Study on Biosynthesis of the Novel Fatty Acids in the Cyanobacterium Synechocystis sp. PCC 6803

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Abstract

Methylesters of fatty acids from microalgae attract attention as a biodiesel. However, most of the fatty acids in microalgae are C16-18 saturated and unsaturated fatty acids. The saturated fatty acids are stable against oxidation under the atmosphere, but they are solidified at the ambient temperature because their melting points are high. Whilst the melting points of the unsaturated fatty acids are low and they are liquid at the temperature. However, C=C double bonds in the carbon skeleton are unstable due to oxidation by the atmospheric oxygen. The cyclopropane fatty acid and mid-chain methyl-branched fatty acid in which certain bacteria compose has low melting-points and stable to the oxidation. In this study, I succeeded in synthesis of these unusual methylated fatty acids in the cyanobacterium, *Synechocystis* sp. PCC 6803. In addition, I examined the effect of synthesis of cyclopropane fatty acids or mid-chain methyl-branched fatty acid in *Synechocystis* on the growth and the photosynthetic activity.

First, I attempted to synthesize cyclopropane fatty acids in *Synechocystis* by heterologous expression of the *cfa* gene for the cyclopropane fatty acid synthase from *Escherichia coli*. I successfully synthesized C19 cyclopropane fatty acid from oleic acid in *Synechocystis* expressing the *cfa* gene. By the co-expression of the *desC2* gene for *sn*-2 specific Δ9 desaturase which converts palmitic acid to palmitoleic acid in the lipids, the cells also produced C17 cyclopropane fatty acid. The growth rates and photosynthetic activities in the wild-type and the *cfa*-expression cells grown at 34°C were equivalent. And also the cells expressed the *cfa* and *desC2* genes produced less amount of palmitoleic acid than the other cells expressed the *desC2* gene. This result indicated that DesC2 might unsaturate palmitic acid attached on the *sn-2* position of the lipids containing C18 unsaturated fatty acid on the *sn-1* position, but not the lipids containing C18 saturated fatty acid or C19 cyclopropane fatty acid.

Second, I attempted to synthesize 10-methyl stearic acid, which is one of the mid-chain methyl-branched fatty acid, in *Synechocystis*. But, the biosynthetic pathway of 10-methyl stearic acid *in vivo* has not been demonstrated clearly yet. It had been speculated that 10-methyl stearic acid is synthesized from oleic acid by *S*-adenosyl-ʟ-methionine-dependent methyltransferase and NADPH-dependent reduction via a methylenated intermediate, 10-methylene stearic acid. I focused on novel *S*-adenosyl-ʟ-methionine-dependent methyltransferases from *Mycobacterium chlorophenolicum* that are involved in 10-methyl stearic acid synthesis and selected candidate protein, WP_048472121, by a comparative genomic analysis. And also I found that the gene encoding WP_048472121 was collocated with another gene encoding WP_048472120, which is a protein containing a domain associated with flavin adenine dinucleotide-binding oxidoreductase activity. The co-expression of these proteins (hereafter called BfaA and BfaB, respectively) led to the biosynthesis of 10-methyl stearic acid in *E. coli* cells via the methylenated intermediate, 10-methylene stearic acid. Then I successfully synthesized 10-methyl stearic acid from oleic acid in *Synechocystis* expressing the *bfaA* and *bfaB* genes.

In this study, I succeeded in synthesizing the cyclopropane fatty acids which is higher industrial availability than saturated or unsaturated fatty acid in *Synechocystis*. This work is first trial to synthesize unusual methylated fatty acids in phototroph. Interestingly, synthesizing of these fatty acids did not affect on photosynthetic activity. So I expect that the industrial production of these fatty acids by microalgae will become possible.

Key words:

Biofuel, Cyclopropane fatty acid, Acyl-lipid desaturase, Mid-chain methyl-branched fatty acids, Tuberculostearic acid, SAM-dependent methyltransferase, *Synechocystis* sp. PCC 6803.

General Introduction

Contemporary society is dependent on large quantities of fossil fuels. The 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 the fossil fuels may accelerate global warming and environmental pollutions. Moreover, demand for the fossil fuels is increasing due to the 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 the 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 (Chisti, 2007; Parmar et al., 2011). Some microalgae accumulate large quantities of oils (mainly triacylglycerols), especially under conditions of stress, such as nitrogen starvation (Hu et al., 2008). Fatty acids in triacylglycerols are hydrolyzed and methylated, and their derivatives are utilized as a biodiesel. Most fatty acids from microalgae are multiply unsaturated; thus, the biofuels derived from them are fluid but vulnerable to oxidation. Thus, direct use of the natural fatty acids from microalgae in the industry may be problematic. Use of fatty acids could be expanded if they were stable in response to oxidation during long-term storage and if they were sufficiently fluid. Saturated fatty acids are stable in response to oxidation, but saturated fatty acids with C16 or C18 chains (which are the major acyl groups of the lipids in the living organisms) solidify at physiological temperatures. Thus, the saturated fatty acids are not suitable for use in biofuel production.

In the living organisms, modified fatty acids are crucial for the functions of the cellular membranes and storage lipids where these 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 Low, 1970; Cronan et al., 1974; Takayama et al., 2005). Some bacteria, such as *Escherichia coli* and *Lactobacillus arabinosus*, synthesize cyclopropane fatty acids, which contain a cyclopropane ring in the acyl group. Cyclopropane fatty acid synthase encoded by the *cfa* gene in *E*. *coli* catalyzes the modification of acyl chains to their cyclopropane derivatives through the methylation of an unsaturated double bond. In this reaction, *S*-adenosyl-ʟ-methionine (SAM) is used as a methyl donor. The enzyme acts on the *cis*-double bond at the $\Delta 9$ or $\Delta 11$ in C16 and C18 fatty acids attached to the membrane lipids (Cronan et al., 1974; Grogan and Cronan, 1997; Law, 1971). The branched-chain fatty acids can be classified into two types, namely *iso*- or *anteiso*-methyl-branched fatty acids and mid-chain methyl-branched fatty acids. The *iso*-methyl-branched fatty acids have the methyl branch on the second carbon from the methyl end of the acyl group, whilst the *anteiso*-methyl-branched fatty acids have the methyl branch on the third carbon from the methyl end. The biosynthetic pathway of these fatty acids is well characterized in *Bacillus subtilis* (Namba et al., 1969; Oku and Kaneda, 1988). Isobutyrate, isovalerate, and 2-methylbutyrate derived from the degradation of the branched-chain amino acids, e.g., valine, leucine, and isoleucine, respectively, are thioesterified with coenzyme A to be activated and utilized as precursors of fatty acid synthesis, and then the *iso*- or *anteiso*-methyl-branched fatty acids are synthesized. As a mid-chain methyl-branched fatty acid, 10-methyl stearic acid (also called tuberculostearic acid or 10-methyl octadecanoic acid, hereafter described as 19:0Me10) is mainly known as a major component of the lipids of the tubercle bacillus (Lennarz et al., 1962). It is hypothesized that 19:0Me10 is synthesized by a two-step biosynthetic pathway (Akamatsu and Low, 1970; Jaureguiberry et al., 1965). The first step of the biosynthesis is the methylenation of oleic acid (18:1Δ9) with SAM as the methyl donor, and the resulting 10-methylene stearic acid (19:1Me10) has been identified from cells of a *Corynebacterium* sp. (Couderc et al.,

1991). The second step is the reduction of the 19:1Me10 to the 19:0Me10 with NADPH as the reducing agent (Akamatsu and Low, 1970). Mycolic acids are also well known as a main component of the lipids in the envelope of mycobacteria and related species, including the genera *Mycobacterium, Nocardia, Rhodococcus,* and *Corynebacterium* (Asselineau and Asselineau, 1978; Barry et al., 1998). Mycolic acids have β-hydroxy-α-alkyl branched structures with a high molecular mass. In *Mycobacteria,* they contain approximately 60 to 90 carbons (Qureshi et al., 1978). Depending on the species, mycolic acids contain a variety of modifications, including carbon double bonds, cyclopropane rings, and methyl branches (Takayama et al., 2005). The identification of some genes encoding the enzymes that catalyze these modifications of mycolic acids have been reported, and among them, it has been unraveled that MmaA1 is involved in the conversion of the unsaturated double bond to the allylic methyl branch using SAM as a methyl donor (Yuan and Barry, 1996; Yuan et al., 1997).

A green sulfur bacterium, *Chlorobaculum tepidum,* also produces cyclopropane fatty acids attached to glycolipids (Mizoguchi et al., 2013). This suggests that heterotrophic bacteria are not the only producers of cyclopropane fatty acids. However, oxygenic photosynthetic organisms do not synthesize unusual methylated fatty acid including cyclopropane fatty acids, branched-chain fatty acids, or mycolic acids. Instead, they produce unsaturated fatty acids to maintain membrane fluidity. Cyanobacterial acyl-lipid desaturases introduce double bonds at specific positions in fatty acids that are esterified to the glycerol backbone of the membrane lipid (Murata et al., 1992). The *desA, desB, desC,* and *desD* genes of *Synechocystis* sp. PCC 6803 encode the acyl-lipid desaturases that introduce double bonds at the Δ12, Δ15, Δ9, and Δ6 positions, respectively, of C18 fatty acids attached to the *sn*-1 position of the lipids. In *Synechocystis*, a saturated C18 fatty acid, stearic acid (18:0), is synthesized and incorporated into the *sn*-1 position of membrane lipids, followed by desaturation. DesC introduces a double bond at the Δ9

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position in the 18:0, and 18:1Δ9 is synthesized. Then, DesA and DesD unsaturate at the Δ12 and Δ6 positions, respectively, of 18:1Δ9, and the Δ12 unsaturated fatty acids are utilized by DesB as substrates to introduce an unsaturated bond at the Δ15 position. The *desA* and *desD* genes were inactivated in the *desAD⁻* strain, and this strain accumulated more 18:1Δ9 than did the wild-type strain (Tasaka et al., 1996). In *Synechocystis*, the endogenous desaturase specifically unsaturates C18 fatty acids attached at the *sn*-1 position of lipids but cannot unsaturate C16 fatty acids attached at the *sn*-2 position. On the other hand, DesC2 from *Nostoc* sp. strain 36 can unsaturate the Δ9 position of C16 fatty acids attached at the *sn*-2 position (Chintalapati et al., 2006).

Fatty acids are the main component to constitute the cell membrane of all organisms. Among fatty acids, unsaturated fatty acids are essential to maintain the membrane fluidity. In *Synechocystis*, the expression level of the *desA*, *desB*, and *desD* genes is induced by low-temperature conditions (Los et al., 1997). The membrane lipids of *Synechocystis* cells are constructed from glycolipids (i.e., monogalactosyl diacylglycerol, digalactosyl diacylglycerol, sulfoquinovosyl diacylglycerol) and a phospholipid, phosphatidylglycerol. Furthermore, the cells of *Synechocystis* possess three types of membranes, which are outer membrane, inner membrane, and thylakoid membrane. Because thylakoid membrane is harboring protein complexes and pigments necessary for photosynthesis, the fatty acid composition which influences on membrane fluidity has a key role for the cellular metabolisms. Especially, it is reported that the existence of the unsaturated fatty acid in the lipid plays an important role to response to the environmental stress such as low temperature, high salinity and strong-light stresses (Allakhverdiev et al., 1998, Gombos et al., 1992). The oxygenic photosynthetic organisms producing unusual methylated fatty acid such as the cyclopropane fatty acids and the mid-chain methyl-branched fatty acid are not found in nature at present. The synthesis of these fatty acids in *Synechocystis* is the

first trial. Therefore, it is very interesting to study the impact of synthesis of the modified

fatty acid except for unsaturated fatty acid on photosynthesis.

Chapter 1: Construction of a Cyanobacterium Synthesizing Cyclopropane Fatty Acids

1.1. Introduction

Derivatives of fatty acids from microalgae are thought to be sources of biofuels. Most of the fatty acids from microalgae are multiply unsaturated, thus, the biofuels from the microalgae are rather fluid, but labile to oxidation. Cyclopropane fatty acid synthase encoded by the *cfa* gene in *E. coli* catalyzes the modification of acyl chains to their cyclopropane derivatives through methylation of an unsaturated double 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 fatty acids attached to lipids in the membrane (Wang et al., 1992).

Cyanobacterial acyl-lipid desaturases introduce double bonds at specific positions in fatty acids that are esterified to the glycerol backbone of the membrane lipid (Murata et al., 1992). The *desA*, *desB*, *desC*, and *desD* genes of *Synechocystis* sp. PCC 6803 encode the acyl-lipid desaturases that introduce double bonds at the $\Delta 12$, $\Delta 15$, $\Delta 9$, and $\Delta 6$ positions, respectively, of C18 fatty acids attached at the *sn*-1 position of the lipids. The *desA* and *desD* genes were inactivated in the *desAD*⁻ strain, and this strain accumulated more oleic acid (18:1$\Delta 9$) than did the wild-type strain (Tasaka et al., 1996). In *Synechocystis*, the endogenous desaturase specifically unsaturates C18 fatty acids attached at the *sn*-1 position of lipids but cannot unsaturate C16 fatty acids attached at the *sn*-2 position. On the other hand, DesC2 from *Nostoc* sp. strain 36 can unsaturate the $\Delta 9$ position of C16 fatty acids attached at the *sn*-2 position (Chintalapati et al., 2006).

In this study, I attempted to synthesize cyclopropane fatty acids in the cyanobacterium *Synechocystis* sp. PCC 6803 by expressing the cyclopropane fatty acid

synthase, Cfa, from *E. coli* to produce fatty acids that are fluid and stable in response to oxidization. To increase the yield of cyclopropane fatty acids *in vivo*, I also expressed the *desC2* gene from *Nostoc* sp. and examined the effects of mutations in the *desA* and *desD* genes of *Synechocystis*. I analyzed the changes in fatty acid composition, cell growth, and respiration, and photosynthesis activities in the *Synechocystis* transformants.

1.2. Materials and Methods

1.2.1. Organisms and culture conditios

The *Synechocystis* sp. PCC 6803 glucose-tolerant strain (Williams, 1988) was used as the wild type in this study. *Synechocystis* cells were grown in BG11 (Stanier et al., 1971) 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 µmole photons m^{-2} s^{-1} and aerated with 1% (v/v) CO_2-enriched air (Wada and Murata, 1989). All transformants were maintained in BG11 medium solidified with 1.5% (w/v) Bacto-agar (BD Biosciences Japan, Tokyo) in the presence of 25 µg/mL kanamycin sulfate, 25 µg/mL spectinomycin dihydrochloride pentahydrate, or 25 µg/mL chloramphenicol, depending on the selection markers used.

      E. coli strain JM109 (Yanisch-Perrron et al., 1985) was grown in 1.5 mL of Luria-Bertani (LB) medium (Bertani, 1951) 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 fatty acids, 18:1Δ9, linoleic acid (18:2Δ9,12), γ-linolenic acid (18:3Δ6,9.12), and α-linolenic acid (18:3Δ9,12,15), to the *E. coli* cells, I cultivated the cells in liquid LB medium containing 1 mM sodium salt of 18:1Δ9 (Tokyo chemical industry, Japan), 18:2Δ9,12 (Funakoshi, Tokyo, Japan), 18:3Δ6,9.12 (Sigma Aldrich,

Missouri, USA), or 18:3Δ9,12,15 (Funakoshi).

1.2.2. Plasmid construction and transformation

For heterologous expression of the *cfa* and *desC2* genes in *Synechocystis* and overexpression of the *cfa* gene in *E. coli*, I constructed four plasmids—pTHT-cfasp, pTHT-Sp, pTHT-desC2Sp, and pTHT-cfadesC2Sp—which are derivatives of an expression vector, pTCHT2031V, for this cyanobacterium (Ishizuka et al., 2006) (Table 2). Figure 1 shows the construction scheme for these plasmids, and Table 1 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 (Ishizuka et al., 2006). The derived plasmids had fragments introduced between 2031up and 2031dn into the *Synechocystis* chromosome through homologous recombination with the coding sequence of the *slr2031* gene, a non-essential gene. At first, to replace the selection marker from the Cm-r with the spectinomycin resistance gene cassette (Sp-r), I constructed a plasmid lacking the Cm-r, 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 (Takara Bio) to obtain the pMD-cfa plasmid, which was confirmed by DNA sequencing. I next amplified a DNA fragment including the Sp-r using pAM1146 (Tsinoremas et al., 1994) as a template. The Sp-r fragment, digested with *BamH*I and *Bgl*II, was inserted into pMD-cfa, and pTHT2031V was cleaved with *Bgl*II 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*II and inserted into pTHT2031V digested

using the same restriction enzymes to obtain pTHT-cfasp. The *desC2* gene from the *Nostoc* sp. strain 36 (Chitalapati et al., 2006) was artificially synthesized (Life Technologies Japan, Tokyo) and optimized for the codon usage of *Synechocystis*. Finally, I 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 fatty acid compositions of these cells were analyzed. pTHT-cfasp, pTHT-desC2Sp, and pTHT-cfadesC2Sp were used to transform cells of the wild-type and *desAD*⁻ strains of *Synechocystis* by homologous recombination (Williams, 1988). After verifying complete segregation of the mutated chromosomes from those possessing the native *slr2031* gene by PCR, fatty acid compositions and photosynthesis and respiration activities were analyzed.

1.2.3. Fatty acid analysis

Profiles of fatty acids in the cells were examined by the method of Kotajima et al. (2014). 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 fatty acid 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 µL of *n*-hexane.

To identify and quantify FAMEs, I applied 1 µl 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. FAMEs of myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), 18:1Δ9, 18:2Δ9,12, 18:3Δ9,12,15, and 18:3Δ6,9,12 were provisionally identified based on retention time and mass spectrums of commercial FAME standards (Nu-Chek Prep, Elysian, MN). To identify *cis*-9,10-methylenehexadecanoic acid (17:1cycloΔ9) and *cis*-9,10-methyleneoctadecanoic acid (19:1cycloΔ9), I 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. I confirmed the retention times and mass spectrums of 17:1cycloΔ9 (Santa Cruz Biotechnology, Texas USA) and 19:1cycloΔ9 (Santa Cruz Biotechnology). *cis*-9,10-methylene-*cis*-12-octadecenoic acid (19:2Δ12cycloΔ9) and *cis*-9,10-methylene-*cis*-12,15-octadecadienoic acid (19:3Δ12,15cycloΔ9) were estimated from the differences in the mass of the parent ion of 19:1cycloΔ9 and pattern of fragmentation seen in the GC-MS results.

In order to analyze fatty acids attached to each lipid, the harvested cells were stored at −80°C and the lipids were extracted with CH_3Cl:methanol (2:1, v/v). The cell debris was sedimented by centrifugation. The resulting supernatant was transferred into new tubes and evaporated. Then, the samples was re-suspended in 300 µl CH_3Cl: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.

*1.2.4. Photosynthesis and respiration activitie*s

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.

1.2.5. 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 OD_{730} 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. (2014).

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, I 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 1). As a reference, I amplified the *rnpB* gene encoding the RNase P subunit B using primers rnpB_RT_F and rnpB_RT_R. As a negative control,

I 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 (Livak and Schmittgen, 2001). The results of the wild-type cells were used for calibration.

1.3. Results

1.3.1. Fatty acid composition of cfa-overexpressing E. coli

E. coli cells synthesize the unsaturated fatty acids 16:1Δ9 and vaccenic acid (18:1Δ11) as components of their membrane lipids, but not 18:1Δ9, 18:2Δ9,12, or 18:3Δ6,9.12, which are the major unsaturated fatty acids in *Synechocystis* cells grown at 34°C. Fatty acid-modifying enzymes, including the acyl-lipid fatty acid desaturases, are position-specific (Chi et al., 2008). It was unclear whether Cfa in *E. coli* introduces cyclopropane groups at the Δ9 and Δ11 positions of the unsaturated fatty acids or specifically at the ω7 position. To determine whether Cfa could modify 18:1Δ9, 18:2Δ9,12, and 18:3Δ6,9.12 *in vivo*, I analyzed the fatty acid compositions of the wild-type and *cfa*-overexpressing *E. coli* strains cultivated in a liquid medium containing 18:1Δ9, 18:2Δ9,12, 18:3Δ9,12,15, or 18:3Δ6,9,12.

