Title

Construction of a cyanobacterium synthesizing cyclopropane fatty acids

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Abstract

Microalgae have received much attention as a next-generation source of biomass energy. However, most of the fatty acids (FAs) from microalgae are multiply unsaturated; thus, the biofuels derived from them are fluid, but vulnerable to oxidation. In this study, we attempted to synthesize cyclopropane FAs in the cyanobacterium *Synechocystis* sp. PCC 6803 by expressing the *cfa* gene for cyclopropane FA synthase from *Escherichia coli* with the aim of producing FAs that are fluid and stable in response to oxidization. We successfully synthesized cyclopropane FAs in *Synechocystis* with a yield of ~30% of total FAs. Growth of the transformants was altered, particularly at low temperatures, but photosynthesis and respiration were not significantly affected. C16:1^{Δ9} synthesis in the *desA*⁻/*desD*⁻ strain by expression of the *desC2* gene for *sn*-2 specific Δ9 desaturase positively affected growth at low temperatures via promotion of various cellular processes, with the exceptions of photosynthesis and respiration. Estimation of the apparent activities of desaturases suggested that some acyl-lipid desaturases might recognize the lipid side chain.

Keywords

Biofuel; Microalgae; Cyclopropane fatty acid; Acyl lipid desaturase; *Synechocystis* sp. PCC 6803

1. Introduction

Contemporary society is dependent on large quantities of fossil fuels. Fossil fuels are used not only as resources for large-scale transportation and generation of electricity, but also as raw materials for the production of various chemicals. However, use of fossil fuels accelerates global warming and environmental pollution. Moreover, the demand for fossil fuels is increasing due to global industrial expansion, while the availability of these fuels is gradually diminishing. Therefore, development of alternative sources of liquid fuels is required to sustain society. In recent years, microalgae have attracted attention as next-generation sources of biomass energy because their production does not directly compete with the production of land crops, which is sources of food, and because their production efficiency is extremely high [5,16]. Some microalgae accumulate large quantities of oils (mainly triacylglycerols), especially under conditions of stress, such as nitrogen starvation [8]. Fatty acids (FAs) in triacylglycerols are hydrolyzed and methylated, and their derivatives are utilized as biofuels. Most FAs from microalgae are multiply unsaturated; thus, the biofuels derived from them are fluid, but vulnerable to oxidation. Thus, direct use of natural FAs from microalgae in industry may be problematic. Use of FAs could be expanded if they were stable in response to oxidation during long-term storage and if they were sufficiently fluid. Saturated FAs are stable in response to oxidation, but saturated FAs with C16 or C18 chains (which are

the major acyl groups of lipids in living organisms) solidify at physiological temperatures. Thus, saturated FAs are not suitable for use in biofuel production.

Some bacteria, such as *Escherichia coli* and *Lactobacillus arabinosus*, synthesize cyclopropane FAs, which contain a cyclopropane ring in the acyl group. For example, 30% of FAs in the total membrane lipids of *E. coli* cells are cyclopropane FAs. A green sulfur bacterium, *Chlorobaculum tepidum*, also produces cyclopropane FAs attached to glycolipids [15]. This suggests that heterotrophic bacteria are not the only producers of cyclopropane FAs. However, oxygenic photosynthetic organisms do not synthesize cyclopropane FAs. Instead, they produce unsaturated FAs to maintain membrane fluidity, suggesting that cyclopropane FAs might negatively affect oxygenic photosynthesis. Cyclopropane FA synthase in *E. coli* catalyzes the modification of acyl chains to their cyclopropane derivatives through methylation of an unsaturated bond. In this reaction, *S*-adenosyl-L-methionine (SAM) is used as a methyl donor. The enzyme acts on the double bond at the $\Delta 9$ or $\Delta 11$ in FAs attached to lipids in the membrane [23]. Unsaturated FAs exist in the membrane lipids of most organisms and maintain membrane fluidity. Additionally, SAM exists in most organisms as a methyl donor for methylation reactions. Thus, organisms harboring $\Delta 9$ or $\Delta 11$ unsaturated FAs in their membrane lipids and in which the cyclopropane FA synthase from *E. coli* is expressed heterologously may have the ability to synthesize cyclopropane FAs. However, this has not

74 been evaluated to date.

75 Cyanobacterial acyl-lipid desaturases introduce double bonds at specific positions in FAs
76 that are esterified to the glycerol backbone of the membrane lipid [14]. The *desA*, *desB*, *desC*,
77 and *desD* genes of *Synechocystis* sp. PCC 6803 encode the acyl-lipid desaturases that
78 introduce double bonds at the $\Delta 12$, $\Delta 15$, $\Delta 9$, and $\Delta 6$ positions, respectively, of C18 FAs
79 attached at the *sn*-1 position of the lipids. In *Synechocystis*, a saturated C18 FA, stearic acid
80 (C18:0), is synthesized and incorporated into the *sn*-1 position of membrane lipids, followed
81 by desaturation. DesC introduces a double bond at the $\Delta 9$ position in the saturated FA. Then,
82 DesA and DesD unsaturate at the $\Delta 12$ and $\Delta 6$ positions, respectively, of oleic acid (C18:1 $^{\Delta 9}$),
83 and the $\Delta 12$ unsaturated FAs are utilized by DesB as substrates to introduce an unsaturated
84 bond at the $\Delta 15$ position. The *desA* and *desD* genes were inactivated in the *desA*⁻/*desD*⁻ strain,
85 and this strain accumulated more C18:1 $^{\Delta 9}$ than did the wild-type strain [18]. In *Synechocystis*,
86 the endogenous desaturase specifically unsaturates C18 FAs attached at the *sn*-1 position of
87 lipids but cannot unsaturate C16 FAs attached at the *sn*-2 position. On the other hand, DesC2
88 from *Nostoc* sp. strain 36 can unsaturate the $\Delta 9$ position of C16 FAs attached at the *sn*-2
89 position [4].

90 In this study, we attempted to synthesize cyclopropane FAs in the cyanobacterium
91 *Synechocystis* sp. PCC 6803 by expressing the cyclopropane FA synthase from *E. coli* to

produce FAs that are fluid and stable in response to oxidization. To increase the yield of cyclopropane FAs *in vivo*, we also expressed the *desC2* gene from *Nostoc* sp. and examined the effects of mutations in *desA* and *desD* of *Synechocystis*. We analyzed the changes in FA composition, cell growth, and respiration, and photosynthesis activities in the *Synechocystis* transformants.

