table 1. Fatty acid composition of *Synechocystis* cells expressing *bfaA* and *bfaB* at 34°C**

506

Strain	Fatty acid (mol %)							
	16:0	16:1Δ9	18:0	18:1Δ9	18:2Δ9,12	18:3Δ6,9,12	19:0Me10	19:1Me10
Wild type	52.8 ± 0.4	2.8 ± 0.1	1.0 ± 0.4	10.3 ± 0.8	24.0 ± 0.4	9.0 ± 0.5	- <sup>a</sup>	-
<i>bfaA</i> <sup>+</sup>	54.2 ± 0.9	2.3 ± 0.4	2.3 ± 0.9	14.6 ± 2.6	18.2 ± 0.9	8.4 ± 1.0	-	-
<i>bfaB</i> <sup>+</sup>	58.8 ± 0.4	2.3 ± 0.5	0.8 ± 0.1	13.9 ± 0.6	16.8 ± 1.5	6.2 ± 0.6	-	1.2 ± 0.1
<i>bfaAB</i> <sup>+</sup>	59.0 ± 1.2	1.7 ± 0.9	1.4 ± 0.3	9.3 ± 2.0	16.5 ± 1.3	10.4 ± 1.2	1.7 ± 0.4	-
<i>desAD</i> <sup>-</sup>	53.8 ± 0.2	1.6 ± 0.9	1.4 ± 0.7	43.1 ± 0.3	-	-	-	-
<i>desAD</i> <sup>-</sup> / <i>bfaA</i> <sup>+</sup>	54.0 ± 3.7	2.5 ± 0.4	2.1 ± 0.1	41.4 ± 3.3	-	-	-	-
<i>desAD</i> <sup>-</sup> / <i>bfaB</i> <sup>+</sup>	55.5 ± 1.6	1.5 ± 0.5	1.4 ± 0.8	39.8 ± 2.0	-	-	-	1.8 ± 0.2
<i>desAD</i> <sup>-</sup> / <i>bfaAB</i> <sup>+</sup>	54.3 ± 0.6	2.9 ± 0.4	2.4 ± 0.3	37.6 ± 0.4	-	-	2.8 ± 0.5	-

507 <sup>a</sup> not detected.

508 **Table 2 Fatty acid composition of *Synechocystis* cells expressing *bfaA* and *bfaB* at 24°C**

509

Strain	Fatty acid (mol %)								
	16:0	16:1Δ9	18:0	18:1Δ9	18:2Δ9,12	18:3Δ6,9,12	18:3Δ9,12,15	18:4Δ6,9,12,15	19:0Me10
Wild type	54.1 ± 0.3	2.8 ± 0.8	1.7 ± 0.4	14.1 ± 4.4	12.9 ± 1.8	10.9 ± 2.1	1.9 ± 0.1	1.6 ± 0.3	- <sup>a</sup>
<i>bfaAB</i> <sup>+</sup>	57.9 ± 2.2	3.4 ± 0.3	1.8 ± 0.6	5.9 ± 0.9	10.0 ± 1.1	13.8 ± 2.6	3.7 ± 0.5	2.7 ± 0.5	0.8 ± 0.1
<i>desAD</i> <sup>-</sup>	54.4 ± 1.3	3.3 ± 0.2	4.6 ± 1.4	37.7 ± 0.4	-	-	-	-	-
<i>desAD</i> <sup>-</sup> / <i>bfaAB</i> <sup>+</sup>	51.3 ± 0.1	2.4 ± 0.4	1.5 ± 0.2	40.8 ± 0.1	-	-	-	-	4.0 ± 0.4

510 <sup>a</sup> not detected.