Addition of each fatty acid to a culture of the wild-type strain of *E. coli* resulted in total fatty acids in membrane lipids comprising 10-30% of 18:1Δ9, 18:2Δ9,12, 18:3Δ9,12,15, and 18:3Δ6,9,12 (Table 3). Wild-type cells cultivated with 18:1Δ9, 18:2Δ9,12, and 18:3Δ9,12,15 exhibited 0.3 ± 0.1%, 0.3 ± 0.1%, and 1.7 ± 0.7% of 19:1cycloΔ9, 19:2Δ12cycloΔ9, and 19:3Δ12,15cycloΔ9, respectively, among the total membrane fatty acids. The total fatty acids in membrane lipids of the *cfa*-overexpressing *E. coli* strain comprised 8-12% of 18:1Δ9, 18:2Δ9,12, 18:3Δ9,12,15, and 18:3Δ6,9,12. Moreover, the *cfa*-overexpressing strain cultivated with 18:1Δ9, 18:2Δ9,12, and 18:3Δ9,12,15 exhibited 14.5 ± 2.9%, 5.4 ± 0.7%, and 2.2 ± 0.2% of 19:1cycloΔ9,

19:2Δ12cycloΔ9, and 19:3Δ12,15cycloΔ9, respectively, among total membrane fatty acids. When both wild-type and *cfa*-overexpressing *E. coli* cells were cultivated with 18:3Δ6,9,12, approximately 10% of it of the total fatty acids was incorporated into the membrane lipids. Both cells synthesized 17:1cycloΔ9 and lactobacillic acid (19:1cycloΔ11), but did not synthesize novel cyclopropane fatty acids, such as *cis*-9,10-methylene-*cis*-6,12-octadecadienoic acid (19:3Δ6,12cycloΔ9). 16:1Δ7, 16:2Δ7,10, 16:3Δ4,7,10, and 16:3Δ7,10,13 were detected when the cells were cultured in media including 18:1Δ9, 18:2Δ9,12, 18:3Δ6,9,12, and 18:3Δ9,12,15, respectively, as exogenous fatty acids. The purities of 18:1Δ9, 18:2Δ9,12, 18:3Δ6,9,12, and 18:3Δ9,12,15 were higher than 97%, 99%, 99%, and 99%, respectively. In addition, when I incubated these fatty acids in LB medium with shaking, these fatty acids were not changed into C16 fatty acids (data not shown). Thus, I concluded that 16:1Δ7, 16:2Δ7,10, 16:3Δ4,7,10, and 16:3Δ7,10,13 were produced from 18:1Δ9, 18:2Δ9,12, 18:3Δ6,9,12, and 18:3Δ9,12,15, respectively, in the *E. coli* cells during the culture, perhaps via β-oxidation.

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

According to the results from fatty acid feeding experiments in *E. coli* overexpressing the *cfa* gene, Cfa can convert 18:1Δ9 and 18:2Δ9,12 to 19:1cycloΔ9 and 19:2Δ12cycloΔ9, respectively. I examined whether the unsaturated fatty acids endogenously synthesized and incorporated into membrane lipids are converted to cyclopropane fatty acids *in vivo* by heterologously expressing *cfa* in wild-type *Synechocystis* cells.

In wild-type *Synechocystis* cells, 18:1Δ9 and 18:2Δ9,12, which are thought to be substrates for Cfa, comprised 18.3 ± 0.6% and 16.6 ± 0.6% of total fatty acids, respectively. 18:3Δ6,9,12, which may not be catalyzed by Cfa, comprised 9.2 ± 0.8% of total fatty acids. In the strain harboring the *cfa* gene (*cfa*[+]), 19:1cycloΔ9 comprised 18.8 ± 0.4% of total fatty acids. Simultaneously, the 18:1Δ9 content was reduced to 5.1 ± 0.4%

(Table 4). Interestingly, 19:2Δ12cycloΔ9 was not produced from 18:2Δ9,12 in *Synechocystis* cells. The abundance of 18:2Δ9,12 in the *cfa*⁺ strain was 10.7 ± 0.7%, which was slightly lower than that in wild-type cells. These results indicated that Cfa was functional in the *Synechocystis* cells, but that only 18:1Δ9 was used as a substrate and that DesA, a Δ12 acyl-lipid desaturase in *Synechocystis*, also uses 18:1Δ9 but not 19:1cycloΔ9 as a substrate. Cfa and DesA compete for use of 18:1Δ9.

1.3.3. Heterologous expression of the cfa gene in desAD⁻ *strain*

Cfa catalyzed the synthesis of 19:1cycloΔ9 only from 18:1Δ9 in *Synechocystis* cells (Table 4). To increase the abundance of 19:1cycloΔ9 in *Synechocystis* cells, I attempted to introduce the *cfa* gene into the *desAD*⁻ strain (*desAD*⁻/*cfa*⁺), which does not produce C18 polyunsaturated fatty acids (Tasaka et al., 1996). Total fatty acids of the *desAD*⁻ strain comprised 49.8 ± 0.5% of 18:1Δ9, which is 2.5-fold greater than that of wild-type cells (Table 4). Although I attempted to increase the abundance of 19:1cycloΔ9 in the *desAD*⁻/*cfa*⁺ strain, it unexpectedly comprised 22.2 ± 0.6% of the total fatty acids (Table 4). First, I hypothesized that Cfa prefers unsaturated fatty acids attached to phospholipids. However, the fatty acid compositions of each lipid class— monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG), and phosphatidylglycerol (PG)— of *desAD*⁻/*cfa*⁺ were not markedly altered (Table 5), suggesting that Cfa does not discriminate among lipid head groups. Excess 19:1cycloΔ9 in the membrane lipids might exert deleterious effects on *Synechocystis* cells.

1.3.4. Heterologous coexpression of the cfa *and* desC2 *genes in* Synechocystis *cells*

In the *desAD*⁻/*cfa*⁺ strain, the abundance of 19:1cycloΔ9 was not increased compared with that in the *cfa*⁺ strain. To increase the abundance of cyclopropane fatty acids in *Synechocystis* cells, I attempted to synthesize a novel cyclopropane fatty acid,

17:1cycloΔ9, from 16:1Δ9 in *Synechocystis*. The endogenous desaturases of *Synechocystis* specifically unsaturate C18 fatty acids attached at the *sn*-1 position of the lipids, but cannot unsaturate C16 fatty acids attached at the *sn*-2 position (Murata et al., 1992). In contrast, DesC2 from *Nostoc* sp. strain 36 specifically unsaturates C16 saturated fatty acid attached at the *sn*-2 position (Chitalapati et al., 2006). I aimed to synthesize 17:1cycloΔ9 by coexpression of the *cfa* and *desC2* genes in the wild-type and *desAD*⁻ strains of *Synechocystis* (*cfa*⁺/*desC2*⁺ and *desAD*⁻/*cfa*⁺/*desC2*⁺). Furthermore, I also constructed the *desC2*⁺ and *desAD*⁻/*desC2*⁺ strains by introducing the corresponding genes into the wild-type and *desAD*⁻ cells, respectively, as controls.

In the *desC2*⁺ and *desAD*⁻/*desC2*⁺ strains, 16:1Δ9 comprised approximately 25% of total fatty acids. Because 19:1cycloΔ9 comprised ~20% of total fatty acids in the *cfa*⁺ and *desAD*⁻/*cfa*⁺ strains, I expected that the *cfa*⁺/*desC2*⁺ and *desAD*⁻/*cfa*⁺/*desC2*⁺ strains would synthesize ~20% 17:1cycloΔ9 in addition to 20% 19:1cycloΔ9. However, the *cfa*⁺/*desC2*⁺ and *desAD*⁻/*cfa*⁺/*desC2*⁺ strains produced only 7.7 ± 0.5% and 5.2 ± 0.4% 17:1cycloΔ9, respectively. Moreover, the 16:1Δ9 abundance was reduced to 2.3 ± 0.1% and 1.8 ± 0.1% in the *cfa*⁺/*desC2*⁺ and *desAD*⁻/*cfa*⁺/*desC2*⁺ strains, respectively. Interestingly, 19:1cycloΔ9 abundance was maintained after introduction of the *desC2* gene, comprising more than 20% in the *cfa*⁺/*desC2*⁺ and *desAD*⁻/*cfa*⁺/*desC2*⁺ strains. The abundance of 17:1cycloΔ9 in the *cfa*⁺/*desC2*⁺ and *desAD*⁻/*cfa*⁺/*desC2*⁺ strains was lower than my estimation. 16:1Δ9 abundance in the *cfa*⁺/*desC2*⁺ and *desAD*⁻/*cfa*⁺/*desC2*⁺ strains was markedly lower than that in the *desC2*⁺ and *desAD*⁻/*desC2*⁺ strains.

1.3.5. *The Cell growth and photosynthesis of* Synechocystis *transformants*

In this study, I constructed *Synechocystis* strains that synthesized cyclopropane fatty acids and unsaturated C16 fatty acids. These are novel fatty acids in *Synechocystis* cells; therefore, I examined the effects of synthesis of cyclopropane fatty acids and unsaturated

C16 fatty acid 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) (Figure 2). The growth rates were not altered by introduction of the *cfa* gene in wild-type cells. The growth of *desAD*^{-} cells at 34°C was reduced compared with that of wild-type cells, whereas growth at 26°C was almost halted, as reported previously (Tasaka et al., 1996). The growth rate of *desAD*[-]/*cfa*^{+} cells was lower than that of *desAD*^{-} cells at both temperatures and these cells were unable to grow at 26°C. 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. Although the growth of the *desAD*[-]/*desC2*[+] strain at 34°C was not changed, growth at 26°C was significantly increased. However, the growth rate of the *desAD*[-]/*cfa*[+]/*desC2*[+] strain decreased to almost the same extent as that of the *desAD*[-] strain.

Modification of fatty acid profiles by expression of *cfa* and *desC2* altered the growth rates of *Synechocystis* transformants, especially at 26°C (Figure 2). To investigate these phenomena, I 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 (Figure 3). 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.

1.3.6. *Apparent activities of desaturases and Cfa in* Synechocystis *transformants*

To compare apparent desaturase and cyclopropane fatty acid synthetase activities, I determined the total abundance of fatty acids synthesized by each enzyme (Table 4, Table 6, and Figure 4).

The total abundance of fatty acids unsaturated by DesC is shown in Figure 4A. The *cfa*[+], *desC2*[+], and *cfa*[+]/*desC2*[+] strains synthesized lower levels of fatty acids

unsaturated by DesC than the wild-type cells did. In addition, the *desAD⁻/cfa⁺*, *desAD⁻/desC2⁺*, and *desAD⁻/cfa⁺/desC2⁺* strains synthesized lower levels of fatty acids unsaturated by DesC than did the *desAD⁻* strain. This might be due to a mechanism regulating membrane fluidity. Figure 4B, C, and D show the total abundance of fatty acids 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. Figure 4E shows the total abundance of fatty acids unsaturated by DesC2. These results demonstrated that cells harboring both the *cfa* and *desC2* genes produced lower levels of fatty acids unsaturated by DesC2 than did cells expressing the *desC2* gene under both the high- and low-temperature conditions. The total abundance of saturated fatty acids is shown in Figure 4G. These results demonstrated that *Synechocystis* cells harboring only *desC2* produced lower levels of saturated fatty acids than did wild-type cells, but that the cells containing both *cfa* and *desC2* accumulated large quantities of saturated fatty acids. Indeed, the levels were almost identical to those in wild-type *Synechocystis* cells.

1.4. Discussion

1.4.1. Substrate specify of Cfa

The *cfa*-overexpressing strain of *E.coli* cultivated with 18:1Δ9, 18:2Δ9,12, and 18:3Δ9,12,15 exhibited 14.5 ± 2.9%, 5.4 ± 0.7%, and 2.2 ± 0.2% of 19:1cycloΔ9, 19:2Δ12cycloΔ9, and 19:3Δ12,15cycloΔ9, respectively, among total membrane fatty acids. When both wild-type and *cfa*-overexpressing *E. coli* cells were cultivated with 18:3Δ6,9,12, approximately 10% of it of the total fatty acids was incorporated into the membrane lipids. Both cells synthesized 17:1cycloΔ9 and lactobacillic acid (19:1cycloΔ11), but did not synthesize novel cyclopropane fatty acids, such as 19:3Δ6,12cycloΔ9. These results suggest that Cfa can specifically modify the Δ9 position

of the C18 unsaturated fatty acids 18:1Δ9, 18:2Δ9,12, and 18:3Δ9,12,15 as substrates for production of cyclopropane fatty acids. However, if both the Δ6 and Δ9 positions of the C18 unsaturated fatty acids were unsaturated, Cfa could not introduce a cyclopropane ring at the Δ9 position.

1.4.2. Synthesis of cyclopropane fatty acids in Synechocystis

Although Cfa converted 18:2Δ9,12 to 19:2Δ12cycloΔ9 in *E. coli* cells, this did not happen in *Synechocystis* cells. The reason for this discrepancy is unclear, but the head groups of the membrane lipids differ between these two organisms. The membrane lipids in *E. coli* are phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine, PG, and cardiolipin) (Cronan, 1968), whereas those in *Synechocystis* are mainly glycolipids (e.g., MGDG, DGDG, and SQDG) and only one phospholipid, PG (Murata, et al., 1992). I separated the total lipids into each membrane lipid (i.e., MGDG, DGDG, SQDG, and PG) by thin-layer chromatography and determined the fatty acid profiles. As shown in Table 5, in the *cfa*[+] strain, 19:1cycloΔ9 was incorporated into all four types of lipids, and the 19:1cycloΔ9 content in SQDG was slightly lower than those in the other three lipids. Because a greater quantity of 16:0 was attached to SQDG than to the other lipids in *Synechocystis* cells, as reported previously (Wada and Murata, 1990), the 19:1cycloΔ9 level in the lipids was lower. Thus, Cfa activities were unaffected by differences in the lipid head groups.

The difference in lipid types does not explain the lack of production of 19:2Δ12cycloΔ9 in *Synechocystis*. According to the literature, Cfa acts on the nonpolar portion of phospholipids dispersed in vesicles (Taylor and Cronan, 1979). Recently, Biller et al. (2014) showed that many microorganisms develop and release small membrane vesicles from cell surfaces. However, the presence of such membrane vesicles in the cytosol of cyanobacterial cells is yet to be demonstrated. At this time, I cannot clearly

identify the substrate of Cfa. In *Synechocystis* cells, there are three cell membranes, i.e., the outer membrane, inner membrane, and thylakoid membrane. In these membranes, the lipids or fatty acids exist evenly (Kim et al., 1999). Thus, I did not consider the presence of a different compartment of $18:2\Delta9,12$ in *Synechocystis*. However, in unidentified vesicles and possible microdomains where Cfa might locate and function, I cannot ignore the possibility. It is unclear why $19:2\Delta12cyclo\Delta9$ was not produced in *Synechocystis*.

1.4.3. Effect of Cfa and DesC2 on the cell growth and photosynthesis

The growth rate of *desAD*⁻*/cfa*⁺ cells was lower than that of *desAD*⁻ cells at both temperatures and these cells were unable to grow at 26°C. These results suggest that $19:1cyclo\Delta9$ synthesis in cells that do not synthesize polyunsaturated fatty acids negatively affected growth, irrespective of temperature. The melting point of $18:1\Delta9$; i.e., 16.3˚C (http://www.sciencelab.com/msds.php?msdsId=9927682), is lower than that of $19:1cyclo\Delta9$ (i.e., 27.8–28.8˚C) (Hofman et al., 1952), suggesting that maintenance of membrane fluidity in cells with membrane lipids possessing $19:1cyclo\Delta9$ instead of $18:1\Delta9$ 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 $16:1\Delta9$ in the wild-type cells negatively affected their growth, particularly at low temperatures. In a previous report (Chitalapati et al., 2006), the unsaturation of the C16 fatty acids 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 might not be the case in *Synechocystis*. Although $16:1\Delta9$ synthesis in wild-type cells resulted in decreased growth at 26°C, growth of the *desAD*⁻*/desC2*⁺ strain was stimulated at this temperature. If the C18 fatty acid at the *sn*-1 position is not multiply unsaturated, unsaturation of the C16 fatty acid at the *sn*-2 position might

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contribute to growth at low temperatures. 16:1Δ9 production in the *desAD⁻* 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 16:1Δ9 at the *sn*-2 position of membrane lipids into 16:1cycloΔ9 might decrease membrane fluidity.

Respiration and photosynthesis activities were not significantly altered in cells grown at 34°C (Figure 3). As reported previously (Tasaka et al 1996), the growth rate of the *desAD⁻* 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 *desAD⁻* strain at 26°C, photosynthesis and respiration activities were lower than those of the *desAD⁻* 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, 16:1Δ9 synthesis in the *desAD⁻* 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 Figure 2 were grown at 70 µmole photons m^{-2} s^{-1} and the photosynthetic activity shown in Figure 3 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 O_2 evolution. I thought that there was a discrepancy between the two experiments due to a difference in the light conditions. Therefore, I further investigated the photosynthetic activity at various light intensities, including 75 µmol photons/m^2/s, which is the closest to the growth condition. As a result, even at a low light intensity, I could not observe clear differences in the photosynthetic activity in the strains (Figure 5). Therefore, modification

of the fatty acid composition might have altered the activities of endogenous desaturase in *Synechocystis* and indirectly affected the growth of the transformants.

1.4.4. Regulation of the expression level of desaturases

The *cfa*$^+$, *desC2*$^+$, and *cfa*$^+$/*desC2*$^+$ strains synthesized lower levels of fatty acids unsaturated by DesC than the wild-type cells did. In addition, the *desAD*$^-$/*cfa*$^+$, *desAD*$^-$/*desC2*$^+$, and *desAD*$^-$/*cfa*$^+$/*desC2*$^+$ strains synthesized lower levels of fatty acids unsaturated by DesC than did the *desAD*$^-$ strain. In accordance with previous reports (Los et al., 1997), 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. I 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. I 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 7A). These results suggest that there might be a novel mechanism for regulating DesC activity, perhaps via post-translationally, to maintain the suitable membrane fluidity.

The apparent activities of DesA, DesD, and DesB were decreased to some extent by introduction of the *desC2* gene, especially at 26°C. The expression of the *desA*, *desD*, and *desB* genes is induced at low temperature to maintain membrane fluidity in *Synechocystis* cells (Wada and Murata, 1990). The expression of the *desC2* gene may downregulate the expression levels of *desA*, *desD*, and *desB* via the regulatory mechanism for maintaining membrane fluidity (Los et al., 1997). I 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. I found that the *desA* and *desD* genes expression levels in the *cfa*$^+$, *desC2*$^+$, and *cfa*$^+$/*desC2*$^+$ strains were not

remarkably altered from those in the wild-type cells (Table 7B, C). Alternatively, DesC2 may compete with DesA, DesD, and DesB, and these endogenous desaturases may specifically unsaturate C18 fatty acids attached at the *sn*-1 position of lipids containing C16-saturated fatty acids at the *sn*-2 position, but not those containing C16-unsaturated fatty acids. Figure 6 summarizes these results, and the data therein suggest that no pathway for unsaturation by DesA, DesD, and DesB after unsaturation by DesC2 exists.

1.4.5. Substrate specify of DesC2

The cells harboring both the *cfa* and *desC2* genes produced lower levels of fatty acids unsaturated by DesC2 than did cells expressing the *desC2* gene under both the high- and low-temperature conditions. Moreover, *Synechocystis* cells harboring only *desC2* produced lower levels of saturated fatty acids than did wild-type cells, but that the cells containing both *cfa* and *desC2* accumulated large quantities of saturated fatty acids. Indeed, the levels were almost identical to those in wild-type *Synechocystis* cells. These results suggested that DesC2 might unsaturate 16:0 attached at the *sn*-2 position of lipids possessing C18 unsaturated fatty acids at the *sn*-1 position but not lipids with C18-saturated fatty acids or C19 cyclopropane fatty acids (Figure 6). The cyclopropane fatty acid yield would be increased if the *sn*-2-specific desaturase could unsaturate 16:0 attached at the *sn*-2 position of lipids containing C18-saturated fatty acids or C19 cyclopropane fatty acids at the *sn*-1 position.