2. Materials and Methods

2.1. Organisms and culture conditions

The *Synechocystis* sp. PCC 6803 glucose-tolerant strain [24] was used as the wild type in this study. *Synechocystis* cells were grown in BG11 [17] buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–NaOH (pH 7.5) at 34°C or 26°C under continuous illumination by white fluorescent lamps at 70 $\mu\text{mole photons m}^{-2} \text{ s}^{-1}$ and aerated with 1% (v/v) CO₂-enriched air [21]. All transformants were maintained in BG11 medium solidified with 1.5% (w/v) Bacto-agar (BD Biosciences Japan, Tokyo) in the presence of 25 $\mu\text{g/mL}$ kanamycin sulfate, 25 $\mu\text{g/mL}$ spectinomycin dihydrochloride pentahydrate, or 25 $\mu\text{g/mL}$ chloramphenicol, depending on the selection markers used.

E. coli strain JM109 [25] was grown in 1.5 mL of LB medium [1] at 37°C with shaking at 200 rpm. All transformants were maintained in LB medium solidified with 1.5% (w/v)

Bacto-agar (BD Biosciences Japan) in the presence of 50 µg/mL sodium ampicillin or 50 µg/mL spectinomycin dihydrochloride pentahydrate, depending on the selection markers used. To supply exogenous FAs, C18:1^{Δ9}, linoleic acid (C18:2^{Δ9,12}), γ-linolenic acid (C18:3^{Δ6,9,12}), and α-linolenic acid (C18:3^{Δ9,12,15}), to the *E. coli* cells, we cultivated the cells in liquid LB medium [1] containing 1 mM sodium salt of C18:1^{Δ9} (Tokyo chemical industry, Japan), C18:2^{Δ9,12} (Funakoshi, Tokyo, Japan), C18:3^{Δ6,9,12} (Sigma Aldrich, Missouri, USA), or C18:3^{Δ9,12,15} (Funakoshi).

2.2. Plasmid construction and transformation

For heterologous expression of the *cfa* and *desC2* genes in *Synechocystis* and overexpression of the *cfa* gene in *E. coli*, we constructed four plasmids—pTHT-cfaSp, pTHT-Sp, pTHT-desC2Sp, and pTHT-cfadesC2Sp—which are derivatives of an expression vector, pTCHT2031V, for this cyanobacterium [9]. Fig. S1 shows the construction scheme for these plasmids, and Table S1 shows the primers used. The plasmid pTCHT2031V contains five DNA fragments in the following order: the sequence upstream of the *slr2031* gene (2031up), a chloramphenicol resistance gene cassette (Cm-r), the *trc* promoter sequence (*trc*), the sequence downstream of the *slr2031* gene (2031dn), and the plasmid backbone of the pUC vector [9]. The derived plasmids had fragments introduced between 2031up and 2031dn

into the *Synechocystis* chromosome through homologous recombination with the coding sequence of *slr2031*, a non-essential gene. At first, to replace the selection marker from the chloramphenicol resistance gene cassette with the spectinomycin resistance gene cassette, we constructed a plasmid lacking the chloramphenicol resistance gene cassette, pTHT2031V, from pTCHT2031V by PCR amplification and using an In-Fusion HD Cloning Kit (Takara Bio, Ohtsu, Japan). The *cfa* gene was amplified by PCR by using *E. coli* chromosomal DNA as the template. The amplified DNA fragment was subcloned into a T-vector pMD19 vector (Takara Bio) to obtain the pMD-cfa plasmid, which was confirmed by DNA sequencing. We next amplified a DNA fragment including the spectinomycin resistance gene (Sp-r) using pAM1146 [20] as a template. The Sp-r fragment, digested with *Bam*HI and *Bgl*III, was inserted into pMD-cfa, and pTHT2031V was cleaved with *Bgl*III in the same orientation as transcription of the *cfa* gene and the *trc* promoter, respectively, to obtain pMD-cfaSp and pTHT-Sp. The fragments containing the *cfa* and Sp-r genes in pMD-cfaSp were excised using *Nde*I and *Bgl*III and inserted into pTHT2031V digested using the same restriction enzymes to obtain pTHT-cfaSp. The *desC2* gene from the *Nostoc* sp. strain 36 [4] was artificially synthesized (Life Technologies Japan, Tokyo) and optimized for the codon usage of *Synechocystis*. Finally, we inserted the *desC2* gene into pTHT-cfaSp and amplified the fragment with two primer sets using an In-Fusion HD Cloning Kit (Takara Bio) to obtain

pTHT-desC2Sp and pTHT-cfadesC2Sp.

pTHT-cfaSp and pTHT-Sp were introduced into *E. coli* strain JM109 to construct the *cfa* overexpression and vector control strains, respectively. The FA compositions of these cells were analyzed. pTHT-cfaSp, pTHT-desC2Sp, and pTHT-cfadesC2Sp were used to transform cells of the wild-type and *desA*⁻/*desD*⁻ strains of *Synechocystis* by homologous recombination [24]. After verifying complete segregation of the mutated chromosomes from those possessing the native *slr2031* gene by PCR, FA compositions and photosynthesis and respiration activities were analyzed.

2.3. FA analysis

Profiles of FAs in the cells were examined by the method of Kotajima *et al.* [11]. Cells were precipitated by centrifugation and re-suspended in 2 mL of methanol. The suspensions were transferred to glass test tubes. After complete drying using a concentrating centrifuge (CC-105, Tomy Seiko, Tokyo, Japan), the pellet was re-suspended in 0.1 M hydrochloric acid methanolic solution (Wako Pure Chemicals, Osaka, Japan). Then, the tubes were tightly capped and incubated at 100°C for 1 h to allow saponification of the acyl-groups in lipids and conversion into FA methyl esters (FAMES). The resultant FAMES were recovered using *n*-hexane. The hexane phases recovered were evaporated, and the residues containing FAMES

were dissolved in 100 μ L of *n*-hexane.

To identify and quantify FAMES, we applied 1 μ l of the hexane solution to a GC-2014 gas chromatograph equipped with a flame-ionization detector (Shimadzu, Kyoto, Japan). Helium was used as a carrier gas at a constant flow rate of 1.25 mL/min in split-less mode. A CP-Sil5 CB column (Agilent Technologies, Santa Clara, CA) was used at the following temperatures: 60°C for 1.5 min, then 130°C at 20°C/min, and a further increase to 230°C at 4°C/min. Most FAMES were provisionally identified based on retention time and confirmed using commercial FAME standards (Nu-Chek Prep, Elysian, MN). To identify *cis*-9,10-methylenehexadecanoic acid (C17:1cyclo ^{Δ 9}) and *cis*-9,10-methyleneoctadecanoic acid (C19:1cyclo ^{Δ 9}), we used a gas chromatograph, GC-2010, equipped with a mass spectrometer, QP-2010 (Shimadzu). Conditions of GC were identical to those used for the FAME quantification, as described above. We confirmed the retention times and mass spectrums of C17:1cyclo ^{Δ 9} (Santa Cruz Biotechnology, Texas USA) and C19:1cyclo ^{Δ 9} (Santa Cruz Biotechnology). *cis*-9,10-methylene-*cis*-12-octadecenoic acid (C19:2 ^{Δ 12}cyclo ^{Δ 9}) and *cis*-9,10-methylene-*cis*-12,15-octadecadienoic acid (C19:3 ^{Δ 12,15}cyclo ^{Δ 9}) were estimated from the differences in the mass of the parent ion of C19:1cyclo ^{Δ 9} and pattern of fragmentation seen in the GC-MS results.