511 **Table 3 Fatty acid composition of *Synechocystis* cells expressing *cobfaA* and *cobfaB***

512

		Fatty acid (mol %)						
Parental cell	Gene type	16:0	16:1Δ9	18:0	18:1Δ9	18:2Δ9,12	18:3Δ6,9,12	19:0Me10
	<i>trc-bfaAB</i>	59.0 ± 1.2	1.7 ± 0.9	1.4 ± 0.3	9.3 ± 2.0	16.5 ± 1.3	10.4 ± 1.2	1.7 ± 0.4
Wild type	<i>trc-cobfaAB</i>	57.1 ± 0.7	2.7 ± 0.4	2.0 ± 0.4	7.5 ± 0.2	19.1 ± 0.2	9.3 ± 0.3	2.3 ± 0.2
	<i>cpc-cobfaAB</i>	55.8 ± 0.6	2.7 ± 0.6	2.0 ± 0.3	8.5 ± 0.1	19.8 ± 0.2	10.0 ± 0.2	1.3 ± 0.1
	<i>trc-bfaAB</i>	54.3 ± 0.6	2.9 ± 0.4	2.4 ± 0.3	37.6 ± 0.4	- <sup>a</sup>	-	2.8 ± 0.5
<i>desAD</i>	<i>trc-cobfaAB</i>	57.8 ± 0.7	1.7 ± 0.5	2.8 ± 0.2	33.6 ± 0.4	-	-	4.1 ± 0.6*
	<i>cpc-cobfaAB</i>	56.1 ± 0.2	2.5 ± 0.7	2.6 ± 0.4	36.1 ± 0.3	-	-	2.7 ± 0.1

513 <sup>a</sup> not detected. The results of wild-type-*trc-bfaAB* and *desAD*<sup>-</sup>-*trc-bfaAB* were the same result  
 514 for strains *bfaAB*<sup>+</sup> and *desAD*<sup>-</sup>/*bfaAB* listed in Table 1. \* Significant difference was indicated  
 515 by Student's t-test when compared with *trc-bfaAB* of Wild type (*P*<0.01).



516 **Table 4. Fatty acid composition of each lipid class in *Synechocystis* cells expressing *bfaA***  
 517 **and *bfaB***

518

Strain	Lipid class	Fatty acid (mol %)						
		16:0	16:1Δ9	18:0	18:1Δ9	18:2Δ9,12	18:3Δ6,9,12	19:0Me10
Wild type	MGDG	50.5 ± 0.7	3.3 ± 0.3	0.4 ± 0.1	4.8 ± 0.2	27.4 ± 0.3	13.5 ± 0.6	- <sup>a</sup>
	DGDG	49.7 ± 0.6	3.8 ± 0.1	0.6 ± 0.2	6.3 ± 0.3	19.8 ± 0.5	20.0 ± 0.7	-
	SQDG	61.0 ± 1.3	6.0 ± 1.0	0.8 ± 0.3	15.4 ± 1.2	16.1 ± 1.0	0.6 ± 0.1	-
	PG	58.4 ± 3.3	5.2 ± 1.1	2.4 ± 1.5	19.4 ± 3.0	13.7 ± 0.6	0.9 ± 0.1	-
	Total <sup>b</sup>	53.8 ± 0.2	4.0 ± 0.8	0.7 ± 0.2	8.8 ± 0.6	22.6 ± 0.3	10.1 ± 0.4	-
<i>bfaAB</i> <sup>+</sup>	MGDG	53.5 ± 0.1	3.0 ± 0.3	0.6 ± 0.1	5.6 ± 1.8	20.0 ± 0.8	17.0 ± 0.9	0.3 ± 0.1
	DGDG	53.7 ± 1.7	3.6 ± 0.2	0.6 ± 0.3	6.2 ± 1.7	14.3 ± 1.6	21.4 ± 1.6	t
	SQDG	72.4 ± 0.7	4.4 ± 0.2	1.0 ± 0.1	9.1 ± 1.5	9.1 ± 0.6	0.6 ± 0.1	3.4 ± 0.4*
	PG	58.6 ± 1.4	2.0 ± 1.3	4.9 ± 2.8	10.8 ± 2.7	17.0 ± 3.4	0.3 ± 0.1	6.4 ± 1.6*
	Total	59.5 ± 0.6	3.0 ± 1.1	0.9 ± 0.2	7.1 ± 1.4	15.7 ± 0.8	12.2 ± 0.5	1.5 ± 0.2
<i>desAD</i> <sup>-</sup>	MGDG	44.2 ± 1.4	4.1 ± 0.8	2.1 ± 0.4	49.6 ± 1.9	-	-	-
	DGDG	49.1 ± 0.4	3.1 ± 0.4	3.2 ± 1.5	44.6 ± 1.5	-	-	-
	SQDG	68.8 ± 4.8	6.2 ± 1.1	0.5 ± 0.2	24.5 ± 5.3	-	-	-
	PG	50.2 ± 0.7	3.0 ± 0.5	3.0 ± 2.4	43.7 ± 2.4	-	-	-
	Total	52.9 ± 2.6	4.4 ± 2.0	1.3 ± 0.4	41.4 ± 4.2	-	-	-
<i>desAD</i> <sup>-</sup> / <i>bfaAB</i> <sup>+</sup>	MGDG	42.9 ± 0.7	3.2 ± 0.7	2.6 ± 0.2	50.7 ± 0.3	-	-	0.6 ± 0.1
	DGDG	46.8 ± 0.6	2.9 ± 0.3	1.6 ± 0.5	48.7 ± 0.3	-	-	t
	SQDG	64.4 ± 2.7	4.7 ± 1.1	0.6 ± 0.3	24.6 ± 2.1	-	-	5.7 ± 1.6**
	PG	54.8 ± 3.2	2.1 ± 1.5	4.0 ± 2.3	30.3 ± 1.7	-	-	8.8 ± 2.2*
	Total	49.7 ± 1.4	3.6 ± 1.0	1.8 ± 0.3	42.1 ± 1.9	-	-	2.7 ± 0.2