Chapter 2: Identification of the mid-Chain Methyl-Branched Fatty Acid Synthase

2.1. Introduction

In living organisms, modified fatty acids are crucial for the functions of the cellular membranes and storage lipids where these 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 Low, 1970; Cronan et al., 1974; Takayama et al., 2005). Some bacteria, such as *Escherichia coli* and *Lactobacillus arabinosus*, synthesize cyclopropane fatty acids, which contain a cyclopropane ring in the acyl group. Cyclopropane fatty acid synthase encoded by the *cfa* gene in *E. coli* catalyzes the modification of acyl chains to their cyclopropane derivatives through the methylation of an unsaturated bond. In this reaction, *S*-adenosyl-ʟ-methionine (SAM) is used as a methyl donor. Branched-chain fatty acids can be classified into two types, namely *iso*- or *anteiso*-methyl-branched fatty acids and mid-chain methyl-branched fatty acids. The biosynthetic pathway of *iso*- or *anteiso*-methyl-branched fatty acids is well characterized in *Bacillus subtilis* (Namba et al., 1969; Oku and Kaneda, 1988). As a mid-chain methyl-branched fatty acids, 10-methyl stearic acid (also called tuberculostearic acid or 10-methyl octadecanoic acid, hereafter described as 19:0Me10) is mainly known as a major component of the lipids of the tubercle bacillus (Lennarz et al., 1962). It is hypothesized that 19:0Me10 is synthesized by a two-step biosynthetic pathway (Akamatsu and Low, 1970; Jaureguiberry et al., 1965). The first step of the biosynthesis is the methylenation of oleic acid (18:1Δ9) with SAM as the methyl donor, and the resulting 10-methylene stearic acid (19:1Me10) has been identified from cells of a *Corynebacterium* sp. (Couderc et al., 1991). The second step is the reduction of the

methylenated fatty acid to the 10-methyl fatty acid with NADPH as the reducing agent (Akamatsu and Low, 1970). Mycolic acids are also well known as a main component of the lipids in the envelope of mycobacteria and related species, including the genera *Mycobacterium, Nocardia, Rhodococcus,* and *Corynebacterium* (Asselineau and Asselineau, 1978; Barry et al., 1998). Depending on the species, mycolic acids contain a variety of modifications, including carbon double bonds, cyclopropane rings, and methyl branches (Takayama et al., 2005). The identification of some genes encoding the enzymes that catalyze these modifications of mycolic acids have been reported, and among them, it has been unraveled that MmaA1 is involved in the conversion of the unsaturated double bond to the allylic methyl branch using SAM as a methyl donor (Yuan and Barry, 1996; Yuan et al., 1997).

It has been speculated that methyltransferases from *Mycobacterium* are involved in the biosynthesis of 19:0Me10. Meena et al. (2013) demonstrated the synthesis of 19:0Me10 by a recombinant protein encoded by the *umaA* gene from *M. tuberculosis* H_{37}Rv. In this study, 18:1Δ9 connected with phosphatidylcholine (PC) was ^{3}H-labeled by incubation with the recombinant UmaA protein, *S*-adenosyl-[methyl ^{3}H] methionine (^{3}H-labeled SAM), and NADPH. Additionally, Meena and Kolattukudy (2013) also demonstrated the synthesis of 19:0Me10 by a recombinant protein encoded by the *ufaA1* gene from *M*. *tuberculosis* H_{37}Rv. In this study, the methylester of 19:0Me10 was detected by gas chromatography (GC) with a flame-ionization detector in a mixture comprising a crude extract from *E. coli* cells expressing UfaA1, PC or phosphatidylethanolamine containing 18:1Δ9, SAM, and NADPH. Both UmaA and UfaA1 are methyltransferases that do not possess any domains functioning in the redox reaction. These findings contradict the two-step biosynthetic pathway predicted previously. In the study that focused on UmaA, ^{3}H-labeled PC was separated by silica gel thin-layer chromatography and detected by fluorography. There has been no direct evidence for the synthesis of

19:0Me10 by UmaA. In the study that focused on UfaA1, the unidentified proteins in the crude extract from *E. coli* cells might have played important roles in the reduction of the methylenated intermediate.

The biosynthetic reactions of cyclopropane fatty acid, mid-chain methyl-branched fatty acids, and mycolic acid are similar processes that include the SAM-dependent methylation of the unsaturated double bond in the unsaturated fatty acids (Takayama et al., 2005). Here, I paid attention to the methyltransferase for the biosynthesis of these modified fatty acids and screened potential methyltransferase candidates for the synthesis of 19:0Me10 through a genomic comparison among various bacteria. I ascertained the existence of the modified fatty acids in the cells of those organisms via a literature survey. The candidate methyltransferases were functionally investigated *in vivo* by heterologous expression in *E. coli* cells as a host. I found that a methyltransferase and a reductase catalyze the two-step reaction for the biosynthesis of 19:0Me10.

2.2. Materials and Methods

2.2.1. Organisms and culture conditions

As host cells, I used two strains of *E. coli*, namely JM109 (Yanisch-Perron et al., 1985) for the construction of plasmids and Rosetta™ 2 (Merck Millipore, Darmstadt, Germany) for the expression of the target genes. The cells were grown in 2 mL of Luria–Bertani (LB) medium (Bertani, 1951) at 37 °C with shaking at 180 rpm. All transformants were maintained in LB medium solidified with 1.5% (w/v) Bacto® agar (BD Biosciences Japan, Tokyo, Japan) in the presence of 100 µg/mL sodium ampicillin, 50 µg/mL chloramphenicol, or 50 µg/mL spectinomycin dihydrochloride pentahydrate, depending on the selection markers on the plasmids. To exogenously supply fatty acids to the culture of *E. coli* cells, 1 mM of each sodium salt of palmitoleic acid (16:1Δ9, Wako Pure Chemicals, Osaka, Japan), 18:1Δ9 (Tokyo Chemical Industry, Tokyo, Japan), linoleic acid (18:2Δ9,12,

Funakoshi, Tokyo, Japan), γ-linolenic acid (18:3Δ6,9.12, Sigma-Aldrich Japan, Tokyo, Japan), γ-linolenic acid (18:3Δ9,12,15, Funakoshi), or vaccenic acid (18:1Δ11, Sigma-Aldrich Japan) was added to the liquid LB medium. *Corynebacterium urealyticum* ATCC 43042 was grown on R agar (http://www.jcm.riken.jp/cgi-bin/jcm/jcm_grmd?GRMD=26) with 0.5% (v/v) TWEEN 80 (Wako Pure Chemicals) and incubated at 37°C for 18 h.

2.2.2. Plasmid construction and transformation

To express the target genes in *E. coli* heterologously, I constructed six plasmids, namely pM1942, pM2121, pFADO-4-M2121, pFADO-M2121-S, pFADO-M2121-I, and pFADO-M2121-PCYC (Table 2), which are derivatives of an expression vector, pTCHT2031V (Ishizuka et al., 2006). Figure 7 shows the processes for the construction of these plasmids. The plasmid pTCHT2031V contains a chloramphenicol resistance gene cassette (Cm^r) and the *trc* promoter sequence (P*trc*). First, to replace the selection marker from the Cm^r with the spectinomycin resistance gene cassette (Sp^r), I constructed a plasmid lacking the Cm^r, pTHT2031V, from pTCHT2031V by the polymerase chain reaction (PCR) amplification of the entire portion of pTCHT2031V except Cm^r using the primers pTCHT_Cm_remove_InF_F and pTCHT_Cm_remove_InF_R (Table 1) and the In-Fusion$^®$ HD cloning kit (Takara Bio, Ōtsu, Japan) for circularization. The candidate genes of the methyltransferases, WP_048471942 and WP_048472121, and a genomic fragment including two genes for the flavin adenine dinucleotide (FAD)-binding oxidoreductase, WP_048472120, and WP_048472121 (FADO-4-M2121), were amplified by PCR from the chromosomal DNA of *Mycobacterium chlorophenolicum* JCM 7439 as the template and primer sets, M1942_Nde_F/M1942_Bam_R, M2121_Nde_F/M2121_Bam_R, and FAD_Nde_F/M2121_Bam_R, respectively. The amplified DNA fragments were subcloned into a T-vector pMD19 simple vector (Takara

Bio) to obtain the plasmids pMD-M1942, pMD-M2121, and pMD-FADO-4-M2121, and the DNA sequences of the inserts were confirmed. I then amplified a DNA fragment including the Sp^r using pAM1146 (Tsinoremas et al., 1994) as a template. The Sp^r fragment digested with *Bgl*II and *BamH*I was inserted into pMD-M1942, pMD-M2121, and pMD-FADO-4-M2121 cleaved with *Bam*HI to obtain pMD-M1942S, pMD-M2121S, and pMD-FADO-4-M2121S, respectively. I chose the plasmids in which the Sp^r fragments were transcribed in the same orientation as the candidate genes. The fragments containing M1942, M2121, or FADO-4-M2121 and the Sp^r genes in these plasmids were excised by *Nde*I and *BamH*I and inserted into pTHT2031V digested with *Nde*I and *Bgl*II to obtain pM1942, pM2121, and pFADO-4-M2121. To insert the canonical Shine–Dalgarno (SD) sequence in *E. coli* between the two open reading frames, pFADO-4-M2121 was amplified using primer sets, SD_add_S_F/SD_add_S_R or SD_add_I_F/SD_add_I_R, and circularized using the In-Fusion® HD cloning kit to obtain pFADO-M2121-S or pFADO-M2121-I, respectively. Finally, a homologous gene to polyketide cyclase in *M. chlorophenolicum* JCM 7439 was also amplified by PCR using the primers PCYC_inf_F and PCYC_inf_R, and inserted into pFADO-M2121-I using the In-Fusion® HD cloning kit to obtain pFADO-M2121-PCYC.

The plasmid for the expression of the *umaA* gene in *E. coli* was also a derivative of an expression vector, pTCHT2031V. The *umaA* gene from *Mycobacterium tuberculosis* (Meena et al., 2013) was artificially synthesized (Life Technologies Japan, Tokyo) and optimized for the codon usage of the cyanobacterium *Synechocystis* sp. PCC 6803. The Sp^r fragment digested with *BamH*I and *Bgl*II was inserted into pTAKN-2-UmaA and then cleaved with *Bgl*II to obtain pTAKN-2-UmaAS. The fragments containing *umaA* and the Sp^r genes in this plasmid were excised by *Nde*I and *Bgl*II and inserted into pTHT2031V digested with *Nde*I and *Bgl*II to obtain pUmaA.

2.2.3. Fatty acid analysis

The profiles of fatty acids in the *E. coli* transformants were carried out using the method described in Chapter 1. To identify fatty acid methyl esters (FAMEs) of myristic acid (14:0), palmitic acid (16:0), 16:1Δ9, stearic acid (18:0), 18:1Δ9, 18:1Δ11, *cis*-9,10-methyleneoctadecanoic acid (19:1cycloΔ9), and 19:0Me10, I used a gas chromatograph, GC-2010, equipped with a mass spectrometer, QP-2010 (Shimadzu). The conditions of GC were identical to those used for the FAME quantification, as described in Chapter 1. I confirmed the retention times and mass spectra of commercial FAME standards (Nu-Chek Prep, Elysian, MN, >99%), 19:1cycloΔ9 (Santa Cruz Biotechnology, ≥98%), 18:1Δ11 (≥97%), and 19:0Me10 (Larodan Fine Chemicals, Malmö, Sweden, ≥97%).

cis-7-hexadecenoic acid (16:1Δ7), lactobacilic acid (19:1cycloΔ11), and *cis*-9,10-methylenehexadecanoic acid (17:1cycloΔ9), were identified by comparing with the mass spectrometry data library. *cis*-9,10-Methylene-*cis*-12-octadecenoic acid (19:2Δ12cycloΔ9), *cis*-9,10-methylene-*cis*-12,15-octadecadienoic acid (C19:3Δ12,15cycloΔ9), and *cis*-11,12-methyleneoctadecanoic acid (19:1cycloΔ11) were identified from the mass (*m/z*) of the parent ions and the pattern of fragmentation in the GC-mass spectrometry results.

2.2.4. Alignments of the homologous proteins and construction of phylogenic tree

The protein sequences were obtained from the genetic information registered in the NCBI GenBank database (www.ncbi.nlm.nih.gov/pubmed). The alignment and construction of a phylogenic tree of the primary structures of the proteins was performed using neighbor-joining method with ClustalX2 (http://www.clustal.org/) and phylogenic tree was drawn using Fig Tree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). The alignment and comparison of the primary sequences of the homologous proteins was performed using

ClustalW (http://www.genome.jp/tools-bin/clustalw) and BoxShade Server (http://www.ch.embnet.org/software/BOX_form.html).

2.2.5. *Protein assay*

Proteins for analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were extracted from *E. coli* cells as follows: The cells were precipitated by centrifugation and re-suspended in 50 mM HEPES-NaOH (pH 7.0). The cell suspensions were treated by French press (model OS, Constant Systems Ltd., Northants, UK) with 45 kpsi to disrupt the cells. Then cellular debris was removed by centrifugation (20,000 x *g*, 20 min, 4°C). Supernatant were stored as soluble fraction. Cellular debris was re-suspended in 50 mM HEPES and then stored as insoluble fraction. Sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8) was added to each fraction, and incubated at 95 °C for 5 min to allow the denaturation of the proteins. After applying of samples to 8% gel and electrophoresis, gel was stained with CBB (0.25% coomassie brilliant blue R-250, 40% methanol and 7% acetic acid) for 2 h and decolorized moderately.

2.3. Results

2.3.1. *Heterologous expression of the* umaA *gene in* E. coli

Meena et al. (2013) previously demonstrated that the recombinant protein encoded by the *umaA* gene from *M. tuberculosis* $H_{37}Rv$ produces 19:0Me10 from 18:1Δ9 attached to PC *in vitro* and the addition of NADPH to the reaction mixture stimulates the production of 19:0Me10. Based on this report, I attempted to synthesize 19:0Me10 *in vivo* by culturing *E. coli* cells expressing the *umaA* gene in a medium supplying 18:1Δ9 as an exogenous fatty acid. According to the report of Meena et al. (2013), the expression of UmaA in *E. coli* may produce 19:0Me10, although I could not observe any novel fatty acids, including

19:0Me10, in cells transformed with the plasmid pUmaA (data not shown). The entire portion of UmaA is occupied by the typical structure of an SAM-dependent methyltransferase and may not have any capable domains involved in the reduction step. There was a discrepancy between the results reported by Meena et al. (2013) and those of previous reports (Akamatsu and Low, 1970; Couderc et al., 1991; Jaureguiberry et al., 1965). To attempt to resolve this discrepancy, I searched for novel genes involved in the biosynthesis of 19:0Me10.

2.3.2. Heterologous expression of the candidate genes in E. coli

Certain bacteria produce methylated fatty acids such as cyclopropane fatty acid, mid-chain methyl-branched fatty acids, and mycolic acid. I surveyed bacteria producing methylated fatty acids from the literature. The genomic data of 15 organisms, i.e., *Salmonella enterica* subsp. serovar Typhimurium ATCC 13311 (Balamurugan, 2010), *E. coli* K-12 (McGarrity and Armstrong, 1975), *Helicobacter pylori* ATCC 43504 (Inamoto et al., 1995), *Campylobacter jejuni* subsp. Jejuni 81-176 (Asakura et al., 2016), *Lactobacillus reuteri* I5007 (Liu et al., 2014), *Clostridium acetobutylicum* ATCC 39057 (Lepage et al., 1987), *Rhodococcus ruber* KCCM 41053 (Hwang et al., 2015), *Amycolatopsis orientalis* DSM 43387 (Majumdar et al., 2006), *Rhodococcus phenolicus* DSM 44812 (Rehfuss and Urban, 2005), *M. chlorophenolicum* JCM 7439 (Apajalahti et al., 1986; Hagglblom et al., 1994), *Rhodococcus erythropolis* DSM 43066 (Koch et al., 1995), *Rhodococcus qingshengii* djl-6 (Xu et al., 2007), *Mycobacterium tuberculosis* H37Rv (Khuller et al., 1982), *Nocardia donostiensis* X1654 (Ercibengoa et al., 2016), and *Corynebacterium urealyticum* ATCC 43042 (Couderc et al., 1991) were selected. These organisms were then categorized into three groups according to their possession of methylated fatty acids, as follows: (I) bacteria producing only cyclopropane fatty acids, (II) bacteria producing only mid-chain methyl-branched fatty acids, and (III) bacteria

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producing mid-chain methyl-branched fatty acids and mycolic acids (Table 8). It has been reported that cyclopropane fatty acid, mid-chain methyl-branched fatty acid, and mycolic acid are synthesized by a methyltransferase using SAM as a donor of the methyl group (Takayama et al., 2005). Therefore, I attempted to search for candidate methyltransferases using the *cfa* gene from *E. coli*, which is well characterized as the gene for cyclopropane fatty acid synthase (Fagan and Palfey, 2010) and possesses an SAM-dependent methyltransferase domain, as a query sequence and the genomic sequences of the organisms listed in Table 8 as the targets for a BLASTP search (Mount, 2007). However, the genomic sequence data of six strains, namely *S. enterica* subsp. serovar Typhimurium ATCC 13311, *C. acetobutylicum* ATCC 39057, *R. qingshengii* djl-6, *R. ruber* KCCM 41053, *R. erythropolis* DSM 43066, and *A. oriental* DSM 43387, were not available from public databases. Therefore, the genomic DNA sequences of closely related strains, namely *S. enterica* subsp. serovar Typhimurium SL1344, *C. acetobutylicum* ATCC 824, *R. qingshengii* BKS 20-40, *R. ruber* BKS20-38, *R. erythropolis* R138, and *A. orientalis* DSM 40040, respectively, were used as surrogates. Then, I selected one potential SAM-dependent methyltransferase that had the highest homology with the query sequence from the genomic information of each organism. The genomic DNA of *M. chlorophenolicum* JCM 7439, which is categorized into group III in Table 8, was available from the National BioResource Center at the Institute of Physics and Chemical Research (RIKEN) and I did not know how similar the three types of methyltransferases involved in the biosynthesis of cyclopropane fatty acid, mid-chain methyl-branched fatty acid, and mycolic acid would be. Thus, while I chose only one methyltransferase from each organism, I chose all the potential methyltransferases from *M. chlorophenolicum* JCM7439. In the chromosome of *M. chlorophenolicum*, seven SAM-dependent methyltransferases were identified (WP_048471080, WP_048471690, WP_048471691, WP_048471942, WP_048472121, WP_048473845, and WP_048473846). Then, I drew a

phylogenetic tree of these protein sequences using the ClustalX2 program (http://www.clustal.org/) (Figure 8). The resultant phylogenic tree showed that the methyltransferases from the organisms classified into group I in Table 8 were included in clade A, those from the organisms classified into group II were included in clade B, and those from the organisms classified into group III were included in clades B and C, suggesting that the enzymes categorized into clades A, B, and C might be related to the synthesis of cyclopropane fatty acids, mid-chain methyl-branched fatty acids, and mycolic acids, respectively. Among the seven SAM-dependent methyltransferases identified in *M. chlorophenolicum*, WP_048471942 and WP_048472121 were included in clade B as shown in Figure 8. Thus, I hypothesized that these two proteins are potential candidate methyltransferases for the biosynthesis of the mid-chain methyl-branched fatty acids in *M. chlorophenolicum*. To investigate their functions, I expressed each gene in *E. coli* cells using the plasmids pM1942 and pM2121 including the genes for WP_048471942 and WP_048472121, respectively, which both possessed the P*trc* promoter with the canonical SD sequence upstream of the initiation codon (Figure 7), and analyzed their fatty acid compositions. As a result, a novel chromatography peak that was not found in the vector control strain was detected in the cells expressing WP_048472121 that were cultured in medium supplemented with 18:1Δ9 (Figure 9 and Figure 10), but not in those expressing WP_048471942. Unfortunately, the retention time and mass spectral pattern of this peak were not identical with those of the commercially obtained mid-chain methyl-branched fatty acid standard, 19:0Me10. I then analyzed the electrophoretic separation pattern of proteins in the cells by SDS-PAGE. I found a protein band corresponding to a molecular mass of 49 kDa, which was not found in the cells of non-transformed cultures, in the lysates of the *E. coli* cells transformed with each methyltransferase gene, WP_048471942 and WP_048472121, whose predicted molecular masses are approximately 49 kDa (Figure 11). The results indicated that the expression of the WP_048471942 and

WP_048472121 methyltransferases was not sufficient to produce mid-chain methyl-branched fatty acid.