In order to analyze FAs attached to each lipid, the harvested cells were stored at -80°C

and the lipids were extracted with CH₃Cl:methanol (2:1, v/v). The cell debris were sedimented by centrifugation. The resulting supernatant was transferred into new tubes and evaporated. Then, the samples was re-suspended in 300 µl CH₃Cl:methanol (2:1, v/v) and applied to a silica gel plate. The lipids were separated by thin-layer chromatography using chloroform/methanol/acetic acid (65:25:8, v/v/v). The lipids were detected by staining with primulin and then the silica gel corresponding to the lipid spots was scraped off and extracted as described above.

2.4. Photosynthesis and respiration activities

Photosynthesis and respiration activities were measured as evolution and absorption of oxygen, respectively, using an oxygen electrode (Oxytherm System, Hansatech, Norfolk, UK). *Synechocystis* cells were grown at 34°C and 26°C for 2 d, and assayed at the same temperatures. Photosynthesis activity in these samples was measured at a light intensity of 600 µmole photons m⁻² s⁻¹, which represented saturated light conditions. Sodium hydrogen carbonate (2.5 mM) was added to the cell suspensions as a carbon source. Respiration activity was measured in the dark.

2.5. RNA extraction and quantification of mRNA

For the RNA extraction, wild-type and transformant cells were cultivated at 34°C under standard growth condition for 3 d, and then the cultures were transferred to 26°C. After 1 d of cultivation, the cells were inoculated into fresh BG11 medium at an OD₇₃₀ of 0.1 and further cultivated at 26°C for 1 d. The total RNAs were isolated by TRIzol Max Bacterial RNA Isolation Kit (Invitrogen, Carlsbad, CA), and purified by RNeasy Minikit (QIAGEN, Hilden, Germany) as previously described by Kotajima *et al.* [11].

The total RNAs extracted from the *Synechocystis* cells were reverse transcribed by PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio) to obtain cDNAs. Real-time PCR was performed using a GoTaq qPCR Master Mix (Promega, Fitchburg, WI) on a PikoPeal 96 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA). The 10 µL of PCR reaction mixture contained 5 ng of cDNAs, 0.2 µL of 10 µM forward primer, and 0.2 µL of 10 µM reverse primer. To determine the expression levels of the *desC*, *desA*, and *desD* genes, we used primer sets *desC_qPCR_F* and *desC_qPCR_R*, *desA_qPCR_F* and *desA_qPCR_R*, and *desD_qPCR_F* and *desD_qPCR_R*, respectively (Table S1). As a reference, we amplified the *rnpB* gene encoding the RNase P subunit B using primers *rnpB_RT_F* and *rnpB_RT_R*. As a negative control, we used total RNA samples that were not reverse transcribed. The threshold cycle (C_T) values were determined by PikoReal Software 2.2 (Thermo Fisher Scientific) following the manual. Relative quantitation was

performed using the comparative C_T method [12]. The results of the wild-type cells were used for calibration.

3. Results and Discussion

3.1. FA composition of *cfa*-overexpressing *E. coli*

E. coli cells synthesize the unsaturated FAs palmitoleic acid (C16:1 $^{\Delta 9}$) and vaccenic acid (C18:1 $^{\Delta 11}$) as components of their membrane lipids, but not C18:1 $^{\Delta 9}$, C18:2 $^{\Delta 9,12}$, or C18:3 $^{\Delta 6,9,12}$, which are the major unsaturated FAs in *Synechocystis* cells grown at 34°C. FA-modifying enzymes, including the acyl-lipid FA desaturases, are position-specific [3]. It was unclear whether Cfa in *E. coli* introduces cyclopropane groups at the $\Delta 9$ and $\Delta 11$ positions of the unsaturated FAs or specifically at the $\omega 7$ position. To determine whether Cfa could modify C18:1 $^{\Delta 9}$, C18:2 $^{\Delta 9,12}$, and C18:3 $^{\Delta 6,9,12}$ *in vivo*, we analyzed the FA compositions of the wild-type and *cfa*-overexpressing *E. coli* strains cultivated in a liquid medium containing C18:1 $^{\Delta 9}$, C18:2 $^{\Delta 9,12}$, C18:3 $^{\Delta 9,12,15}$, or C18:3 $^{\Delta 6,9,12}$.

Addition of each FA to a culture of the wild-type strain of *E. coli* resulted in total FAs in membrane lipids comprising 10-30% of C18:1 $^{\Delta 9}$, C18:2 $^{\Delta 9,12}$, C18:3 $^{\Delta 9,12,15}$, and C18:3 $^{\Delta 6,9,12}$ (Table 1). Wild-type cells cultivated with C18:1 $^{\Delta 9}$, C18:2 $^{\Delta 9,12}$, and C18:3 $^{\Delta 9,12,15}$ exhibited $0.3 \pm 0.1\%$, $0.3 \pm 0.1\%$, and $1.7 \pm 0.7\%$ of C19:1cyclo $^{\Delta 9}$, C19:2 $^{\Delta 12}$ cyclo $^{\Delta 9}$, and C19:3 $^{\Delta 12,15}$ cyclo $^{\Delta 9}$,

236 respectively, among the total membrane FAs. The total FAs in membrane lipids of the
 237 *cfa*-overexpressing *E. coli* strain comprised 8-12% of C18:1^{Δ9}, C18:2^{Δ9,12}, C18:3^{Δ9,12,15}, and
 238 C18:3^{Δ6,9,12}. Moreover, the *cfa*-overexpressing strain cultivated with C18:1^{Δ9}, C18:2^{Δ9,12}, and
 239 C18:3^{Δ9,12,15} exhibited 14.5 ± 2.9%, 5.4 ± 0.7%, and 2.2 ± 0.2% of C19:1cyclo^{Δ9},
 240 C19:2^{Δ12}cyclo^{Δ9}, and C19:3^{Δ12,15}cyclo^{Δ9}, respectively, among total membrane FAs. When both
 241 wild-type and *cfa*-overexpressing *E. coli* cells were cultivated with C18:3^{Δ6,9,12},
 242 approximately 10% of it of the total FAs was incorporated into the membrane lipids. Both
 243 cells synthesized C17:1cyclo^{Δ9} and lactobacillic acid (C19:1cyclo^{Δ11}), but did not synthesize
 244 novel cyclopropane FAs, such as *cis*-9,10-methylene-*cis*-6,12-octadecadienoic acid
 245 (C19:3^{Δ6,12}cyclo^{Δ9}). C16:1^{Δ7}, C16:2^{Δ7,10}, C16:3^{Δ4,7,10}, and C16:3^{Δ7,10,13} were detected when the
 246 cells were cultured in media including C18:1^{Δ9}, C18:2^{Δ9,12}, C18:3^{Δ6,9,12}, and C18:3^{Δ9,12,15},
 247 respectively, as exogenous FAs. The purities of C18:1^{Δ9}, C18:2^{Δ9,12}, C18:3^{Δ6,9,12}, and
 248 C18:3^{Δ9,12,15} were higher than 97%, 99%, 99%, and 99%, respectively. In addition, when we
 249 incubated these FAs in LB medium with shaking, these FAs were not changed into C16 FAs
 250 (data not shown). Thus, we concluded that C16:1^{Δ7}, C16:2^{Δ7,10}, C16:3^{Δ4,7,10}, and C16:3^{Δ7,10,13}
 251 were produced from C18:1^{Δ9}, C18:2^{Δ9,12}, C18:3^{Δ6,9,12}, and C18:3^{Δ9,12,15}, respectively, in the *E.*
 252 *coli* cells during the culture, perhaps via β-oxidation.

