Characterization of cyanobacterial cells synthesizing 10-methyl stearic acid

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Abstract

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

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

Keywords

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

Abbreviations

MGDG; monogalactosyldiacylglycerol, DGDG; digalactosyldiacylglycerol, SQDG; sulfoquinovosyldiacylglycerol, PG; phosphatidylglycerol, co*bfaAB*; codon optimized *bfaA* and *bfaB*, GC; gas chromatography, FAME; fatty acid methyl ester, 16:0; palmitic acid, 16:1Δ9; palmitoleic acid, 18:0; stearic acid, 18:1Δ9; oleic acid, 18:2Δ9,12; linoleic acid, 18:3Δ6,9,12; γ-linolenic acid, 18:3Δ9,12,15; α-linolenic acid, 18:4Δ6,9,12,15; stearidonic acid, 19:0Me10; 10-methyl stearic acid, 19:1ΔMe10; 10-methylene stearic acid.

Introduction

Contemporary society is dependent on the consumption of enormous quantities of fossil fuels. The fossil fuels are used not only as resources for transportation and generation of electricity at the large-scale but also as raw materials for the production of various chemicals. However, the use of fossil fuels is thought to accelerate global warming and increase environmental pollution. Moreover, the demand for fossil fuels is rising due to global industrial expansion, while the availability of these fuels is gradually diminishing. Therefore, exploitation of alternative sources of liquid fuels is required to meet the needs of the society. In recent years, microalgae have attracted attention as next-generation sources of biomass energy because of their high productivity and because they do not compete directly with the production of land crops, which are the primary sources of foods (Chisti 2007; Parmar et al. 2011). Methyl esters of fatty acids from microalgae are primarily expected to serve as biodiesel. However, most of the fatty acids in microalgae are C16–22 saturated and unsaturated fatty acids. The saturated fatty acids are stable against atmospheric oxidation, but they solidify at ambient temperatures due to their melting points being high. In contrast, the melting points of the polyunsaturated fatty acids are relatively low, and they are fluid at ambient temperatures. However, carbon-carbon double bonds in the carbon skeleton of polyunsaturated fatty acids are unstable due to susceptibility to

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

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

10-methylene stearic acid (19:1ΔMe10) formed has been identified in cells of *Corynebacterium urealyticum* (Couderc et al. 1991). The second step is the reduction of 19:1ΔMe10 to 19:0Me10, with NADPH as the reducing agent (Akamatsu and Law 1970). Our previous study revealed that BfaB and BfaA from *Mycobacterium chlorophenolicum* catalyze these two steps, respectively (Machida et al. 2017). By heterologous expression of *bfaA* and *bfaB* in *Escherichia coli*, 19:0Me10 is synthesized *in vivo* from 18:1Δ9, which was supplemented in the media; cells expressing only *bfaB* produce 19:1ΔMe10.

Fatty acids are the main constituents of cell membranes in all living organisms. The unsaturated fatty acids are essential to maintaining membrane fluidity, which is critical for membrane function. Acyl-lipid desaturases of cyanobacteria introduce double bonds at specific positions in the fatty acids that are esterified with the glycerol backbone of the membrane lipids (Murata et al. 1992). The genes *desA*, *desB*, *desC*, and *desD* of *Synechocystis* sp. PCC 6803 encode the acyl-lipid desaturases that introduce double bonds at the positions Δ12, Δ15, Δ9, and Δ6, respectively, of the C18 fatty acids bound at the *sn*-1 position of the lipids. In *Synechocystis*, the expression of *desA*, *desB*, and *desD* is induced under low-temperature conditions (Los et al. 1997). The membrane lipids of *Synechocystis* cells are composed of glycolipids, such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG) and a single phospholipid,

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

In this study, we synthesized the fatty acid 19:0Me10 which is saturated and has a low melting point in *Synechocystis* by expressing the *M. chlorophenolicum* genes, *bfaA* and *bfaB*. To increase the yield of 19:0Me10 *in vivo*, we also examined the effects of mutations in *desA* and *desD* in *Synechocystis*, which lead to accumulation of 18:1Δ9, such that 18:1Δ9 comprises more than 40% of the total fatty acid content (Tasaka et al. 1996). We analyzed changes in fatty acid composition, growth, and respiratory and photosynthetic activities in the *Synechocystis* transformants.