2.3.3. Heterologous expression of WP_048472120 and WP_048472121 in E. coli

In a previous study, it was reported that biosynthetic pathway of mid-chain methyl-branched fatty acids consists of a two-step reaction that includes a methylenation reaction using SAM as a donor of the methylene group to synthesize intermediates and a reduction reaction using NADPH as a cofactor to produce the saturated methyl-branched fatty acid (Oku and Kaneda, 1988). The SAM-binding site and methylase domain occupy the entirety of both the WP_048471942 and WP_048472121 proteins and they do not seem to possess any domains associated with redox reactions. Thus, I anticipated that a gene encoding another protein that catalyzes the reduction reaction might exist elsewhere in the *M. chlorophenolicum* genome. I performed a comparative genomic analysis of the structures of the genes for WP_048471942 and WP_048472121 with sequences available in public databases and found that a gene for a protein of unknown function, which is annotated as "an FAD-binding oxidoreductase" (WP_048472120), was located upstream of the gene for WP_048472121. The termination codon of the gene for the FAD-binding oxidoreductase-like protein and the initiation codon of the gene for WP_048472121 overlapped by 4 base pairs. Therefore, it seems possible that these genes are co-transcribed as an operon from the chromosome in *M. chlorophenolicum*, in which case the two proteins are more likely to be involved in the same reaction. Furthermore, the orthologous genes were also conserved in the genomic sequences of the organisms categorized into clade B and C as shown in Figure 8, and the genes in *R. ruber* BKS 20-38 *R. erythropolis* R138, *R. qingshengii* BKS 20-40, and *N. donostiensis* X 1654 also overlapped by 4 base pairs, like those in *M. chlorophenolicum* JCM 7439 (Figure 12). To investigate whether the FAD-binding oxidoreductase-like protein and the SAM

methyltransferase are involved in mid-chain methyl-branched fatty acid synthesis, I constructed *E. coli* transformants to express both genes using the plasmid pFADO-4-M2121 (Figure 7). However, I could not detect the synthesis of mid-chain methyl-branched fatty acid. Then, I analyzed the expression of the recombinant proteins by SDS-PAGE. I found a 52 kDa band corresponding to the FAD-binding oxidoreductase-like protein, but no 49 kDa band for WP_048472121 methyltransferase, in the lysates of the cells transformed with the two overlapped genes (data not shown). Then, I looked at the upstream sequence of the WP_048472121 gene (−15)-GGCGGTACGGC-(−5) and found that the sequence was somewhat different from the canonical SD sequence in *E. coli*, i.e., (−15)-AAGGAGGAATA-(−5) (Figure 13A). I expected that this sequence was not recognized as an SD sequence in *E. coli*. Therefore, the sequence upstream of WP_048472121 was modified by two approaches. First, the sequence was modified by substitution of the upstream nucleotide sequences without changing the encoding amino acids and the 4 overlapping base pairs to produce the plasmid pFADO-M2121-S (Figure 13B). Second, the sequence was modified by the insertion of the SD sequence with the removal of the overlap between the genes without changing the nucleotide sequence of the gene for FAD-binding oxidoreductase-like protein and creating a termination codon from the overlapped ATG to produce the plasmid pFADO-M2121-I (Figure 13C). The two resultant plasmids were then transformed into the *E. coli* cells. The results of an analysis of fatty acid composition showed that the cells expressing both types of modified genes accumulated a novel mid-chain methyl-branched fatty acid, 19:0Me10, when 18:1Δ9 was added into the culture as an exogenous fatty acid. Note that only the results for the cells transformed with the plasmid pFADO-M2121-I are shown in Table 9. And by the SDS-PAGE analysis, 52 kDa band for the FAD-binding oxidoreductase-like protein and 49 kDa band for WP_048472121 methyltransferase were detected from the proteins extracted from *E. coli* cell introduced the plasmid

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pFADO-M2121-I (Figure 11). These results indicated that the FAD-binding oxidoreductase-like protein and WP_048472121 are involved in the synthesis of 19:0Me10. Hereafter, I call them the *bfaA* and *bfaB* genes, respectively. In general, the 4-bp overlapping genes are co-transcribed as dicistronic genes in the bacteria, as an example of the ssl2245-sll1130 genes in the cyanobacterium *Synechocystis* sp. PCC 6803 (Srikumar et al., 2017). I speculated that the *bfaA* and *bfaB* genes are an operon in *M. chlorophenolicum*.

2.3.4. *Heterologous expression of WP_048472873 in* E. coli

As shown in Figure 13, the third conserved gene for WP_048472873 was located at the upstream of *bfaA* on the opposite coding strand. The orthologs of this gene were found in the same genetic locus on the chromosomes of the organisms categorized into clade B, as shown in Figure 12. Genes that adjoin on a chromosome in a head-to-head arrangement are often expressed concomitantly, perhaps due to sharing regulatory elements. Furthermore, the products of such genes may function together. The upstream gene was annotated to encode a homolog of polyketide cyclase, which has a "hydrophobic ligand binding site" and may interact with the hydrocarbon chains containing carboxyl or hydroxyl groups. This genomic information may indicate that this gene has the potential of assistant for biosynthesis of 19:0Me10 and the expression of this gene improve the productivity of 19:0Me10. To evaluate the possibility that the third gene product is involved in the formation of 19:0Me10, I expressed the gene for polyketide cyclase together with the *bfaA* and *bfaB* genes in *E. coli* cells using the plasmid pFADO-M2121-PCYC (Figure 7). However, the abundance of mid-chain methyl-branched fatty acid in the cells was not altered compared with that in the cells expressing the *bfaA* and *bfaB* genes (data not shown), indicating that the third gene located upstream of the *bfaA* and *bfaB* genes does not seem to be an essential factor for mid-chain

methyl-branched fatty acid synthesis or does not function in *E. coli* cells.

2.4. Discussion

2.4.1. Two-step reaction for the synthesis of mid-chain methyl-branched fatty acid

In this study, it was indicated that BfaA and BfaB are involved in the synthesis of 19:0Me10. It has been reported that some *Corynebacterium* species, e.g., ATCC 43042, ATCC 43043, and ATCC 43044, accumulate 19:1Me10 as an intermediate of 19:0Me10 (Couderc et al., 1991). 19:1Me10 is thought to be produced by the methylation of 18:1Δ9 and then reduced to 19:0Me10. If BfaB and BfaA catalyze the two reaction steps of methylenation and reduction, respectively, then the cells expressing only BfaB should accumulate 19:1Me10. The results of a fatty acid analysis showed that a peak corresponding to a novel fatty acid was detected in the *E. coli* cells transformed with the *bfaB* gene using the plasmid pM2121 (Figure 9). This fatty acid could not be clearly identified as 19:1Me10, because an authentic standard for this fatty acid was not available and the fragment patterns were also not available in the mass spectrometry data library. However, one of the fatty acid extracted from *C. urealyticum* ATCC 43042 showed same retention time and mass spectra with the characteristic fatty acid extracted from the *E. coli* cell expressing *bfaB* gene (Figure 9). 19:1Me10 was detected from *C. urealyticum* and the content of the fatty acid was approximately 3.5 % of the total fatty acids as shown in the previous literalities (Couderc et al., 1991). And the mass of the parent ion of this peak was 310, which corresponds to the calculated molecular mass of the methyl ester of 19:1Me10 (Figure 10). From these results, I expected that these peaks were for 19:1Me10.

It has been speculated that the second step of 19:0Me10 biosynthesis includes a reduction reaction using NADPH as a cofactor (Akamatsu and Low, 1970). My results indicated that BfaA might catalyze the reduction of 19:1Me10, since this protein is annotated as an "FAD-linked oxidoreductase". FAD is a cofactor for numerous enzymes

that mediate redox reactions. For instance, UDP-*N*-acetylmuramate:NADP$^+$ oxidoreductase (MurB) is an FAD-binding enzyme that catalyzes the NADPH-dependent reduction of UDP-*N*-acetyl-3-*O*-(1-carboxyvinyl)-α-ᴅ-glucosamine to the corresponding ᴅ-lactyl compound, UDP-*N*-acetyl-α-ᴅ-muramate. First, NADPH reduces the FAD of the enzyme to NADP$^+$, which dissociates from the enzyme. Then, the enzyme interacts with the substrate, UDP-*N*-acetyl-3-*O*-(1-carboxyvinyl)-α-ᴅ- glucosamine, and the reduced FAD transfers an electron to the substrate (Fagan and Palfey, 2010). I speculate that a similar reaction mechanism might occur in the case of BfaA.

2.4.2. *Substrate specify of BfaA and BfaB*

E. coli cells synthesize the unsaturated fatty acids 16:1Δ9 and 18:1Δ11 as components of their membrane lipids, but they do not synthesize 18:1Δ9, 18:2Δ9,12, or 18:3Δ6,9,12. In general, fatty acid-modifying enzymes, including the acyl-lipid fatty acid desaturases, react with the fatty acids in a position-specific manner (Los and Murata, 1998). I also reported that the cyclopropane fatty acid synthase in *E. coli* introduced a cyclopropane ring into the natural substrates 16:1Δ9 and 18:1Δ11, and the artificial substrates 18:1Δ9, 18:2Δ9,12, and 18:3Δ9,12,15, but not 18:3Δ6,9,12 as described in Chapter 1. According to the results in Table 8, BfaA and BfaB specifically react with 18:1Δ9, but not 16:1Δ9 and 18:1Δ11, in *E. coli*. It was unclear whether BfaA and BfaB introduce a branched chain at the Δ9 position or other positions in various unsaturated fatty acids. I analyzed the fatty acid compositions of the vector control and BfaA and BfaB-transformed strains cultivated in liquid media supplemented with 1 mM each of 16:1Δ9, 18:1Δ11, 18:2Δ9,12, 18:3Δ9,12,15, and 18:3Δ6,9.12 as exogenous fatty acids. The results showed that the fatty acid composition of the BfaA and BfaB-transformed strains was not different from that of the vector control strain and no novel mid-chain methyl-branched fatty acids were detected (Table 10). These results suggest that BfaA and BfaB can specifically modify

only the Δ9 position of the C18 mono-unsaturated fatty acid, 18:1Δ9, as a substrate for the production of mid-chain methyl-branched fatty acid. *M. chlorophenolicum* has 16:1Δ9 and 18:1Δ9 as unsaturated fatty acids and synthesizes 19:0Me10 (Hagglblom et al., 1994). Based on these phenomena, it is concluded that 18:1Δ9 produced by the desaturation of 18:0 might be converted to 19:0Me10 by BfaA and BfaB.

2.4.3. Homologous proteins of BfaA and BfaB

From my current study, it was suggested that BfaA and BfaB encoding the FAD-binding oxidoreductase and the SAM-dependent methyltransferase, respectively, are involved in the synthesis of 19:0Me10 in *M. chlorophenolicum*. As shown in Figure 12, the paralogs of the *bfaA* and *bfaB* operon were conserved in other bacteria producing 19:0Me10 and similarities of paralogues to BfaA and BfaB were more than 50%, except *R. phenolicus* DSM 44812. In this organism, the genes encoding the most similar enzymes for BfaA and BfaB, i.e., WP_068165945 and WP_068165842, were separately located in the chromosome and the similarity of these proteins to the other homologues were rather low in Figure 12. I, then, compared the primary sequences of the homologous proteins of BfaA and BfaB in these bacteria except *R. phenolicus* DSM 44812 (Figure 14A and 14B). These proteins were very similar to each other, although the FAD-binding oxidoreductase and the SAM-dependent methyltransferase from *C. urealyticum* ATCC 43042 possess the unique short-inserted sequences of 11 and 19 amino acid residues at 398 and 176 amino acids, respectively. According to the previous publications, only from *C. urealyticum* ATCC 43042, 19:1Me10 is detected as the intermediate of 19:0Me10 synthesis (Couderc et al., 1991). The unique extensions in the enzymes in *C. urealyticum* ATCC 43042 may extremely enhance the activity of the SAM-dependent methyltransferase producing 19:1Me10 and/or significantly decrease the activity of the FAD-binding oxidoreductase processing 19:1Me10 to 19:0Me10, and then 19:1Me10 might be accumulated.

General Discussion

First, I successfully synthesized cyclopropane fatty acids in *Synechocystis* sp. PCC 6803 at a level comprising ~30% of total fatty acids. Growth of *Synechocystis* cells harboring the *cfa* and *desC2* genes was altered, particularly under low-temperature conditions, but photosynthetic and respiratory activities were not significantly affected. These results suggest that Cfa and desaturases may recognize lipid side chains. Especially, DesC2 might distinguish a fatty acid class binding to *sn*-1 position and unsaturate a fatty acid binding to *sn*-2 position. This possibility provides new knowledge for a role of the desaturase and mechanism of the substrate recognition. Second, I successfully synthesized 10-methyl stearic acid in *E. coli* by the heterologous expression of BfaA and BfaB from *M. chlorophenolicum*. Cells expressing the *bfaB* gene alone might produce 10-methylene stearic acid as an intermediate of 10-methyl stearic acid production. It seems that an FAD-linked oxidoreductase encoded by the *bfaA* gene contributes to the reduction reaction that is required for the conversion of 10-methylene stearic acid to 10-methyl stearic acid. I expect that these findings of a novel 10-methyl stearic acid biosynthetic system will be helpful in healthcare or industry.

There are three types of fatty acid desaturase, acyl-lipid, acyl-CoA, and acyl-ACP desaturase (Murata and Wada, 1995). The acyl-lipid desaturases can introduce unsaturated double bond into fatty acids binding to lipid (Murata et al., 1992, Murata and Wada, 1995), and are harboring the transmembrane domain (e.g. DesA, DesB, DesC, and DesD). On the other hand, the acyl-CoA and acyl-ACP desaturases recognize acyl group bound to coenzyme A or acyl-carrier protein as substrates, respectively (Murata et al., 1992, Murata and Wada, 1995). Especially, stearoyl-ACP desaturase, one of the acyl-ACP desaturase, is harboring the transmembrane domain (e.g. AC002333 from *Arabidopsis thaliana*). From these features of fatty acid desaturases, it is predicted that the enzymes which can modify

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the fatty acid binding to the lipid are insoluble, and the enzymes which can modify the fatty acid binding to ACP are soluble. BfaA and BfaB identified in my study do not harbor transmembrane domain. Thus, it is expected that the possibility that they may recognize oleic acid binding to ACP. In chapter 3, the introduction of the *bfaA* and *bfaB* genes into the *desAD⁻* strain which accumulates a large amount of oleic acid did not show significant improvement of the productivity of 10-methyl stearic acid compared with that in the wild-type strain. This result indicates that in the *Synechocystis* cells, oleic acid synthesized on the membrane lipids might be arduous to be transferred into ACP and retained in the lipids. Generally, it is considered that fatty acids are transferred in ACP-form, lipid-form, and free-fatty acid, which are catalyzed by acyl-ACP synthase, acyl transferase, and hydrolase, respectively. However, Gao et al., (2012) demonstrated that the abundance of the free-oleic acid was much lower than the other free-fatty acids in the *Synechocystis* cells disrupted the *slr1609* gene encoding acyl-ACP synthase, which cells accumulate free-fatty acids. This result does not contradict my hypothesis that oleic acid might stay on the lipid-form and not easily transfer to the ACP-form. Therefore, the abundance of oleic acid binding to ACP which can be recognized by BfaA and BfaB may be small in the cells and productivity of 10-methyl stearic acid was not improved in the *desAD⁻* strain. Next, Cfa is not harboring transmembrane domain. However, in the previous study, it is reported that Cfa can modify the oleic acid binding to phospholipid (Grogan and Cronan, 1997). This report is inconsistent with my hypothesis about the relationship of the transmembrane domain and substrate specificity. Even protein without a transmembrane domain may modify the fatty acid binding to the lipid.

The homolog of BfaA and BfaB in *M. tuberculosis*, tubercle bacillus, is WP_003917236 and WP_003420415, respectively (Figure 12), and it is predicted that these proteins are also involved in the synthesis of 10-methyl stearic acid in *M. tuberculosis* cells. 10-methyl stearic acid which is also called tuberculostearic acid is a

main component of the cell membrane in *M. tuberculosis*. The unusual cell architecture of this organism is helpful to protect the cells from noxious chemicals. Additionally, tuberculostearic acid is one of the important constituents to build the highly complex cell wall (Dmitriev et al., 2000). Although it has been reported that UmaA and UfaA1 are involved in the synthesis of tuberculostearic acid, UmaA showed no activity *in vivo* in my study. BfaA and BfaB which I discovered in this study showed an activity to synthesize tuberculostearic acid *in vivo*. I expect that this discovery will be utilized in not only the biological field but also the medical field in future.

The production of fatty acid from microalgae is paid attention. In this study, cyanobacterium, *Synechocystis*, was used as host cells. But this organism synthesizes fatty acid for the component of the membrane lipid mainly, and cannot accumulate the fatty acids as a reserve substance. Thus, the construction of the production system of fatty acid is not realistic in this cell. However, green algae, (e.g. *Chlamydomonas reinhardtii*) and diatom (e.g. *Phaeodactylum tricornutum*) which can accumulate the triacylglycerol as lipid droplet are regarded as a viable prospect. Results of my study indicate that the synthesis of cyclopropane fatty acids and mid-chain methyl-branched fatty acid has no effect on photosynthesis. I anticipate that the construction of production system of these fatty acids employing eukaryotic phototroph is possible. Additionally, in the cyanobacterium, free fatty acid secretion system attracts attention. This system is achieved by overexpression of the acyl ACP synthase and disruption of the thioesterase (Kato et al., 2016). Because the free fatty acid can be secreted to outside of the cells, we do not need to break the cells and collect a fatty acid like milking. It might be effective to emulate this system and let release the cyclopropane fatty acids or mid-chain methyl-branched fatty acids to outside of the cyanobacterium cells. The studies described here are the first attempt at synthesizing unusual methylated fatty acids in phototroph. At this moment, microalgae producing useful compound are isolated from nature and cultivated in large

scale. But in this strategy, it is difficult to design the system to produce the target compound. This study has the potential to design the artificial phototroph synthesizing useful compound and develop into industrial production. I anticipate that my study will become the fundamental base to establish new industry using microalgae fixing carbon dioxide and sustain society.