253 These results suggest that Cfa can specifically modify the Δ9 position of the C18

unsaturated FAs C18:1^{Δ9}, C18:2^{Δ9,12}, and C18:3^{Δ9,12,15} as substrates for production of cyclopropane FAs. However, if both the Δ6 and Δ9 positions of the C18 unsaturated FAs were unsaturated, Cfa could not introduce a cyclopropane ring at the Δ9 position. These results suggested that C18:1^{Δ9}, C18:2^{Δ9,12}, and C18:3^{Δ9,12,15} are candidate substrates for Cfa in *cfa*-expressing *Synechocystis* cells.

3.2. FA compositions of *Synechocystis* transformants

3.2.1. Heterologous expression of the *cfa* gene in wild-type *Synechocystis*

According to the results from FA feeding experiments in *E. coli* expressing the *cfa* gene, Cfa can convert C18:1^{Δ9} and C18:2^{Δ9,12} to C19:1cyclo^{Δ9} and C19:2^{Δ12}cyclo^{Δ9}, respectively. We examined whether the unsaturated FAs endogenously synthesized and incorporated into membrane lipids are converted to cyclopropane FAs *in vivo* by heterologously expressing *cfa* in wild-type *Synechocystis* cells.

In wild-type *Synechocystis* cells, C18:1^{Δ9} and C18:2^{Δ9,12}, which are thought to be substrates for Cfa, comprised $18.3 \pm 0.6\%$ and $16.6 \pm 0.6\%$ of total FAs, respectively. C18:3^{Δ6,9,12}, which may not be catalyzed by Cfa, comprised $9.2 \pm 0.8\%$ of total FAs. In the strain harboring the *cfa* gene (*cfa*⁺), C19:1cyclo^{Δ9} comprised $18.8 \pm 0.4\%$ of total FAs. Simultaneously, the C18:1^{Δ9} content was reduced to $5.1 \pm 0.4\%$ (Table 2). Interestingly,

272 C19:2^{Δ12}cyclo^{Δ9} was not produced from C18:2^{Δ9,12} in *Synechocystis* cells. The abundance of
273 C18:2^{Δ9,12} in the *cfa*⁺ strain was $10.7 \pm 0.7\%$, which was slightly lower than that in wild-type
274 cells. These results indicated that Cfa was functional in the *Synechocystis* cells, but that only
275 C18:1^{Δ9} was used as a substrate and that DesA, a $\Delta 12$ acyl-lipid desaturase in *Synechocystis*,
276 also uses C18:1^{Δ9} but not C19:1cyclo^{Δ9} as a substrate. Cfa and DesA compete for use of
277 C18:1^{Δ9}.

278 Although Cfa converted C18:2^{Δ9,12} to C19:2^{Δ12}cyclo^{Δ9} in *E. coli* cells, this did not happen
279 in *Synechocystis* cells. The reason for this discrepancy is unclear, but the head groups of the
280 membrane lipids differ between these two organisms. The membrane lipids in *E. coli* are
281 phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol
282 [PG], and cardiolipin) [6], whereas those in *Synechocystis* are mainly glycolipids (e.g.,
283 monogalactosyl diacylglycerol [MGDG], digalactosyl diacylglycerol [DGDG], and
284 sulfoquinovosyl diacylglycerol [SQDG]) and only one phospholipid, PG [14]. We next
285 separated the total lipids into each membrane lipid (i.e., MGDG, DGDG, SQDG, and PG) by
286 thin-layer chromatography and determined the FA profiles. As shown in Table S2, in the *cfa*⁺
287 strain, C19:1cyclo^{Δ9} was incorporated into all four types of lipids, and the C19:1cyclo^{Δ9}
288 content in SQDG was slightly lower than those in the other three lipids. Because a greater
289 quantity of palmitic acid (C16:0) was attached to SQDG than to the other lipids in

290 *Synechocystis* cells, as reported previously [22], the C19:1cyclo^{Δ9} level in the lipids was lower.

291 Thus, Cfa activities were unaffected by differences in the lipid head groups.

292 The difference in lipid types does not explain the lack of production of C19:2^{Δ12}cyclo^{Δ9} in
293 *Synechocystis*. According to the literature, Cfa acts on the nonpolar portion of phospholipids
294 dispersed in vesicles [19]. Recently, S. J. Biller *et al.* [2] showed that many microorganisms
295 develop and release small membrane vesicles from cell surfaces. However, the presence of
296 such membrane vesicles in the cytosol of cyanobacterial cells is yet to be demonstrated. At
297 this time, we cannot clearly identify the substrate of Cfa. In *Synechocystis* cells, there are
298 three cell membranes, i.e., the outer membrane, inner membrane, and thylakoid membrane. In
299 these membranes, the lipids or FAs do not exist unevenly [10]. Thus, we did not consider the
300 presence of a different compartment of C18:2^{Δ9,12} in *Synechocystis*. However, in unidentified
301 vesicles and possible microdomains where Cfa might locate and function, we cannot ignore
302 the possibility. It is unclear why C19:2^{Δ12}cyclo^{Δ9} was not produced in *Synechocystis*.

304 3.2.2. Heterologous expression of *cfa* in *desA*⁻/*desD*⁻ *Synechocystis* cells

305 Cfa catalyzed the synthesis of only C19:1cyclo^{Δ9} from C18:1^{Δ9} in *Synechocystis* cells
306 (Table 2). To increase the abundance of C19:1cyclo^{Δ9} in *Synechocystis* cells, we attempted to
307 introduce the *cfa* gene into the *desA*⁻/*desD*⁻ strain (*desA*⁻/*desD*⁻/*cfa*⁺), which does not produce

C18 polyunsaturated FAs [18]. Total FAs of the *desA⁻/desD⁻* strain comprised $49.8 \pm 0.5\%$ of C18:1^{Δ⁹}, which is 2.5-fold greater than that of wild-type cells (Table 2). Although we attempted to increase the abundance of C19:1cyclo^{Δ⁹} in the *desA⁻/desD⁻/cfa⁺* strain, it unexpectedly comprised $22.2 \pm 0.6\%$ of the total FAs (Table 2). First, we hypothesized that Cfa prefers unsaturated FAs attached to phospholipids. However, the FA compositions of each lipid class—MGDG, DGDG, SQDG, and PG—of *desA⁻/desD⁻/cfa⁺* were not markedly altered, suggesting that Cfa does not discriminate among lipid head groups (Table S2). Excess C19:1cyclo^{Δ⁹} in the membrane lipids might exert deleterious effects on *Synechocystis* cells.