Materials and Methods

Organisms and culture conditions

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

1971) buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–NaOH (pH 7.5) at 34°C or 24°C under continuous illumination at 70 µmole photons m^{-2} s^{-1} by white fluorescent lamps and aerated with 1% (v/v) CO_2-enriched air (Wada and Murata 1989). For screening of transformants and maintaining of *Synechocystis* cells, we used BG11 medium solidified with 1.5% (w/v) Bacto-agar (BD Biosciences Japan, Tokyo, Japan) including the 25 µg/mL kanamycin sulfate, 25 µg/mL spectinomycin dihydrochloride pentahydrate, or 25 µg/mL chloramphenicol (Wako Pure Chemicals, Osaka, Japan), depending on the selection markers. For growth under microoxic conditions, liquid cultures were bubbled with 1% (v/v) CO_2-mixed nitrogen gas (Japan Fine Products, Kanagawa, Japan).

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

Plasmid construction and transformation

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 (Cm^r),
150 the *trc* promoter sequence (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 Cm^r with the spectinomycin resistance gene cassette (Sp^r), we constructed pTHT2031, a
153 plasmid lacking Cm^r, from pTCHT2031v by polymerase chain reaction (PCR) amplification of
154 the entire sequence of pTCHT2031v, except the 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® 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® Terminator v.3.1 (Life Technologies, Foster City, CA, USA)

and ABI 3130 Genetic Analyzer (Life Technologies). We then performed PCR to amplify a DNA fragment containing the Sp^r cassette using pAM1146 (Tsinoremas et al. 1994) as the template and the primer set, Sp_Bgl_F and Sp_Bam_R. The Sp^r fragment was digested with *Bgl*II and *Bam*HI, inserted into *Bam*HI-cleaved pMD-bfaB and pMD-bfaA-4-bfaB, to obtain pMD-bfaB-S and pMD-bfaA-4-bfaB-S, respectively. We selected plasmids in which the Sp^r fragments were transcribed in the same orientation as the genes of interest that were to be expressed. The fragments containing bfaB-Sp^r and bfaA-4-bfaB-Sp^r were excised from these plasmids by *Nde*I and *BamH*I digestion, and inserted into pTHT2031 digested with the same restriction enzymes, to obtain pTHT2031-bfaB-S and pTHT2031-bfaA-4-bfaB-S, respectively. The native sequence of *bfaA* and *bfaB* shows a 4-bp overlap between the genes. We previously observed that the Shine–Dalgarno (SD) sequence is essential for the stable translation of the BfaB protein in *E. coli* cells (Machida et al. 2017). To insert the canonical SD sequence (5′-AGGAGGAATAAACC-3′), which is also present in the *trc* promoter region of the original pTCHT2031v (Ishizuka et al. 2006) between the two open reading frames of *bfaA* and *bfaB*, pTHT2031-bfaA-4-bfaB-S was amplified using the primer set, SD_add_I_F and SD_add_I_R, and circularized using In-Fusion® HD cloning kit to obtain pTHT2031-bfaAB-S. To construct pTHT2031-bfaA-S, pTHT2031-bfaA-4-bfaB-S was linearized by PCR using the primer set, Sp_up_F and bfaA_dn_inf_R, and circularized using the In-Fusion® HD cloning kit.

Both *bfaA* and *bfaB* were artificially synthesized (Life Technologies Japan, Tokyo), and optimized for codon usage by *Synechocystis* (cobfaAB) (Fig. S2). The cobfaAB fragment was amplified from pEX-cobfaAB using the primer set, coBfaAB_trc_inf_F and coBfaAB_Sp_inf_R. After linearization of pTHT2031-bfaAB-S, using the primer set, Sp_up_F and pTHT_trcdn_R, it was ligated with the cobfaAB fragment using In-Fusion® HD cloning kit, resulting in the formation of pTHT2031-coBfaAB-S.