Acknowledgements

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Tables and Figures

Table 1. Primers used in this study

No.	Name	Sequence (5'-3')
1	pTCHT_Cm_remove_InF_F	TTTTTGCTTCATCGCTTAAGGCAGTTATTG
2	pTCHT_Cm_remove_InF_R	ACTGCCTTAAAAAAAGAAGCAAAAAGCCTA
3	cfa_Nde_F	CATATGATGAGTTCATCGTGTATAGA
4	cfa_Bgl_R	AGATCTTTAGCGAGCCACTCGAAGGC
5	Sp_BamHI_F	GGGGATCCATCAATTCCCCTGCTCGCGC
6	Sp_Bgl_R	GGAGATCTTCCCAATTTGTGTAGGGCTT
7	desC2_F	TCAAGTAGGAGATTAATTCA
8	desC2_R	TTAGCCATGAGTTGCACCTT
9	pTHT_cfaup_inf_R	TAATCTCCTACTTGACATATGATCCTTATCG
10	pTHT_cfadn_inf_R	TAATCTCCTACTTGAGGATCTTTAGCGAGCCACTC
11	pTHT_Sprup_inf_F	GCAACTCATGGCTAAATCAATTCCCCTGCTCGCGC
12	desC_qPCR_F	AAATGCCCCAAATAACGAAGG
13	desC_qPCR_R	AAAGCTGATATTCCCCGCTACA
14	desA_qPCR_F	TATCCCCGTTGGGTGGAA
15	desA_qPCR_R	AAGGTGCGCTCGTAAAGAAAG
16	desD_qPCR_F	GGTTGATGTGGGGATTGGA
17	desD_qPCR_R	CCTACTGGGTTGTATGGTTTTGG
18	rnpB_RT_F	GTAAGAGCGCACCAGCAGTATC
19	rnpB_RT_R	TCAAGCGGTTCCACCAATC
20	pTCHT_Cm_remove_InF_F	TTTTTGCTTCATCGCTTAAGGCAGTTATTG
21	pTCHT_Cm_remove_InF_R	ACTGCCTTAAAAAAAGAAGCAAAAAGCCTA
22	M1942_Nde_F	CATATGATGACGACCGCTGAAACGG
23	M1942_Bam_R	GGATCCTCACGATTTCGATGCCCGCG
24	M2121_Nde_F	CATATGATGACGACTTTTCGGGAACG
25	M2121_Bam_R	GGATCCTCAGGCGGTCCACCAAGGCC
26	FAD_Nde_F	CATATGATGTCTGTTCCCGCAACCGA
27	Sp_Bgl_F	GGAGATCTATCAATTCCCCTGCTCGCGC
28	Sp_Bam_R	GGGGATCCTCCCAATTTGTGTAGGGCTT
29	SD_add_S_F	AGGAGACAATGACGACTTTTCGGG
30	SD_add_S_R	TCGTCATTGTCTCCTTACCGCCTTCGCATACAGG
31	SD_add_I_F	AGGAGGAATAAACCATGACGACTTTTCGGGAACGACCG
32	SD_add_I_R	TGGTTTATTCCTCCTTCATTGTCGCCGTACCGCC
33	PCYC_inf_F	TGGTGGACCGCCTGAGAGGAGTTCGCACCGATGGG

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34	PCYC_inf_R	AGCAGGGGAATTGATCTACACGTCCTGGACGGCCTC
35	Sp_Bam_F	GGGGATCCATCAATTCCCCTGCTCGCGC
36	Sp_Bgl_R	GGAGATCTTCCCAATTTGTGTAGGGCTT

Table 2. Plasmids used in this study

Plasmids	Description	Reference
pTCHT2031V	Carrying *trc* promoter, Amp^r, and Cm^r	Ishizuka et al., 2006
pTHT2031V	Carrying *trc* promoter, and Amp^r	This study
pAM1146	Carrying Amp^r, and Sp^r	Tsinoremas et al., 1994
pMD-M1942	pMD19 with WP_048471942, carrying Amp^r	This study
pMD-M2121	pMD19 with WP_048472121, carrying Amp^r	This study
pMD-FADO-4-M2121	pMD19 with WP_048472021 and WP_048472121 with overlap of 4 base pair, carrying Amp^r	This study
pMD-M1942S	pMD-M1942 with Sp^r	This study
pMD-M2121S	pMD-M2121 with Sp^r	This study
pMD-FADO-4-M2121S	pMD-FADO-4-M2121 with Sp^r	This study
pM1942	Carrying *trc* promoter, WP_048471942, Sp^r, and Amp^r	This study
pM2121	Carrying *trc* promoter, WP_048472121, Sp^r, and Amp^r	This study
pFADO	Carrying *trc* promoter, WP_048472120, Sp^r, and Amp^r	This study
pFADO-4-M2121	Carrying *trc* promoter, WP_048472021 and WP_048472121 with overlap of 4 base pair, Sp^r, and Amp^r	This study
pFADO-M2121-I	pFADO-4-M2121 with insertion of SD sequence between WP_048472021 and WP_048472121	This study
pFADO-M2121-S	pFADO-4-M2121 with substitution of SD sequence between WP_048472021 and WP_048472121	This study
pFADO-M2121-PCYC	pFADO-M2121-I with PCYC	This study
pTAKN-2-UmaA	pTAKN-2 with UmaA, carrying Kan^r	Artificially synthesized
pTAKN-2-UmaAS	pTAKN-2-UmaA with Sp^r	This study
pUmaA	Carrying *trc* promoter, UmaA, Sp^r, and Amp^r	This study

Table 3. Fatty acid composition of total lipids from wild-type and *cfa*-overexpressing *E. coli* strains

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 experiments, expressed as mol % of total fatty acids. "-", not detected. "*", estimated from comparison with 19:1cyclo$^{\Delta 9}$.

	Fatty acid (mol %)									
	Wild type					*cfa*-overexpressing strain				
	Exogenous fatty acid					Exogenous fatty acid				
FA	-	18:1$^{\Delta 9}$	18:2$^{\Delta 9,12}$	18:3$^{\Delta 9,12,15}$	18:3$^{\Delta 6,9,12}$	-	18:1$^{\Delta 9}$	18:2$^{\Delta 9,12}$	18:3$^{\Delta 9,12,15}$	18:3$^{\Delta 6,9,12}$
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$^{\Delta 9}$	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$^{\Delta 7}$	-	2.5 ± 1.2	-	-	-	-	3.8 ± 1.1	-	-	-
16:2$^{\Delta 7,10}$	-	-	2.9 ± 1.0	-	-	-	-	2.1 ± 0.1	-	-
16:3$^{\Delta 7,10,13}$	-	-	-	5.2 ± 0.4	-	-	-	-	4.5 ± 0.7	-
16:3$^{\Delta 4,7,10}$	-	-	-	-	2.6 ± 1.3	-	-	-	-	2.5 ± 1.0
17:1cyclo$^{\Delta 9}$	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$^{\Delta 11}$	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$^{\Delta 9}$	-	29.4 ± 1.5	-	-	-	-	12.3 ± 3.6	-	-	-
18:2$^{\Delta 9,12}$	-	-	12.9 ± 3.7	-	-	-	-	7.8 ± 1.1	-	-
18:3$^{\Delta 9,12,15}$	-	-	-	17.6 ± 0.4	-	-	-	-	12.3 ± 1.2	-
18:3$^{\Delta 6,9,12}$	-	-	-	-	10.9 ± 2.3	-	-	-	-	8.2 ± 0.6
19:1cyclo$^{\Delta 11}$	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$^{\Delta 9}$	-	0.3 ± 0.1	-	-	-	-	14.5 ± 2.9	-	-	-
*19:2$^{\Delta 12}$cyclo$^{\Delta 9}$	-	-	0.3 ± 0.1	-	-	-	-	5.4 ± 0.7	-	-
*19:3$^{\Delta 12,15}$cyclo$^{\Delta 9}$	-	-	-	1.7 ± 0.7	-	-	-	-	2.2 ± 0.2	-
*19:3$^{\Delta 6,12}$cyclo$^{\Delta 9}$	-	-	-	-	-	-	-	-	-	-

Table 4. Fatty acid compositions of total lipids from *Synechocystis* cells expressing the *cfa* and *desC2* genes

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 acids. "-", not detected

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	-	-	-
cfa+	55.5 ± 0.8	-	-	2.1 ± 0.1	5.1 ± 0.4	10.7 ± 0.7	7.8 ± 0.6	-	-	18.8 ± 0.4
desC2+	31.3 ± 0.8	25.3 ± 0.7	-	2.6 ± 0.4	15.2 ± 0.7	19.3 ± 1.2	6.3 ± 0.8	-	-	-
cfa+/*desC2*+	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
desAD-	49.0 ± 0.4	-	-	1.2 ± 0.2	49.8 ± 0.5	-	-	-	-	-
desAD-/*cfa*+	59.7 ± 0.4	-	-	3.5 ± 0.2	14.6 ± 0.8	-	-	-	-	22.2 ± 0.6
desAD-/*desC2*+	35.8 ± 0.4	26.2 ± 1.0	-	4.1 ± 1.6	33.9 ± 1.0	-	-	-	-	-
desAD-/*cfa*+/*desC2*+	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	-
cfa+	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
desC2+	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	-
cfa+/*desC2*+	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
desAD-	54.0 ± 0.7	-	-	1.3 ± 0.3	44.7 ± 0.8	-	-	-	-	-
desAD-/*cfa*+	54.7 ± 1.1	-	-	7.2 ± 3.7	18.8 ± 2.5	-	-	-	-	19.3 ± 1.0
desAD-/*desC2*+	28.7 ± 0.7	25.4 ± 1.2	-	2.9 ± 0.2	43.1 ± 0.3	-	-	-	-	-
desAD-/*cfa*+/*desC2*+	51.5 ± 1.0	3.3 ± 0.5	2.8 ± 0.1	2.7 ± 0.2	16.3 ± 1.8	-	-	-	-	23.4 ± 1.1

Table 5. Fatty acid composition in each lipid classes from the *Synechocystis* cells expressing the *cfa* and *desC2* genes

The cells were grown at 34˚C for 3 d. Mean ± S.D. values of three independent experiments, expressed as mol % of total fatty acids. "-", not detected and "T", trace amount.

Strain	Lipid class	Fatty acid (mol %)							
		16:0	16:1$^{\Delta 9}$	17:1cyclo$^{\Delta 9}$	18:0	18:1$^{\Delta 9}$	18:2$^{\Delta 9,12}$	18:3$^{\Delta 6,9,12}$	19:1cyclo$^{\Delta 9}$
Wild type	MGDG	56.6± 7.5	-	-	5.9 ± 3.6	18.3 ± 13.2	15.6 ± 4.5	4.1 ± 1.5	-
	DGDG	64.1 ± 14.2	-	-	11.7 ± 10.8	11.2 ± 5.4	7.9 ± 3.6	2.8 ± 1.9	-
	SQDG	74.4 ± 5.2	-	-	1.3 ± 0.1	18.2 ± 5.0	7.1 ± 1.7	T	-
	PG	80.4 ± 4.6	-	-	4.7 ± 0.9	8.5 ± 3.6	6.4 ± 2.4	T	-
	total	58.0 ± 3.9	-	-	3.8 ± 2.5	19.3 ± 2.1	13.6 ± 1.7	5.2 ± 0.7	-
cfa⁺	MGDG	55.2 ± 6.7	-	-	5.1 ± 1.8	1.8 ± 0.1	2.2 ± 2.5	2.4 ± 1.5	34.4 ± 7.9
	DGDG	57.7 ± 7.1	-	-	3.1 ± 0.4	1.3 ± 0.1	2.4 ±2.2	2.1 ± 1.8	33.9 ±10.6
	SQDG	67 ± 4.1	-	-	5.2 ± 0.6	5.1 ± 3.4	6.6 ± 0.3	1.1 ± 0.4	16 ± 5.0
	PG	59.7 ± 4.0	-	-	15.6 ± 1.6	T	T	T	24.6 ± 4.4
	total	59.5 ± 2.1	-	-	2.1 ± 0.4	5.1 ± 0.9	6.7 ± 1.4	3.8 ± 0.8	22.8 ± 6.4
desC2⁺	MGDG	25.8 ± 0.3	23.1 ± 1.5	-	7.9 ± 2.3	15.1 ± 1.3	18.3 ± 0.6	10.6 ±1.4	-
	DGDG	30.9 ± 1.1	25.9 ± 0.8	-	6.2 ± 1.9	13.9 ± 1.2	15.0 ± 3.4	5.7 ± 3.1	-
	SQDG	56.6 ± 3.0	14.5 ± 2.3	-	2.7 ± 1.0	20.8 ± 3.2	2.0 ± 0.4	1.7 ± 1.1	-
	PG	53.3 ± 2.3	11.1 ± 1.3	-	13.2 ± 1.1	13.3 ± 3.0	6.5 ± 2.6	1.4 ± 1.2	-
	total	31.2 ± 2.9	23.5 ± 1.0	-	4.5 ± 0.8	15.6 ± 1.3	16.7 ± 2.1	8.1 ± 1.3	-
cfa⁺/*desC2*⁺	MGDG	38.5 ± 3.0	1.7 ± 0.8	16.4 ± 1.3	2.8 ±0.3	1.9 ±0.2	8.6 ± 1.5	4.1 ± 1.3	24.9 ± 2.7
	DGDG	45.0 ± 1.2	1.1 ± 0.6	11.8 ± 0.9	3.8 ± 0.3	1.6 ± 0.5	11.1 ± 0.7	T	25.7 ± 2.0
	SQDG	72.9 ± 2.0	T	4.6 ± 1.9	4.6 ± 0.5	2.8 ± 0.8	6.5 ± 1.4	T	8.6 ± 4.4
	PG	43.4 ± 4.5	10.7 ± 5.4	3.3 ± 1.3	15.8 ± 12.2	2.8 ± 1.0	T	T	23.9 ± 1.0
	total	44.5 ± 2.3	2.3 ± 1.9	7.7 ± 0.4	5.6 ± 1.5	1.9 ± 0.8	11.1 ± 1.0	6.2 ± 2.2	20.1 ± 2.8
desAD⁻	MGDG	46.3 ± 5.1	-	-	4.4 ± 1.4	49.3 ± 5.4	-	-	-
	DGDG	44.0 ± 2.5	-	-	5.7 ± 1.6	50.7 ± 3.1	-	-	-
	SQDG	82.4 ± 5.0	-	-	2.4 ± 0.8	15.4 ± 5.8	-	-	-
	PG	54.8 ± 2.9	-	-	20.8 ± 5.3	24.3 ± 2.5	-	-	-
	total	48.3 ± 2.1	-	-	1.9 ± 1.2	49.2 ± 2.6	-	-	-
desAD⁻ /*cfa*⁺	MGDG	51.9 ± 2.0	-	-	5.2 ± 0.8	11.8 ± 5.0	-	-	29.9 ± 7.6
	DGDG	57.2 ± 4.9	-	-	4.4 ± 0.8	4.9 ± 2.5	-	-	31.1 ± 6.8
	SQDG	68.9 ± 4.7	-	-	6.2 ± 3.2	15.1 ± 3.1	-	-	10.9 ± 3.2
	PG	53.6 ± 4.8	-	-	19.2 ± 7.4	T	-	-	27.9 ± 6.6
	total	53.7 ± 2.9	-	-	4.5 ± 1.1	13.6 ± 1.9	-	-	28.2 ± 2.8

Genotype	Lipid								
desAD⁻ /*desC2⁺*	MGDG	22.4 ± 8.2	29.2 ± 3.8	-	7.3 ± 3.3	38.4 ± 7.3	-	-	-
	DGDG	21.1 ± 0.2	32.3 ± 1.4	-	6.9 ± 3.5	36.9 ± 2.2	-	-	-
	SQDG	79.8 ± 2.8	6.2 ± 0.1	-	3.7 ± 1.7	9.7 ± 1.4	-	-	-
	PG	71.5 ± 4.2	11.1 ± 0.6	-	9.2 ± 4.7	10.1 ± 0.9	-	-	-
	total	31.1 ± 3.9	25.5 ± 1.2	-	4.7 ± 1.7	32.4 ± 1.8	-	-	-
desAD⁻ /*cfa⁺*/*desC2⁺*	MGDG	49.4 ± 1.1	1.6 ± 1.0	7.9 ± 1.5	2.8 ± 1.0	3.9 ± 0.1	-	-	34.2 ± 0.7
	DGDG	47.8 ± 1.4	T	6.9 ± 1.1	4.1 ± 0.9	5.1 ± 0.7	-	-	36.1 ± 0.8
	SQDG	78.5 ± 1.6	T	2.1 ± 0.3	5.1 ± 1.0	3.1 ± 1.6	-	-	14.8 ± 2.7
	PG	40.9 ± 4.5	6.7 ± 2.6	1.7 ± 0.1	6.2 ± 1.2	6.7 ± 2.4	-	-	40.7 ± 2.2
	total	51.0 ± 1.8	2.9 ± 1.1	5.7 ± 0.8	4.4 ± 0.7	3.7 ± 0.6	-	-	31.9 ± 1.1

Table 6. Relative amounts of fatty acids produced by each desaturase and cyclopropane

fatty acid synthetase

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 acids. "-", not detected.

34˚C	Percentage of total fatty acids (mol %)						
Strain	DesC	DesA	DesD	DesB	DesC2	Cfa	saturated
Wild type	44.2 ± 1.5	25.9 ± 1.4	9.2 ± 0.8	-	-	-	55.8 ± 1.5
cfa^+	42.4 ± 0.8	18.5 ± 1.1	7.8 ± 0.6	-	-	18.8 ± 0.4	57.6 ± 0.8
$desC2^+$	40.8 ± 1.8	25.6 ± 2.3	6.3 ± 1.0	-	25.3 ± 0.8	-	33.8 ± 1.3
$cfa^+/desC2^+$	39.5 ± 1.3	17.5 ± 0.8	6.2 ± 0.4	-	10.0 ± 0.6	27.9 ± 1.2	50.5 ± 1.6
$desAD^-$	49.8 ± 0.5	-	-	-	-	-	50.2 ± 0.5
$desAD^-/cfa^+$	36.8 ± 0.2	-	-	-	-	22.2 ± 0.6	63.2 ± 0.2
$desAD^-/desC2^+$	33.9 ± 1.2	-	-	-	26.2 ± 1.1	-	39.9 ± 2.2
$desAD^-/cfa^+/desC2^+$	31.9 ± 2.7	-	-	-	7.1 ± 0.5	33.5 ± 2.3	61 ± 2.6

26˚C	Percentage of total fatty acids (mol %)						
Strain	DesC	DesA	DesD	DesB	DesC2	Cfa	saturated
Wild type	46.8 ± 0.2	42.0 ± 0.8	23.9 ± 0.8	7.1 ± 0.8	-	-	53.2 ± 0.2
cfa^+	44.0 ± 1.5	32.5 ± 3.1	18.8 ± 2.5	5.1 ± 1.0	-	7.2 ± 2.4	56.0 ± 1.5
$desC2^+$	42.5 ± 0.9	31.1 ± 0.7	13.0 ± 0.7	2.3 ± 0.3	27.5 ± 0.7	-	30.0 ± 0.5
$cfa^+/desC2^+$	41.3 ± 1.5	27.8 ± 1.3	13.4 ± 0.5	2.2 ± 0.4	12.2 ± 0.8	17.6 ± 1.9	46.5 ± 0.9
$desAD^-$	44.7 ± 0.8	-	-	-	-	-	55.3 ± 0.8
$desAD^-/cfa^+$	38.1 ± 2.6	-	-	-	-	19.3 ± 1.0	61.9 ± 2.6
$desAD^-/desC2^+$	43.1 ± 0.4	-	-	-	25.4 ± 1.4	-	31.6 ± 1.0
$desAD^-/cfa^+/desC2^+$	39.7 ± 1.1	-	-	-	6.1 ± 0.5	26.2 ± 1.3	54.2 ± 1.4

Table 7. Relative quantification of mRNA of *Synechocystis* cells expressing the *cfa* and

   ***desC2* genes**

The cells were grown at 26˚C for 1 d. Mean ± S.D. values of three independent experiments. A, B, and C show treatment of data when the *desC*, *desA*, and *desD* genes were targeted, respectively. $C_{T\text{ target}}$ and $C_{T\text{ rnpB}}$ mean the C_T values of the target gene and the *rnpB* gene, respectively. $\Delta C_T = C_{T\text{ target}} - C_{T\text{ rnpB}}$. $\Delta\Delta C_T = \Delta C_T - \Delta C_{T\text{ wild type}}$. Relative amounts were calculated from $2^{-\Delta\Delta CT}$ [12].

A

Strain	$C_{T\text{ target}}$	$C_{T\text{ rnpB}}$	ΔC_T	$\Delta\Delta C_T$	Relative amount
Wild type	20.26 ± 2.28	19.18 ± 1.47	1.09 ± 0.85	0	1.00
cfa+	20.27 ± 1.99	19.41 ± 1.19	0.86 ± 0.81	-0.22 ± 0.23	1.18 ± 0.15
desC2+	20.01 ± 2.42	19.27 ± 1.48	0.74 ± 1.00	-0.35 ± 0.16	1.28 ± 0.12
cfa+/desC2+	20.33 ± 2.50	19.56 ± 1.57	0.77 ± 1.36	-0.31 ± 0.60	1.32 ± 0.44

B

Strain	$C_{T\text{ target}}$	$C_{T\text{ rnpB}}$	ΔC_T	$\Delta\Delta C_T$	Relative amount
Wild type	21.37 ± 1.78	19.18 ± 1.47	2.20 ± 0.74	0	1.00
cfa+	21.32 ± 1.73	19.41 ± 1.19	1.92 ± 0.68	-0.28 ± 0.45	1.25 ± 0.34
desC2+	21.14 ± 1.59	19.27 ± 1.48	1.86 ± 0.72	-0.33 ± 0.13	1.26 ± 0.09
cfa+/desC2+	21.20 ± 1.79	19.56 ± 1.57	1.64 ± 0.35	-0.55 ± 0.57	1.54 ± 0.44

C

Strain	$C_{T\text{ target}}$	$C_{T\text{ rnpB}}$	ΔC_T	$\Delta\Delta C_T$	Relative amount
Wild type	22.27 ± 2.18	19.18 ± 1.47	3.10 ± 0.71	0	1.00
cfa+	22.24 ± 2.51	19.41 ± 1.19	2.84 ± 1.32	-0.26 ± 61	1.27 ± 0.43
desC2+	22.19 ± 2.28	19.27 ± 1.48	2.92 ± 0.81	-0.18 ± 0.17	1.14 ± 0.11
cfa+/desC2+	22.61 ± 2.47	19.56 ± 1.57	3.05 ± 0.98	-0.05 ± 0.31	1.05 ± 0.17

Table 8. Existence of each methylated fatty acid in each organism

"+", Existence had been reported; "-", Existence had not been reported; "CFA", cyclopropane fatty acid; "mBFA", mid-chain methyl-branched fatty acid; "MA", Mycolic acid.