3.2.3. Heterologous coexpression of *cfa* and *desC2* in *Synechocystis* cells

In the *desA⁻/desD⁻/cfa⁺* strain, the abundance of C19:1cyclo^{Δ⁹} was not increased compared with that in the *cfa⁺* strain. To increase the abundance of cyclopropane FAs in *Synechocystis* cells, we attempted to synthesize a novel cyclopropane FA, C17:1cyclo^{Δ⁹}, from C16:1^{Δ⁹} in *Synechocystis*. The endogenous desaturases of *Synechocystis* specifically unsaturate C18 FAs attached at the *sn*-1 position of the lipids, but cannot unsaturate C16 FAs attached at the *sn*-2 position [14]. In contrast, DesC2 from *Nostoc* sp. strain 36 specifically unsaturates C16 saturated FA attached at the *sn*-2 position [4]. We aimed to synthesize C17:1cyclo^{Δ⁹} by coexpression of the *cfa* and *desC2* genes in the wild-type and *desA⁻/desD⁻*

strains of *Synechocystis* ($cfa^+/desC2^+$ and $desA^-/desD^-/cfa^+/desC2^+$). Furthermore, we also constructed the $desC2^+$ and $desA^-/desD^-/desC2^+$ strains by introducing the corresponding genes into the wild-type and $desA^-/desD^-$ cells, respectively, as controls.

In the $desC2^+$ and $desA^-/desD^-/desC2^+$ strains, C16:1 $^{\Delta 9}$ comprised approximately 25% of total FAs. Because C19:1cyclo $^{\Delta 9}$ comprised ~20% of total FAs in the cfa^+ and $desA^-/desD^-/cfa^+$ strains, we expected that the $cfa^+/desC2^+$ and $desA^-/desD^-/cfa^+/desC2^+$ strains would synthesize ~20% C17:1cyclo $^{\Delta 9}$ in addition to 20% C19:1cyclo $^{\Delta 9}$. However, the $cfa^+/desC2^+$ and $desA^-/desD^-/cfa^+/desC2^+$ strains produced only $7.7 \pm 0.5\%$ and $5.2 \pm 0.4\%$ C17:1cyclo $^{\Delta 9}$, respectively. Moreover, the C16:1 $^{\Delta 9}$ abundance was reduced to $2.3 \pm 0.1\%$ and $1.8 \pm 0.1\%$ in the $cfa^+/desC2^+$ and $desA^-/desD^-/cfa^+/desC2^+$ strains, respectively. Interestingly, C19:1cyclo $^{\Delta 9}$ abundance was maintained after introduction of the *desC2* gene, comprising more than 20% in the $cfa^+/desC2^+$ and $desA^-/desD^-/cfa^+/desC2^+$ strains. The abundance of C17:1cyclo $^{\Delta 9}$ in the $cfa^+/desC2^+$ and $desA^-/desD^-/cfa^+/desC2^+$ strains was lower than our estimation. C16:1 $^{\Delta 9}$ abundance in the $cfa^+/desC2^+$ and $desA^-/desD^-/cfa^+/desC2^+$ strains was markedly lower than that in the $desC2^+$ and $desA^-/desD^-/desC2^+$ strains.

3.3. Growth of the *Synechocystis* transformants

In this study, we constructed *Synechocystis* strains that synthesized cyclopropane FAs and

unsaturated C16 FAs. These are novel FAs in *Synechocystis* cells; therefore, we examined the effects of synthesis of cyclopropane FAs and unsaturated C16 FA on the growth of *Synechocystis* cells.

Both wild-type and *cfa*⁺ cells grew well at high and low temperatures (34°C and 26°C, respectively) (Fig. 1). The growth rates were not altered by introduction of the *cfa* gene in wild-type cells. The growth of *desA*⁻/*desD*⁻ cells at 34°C was reduced compared with that of wild-type cells, whereas growth at 26°C was almost halted, as reported previously [18]. The growth rate of *desA*⁻/*desD*⁻/*cfa*⁺ cells was lower than that of *desA*⁻/*desD*⁻ cells at both temperatures, and these cells were unable to grow at 26°C. These results suggest that C19:1cyclo^{Δ9} synthesis in cells that do not synthesize polyunsaturated FAs negatively affected growth, irrespective of temperature. The melting point of C18:1^{Δ9}; i.e., 16.3°C (<http://www.sciencelab.com/msds.php?msdsId=9927682>), is lower than that of C19:1cyclo^{Δ9} (i.e., 27.8–28.8°C) [7], suggesting that maintenance of membrane fluidity in cells with membrane lipids possessing C19:1cyclo^{Δ9} instead of C18:1^{Δ9} might be difficult.

The growth rate at 34°C of wild-type cells harboring the *desC2* gene was slightly decreased compared with that of wild-type cells, and growth at 26°C was significantly repressed. These results suggest that synthesis of C16:1^{Δ9} in the wild-type cells negatively affected their growth, particularly at low temperatures. In a previous report [4], the

unsaturation of the C16 FAs attached at the *sn*-2 position was suggested to contribute to the growth of a *Nostoc* strain living in an Antarctic lake at low temperatures, but this was not so in the case of *Synechocystis*. Although the growth of the *desA*⁻/*desD*⁻/*desC2*⁺ strain at 34°C was not changed, growth at 26°C was significantly increased. However, the growth rate of the *desA*⁻/*desD*⁻/*cfa*⁺/*desC2*⁺ strain decreased to almost the same extent as that of the *desA*⁻/*desD*⁻ strain. Although C16:1^{Δ9} synthesis in wild-type cells resulted in decreased growth at 26°C, growth of the *desA*⁻/*desD*⁻ strain was stimulated at this temperature. If the C18 FA at the *sn*-1 position is not multiply unsaturated, unsaturation of the C16 FA at the *sn*-2 position might contribute to growth at low temperatures. C16:1^{Δ9} production by the *desA*⁻/*desD*⁻ strain might have maintained the membrane fluidity under low-temperature conditions. Co-introduction of the *cfa* gene abrogated the positive effects of the *desC2* gene on growth at low temperature. Therefore, the conversion of C16:1^{Δ9} at the *sn*-2 position of membrane lipids into C16:1cyclo^{Δ9} might decrease membrane fluidity.