Two fragments, which were amplified from pTHT2031 and *Synechocystis* chromosomal DNA using the primer sets, pTHT_cpc_inf_F and pTHT_cpc_inf_R and cpc560_F and cpc560_R, respectively, were ligated using the In-Fusion® HD cloning kit to produce pTC2031. We then performed PCR to amplify a DNA fragment containing the Sp^r cassette using pAM1146 as the template and the primer set, Sp_Bgl_F and Sp_Bam_R. After digestion of pTC2031 and the amplified fragment containing Sp^r with *Nde*I and *Bgl*II, we constructed pTC2031-S by ligation. To obtain plasmid pTC2031-cobfaAB-S, pTC2031-S was linearized by PCR using the primer set, Sp_up_F and cpc560_R, and ligated with the cobfaAB fragment using primer set coBfaAB_cpc_inf_F and coBfaAB_Sp_inf_R.

Fatty acid analysis

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

spectra using commercial FAME standards (Nu-Chek Prep, Elysian, MN, >99%) and methylesterified standard of 19:0Me10 (Larodan Fine Chemicals, Malmö, Sweden, ≥97%). To quantify the FAMEs, we applied 1 µL of the hexane solution to a GC-2014 gas chromatograph equipped with a flame ionization detector (Shimadzu). The conditions of GC were identical to those used for FAME identification.

Separation of lipid classes

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

Photosynthetic and respiratory activities

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

Results

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

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

study (Machida et al. 2017), we demonstrated that BfaA and BfaB from *M. chlorophenolicum* are involved in the biosynthesis of 19:0Me10 using 18:1Δ9 as a substrate, which is one of the main components of fatty acids in *Synechocystis*. In the present study, we examined whether 19:0Me10 was synthesized from 18:1Δ9 and incorporated into membrane lipids *in vivo* by the heterologous expression of *bfaA* and *bfaB* in wild-type *Synechocystis*.

In wild-type *Synechocystis* cells, 18:1Δ9, which is believed to be the substrate for BfaB, comprised 10.3 ± 0.8% of the total fatty acid content (Table 1). In the strain containing *bfaA* and *bfaB* (*bfaAB*$^{+}$), 19:0Me10 comprised 1.7 ± 0.4% of the total fatty acid content. Simultaneously, the 18:1Δ9 content was slightly decreased to 9.3 ± 2.0%. The relative amount of 18:2Δ9,12 in the *bfaAB*$^{+}$ strain was 16.5 ± 1.3%, which was also lower than that in the wild-type cells (24.0 ± 0.4%). These results indicated that BfaA and BfaB were functional in the *Synechocystis* cells and that 18:1Δ9 was used as the substrate.

To increase the amount of 19:0Me10 in the *Synechocystis* cells, we attempted to introduce *bfaA* and *bfaB* into the *desAD*$^{-}$ strain, in which *desA* and *desD*, encoding for Δ12 and Δ6 desaturases, respectively, are disrupted and does not produce C18 polyunsaturated fatty acids (Tasaka et al. 1996). 18:1Δ9 constituted 43.1 ± 0.3% of the total fatty acid content of the *desAD*$^{-}$ strain. Although we attempted to increase the amount of 19:0Me10 in the *desAD*$^{-}$*/bfaAB*$^{+}$ strain, it unexpectedly constituted only 2.8 ± 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*$^{+}$ and *desAD*$^{-}$*/bfaAB*$^{+}$ 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*$^{+}$ and *desAD*$^{-}$/*bfaAB*$^{+}$ 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*$^{+}$ and *desAD*$^{-}$/*bfaAB*$^{+}$ cells, at the 34°C, was reduced
282 compared to those of the wild-type and *desAD*$^{-}$ 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*$^{+}$ 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*$^{-}$ strain scarcely grew at
287 24°C, whereas the growth of the *desAD*$^{-}$/*bfaAB*$^{+}$ strain was slightly recovered (Fig. 2D) and
288 was similar to the growth of the *bfaAB*$^{+}$ strain (Fig. 2C). It seems that the synthesis of 19:0Me10