Group	Organisms	CFA	mBFA	MA	References
I	*Salmonella enterica* subsp. serovar Typhimurium ATCC 13311	+	-	-	Balamurugan, 2010
	Escherichia coli K-12	+	-	-	McGarrity and Armstrong, 1975
	Helicobacter pylori ATCC 43504	+	-	-	Inamoto et al., 1995
	Campylobacter jejuni subsp. Jejuni 81-176	+	-	-	Asakura et al., 2016
	Lactobacillus reuteri I5007	+	-	-	Liu et al., 2014
	Clostridium acetobutylicum ATCC 39057	+	-	-	Lepage et al., 1987
II	*Rhodococcus ruber* KCCM 41053	-	+	-	Hwang et al., 2015
	Amycolatopsis orientalis DSM 43387	-	+	-	Majumdar et al., 2006
	Rhodococcus phenolicus DSM 44812	-	+	-	Rehfuss and Urban, 2005
III	*Mycobacterium chlorophenolicum* JCM 7439	-	+	+	Apajalahti et al., 1986; Hagglblom et al., 1994
	Rhodococcus erythropolis DSM 43066	-	+	+	Koch et al., 1995
	Rhodococcus qingshengii djl-6	-	+	+	Xu et al., 2007
	Mycobacterium tuberculosis H37Rv	-	+	+	Khuller et al., 1982
	Nocardia donostiensis X1654	-	+	+	Ercibengoa et al., 2016
	Corynebacterium urealyticum ATCC 43042	-	+	+	Couderc et al., 1991

Table 9. Fatty acid composition of *E. coli* cells expressing the *bfaA* and *bfaB* genes
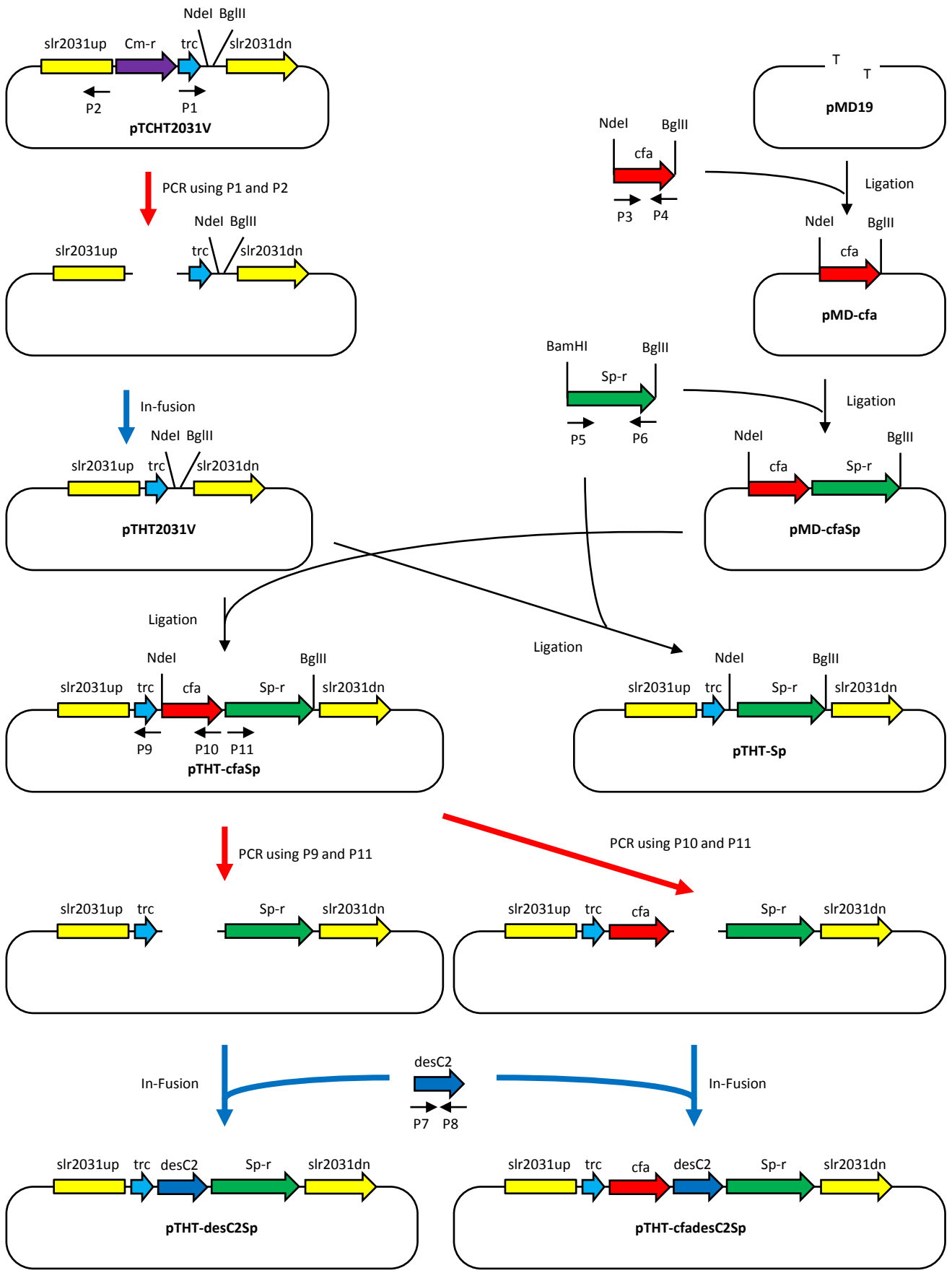
The fatty acid composition of total lipids from the vector control and BfaA and BfaB-expressing *E. coli* strains. The cells were grown at 37 °C for 18 h. As an exogenous fatty acid, 18:1Δ9 (1 mM) was added to the culture. The results were expressed as the mol % of total fatty acids and represent the mean ± standard deviation of three independent experiments. "-", not detected.

	Fatty acid (mol %)							
	Vector control		*bfaA*⁺		*bfaB*⁺		*bfaAB*⁺	
	Exogenous FA 18:1Δ9		Exogenous FA 18:1Δ9		Exogenous FA 18:1Δ9		Exogenous FA 18:1Δ9	
	-	+	-	+	-	+	-	+
14:0	11.7 ± 1.7	5.3 ± 0.2	6.8 ± 0.0	5.2 ± 0.8	6.9 ± 0.3	4.7 ± 0.5	7.9 ± 0.8	5.6 ± 0.2
16:0	42.3 ± 1.6	39.3 ± 1.5	44.2 ± 0.7	38.1 ± 0.0	43.5 ± 0.2	37.0 ± 0.8	44.6 ± 0.4	38.5 ± 2.0
16:1Δ7	-	4.6 ± 0.2	-	4.1 ± 0.1	-	4.2 ± 0.2	-	4.4 ± 0.1
16:1Δ9	3.2 ± 0.5	1.6 ± 0.1	3.6 ± 1.1	1.3 ± 0.1	4.4 ± 0.4	1.5 ± 0.4	3.8 ± 0.4	1.2 ± 0.2
17:1cycloΔ9	22.3 ± 1.1	10.1 ± 0.5	22.5 ± 1.4	11.1 ± 0.3	21.0 ± 0.4	9.3 ± 0.6	21.3 ± 1.0	10.6 ± 0.9
18:0	1.6 ± 0.4	1.2 ± 0.2	1.2 ± 0.1	1.6 ± 0.0	0.96 ± 0.1	2.5 ± 0.9	1.1 ± 0.1	1.4 ± 0.1
18:1Δ9	-	19.8 ± 2.6	-	18.7 ± 1.3	-	23.8 ± 2.1	-	18.0 ± 2.7
18:1Δ11	11.7 ± 1.3	7.1 ± 0.5	14.4 ± 2.4	8.2 ± 0.4	17.6 ± 0.3	10.1 ± 0.5	15.4 ± 1.0	9.2 ± 0.4
19:1cycloΔ9	-	4.8 ± 0.0	-	4.4 ± 0.0	-	3.0 ± 0.5	-	3.6 ± 0.2
19:1cycloΔ11	7.3 ± 1.3	5.6 ± 0.2	7.4 ± 1.4	6.6 ± 0.6	5.7 ± 0.5	4.5 ± 0.5	6.0 ± 0.6	6.1 ± 0.8
19:0Me10	-	-	-	-	-	-	-	0.7 ± 0.0
19:1Me10	-	-	-	-	-	0.5 ± 0.1	-	-

Table 10. Fatty acid composition of *E. coli* cells expressing the *bfaA* and *bfaB* genes with various fatty acids

As exogenous fatty acids, 16:1Δ9, 18:1Δ11, 18:2Δ9,12, 18:3Δ9,12,15, and 18:3Δ6,9,12 (1 mM) were added to the culture. The results were expressed as the mol % of total fatty acids and represent the mean ± standard deviation of three independent experiments. "-", not detected.

	Fatty acid (mol %)									
	Vector control					$bfaAB^+$				
	Exogenous fatty acid					Exogenous fatty acid				
	16:1Δ9	18:1Δ11	18:2Δ9,12	18:3Δ9,12,15	18:3Δ6,9,12	16:1Δ9	18:1Δ11	18:2Δ9,12	18:3Δ9,12,15	18:3Δ6,9,12
14:0	5.5 ± 0.4	5.4 ± 0.3	8.4 ± 2.1	8.3 ± 2.1	5.2 ± 1.7	4.5 ± 0.7	5.6 ± 0.3	8.1 ± 1.9	7.8 ± 1.4	5.1 ± 1.4
16:0	42.0 ± 1.6	36.1 ± 1.0	36.6 ± 0.9	38.5 ± 1.9	39.3 ± 0.2	40.3 ± 1.4	34.7 ± 1.3	37.3 ± 2.3	38.6 ± 1.1	40.1 ± 0.4
16:1Δ9	7.6 ± 1.8	4.5 ± 0.3	5.2 ± 1.3	3.2 ± 1.1	6.2 ± 1.0	8.1 ± 0.9	4.6 ± 0.3	5.6 ± 1.2	3.0 ± 1.0	6.3 ± 1.1
16:2Δ7,10	-	-	4.4 ± 1.6	-	-	-	-	4.1 ± 1.4	-	-
16:3Δ7,10,13	-	-	-	4.9 ± 0.3	-	-	-	-	4.8 ± 0.2	-
16:3Δ4,7,10	-	-	-	-	2.8 ± 0.7	-	-	-	-	2.7 ± 0.8
17:1cycloΔ9	24.9 ± 1.9	15.3 ± 0.6	14.4 ± 2.3	9.2 ± 3.8	17.2 ± 2.6	23.8 ± 0.9	14.8 ± 0.4	14.3 ± 1.8	9.3 ± 3.8	16.4 ± 1.9
18:0	1.0 ± 0.2	0.9 ± 0.1	3.4 ± 0.6	1.9 ± 0.5	1.9 ± 0.7	1.0 ± 0.1	0.9 ± 0.2	3.5 ± 0.7	1.8 ± 0.4	1.7 ± 0.7
18:1Δ11	15.4 ± 1.4	29.3 ± 1.6	11.4 ± 2.3	11.9 ± 1.1	14.1 ± 0.5	18.9 ± 1.8	32.0 ± 0.9	11.6 ± 2.8	12.3 ± 1.3	13.8 ± 0.6
18:2Δ9,12	-	-	11.5 ± 1.0	-	-	-	-	11.3 ± 0.8	-	-
18:3Δ9,12,15	-	-	-	13.9 ± 0.7	-	-	-	-	14.9 ± 0.8	-
18:3Δ6,9,12	-	-	-	-	11.8 ± 1.2	-	-	-	-	11.1 ± 1.0
19:1cycloΔ11	3.6 ± 0.8	8.5 ± 0.8	4.1 ± 2.1	6.1 ± 1.8	2.3 ± 1.0	3.4 ± 0.6	7.4 ± 0.6	3.8 ± 1.9	5.7 ± 2.3	2.2 ± 0.9
19:2Δ12cycloΔ9	-	-	0.9 ± 0.3	-	-	-	-	0.8 ± 0.3	-	-
19:3Δ12,15cycloΔ9	-	-	-	2.2 ± 1.1	-	-	-	-	2.0 ± 0.9	-
mBFAs	-	-	-	-	-	-	-	-	-	-

Figure 1. Construction of the plasmids used in Chapter 1

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.
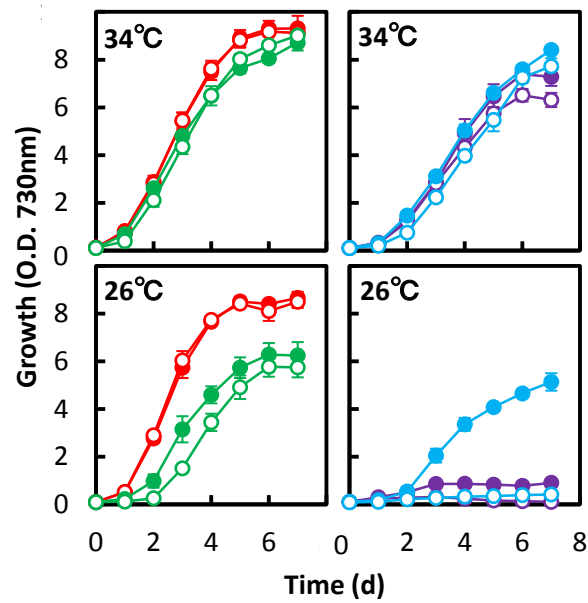
Figure 2. Growth of *Synechocystis* cells expressing the *cfa* and *desC2* genes at 34°C and 26°C

Mean ± 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, *desAD*- cells; open purple circle, *desAD*-/*cfa*+ cells; closed light blue circle, *desAD*-/*desC2*+ cells; and open light blue circle, *desAD*-/*cfa*+/*desC2*+ cells.
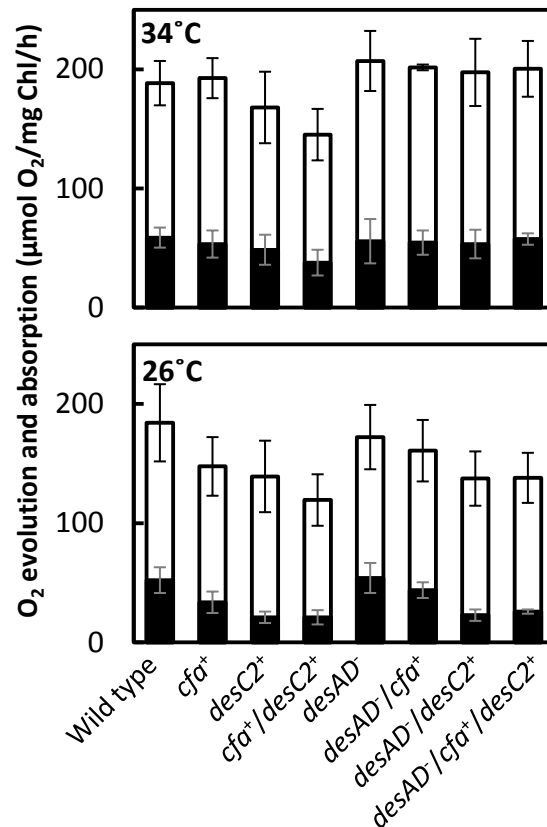
Figure 3. O$_2$ evolution and absorption of *Synechocystis* cells expressing the *cfa* and *desC2* genes at 34°C and 26°C

Cells were cultured at 34°C and 26°C for 2 days, and the activities were measured at the same temperature using an O$_2$ electrode. Mean ± S.D. values of three independent experiments. Black and white bars show respiratory and photosynthetic activities, respectively.
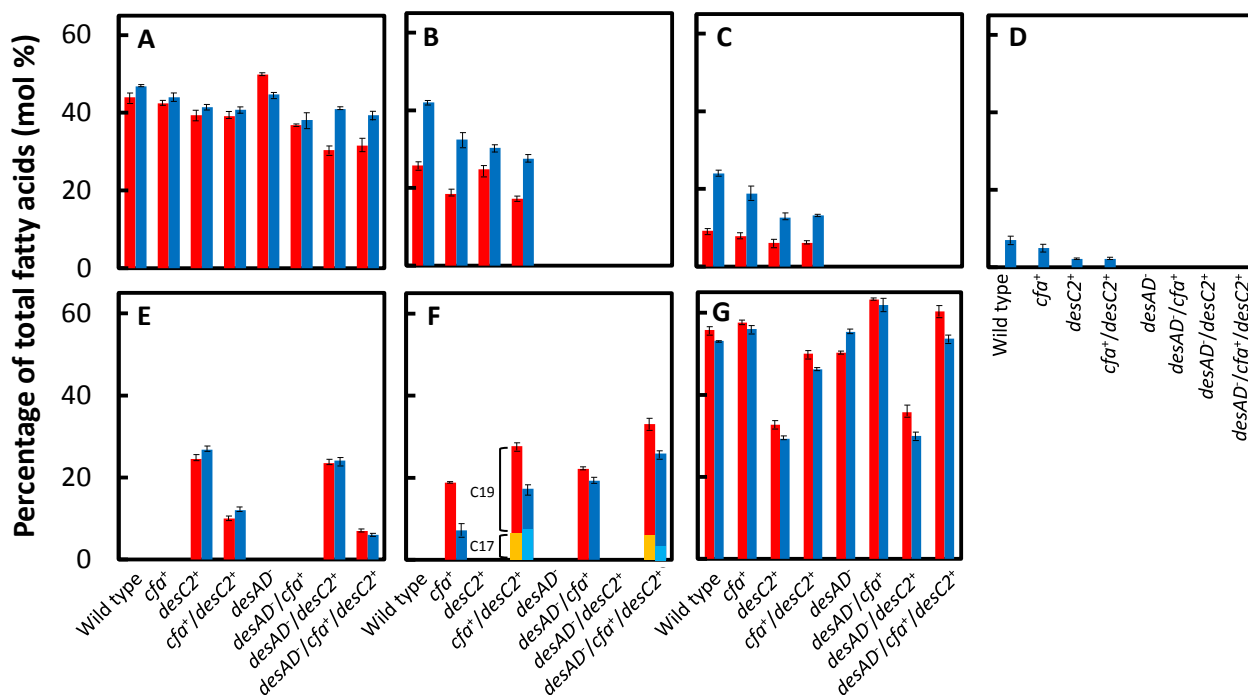
Figure 4. Relative amounts of fatty acids produced by each desaturase and cyclopropane fatty acid synthetase

Mean ± 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 Δ9 unsaturated C18 fatty acids by DesC, the sum of 18:1Δ9, 18:2Δ9,12, 18:3Δ9,12,15, 18:3Δ6,9,12, 18:4Δ6,9,12,15, and 19:1cycloΔ9; B, total production of Δ12 unsaturated C18 fatty acids by DesA, the sum of 18:2Δ9,12, 18:3Δ9,12,15, 18:3Δ6,9,12, and 18:4Δ6,9,12,15; C, total production of Δ6 unsaturated C18 fatty acids by DesD, the sum of 18:3Δ6,9,12 and 18:4Δ6,9,12,15; D, total production of Δ15 unsaturated C18 fatty acids by DesB, the sum of 18:3Δ9,12,15 and 18:4Δ6,9,12,15; E, total production of Δ9 unsaturated C16 fatty acids by DesC2, the sum of 16:1Δ9 and 17:1cycloΔ9; F, total production of cyclopropane fatty acids by Cfa, the sum of 17:1cycloΔ9 and 19:1cycloΔ9; and G, total production of saturated fatty acids, the sum of 16:0 and 18:0.
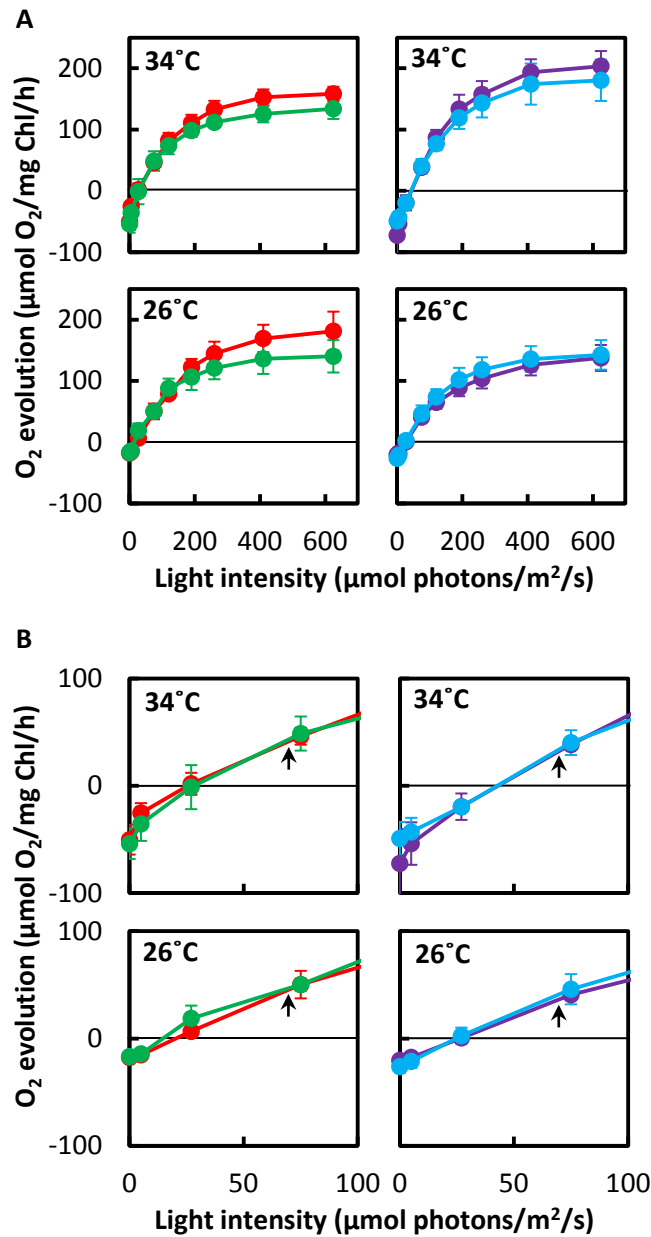
Figure 5. Photosynthesis activities of *Synechocystis* cells expressing the *cfa* and *desC2* genes