3.4. Effect of cyclopropane FA synthesis on photosynthesis and respiration activities

Modification of FA profiles by expression of *cfa* and *desC2* altered the growth rates of *Synechocystis* transformants, especially at 26°C (Fig. 1). To investigate these phenomena, we analyzed photosynthesis and respiration activities in *Synechocystis* cells grown at 34°C and

26°C. Respiration and photosynthesis activities were not significantly altered in cells grown at 34°C (Fig. 2). As reported previously [18], the growth rate of the *desA*⁻/*desD*⁻ strain was severely retarded at low temperature, although photosynthesis and respiration activities were not significantly different from those of wild-type cells. However, the respiration and photosynthesis activities in the wild-type cells harboring the *cfa* or *desC2* gene were decreased to some extent, particularly at 26°C. Although expression of the *desC2* gene resulted in recovery of the growth rate of the *desA*⁻/*desD*⁻ strain at 26°C, photosynthesis and respiration activities were lower than those of the *desA*⁻/*desD*⁻ strain. These results suggest that *cfa* and *desC2* expression in wild-type cells slightly decreased the photosynthesis and respiration activities, which may be related to suppression of the growth rate of the transformants. However, C16:1^{Δ9} synthesis in the *desA*⁻/*desD*⁻ strain led to recovery of the growth rate at 26°C via promotion of various cellular processes other than photosynthesis and respiration.

The cultures in Fig. 1 were grown at 70 μmole photons m⁻² s⁻¹ and the photosynthetic activity shown in Fig. 2 was measured at 600 μmole photons m⁻² s⁻¹, which is near the light saturation point in cells, to observe the maximum rate of O₂ evolution. We thought that there was a discrepancy between the two experiments due to a difference in the light conditions. Therefore, we further investigated the photosynthetic activity at various light intensities,

including 75 $\mu\text{mol photons/m}^2/\text{s}$, which is the closest to the growth condition. As a result, even at a low light intensity, we could not observe clear differences in the photosynthetic activity in the strains (Fig. S2). Therefore, modification of the FA composition might have altered the activities of endogenous desaturase in *Synechocystis* and indirectly affected the growth of the transformants.

3.5. Apparent desaturase and cyclopropane FA synthetase activities in *Synechocystis* strains

To compare apparent desaturase and cyclopropane FA synthetase activities, we determined the total abundance of FAs synthesized by each enzyme (Table 2, Table S3, and Fig. 3). The total abundance of FAs unsaturated by DesC is shown in Fig. 3A. The *cfa*⁺, *desC2*⁺, and *cfa*⁺/*desC2*⁺ strains synthesized lower levels of FAs unsaturated by DesC than the wild-type cells did. In addition, the *desA*⁻/*desD*⁻/*cfa*⁺, *desA*⁻/*desD*⁻/*desC2*⁺, and *desA*⁻/*desD*⁻/*cfa*⁺/*desC2*⁺ strains synthesized lower levels of FAs unsaturated by DesC than did the *desA*⁻/*desD*⁻ strain. This might be due to a mechanism regulating membrane fluidity.

In accordance with previous reports [13], the *desC* gene in *Synechocystis* is constitutively expressed irrespective of culture conditions. However, these findings suggest the existence of a novel mechanism of regulating *desC* expression to prevent excess membrane fluidity. We analyzed expression levels of the *desC* gene by qPCR using total RNA extracted from the

cells of wild-type, *cfa*⁺, *desC2*⁺, and *cfa*⁺/*desC2*⁺ *Synechocystis* strains cultivated at 26°C. We found that the *desC* gene expression levels in the *cfa*⁺, *desC2*⁺, and *cfa*⁺/*desC2*⁺ strains were not remarkably different from those in the wild-type cells (Table S4A). These results suggest that there might be a novel mechanism for regulating DesC activity, perhaps via post-translationally, to maintain the suitable membrane fluidity..

Fig. 3B, C, and D show the total abundance of FAs unsaturated by DesA, DesD, and DesB, respectively. These results demonstrated that the apparent activities of DesA, DesD, and DesB were decreased to some extent by introduction of the *desC2* gene, especially at 26°C. *desA*, *desD*, and *desB* expression is induced at low temperature to maintain membrane fluidity in *Synechocystis* cells [22]. *desC2* expression may downregulate the expression levels of *desA*, *desD*, and *desB* via the regulatory mechanism for maintaining membrane fluidity [13]. We analyzed expression levels of the *desA* and *desD* genes by qPCR using total RNA extracted from the cells of wild-type, *cfa*⁺, *desC2*⁺, and *cfa*⁺/*desC2*⁺ *Synechocystis* strains cultivated at 26°C. We found that the *desA* and *desD* gene expression levels in the *cfa*⁺, *desC2*⁺, and *cfa*⁺/*desC2*⁺ strains were not remarkably altered from those in the wild-type cells (Table S4B, C). Alternatively, DesC2 may compete with DesA, DesD, and DesB, and these endogenous desaturases may specifically unsaturate C18 FAs attached at the *sn*-1 position of lipids containing C16-saturated FAs at the *sn*-2 position, but not those containing

C16-unsaturated FAs. Fig. 4 summarizes these results, and the data therein suggest that no pathway for unsaturation by DesA, DesD, and DesB after unsaturation by DesC2 exists.

Fig. 3E shows the total abundance of FAs unsaturated by DesC2. These results demonstrated that cells harboring both the *cfa* and *desC2* genes produced lower levels of FAs unsaturated by DesC2 than did cells expressing the *desC2* gene under both the high- and low-temperature conditions. The total abundance of saturated FAs are shown in Fig. 3G. These results demonstrated that *Synechocystis* cells harboring only *desC2* produced lower levels of saturated FAs than did wild-type cells, but that the cells containing both *cfa* and *desC2* accumulated large quantities of saturated FAs. Indeed, the levels were almost identical to those in wild-type *Synechocystis* cells. These results suggested that DesC2 might unsaturate C16:0 attached at the *sn*-2 position of lipids possessing C18 unsaturated FAs at the *sn*-1 position but not lipids with C18-saturated FAs or C19 cyclopropane FAs (Fig. 4). The cyclopropane FA yield would be increased if the *sn*-2-specific desaturase could unsaturate C16:0 attached at the *sn*-2 position of lipids containing C18-saturated FAs or C19 cyclopropane FAs at the *sn*-1 position.

4. Conclusions

We successfully synthesized cyclopropane FAs in *Synechocystis* at a level comprising

~30% of total FAs. Growth of *Synechocystis* cells harboring the *cfa* and *desC2* genes was altered, particularly under low-temperature conditions, but photosynthesis and respiration activities were not significantly affected. C16:1^{Δ9} synthesis in the *desA⁻/desD⁻* strain positively affected growth under low-temperature conditions by promoting various cellular processes other than photosynthesis and respiration. Our data suggest that Cfa and desaturases may recognize lipid side chains. The cyclopropane FA yield would be increased if the *sn*-2-specific desaturase could unsaturate C16:0 attached at the *sn*-2 position of lipids containing C18-saturated FAs or C19 cyclopropane FAs at the *sn*-1 position.