289 in the *desAD*$^{-}$ 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*$^{+}$ and *desAD*$^{-}$/*bfaB*$^{+}$, which were transformed with only the *bfaB* gene, produced
301 1.2 ± 0.1% and 1.8 ± 0.2%, respectively, of 19:1ΔMe10 relative to the total fatty acid content
302 (Table 1). The cells of *bfaA*$^{+}$ and *desAD*$^{-}$/*bfaA*$^{+}$ 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*$^{+}$ and
305 *desAD*$^{-}$/*bfaA*$^{+}$ strains was also almost the same as that of the wild-type and *desAD*$^{-}$ cells,
306 respectively (Fig. 2A and B). In contrast, *bfaB*$^{+}$ and *desAD*$^{-}$/*bfaB*$^{+}$ 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*$^+$ and *desAD*$^-$/*bfaAB*$^+$) was slightly better than that
309 of the cells expressing only *bfaB* (*bfaB*$^+$ and *desAD*$^-$/*bfaB*$^+$). 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_2) 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_2 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_2 mixed in nitrogen gas.
322 Fig. 2E shows the cell growth in microoxic conditions. The decreased growth of *bfaB*$^+$
323 and *bfaAB*$^+$ 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

small amount of oxygen synthesized during photosynthesis may trigger oxidative stress in the cells producing 19:1ΔMe10. The growth of the wild-type cells in microoxic conditions was slightly slower than that in aerobic conditions. It is speculated that the cells cultivated in the microoxic conditions may have interfered with respiration, which subsequently retarded the growth rate. Moreover, analysis of the fatty acid composition of the cells cultured under microoxic conditions revealed no significant difference compared with that of cells cultured under aerobic conditions (Table S3).

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

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

Plasmids pTHT2031-cobfaAB-S, in which the codon-optimized *bfaA* and *bfaB* genes are driven by *trc* promoter, and pTC2031-cobfaAB-S, in which these genes driven by *cpc* promoter, were transformed into wild-type *Synechocystis* and *desAD*$^{-}$ cells. As a result, in the wild-type cells expressing both co*bfaA* and co*bfaB* under the regulation of *trc* and *cpc* promoters, 19:0Me10 constituted 2.3 ± 0.2% and 1.3 ± 0.1% of the total fatty acid content, respectively (Table 3). In contrast, in the *desAD*$^{-}$ cells expressing the co*bfaA* and co*bfaB* under the regulation of *trc* and *cpc* promoters, 19:0Me10 consisted of 4.1 ± 0.6% and 2.7 ± 0.1% of the total fatty acid content, respectively. The *desAD*$^{-}$ strain expressing both co*bfaA* and co*bfaB* under the regulation of *trc* promoter showed the highest production of 19:0Me10 in the study, which was approximately two times higher than that in the wild-type cells expressing both *bfaA* and *bfaB* under regulation of *trc* promoter at 1.7 ± 0.4%. While the production of 19:0Me10 in the cells expressing co*bfaA* and co*bfaB* under the control of *cpc* promoter was lower than that in the cells expressing *bfaA* and *bfaB* under the control of *trc* promoter. These results indicated that optimization of codon usage improved the efficiency of translation of BfaA and BfaB, and contributed to increased production of 19:0Me10.

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

361 The highest production of 19:0Me10 reported in this study was 4.1 ± 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 18:1Δ9 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 18:1Δ9 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*$^{+}$ 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 ± 0.1% of the total fatty acid
382 content and it esterified to DGDG was trace amount in the *bfaAB*$^{+}$ strain, whereas for SQDG
383 and PG, it was 3.4 ± 0.4 and 6.4 ± 1.6% (Table 4). Moreover, in the *desAD*$^{-}$/*bfaAB*$^{+}$ strain,
384 MGDG was included only 0.6 ± 0.1% of 19:0Me10, while SQDG and PG were included 5.7 ±
385 1.6% and 8.8 ± 2.2%. In both the *bfaAB*$^{+}$ and *desAD*$^{-}$/*bfaAB*$^{+}$ 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*$^{+}$
388 and *desAD*$^{-}$/*bfaAB*$^{+}$ 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 18:1Δ9 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*$^{+}$ and *desAD*$^{-}$/*bfaAB*$^{+}$ strains. These results indicated that 18:1Δ9 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**