A: Cells were cultured at 34°C and 26°C for 2 d, and the activities were measured at the same temperature using an O_2 electrode. Mean ± S.D. values of three independent experiments. Red, wild-type cells; green, *desC2*⁺ cells; purple, *desAD*⁻ cells; and light blue, *desAD*⁻/*desC2*⁺ cells. B: Magnification of ranges from 0 to 100 µmole photons m⁻² s⁻¹ of the graphs in A. Black allows indicate light intensity at 70 µmole photons m⁻² s⁻¹ for cultivation.
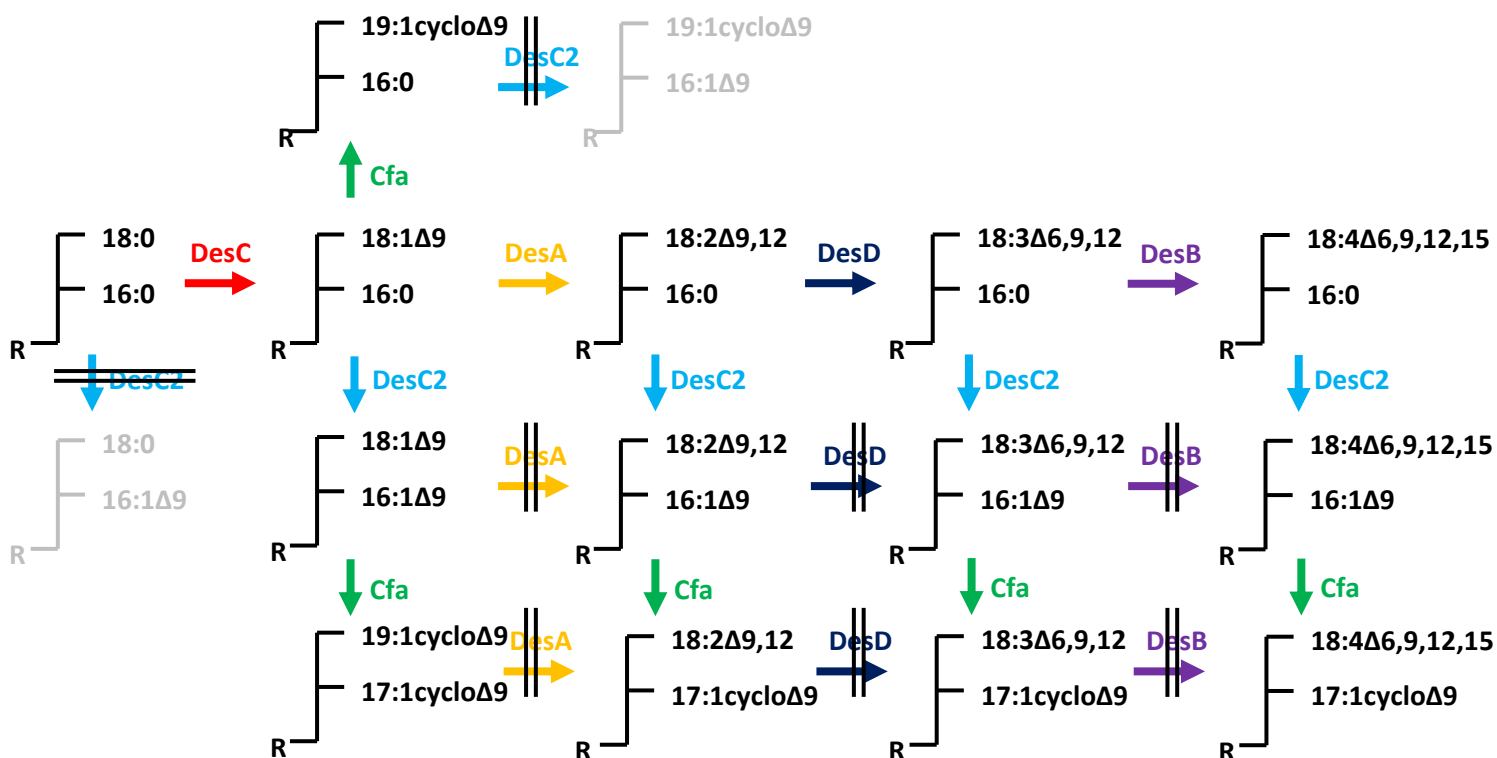
Figure 6. Putative pathways of the desaturases and cyclopropane fatty acid synthetase

Arrows indicate unsaturation by DesA, DesB, DesC, DesC2, and DesD and modification by Cfa. Doublets indicate that a reaction did not occur.
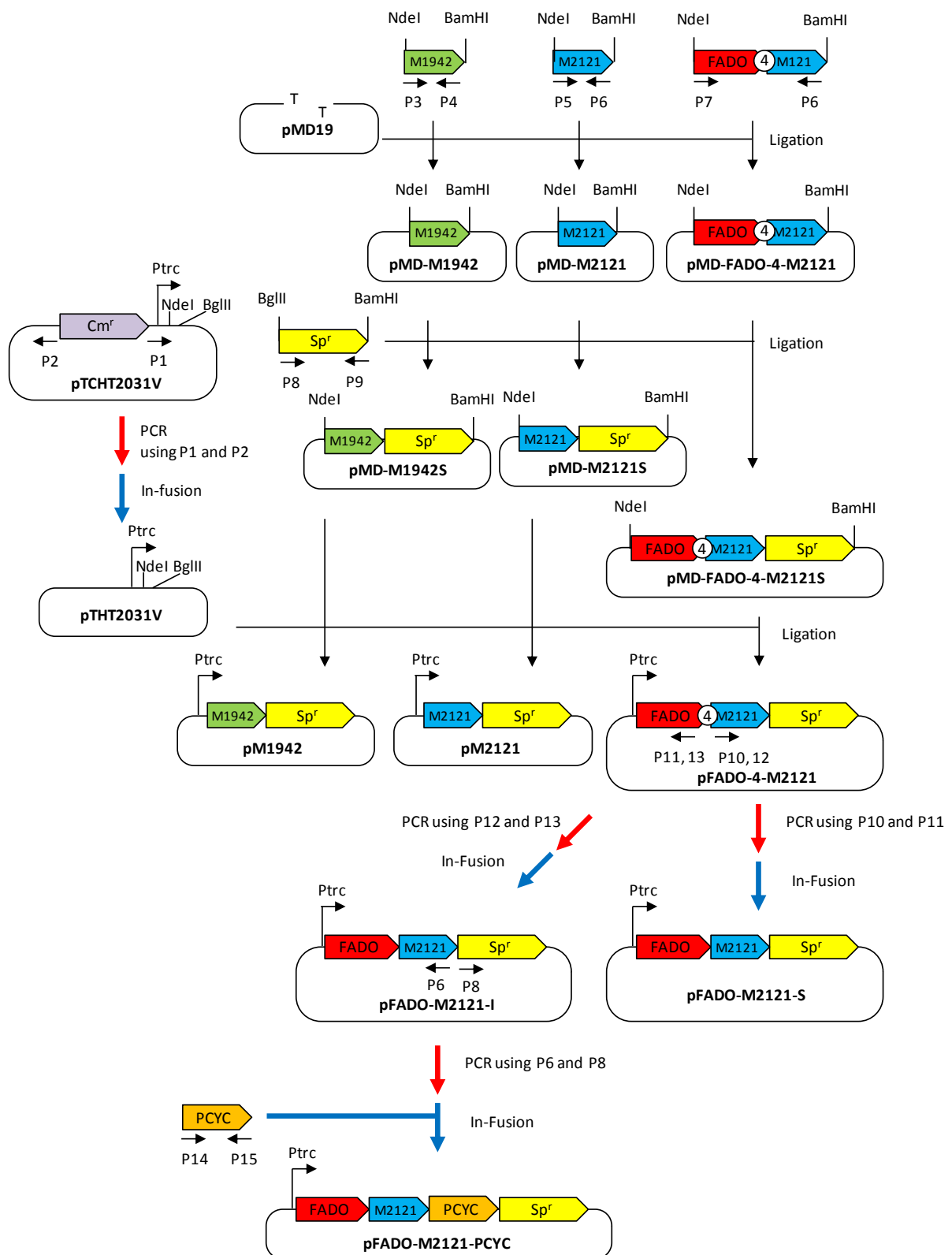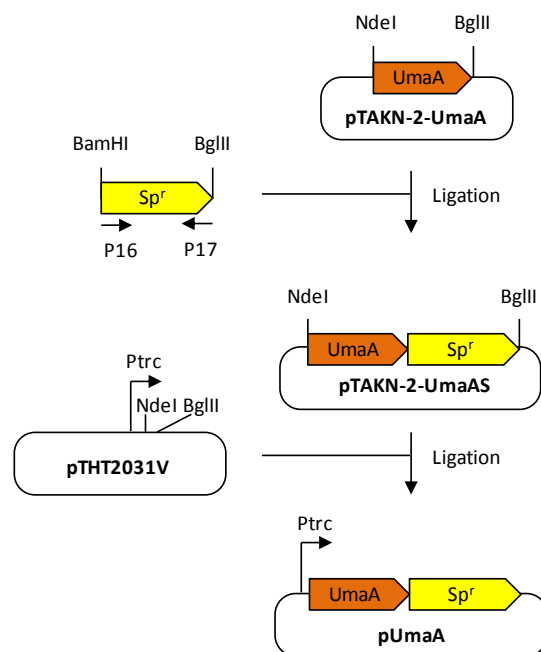
Figure 7. Construction of the plasmids used in Chapter 2

Scheme of the engineered DNA fragment for expression in *E. coli*. The fragments encoding WP_048471942, WP_048472121, flavin adenine dinucleotide (FAD)-binding oxidoreductase, and polyketide cyclase were amplified from *Mycobacterium chlorophenolicum* and the target genes were expressed under the control of the *trc* promoter. M1942, WP_048471942; M2121, WP_0484712121 (BfaB); FADO, FAD-binding oxidoreductase; PCYC, polyketide cyclase. The number "4" in the circle indicates the overlap of 4 base pairs between the two tandem genes.

Accsession No.	Organism	Identity with Cfa	Group No. in Table 1
WP_000098879	*Salmonella enterica* SL1344	90%	I
AAA23562 (Cfa)	*Escherichia coli* K-12	100%	I
WP_000623797	*Helicobacter pylori* ATCC 43504	37%	I
WP_009882805	*Campylobacter jejuni* subsp. Jejuni 81-176	38%	I
WP_003675910	*Lactobacillus reuteri* I5007	36%	I
WP_010964195	*Clostridium acetobutylicum* ATCC 824	45%	I
WP_068165842	*Rhodococcus phenolicus* DSM44812	40%	II
WP_029255513	*Rhodococcus erythropolis* R138	36%	III
WP_048471942	*Mycobacterium chlorophenolicum* JCM 7439	36%	III
WP_048472121	*Mycobacterium chlorophenolicum* JCM 7439	33%	III
WP_003935379	*Rhodococcus ruber* BKS20-38	35%	II
WP_077118797	*Nocardia donostiensis* X1654	35%	III
WP_012359408	*Corynebacterium urealyticm* ATCC 43042	32%	III
WP_037303124	*Amycolatopsis orientalis* DSM 40040	33%	II
WP_007731301	*Rhodococcus qingshengii* BKS 20-40	33%	III
WP_048471690	*Mycobacterium chlorophenolicum* JCM 7439	33%	III
WP_003900985	*Mycobacterium tuberculosis* H37Rv	35%	III
WP_048471080	*Mycobacterium chlorophenolicum* JCM 7439	34%	III
WP_048473845	*Mycobacterium chlorophenolicum* JCM 7439	35%	III
WP_048471691	*Mycobacterium chlorophenolicum* JCM 7439	29%	III
WP_048473846	*Mycobacterium chlorophenolicum* JCM 7439	34%	III

0.05

Figure 8. Phylogenetic tree of the *S*-adenosyl methionine-dependent methyltransferases

Amino acid sequences of 21 SAM-dependent methyltransferases from 15 organisms were analyzed and classified into three clades: A, B, and C.
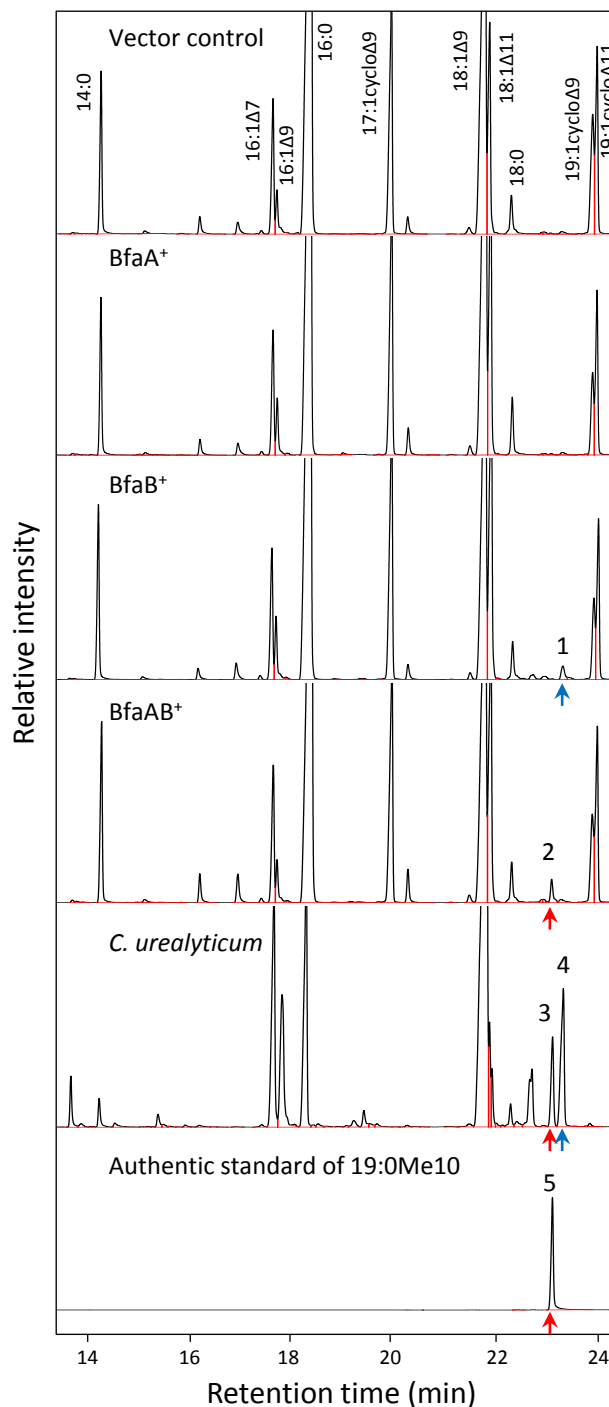
Figure 9. Analysis of fatty acid methylesters extracted from *E. coli* cells expressing the *bfaA* and *bfaB* genes by gas chromatography with a flame-ionization detector

Fatty acids were extracted from *E. coli* and *C. urealyticum* cells, methylesterified, and analyzed by GC-FID. 18:1Δ9 (1 mM) was exogenously added to the culture of *E. coli*. BfaA and BfaB indicate the FAD-binding oxidoreductase and WP_048472121, respectively. Bottom panel indicate the result of the authentic standard of 19:0Me10. Peaks of the identified fatty acid methylesters were indicated as corresponding fatty acids. The retention times of methylesters of 19:0Me10 and 19:1Me10 were indicated by red and blue arrows, respectively.
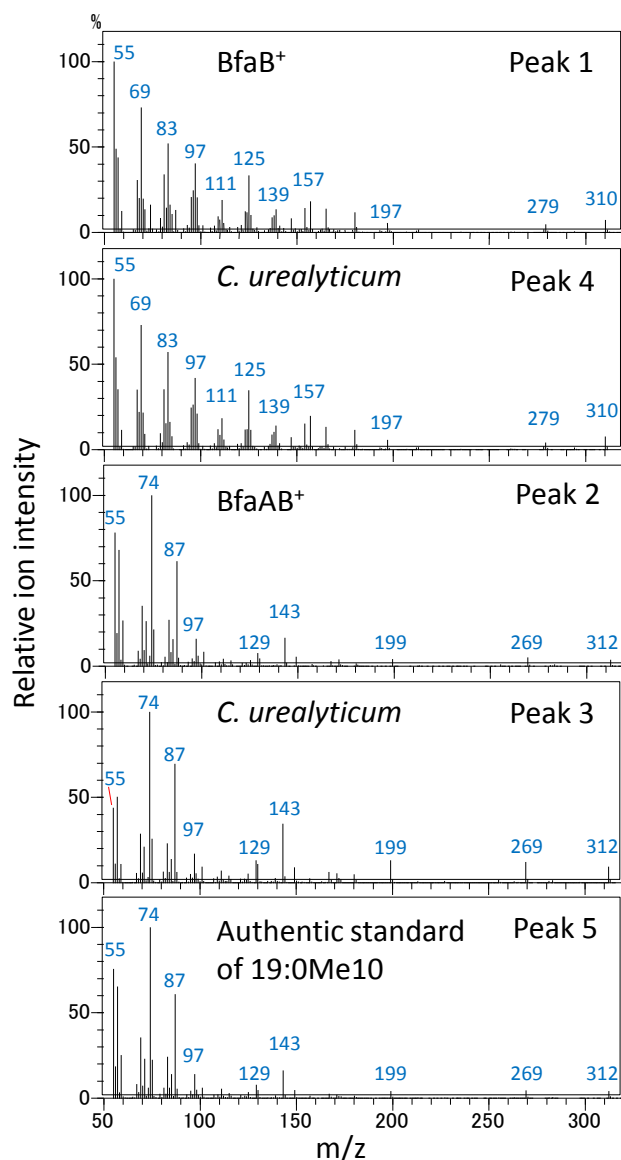
Figure 10. Analysis of fatty acid methylesters extracted from *E. coli* cells expressing the *bfaA* and *bfaB* genes and *C. urealyticum* by gas chromatography with a mass spectrometer