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Figure Captions

Fig. 1. Growth of wild-type and mutant cells at 34°C and 26°C. Mean $\pm$ S.D. values of three independent experiments. Closed red circle, Wild-type cells; open red circle, *cfa*⁺ cells; closed green circle, *desC2*⁺ cells; open green circle, *cfa*⁺/*desC2*⁺ cells; closed purple circle, *desA*⁻/*desD*⁻ cells; open purple circle, *desA*⁻/*desD*⁻/*cfa*⁺ cells; closed light blue circle, *desA*⁻/*desD*⁻/*desC2*⁺ cells; and open light blue circle, *desA*⁻/*desD*⁻/*cfa*⁺/*desC2*⁺ cells.

Fig. 2. O₂ evolution and absorption of wild-type and mutant cells at 34°C and 26°C. Cells were cultured at 34°C and 26°C for 2 days, and the activities were measured at the same temperature using an O₂ electrode. Mean $\pm$ S.D. values of three independent experiments. Black and white bars show respiratory and photosynthetic activities, respectively.

Fig. 3. Relative amounts of fatty acids produced by each desaturase and cyclopropane FA synthetase. Mean $\pm$ S.D. values of three independent experiments, expressed as mol % of total fatty acids. Red and blue bars indicate cells cultivated at 34°C and 26°C, respectively. A, total production of Δ^9 unsaturated C18 FAs by DesC, the sum of C18:1 ^{Δ^9} , C18:2 ^{$\Delta^9,12$} , C18:3 ^{$\Delta^9,12,15$} , C18:3 ^{$\Delta^6,9,12$} , C18:4 ^{$\Delta^6,9,12,15$} , and C19:1cyclo ^{Δ^9} ; B, total production of Δ^{12} unsaturated C18 FAs by DesA, the sum of C18:2 ^{$\Delta^9,12$} , C18:3 ^{$\Delta^9,12,15$} , C18:3 ^{$\Delta^6,9,12$} , and C18:4 ^{$\Delta^6,9,12,15$} ; C, total

production of $\Delta 6$ unsaturated C18 FAs by DesD, the sum of C18:3 $^{\Delta 6,9,12}$ and C18:4 $^{\Delta 6,9,12,15}$; D, total production of $\Delta 15$ unsaturated C18 FAs by DesB, the sum of C18:3 $^{\Delta 9,12,15}$ and C18:4 $^{\Delta 6,9,12,15}$; E, total production of $\Delta 9$ unsaturated C16 FAs by DesC2, the sum of C16:1 $^{\Delta 9}$ and C17:1cyclo $^{\Delta 9}$; F, total production of cyclopropane FAs by Cfa, the sum of C17:1cyclo $^{\Delta 9}$ and C19:1cyclo $^{\Delta 9}$; and G, total production of saturated FAs, the sum of C16:0 and C18:0.

Fig. 4. Putative pathways of the desaturases and cyclopropane FA synthetases. Arrows indicate unsaturation by DesA, DesB, DesC, DesC2, and DesD and modification by Cfa. Doublets indicate that a reaction did not occur.

Fig. S1. Construction of the plasmids used in this study. Red, blue, and black arrows indicate PCR amplification using the indicated primers, conjugation, and cyclization of the fragment and vector using the In-Fusion HD Cloning Kit (Takara Bio), and ligation after digestion by the indicated restriction enzymes.

Fig. S2. Photosynthesis activities of wild-type and mutant cells. A: Cells were cultured at 34°C and 26°C for 2 d, and the activities were measured at the same temperature using an O₂ electrode. Mean $\pm$ S.D. values of three independent experiments. Red, wild-type cells; green,

572 *desC2⁺* cells; purple, *desA⁻/desD⁻* cells; and light blue, *desA⁻/desD⁻/desC2⁺* cells. B:
573 Magnification of ranges from 0 to 100 $\mu\text{mole photons m}^{-2} \text{ s}^{-1}$ of the graphs in A. Black allows
574 indicate light intensity at 70 $\mu\text{mole photons m}^{-2} \text{ s}^{-1}$ for cultivation.
575

576 **Table 1**577 Fatty acid composition of total lipids from wild-type and *cfa*-overexpressing *E. coli* strains

FA	Fatty acid (mol %)									
	Wild type					<i>cfa</i> -overexpressing strain				
	Exogenous FA					Exogenous FA				
	-	18:1 ^{Δ9}	18:2 ^{Δ9,12}	18:3 ^{Δ9,12,15}	18:3 ^{Δ6,9,12}	-	18:1 ^{Δ9}	18:2 ^{Δ9,12}	18:3 ^{Δ9,12,15}	18:3 ^{Δ6,9,12}
14:0	6.8 ± 0.2	2.8 ± 1.5	5.3 ± 1.3	5.8 ± 0.6	4.8 ± 1.6	5.5 ± 1.2	3.9 ± 1.2	5.2 ± 0.9	6.4 ± 0.5	4.5 ± 1.0
16:0	44.9 ± 0.5	44.6 ± 1.3	58.7 ± 2.4	49.4 ± 2.0	41.3 ± 2.6	45.0 ± 0.2	35.8 ± 2.2	53.6 ± 3.1	47.8 ± 1.1	40.5 ± 3.2
16:1 ^{Δ9}	16.8 ± 4.4	3.0 ± 1.4	3.9 ± 0.9	4.7 ± 1.6	11.0 ± 3.6	3.7 ± 0.8	1.8 ± 0.1	2.4 ± 0.9	2.3 ± 1.0	4.5 ± 1.9
16:1 ^{Δ7}	-	2.5 ± 1.2	-	-	-	-	3.8 ± 1.1	-	-	-
16:2 ^{Δ7,10}	-	-	2.9 ± 1.0	-	-	-	-	2.1 ± 0.1	-	-
16:3 ^{Δ7,10,13}	-	-	-	5.2 ± 0.4	-	-	-	-	4.5 ± 0.7	-
16:3 ^{Δ4,7,10}	-	-	-	-	2.6 ± 1.3	-	-	-	-	2.5 ± 1.0
17:1cyclo ^{Δ9}	11.1 ± 5.0	2.1 ± 1.3	6.0 ± 3.8	4.9 ± 2.8	6.4 ± 3.9	22.8 ± 2.1	5.4 ± 1.8	9.5 ± 2.1	10.6 ± 2.5	19.3 ± 3.4
18:0	3.3 ± 0.7	2.5 ± 0.7	2.7 ± 0.4	3.0 ± 0.8	8.8 ± 5.6	6.8 ± 2.0	6.1 ± 2.2	3.9 ± 0.7	4.2 ± 1.5	5.3 ± 1.9
18:1 ^{Δ11}	15.9 ± 1.4	12.3 ± 2.4	5.5 ± 0.1	5.9 ± 0.6	13.1 ± 1.1	5.5 ± 1.8	12.6 ± 5.7	4.2 ± 1.0	3.3 ± 0.6	7.4 ± 2.0
18:1 ^{Δ9}	-	29.4 ± 1.5	-	-	-	-	12.3 ± 3.6	-	-	-
18:2 ^{Δ9,12}	-	-	12.9 ± 3.7	-	-	-	-	7.8 ± 1.1	-	-
18:3 ^{Δ9,12,15}	-	-	-	17.6 ± 0.4	-	-	-	-	12.3 ± 1.2	-
18:3 ^{Δ6,9,12}	-	-	-	-	10.9 ± 2.3	-	-	-	-	8.2 ± 0.6
19:1cyclo ^{Δ11}	1.2 ± 1.0	0.4 ± 0.1	1.8 ± 1.3	1.7 ± 0.7	1.1 ± 0.9	10.6 ± 1.0	4.0 ± 1.0	5.9 ± 0.8	6.5 ± 0.5	7.7 ± 1.7
19:1cyclo ^{Δ9}	-	0.3 ± 0.1	-	-	-	-	14.5 ± 2.9	-	-	-
*19:2 ^{Δ12} cyclo ^{Δ9}	-	-	0.3 ± 0.1	-	-	-	-	5.4 ± 0.7	-	-
*19:3 ^{Δ12,15} cyclo ^{Δ9}	-	-	-	1.7 ± 0.7	-	-	-	-	2.2 ± 0.2	-
*19:3 ^{Δ6,12} cyclo ^{Δ9}	-	-	-	-	-	-	-	-	-	-