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

We succeeded in synthesizing 19:0Me10 *in vivo* by the introduction of *bfaA* and *bfaB* into *Synechocystis*, with 18:1Δ9 accumulation in the cells being slightly decreased (Table 1). To increase the total relative amount of 19:0Me10 in the cells, we introduced *bfaA* and *bfaB* into the *desAD*$^{-}$ strain, which accumulates 18:1Δ9 to a much higher level than do the wild-type cells. However, the amount of 19:0Me10 in *bfaA*$^{+}$ and *desAD*$^{-}$/*bfaA*$^{+}$ strains showed no significant difference. Additionally, we attempted to employ codon optimized *bfaA* and *bfaB*, and the *cpc* promoter, but the amount of 19:0Me10 in the transformants remained at less than 5% of the total fatty acid content (Table 3). We predicted that differences in the lipid composition between *Synechocystis* and *M. chlorophenolicum*, the source of *bfaA* and *bfaB*, might be problematic. Fatty acid analysis of each lipid type revealed the amount of 19:0Me10 incorporated in SQDG and PG was remarkably higher than that in MGDG and DGDG of the *Synechocystis* transformants (Table 4). These results suggest that BfaA and BfaB may specifically modify the 18:1Δ9 that is bound to SQDG and PG, and the production of 19:0Me10 may be limited in *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, stearoyl-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 Δ9,12; and 18:3
443 Δ6,9,12 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*.

Effect of synthesizing 10-methylene stearic acid (19:1ΔMe10) on cell growth

Although the growth of the cells transformed with *bfaA* and *bfaB* was lower than that of the parental strain expressing neither *bfaA* nor *bfaB*, the cells transformed with the only *bfaA* exhibited no significant difference in growth compared with the parental strain at 34˚C (Fig. 2A, B). The cells expressing only *bfaB* accumulated 19:1ΔMe10 (Table 1), and showed a lower growth rate than the cells transformed with both *bfaA* and *bfaB* at 34˚C. These results indicate that 19:1ΔMe10 produced by BfaB may be toxic to the cells, and may, thereby, inhibit cell growth. In contrast, the growth of the cells expressing both *bfaA* and *bfaB* was slightly better than that of the cells expressing only *bfaB* at 34˚C. From this result, it is hypothesized that the toxicity of 19:1ΔMe10 was reduced by the activity of BfaA. Because the photosynthetic and respiratory activities of four strains, wild-type, *bfaAB*$^{+}$, *desAD*$^{-}$, and *desAD*$^{-}$/*bfaAB*$^{+}$, did not show any significant differences (Fig. 1), the toxicity of 19:1ΔMe10 may have a negative influence on a physiological response other than photosynthesis. Moreover, because the reduction in the growth of cells transfected with *bfaB* was mitigated by microoxic conditions, it is assumed that 19:1ΔMe10 induced oxidative stress in the cells. To produce 19:0Me10 efficiently, the expression level of *bfaA* and *bfaB* should be regulated, and the toxicity of

19:1ΔMe10 must be avoided.

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

Conclusions

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

growth was decreased, but the decline was mitigated by microoxic conditions. This indicates that 19:1ΔMe10 caused oxidative stress on the cells. The amount of 19:0Me10 present in SQDG and PG in the *Synechocystis* transformants was remarkably higher than that in MGDG and DGDG. This suggests that the substrate specificity of BfaA and BfaB is for oleic acid bound to SQDG and PG. Based on our findings, it is expected that the efficient production of 19:0Me10 in microalgae can be achieved through the regulation of *bfaA* and *bfaB* expression levels, and the modification of the substrate recognition site for BfaA and BfaB. Finally, our study indicates that by genetic manipulations via the action of photosynthesis, photosynthetic organisms can produce unconventional modified fatty acids that are not found naturally in the cells. This technique will be key in the production of useful compounds related to the fatty acids in the microalgae.

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Tables

Table 1. Fatty acid composition of *Synechocystis* cells expressing *bfaA* and *bfaB* at 34˚C

	Fatty acid (mol %)							
Strain	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	-[a]	-
bfaA$^+$	54.2 ± 0.9	2.3 ± 0.4	2.3 ± 0.9	14.6 ± 2.6	18.2 ± 0.9	8.4 ± 1.0	-	-
bfaB$^+$	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
bfaAB$^+$	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	-
desAD$^-$	53.8 ± 0.2	1.6 ± 0.9	1.4 ± 0.7	43.1 ± 0.3	-	-	-	-
desAD$^-$/*bfaA*$^+$	54.0 ± 3.7	2.5 ± 0.4	2.1 ± 0.1	41.4 ± 3.3	-	-	-	-
desAD$^-$/*bfaB*$^+$	55.5 ± 1.6	1.5 ± 0.5	1.4 ± 0.8	39.8 ± 2.0	-	-	-	1.8 ± 0.2
desAD$^-$/*bfaAB*$^+$	54.3 ± 0.6	2.9 ± 0.4	2.4 ± 0.3	37.6 ± 0.4	-	-	2.8 ± 0.5	-

[a] not detected.