519 <sup>a</sup> not detected. <sup>b</sup> results from lipids analyzed prior to fractionation. <sup>c</sup> trace amount (less than  
 520 0.04%). \* Significant difference was indicated by Student's t-test when compared with total  
 521 lipid ( $P < 0.02$ ). \*\* ( $P < 0.05$ )

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615 **Figure Captions**

616 **Fig. 1. O<sub>2</sub> evolution and absorption of *Synechocystis* cells expressing *bfaA* and *bfaB***

617 Black and white bars show respiratory and photosynthetic activities, respectively. The panels A  
618 and B are results at 34°C and 24°C, respectively.

619

620 **Fig. 2. Growth of *Synechocystis* cells expressing *bfaA* and *bfaB***

621 The cells shown in panels A, B, C, and D were cultured in aerobic conditions. The cells shown  
622 in panel E were cultured in microoxic conditions. The cells shown in panels A, B, and E were  
623 cultured at 34°C. The cells shown in panels C and D were cultured at 24°C. Closed red circle,  
624 wild-type cells; open red circle, *bfaA*<sup>+</sup> cells; closed green circle, *bfaB*<sup>+</sup> cells; open green circle,  
625 *bfaAB*<sup>+</sup> cells; closed purple circle, *desAD*<sup>-</sup> cells; open purple circle, *desAD*<sup>-</sup>/*bfaA*<sup>+</sup> cells; closed  
626 light blue circle, *desAD*<sup>-</sup>/*bfaB*<sup>+</sup> cells; and open light blue circle, *desAD*<sup>-</sup>/*bfaAB*<sup>+</sup> cells.



Figure 1

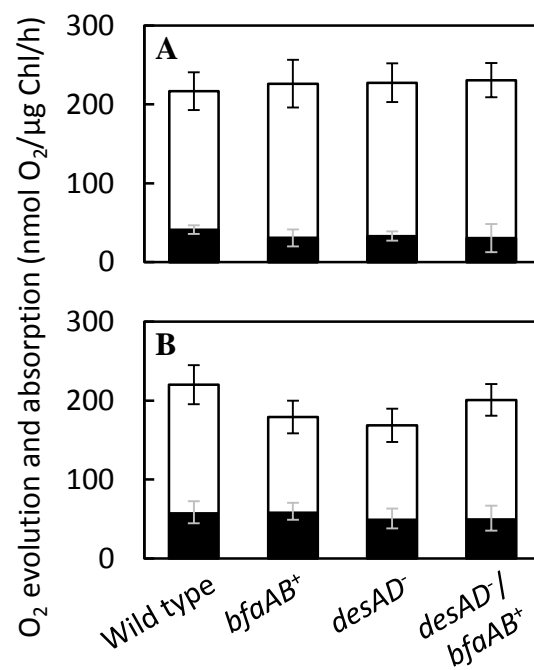


Figure 2