578 The cells were grown at 37°C for 18 h. Exogenous fatty acids (1 mM) were added to the culture. Mean ± S.D. values of three independent
579 experiments, expressed as mol % of total fatty acids. “-”, not detected. “*”, estimated from comparison with 19:1cyclo^{Δ9}.

581 **Table 2**582 Fatty acid compositions of total lipids from *Synechocystis* wild-type and transformants

34°C		Fatty acid (mol %)								
Strain	16:0	16:1Δ9	17:1cycloΔ9	18:0	18:1Δ9	18:2Δ9,12	18:3Δ6,9,12	18:3Δ9,12,15	18:4Δ6,9,12,15	19:1cycloΔ9
Wild type	55.0 ± 1.6	-	-	0.8 ± 0.1	18.3 ± 0.6	16.6 ± 0.6	9.2 ± 0.8	-	-	-
<i>cfa</i> ⁺	55.5 ± 0.8	-	-	2.1 ± 0.1	5.1 ± 0.4	10.7 ± 0.7	7.8 ± 0.6	-	-	18.8 ± 0.4
<i>desC2</i> ⁺	31.3 ± 0.8	25.3 ± 0.7	-	2.6 ± 0.4	15.2 ± 0.7	19.3 ± 1.2	6.3 ± 0.8	-	-	-
<i>cfa</i> ⁺ / <i>desC2</i> ⁺	44.9 ± 0.4	2.3 ± 0.1	7.7 ± 0.5	5.6 ± 1.5	1.9 ± 0.1	11.2 ± 0.3	6.2 ± 0.4	-	-	20.2 ± 0.7
<i>desA</i> ⁻ / <i>desD</i> ⁻	49.0 ± 0.4	-	-	1.2 ± 0.2	49.8 ± 0.5	-	-	-	-	-
<i>desA</i> ⁻ / <i>desD</i> ⁻ / <i>cfa</i> ⁺	59.7 ± 0.4	-	-	3.5 ± 0.2	14.6 ± 0.8	-	-	-	-	22.2 ± 0.6
<i>desA</i> ⁻ / <i>desD</i> ⁻ / <i>desC2</i> ⁺	35.8 ± 0.4	26.2 ± 1.0	-	4.1 ± 1.6	33.9 ± 1.0	-	-	-	-	-
<i>desA</i> ⁻ / <i>desD</i> ⁻ / <i>cfa</i> ⁺ / <i>desC2</i> ⁺	54.6 ± 0.6	1.8 ± 0.1	5.2 ± 0.4	6.4 ± 1.8	3.7 ± 0.7	-	-	-	-	28.2 ± 1.9
26°C		Fatty acid (mol %)								
Strain	16:0	16:1Δ9	17:1cycloΔ9	18:0	18:1Δ9	18:2Δ9,12	18:3Δ6,9,12	18:3Δ9,12,15	18:4Δ6,9,12,15	19:1cycloΔ9
Wild type	52.1 ± 0.1	-	-	1.1 ± 0.3	4.8 ± 0.7	13.5 ± 0.6	21.4 ± 1.0	4.5 ± 0.6	2.6 ± 0.3	-
<i>cfa</i> ⁺	53.9 ± 0.9	-	-	2.1 ± 0.6	4.3 ± 0.9	10.6 ± 0.8	16.9 ± 2.3	3.1 ± 0.6	1.9 ± 0.4	7.2 ± 2.4
<i>desC2</i> ⁺	26.5 ± 0.3	27.5 ± 0.6	-	3.5 ± 0.3	11.4 ± 1.0	16.7 ± 0.9	12.0 ± 0.7	1.5 ± 0.2	0.9 ± 0.1	-
<i>cfa</i> ⁺ / <i>desC2</i> ⁺	42.5 ± 0.4	4.0 ± 0.2	8.2 ± 0.5	4.1 ± 0.4	4.0 ± 0.3	13.1 ± 1.0	12.5 ± 0.6	1.3 ± 0.2	0.9 ± 0.2	9.4 ± 2.0
<i>desA</i> ⁻ / <i>desD</i> ⁻	54.0 ± 0.7	-	-	1.3 ± 0.3	44.7 ± 0.8	-	-	-	-	-
<i>desA</i> ⁻ / <i>desD</i> ⁻ / <i>cfa</i> ⁺	54.7 ± 1.1	-	-	7.2 ± 3.7	18.8 ± 2.5	-	-	-	-	19.3 ± 1.0
<i>desA</i> ⁻ / <i>desD</i> ⁻ / <i>desC2</i> ⁺	28.7 ± 0.7	25.4 ± 1.2	-	2.9 ± 0.2	43.1 ± 0.3	-	-	-	-	-
<i>desA</i> ⁻ / <i>desD</i> ⁻ / <i>cfa</i> ⁺ / <i>desC2</i> ⁺	51.5 ± 1.0	3.3 ± 0.5	2.8 ± 0.1	2.7 ± 0.2	16.3 ± 1.8	-	-	-	-	23.4 ± 1.1

583 The cells were grown at 34°C and 26°C for 3 d. Mean ± S.D. values of three independent experiments, expressed as mol % of total fatty

584 acids. “-”, not detected.

Fig. 1

Fig. 2

Fig. 3

Fig. 4