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

509

	Fatty acid (mol %)								
Strain	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	-[a]
bfaAB^{+}	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
desAD^{-}	54.4 ± 1.3	3.3 ± 0.2	4.6 ± 1.4	37.7 ± 0.4	-	-	-	-	-
desAD^{-}/*bfaAB*$^{+}$	51.3 ± 0.1	2.4 ± 0.4	1.5 ± 0.2	40.8 ± 0.1	-	-	-	-	4.0 ± 0.4

510 [a] not detected.

511 **Table 3 Fatty acid composition of *Synechocystis* cells expressing co*bfaA* and co*bfaB***

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
Wild type	*trc-bfaAB*	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
	trc-cobfaAB	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
	cpc-cobfaAB	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
desAD$^-$	*trc-bfaAB*	54.3 ± 0.6	2.9 ± 0.4	2.4 ± 0.3	37.6 ± 0.4	-[a]	-	2.8 ± 0.5
	trc-cobfaAB	57.8 ± 0.7	1.7 ± 0.5	2.8 ± 0.2	33.6 ± 0.4	-	-	4.1 ± 0.6*
	cpc-cobfaAB	56.1 ± 0.2	2.5 ± 0.7	2.6 ± 0.4	36.1 ± 0.3	-	-	2.7 ± 0.1

513 [a] not detected. The results of wild-type-*trc-bfaAB* and *desAD*$^-$*-trc-bfaAB* were the same result
514 for strains *bfaAB*$^+$ and *desAD*$^-$/*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).

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

		Fatty acid (mol %)						
Strain	Lipid class	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	-[a]
	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[b]	53.8 ± 0.2	4.0 ± 0.8	0.7 ± 0.2	8.8 ± 0.6	22.6 ± 0.3	10.1 ± 0.4	-
bfaAB$^+$	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
desAD$^-$	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	-	-	-
desAD$^-$/	MGDG	42.9 ± 0.7	3.2 ± 0.7	2.6 ± 0.2	50.7 ± 0.3	-	-	0.6 ± 0.1
bfaAB$^+$	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

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

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615 **Figure Captions**

616 **Fig. 1. O_2 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*$^+$ cells; closed green circle, *bfaB*$^+$ cells; open green circle,

625 *bfaAB*$^+$ cells; closed purple circle, *desAD*$^-$ cells; open purple circle, *desAD*$^-$/*bfaA*$^+$ cells; closed

626 light blue circle, *desAD*$^-$/*bfaB*$^+$ cells; and open light blue circle, *desAD*$^-$/*bfaAB*$^+$ cells.

Figure 1

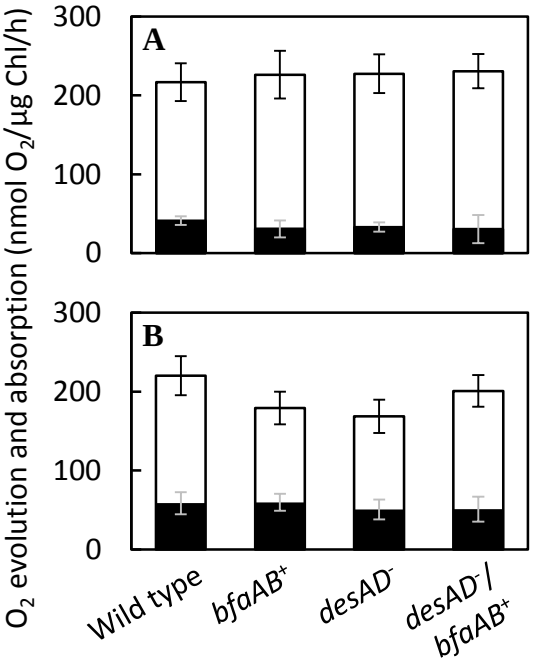

Figure 2