Mass spectra of fatty acid methylester were analyzed by gas chromatography with a mass spectrometer. Peak 1, fatty acid extracted from *bfaB*[+] strain; peak 2, fatty acid extracted from *bfaAB*[+] strain; Peak 3 and 4, fatty acids extracted from *C. urealyticum* cells; and peak 5, authentic standard of 10-methyl stearic acid. The number of peak is linked with the peaks in Fig. 4. BfaA and BfaB indicate FAD-binding oxidoreductase and WP_048472121, respectively.
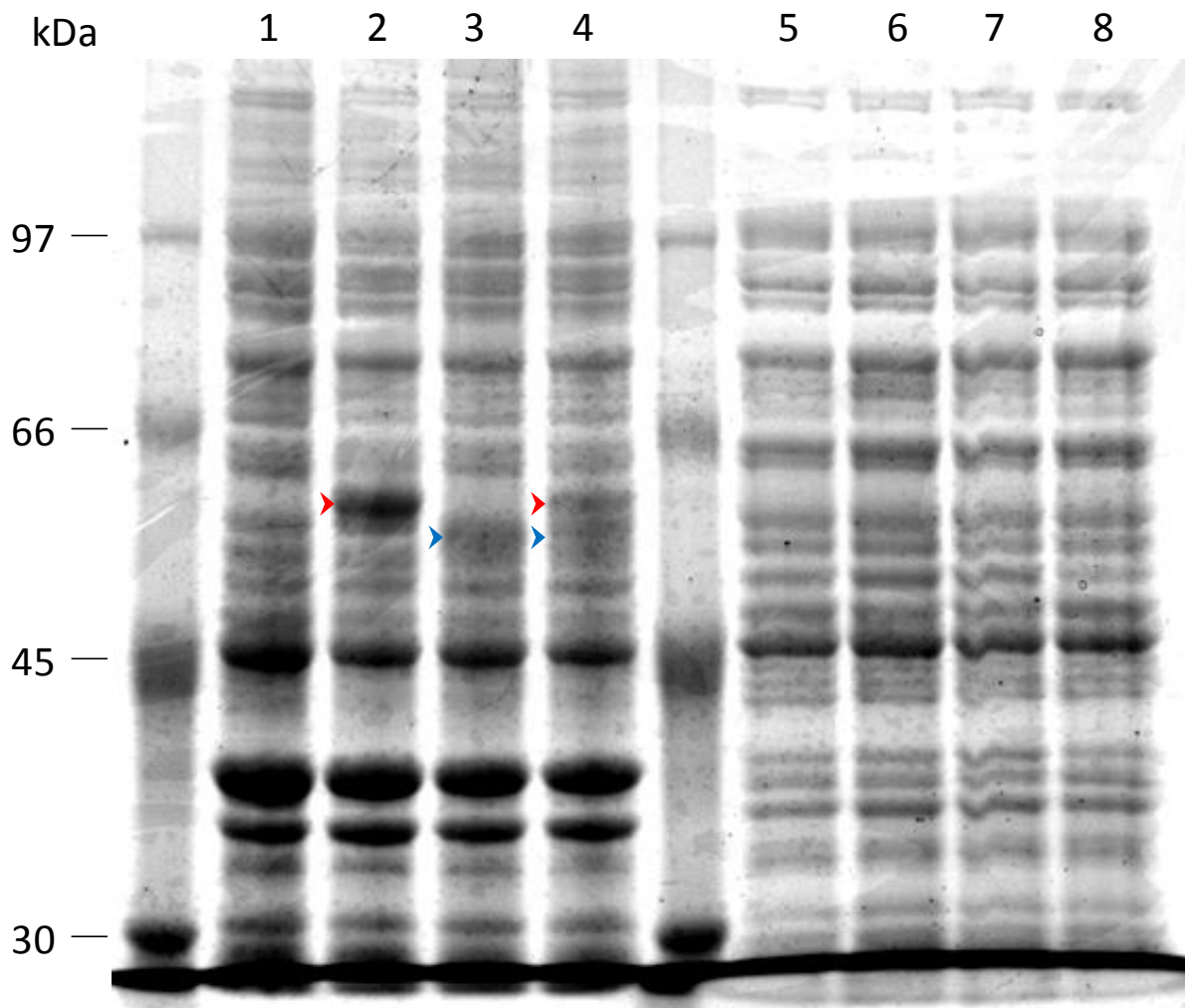
Figure 11. SDS-PAGE analysis of *E. coli* cells expressing the *bfaA* and *bfaB* genes

Proteins extracted from the *E. coli* cells were used for SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Red and blue arrows indicate estimated band for BfaA (52kDa) and BfaB (49kDa), respectively. Lane 1, insoluble fraction of vector control strain; Lane 2, insoluble fraction of *bfaA⁺* strain; Lane 3, insoluble fraction of *bfaB⁺* strain; Lane 4, insoluble fraction of *bfaAB⁺* strain; Lane 5, soluble fraction of vector control strain; Lane 6, soluble fraction of *bfaA⁺* strain; Lane 7, soluble fraction of *bfaB⁺* strain; Lane 8, soluble fraction of *bfaAB⁺* strain.

Mycobacterium chlorophenolicum JCM 7439 (III) WP_048472120 WP_048472121

RecR YE ALA-A PCYC FADO 4 SAMM MP

Rhodococcus ruber BKS 20-38 (II) WP_003935378: 70% WP_003935379: 74%

RecR YE ALA-A PCYC FADO 4 SAMM FOCS

Rhodococcus erythropolis R138 (III) WP_019746543: 70% WP_019746542: 73%

PCYC HYPO FADO 4 SAMM HYPO

Rhodococcus qingshengii BKS 20-40 (III) WP_007731548: 70% WP_003943932: 72%

PCYC HYPO FADO 4 SAMM HYPO

Nocardia donostiensis X 1654 (III) WP_077118793: 71% WP_077118797: 69%

PCYC FADO 4 SAMM P-KIN

Mycobacterium tuberculosis H37Rv (III) WP_003917236: 76% WP_003420415: 80%

RecR YE ALA-A PCYC FADO SAMM DPIII

Amycolatopsis orientalis DSM 40040 (II) WP_037303122 : 57% WP_037303124: 55%

SGNH-H FADO SAMM ALA-S CYC

Corynebacterium urealyticum ATCC 43042 (III) WP_012359407: 63% WP_012359408: 52%

HAL-D FADO SAMM U-GPM

Rhodococcus phenolicus DSM 44812 (II) WP_068165825: 26% WP_068165842: 44%

FADO CHO-T SAMM SAMM HYPO

WP_068165945: 39%

500 bp

Figure 12. Arrangements of the orthologous genes of the FAD-binding oxidoreductase WP_048472120 and SAM-dependent methyltransferase WP_048472121 among microorganisms that produce mid—chain methyl-branched fatty acids

The orthologous genes of the FAD-binding oxidoreductase WP_048472120 and the SAM-dependent methyltransferase WP_048472121 were identified from the genomic sequences of *Mycobacterium chlorophenolicum* JCM 7439 (NZ_BCQY01000005), *Rhodococcus ruber* BKS 20-38 (NZ_AOEX01000025), *Rhodococcus erythropolis* R138 (NZ_CP007255), *Rhodococcus qingshengii* BKS 20-40 (NZ_AODN01000039), *Nocardia donostiensis* X 1654 (NZ_MUKP01000048), *Mycobacterium tuberculosos* H37Rv (NC_018143), *Amycolatopsis orientalis* DSM 40040 (NZ_ASJB01000001), *Corynebacterium urealyticum* ATCC 43042 (NC_010545), and *Rhodococcus phenolicus* DSM 44812 (NZ_LRRH01000116). The genes located near the orthologs of WP_048472121 were drawn schematically. The directions of the arrows indicate the orientation of transcription of the genes. The number "4" in the circle indicates the overlap of 4 base pairs between the two tandem genes. The group number in Table 1 is shown next to the name of strain. The accession number of each FAD-binding oxidoreductase and SAM-dependent methyltransferase is shown above the arrow indicating each gene. The percentage values indicate the identities of the protein sequences with WP_08472120 or WP_048472121. RecR, recombination protein; YE, YbaB/Ebf family (Jutras et al., 2012); ALA-A, *N*-acetylmuramoyl-L-alanine amidase; PCYC, polyketide cyclase; FADO, FAD-binding oxidoreductase; SAMM, SAM-dependent methyltransferase; MP, membrane protein; FOCS, fatty oxidation complex subunit a; P-KIN, protein kinase; SGNH-H, SGNH hydrolase; ALA-S, acetolactate synthase; CYC, cyclase; HAL-D, haloacid dehalogenase; U-GPM, UDP-galactopyranose mutase; CHO-T, choline transporter; HYPO, hypothetical protein; DPIII, DNA polymerase III.

```
A
"FADO"->
 Met-•••••-AlaValArgArgGln***
 ATG-•••-GGCGGTACGGCGACAATGACGACTTTTCGG-•••-TGA
        -15          -5    MetThrThrPheArg-•••-***
                              "WP_048472121"->
B
"FADO"->
 Met-•••••-AlaValArgArgGln***
 ATG-•••-GGCGGTAAGGAGACAATGACGACTTTTCGG-•••-TGA
        -15          -5    MetThrThrPheArg-•••-***
                              "WP_048472121"->
C
"FADO"->
 Met-•••••-AlaValArgArgGln***
 ATG-•••-GGCGGTACGGCGACAATGAAGGAGGAATAAACCATGACGACTTTTCGG-•••-TGA
                   -15          -5    MetThrThrPheArg-•••-***
                          (MetLysGluGlu***) "WP_048472121"->
```

Figure 13. Modification of the Shine–Dalgarno (SD) sequence upstream of the coding sequence of the methyltransferase WP_048472121

A. The native genomic sequences encoding the FAD-binding oxidoreductase (FADO) WP_08472120 and the SAM-dependent methyltransferase WP_048472121 was shown. B. The genomic sequences substituted the two nucleotides in upstream of WP_048472121 without alteration of the encoded polypeptide sequence was shown. The substituted nucleotides are shown by a bold, red-colored font. C. The genomic sequences inserted 14 nucleotides including the canonical SD sequence from *E. coli* between FADO and WP_048472121. The inserted nucleotides are shown by a bold, blue-colored font. The translated amino acid sequences are shown. *** indicates the stop codons. −15 and −5 indicate positions from the translational start codon of WP_048472121. Putative SD sequences were underlined.

A. FAD-binding oxidoreductases

```
R_eryth     1  ------------------------------------------------MGFEQHREGVERLLASYRAIPADASVRLAKK
R_qingsh    1  ------------------------------------------------MGFEQHREGVERLLASYRAIPADASVRLAKK
R_ruber     1  -----------------------------MQKPKSVVVPSGESAHRAGVERLLRSYRAIPADAPVRLAKK
N_donost    1  ---------------------MSLLGKAGHDPAAYESGFAAYRAGVSRLLESYRAIPAGADVRLAKK
M_chloro    1  ---------------------------MSVPATDARAAHADGVERLLASYRAIPPGATVRLAKP
M_tuber     1  ----------------------MQGQLSRTRVYTVPVPGSAQSAYACGVERLLASYRSIPATASIRLAKP
C_urealy    1  MVGDMTVLGALKNIAVETVGKTTDALRIGNGLDARELQPVGWFAHGQAVEKLRASYEAIPPGENVRLAKK
A_orient    1  ------------------------MASGDGGPRATARLVRFAEHEARADALRAQLAGLHG--TIRLAKR

R_eryth    32  TSNLFRARAKTSAPGLDVSGLAGVIAVDAQAHTADVAGMCTYEDLVDATLPYGLAPLVVPQLKTITLGGA
R_qingsh   32  TSNLFRARAKTSAPGLDVSGLAGVIAVDAQARTADVAGMCTYEDLVDATLPYGLAPLVVPQLKTITLGGA
R_ruber    42  TSNLFRARARVNAPGLDVSGLGGVVAVDPVARTADVAGMCTYEDLVDATLPHGLAPLVVPQLKTITLGGA
N_donost   47  TSNLFRARARNTAPGLDVSGLTKVIAVDPDARTADVAGMTTYEDLVATTLPYGLAPLVVPQLKTITLGGA
M_chloro   38  TSNLFRARAKSTAPGLDVSGLTDVISVDPVARTADVAGMCTYEDLVAATLPHGLSPLVVPQLKTITLGGA
M_tuber    49  TSNLFRARVKHDARGLDASGLTGVIGIDPEARTADVAGMCTYEDLIAATLHYGLSPLVVPQLRTITLGGA
C_urealy   71  TSNLFRGRNQSTTPGLDVSGLGGVIAVDPVAGTADVQGMCTYEDLVDTVLPYGYSPTVVPQLKTITLGGA
A_orient   44  TSNLFRARAATSTPGLDVSGFTHVLDVDPLARTADVEGMVTYEQLVDATLPHGLMPVVPQLKTITLGGA

R_eryth   102  VTGLGIESTSFRNGLPHESVLEIDVLTGSGEIITATPDGEHAELFFGFPNSYGTLGYSTRLKIALEPVKK
R_qingsh  102  VTGLGIESTSFRNGLPHESVLEIDVLTGSGEIITATPDGEHAELFFGFPNSYGTLGYSTRLKIALEPVKK
R_ruber   112  VTGLGIESTSFRNGLPHESVLEMDILTGAGEIVTATPDGEHADLFPGFPNSYGTLGYATRLRIELEAVKP
N_donost  117  VTGLGIESTSFRNGLPHESVLELDVLTGAGEIVTATPDGEHADLFRGFPNSYGTLGYSTRLKIELEPVEP
M_chloro  108  VTGLGIESASFRNGLPHESVLEMDILTGTGDVVRASP-TENPDLFHAFPNSYGTLGYSVRLKIELEPVKP
M_tuber   119  VTGLGIESASFRNGLPHESVLEMDILTGAGELLTVSP-GQHSDLYRAFPNSYGTLGYSTRLRIQLEPVRP
C_urealy  141  VTGLGVESACFRNGLPHEALIEMDVLTGTGEVVTCSP-TQNVDLFRGFPNTYGSLGYAVRLKIELEKVKD
A_orient  114  VTGLGIESSSFRNGLVHESVLEMELLTGDGRIVVARADNEHSDLFHGFPNSYGTLGYALRLKIELEPVKP

R_eryth   172  YVALRHVRFDSLKKLEETMDRIVTEREYDGIAVDYLDGVVFTDSESYLTLGVQTDEEGPVSDYTDQDIFY
R_qingsh  172  YVALRHVRFDSLKKLEETMDRIVTEREYDGIAVDYLDGVVFTDSESYLTLGVQTDEEGPVSDYTDQDIFY
R_ruber   182  YVALRHLRFDTLDEMQSALDRIATERVHAGVPVDYLDGVMFSATESYLTLGTQTDAPGPVSDYTGEHIYY
N_donost  187  YVALRHLRFHDLGELEAALTRIVTERDHDGERVDYLDGVVFSAGESYLTLGTQTDEPGPVSDYTGMDIYY
M_chloro  177  FVALRHVRFHAIDALIEAMDRIIETGGWNGERVDYLDGVVFSADESYLCLGVQSATPGPVSDYTGQQIYY
M_tuber   188  FVALRHIRFSSLTAMVAAMERIIDTGGLDGESVDYLDGVVFSADESYLCIGMQTSVPGPVSDYTGQDIYY
C_urealy  210  YVELRHVRFHDVHALTETMEQIIETGEYDGEAVDYLDGAVFSLEECYLMLGRQTDEPGPTSDYTRDRIYY
A_orient  184  YVRLDHVRYDDTEEYFAALGEACRTG-----AADFVDGTVFGPGEQYLTLGTFTTSAPATSDYTWLDIYY

R_eryth   242  RSIQHPSLTQPKTDRLTIRDYLWRWDTDWFWCSRAFGAQNPKIRRFWPKQYLRSSFYWKLIALDHKYDIG
R_qingsh  242  RSIQHSSLTQPKTDRLTIRDYLWRWDTDWFWCSRAFGAQNPKIRRFWPKQYLRSSFYWKLIALDHKYDIG
R_ruber   252  RSIQHASVNHPRTDRLTVRDYLWRWDTDWFWCSRAFGAQNPRIRRLWPKRLLRSSFYWKLIALDHRYGVA
N_donost  257  RSLQHEGG-TPKHDRLTIHDYLWRWDTDWFWCSRAFGTQNPKIRRFWPKRYRRSSFYWKLIALDHRYHIG
M_chloro  247  RSIQHPSG--EKHDRLTIHDYLWRWDTDWFWCSRAFGAQDPRIRRFWPRRLRRSSFYWKLIGYDQRFDIA
M_tuber   258  RSIQHEAG--IKEDRLTIHDYFWRWDTDWFWCSRSFGAQNPRLRRWWPRRYRRSSVYWRLMALDQRFGIA
C_urealy  280  RSIQHPEG--VLRDRLSIRDYLWRWDVDWFWASRAFGTQNPTIRRMWPRDLLRSSFYWKIIGWDRKYDLA
A_orient  249  KSIRERET-----DHLSVRDYLWRWDTDWFWCSRAFGVQHRLPRLLGRRLLRSSVYWKAVALDRRFGIA

R_eryth   312  DRLEKRKGKPPRERVVQDVEVPIERTADFVSWFLEEIPIEPLWLCPLRLR-----------EPSPAAASA
R_qingsh  312  DRLEKRKGKPPRERVVQDVEVPIERTADFVSWFLEEIPIEPLWLCPLRLR-----------EPSPAAASA
R_ruber   322  DRIERRKGNLPRERVVQDIEVPIERTAEFLRWFLDEIPIEPVWLCPLRLR-----------EGGPAARDA
N_donost  326  DKLEARKGNPPRERVVQDIEVPVERTADFMEWFLREIPIEPIWLCPLRLR-----------RTG--SGGS
M_chloro  315  DRIEKRNGRPPRERVVQDVEVPIERTAEFVEWFLATVPIEPIWLCPLRLR---------------DAPNS
M_tuber   326  DRFENSRGRPARERVVQDIEVPIERTCEFLEWFGENVPISPIWLCPLRLR---------------DHAG-
C_urealy  348  DRIEAANNRPARERVVQDIEVTPEHLPEFLEWFFHSCEIEPVWLCPIRIRGTGADGNELVGEGETLGADK
A_orient  314  AKILKLRGLPPEETIVQDIEVPLSRAAEFLDFFRREIPISPVWICPLKQR----------------PG

R_eryth   371  SRPWPLYPLEPKRTYVNIGFWSSVPIVPGER---EGAANRLIEEKVSDFDGHKSLYSDSYYSKEDFEELY
R_qingsh  371  SRPWPLYPLEPKRTYVNIGFWSSVPIVPGER---EGAANRLIEEKVSDFDGHKSLYSDSYYSKEDFEELY
R_ruber   381  QRPWPLYPLEPKRTYVNVGFWSSVPTVAGEI---EGAANRRIEHKVAELDGHKSLYSDSYYTRAEFAQLY
N_donost  383  ARPWPLYPLHPDRTYVNAGFWSSVPITSGEI---EGAANRAIEKAVTDFGGHKSLYSDSYYRQDEFADLY
M_chloro  370  TGGWPLYPIRPHHSYVNVGFWSSVPVGPE-----EGYTNRMIERKVSDLDGHKSLYSDAFYSPEEFDELY
M_tuber   380  ---WPLYPIRPDRSYVNIGFWSSVPVGAT-----EGATNRKIENKVSALDGHKSLYSDSFYTREEFDELY
C_urealy  418  EHPWPLYPLTVGQTWVNVGFWSSVPVDLLGKDAPGAFNKLVEEKVSALGGHKSLYSEAFYDRETFEQLY
A_orient  366  GVNSPLYELDPETLYVNFGFWSAVPLDPGEE---PDTHNRLIEAEVTRLGGRKSLYSDSFYTEDEFWRLY

R_eryth   438  YGGDRYIGLKERYDPKSRLLDLFSKAVQRK
R_qingsh  438  YCGDRYIGLKERYDPKSRLLDLFSKAVQRK
R_ruber   448  YGGGDYPTLKKRYDPESRLLDLFSKAVHNK
N_donost  450  GG-DDYIQLKKRYDPDQRLLDLYSKAVQRK
M_chloro  435  GG-EVYTTVKKIYDPDSRFLDLYAKAVRRQ
M_tuber   442  GG-ETYNTVKKAYDPDSRLLDLYAKAVQRR
C_urealy  488  GG-SFPAQLKEVYDPQGRFPGLYEKTVDWA
A_orient  433  NG-DAYRKLKTAYDPDGRLLDLYAKCVRRR
```

B. SAM-dependent methyltransferases

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M_chloro    1   MTTFRERPTDAANPADGRLTLAEILEIFASG-TRPLKFTAYDGSSAGPDDAALGLDLLTPRGTTYLATAP
M_tuber     1   -------------------MAEILEIFTATGQHPLKFTAYDGSTAGQDDATLGLDLRTPRGATYLATAP
R_eryth     1   -MTTFKDSK----SQD-KLSIAEILETLSDG-MLPLRFTAYDGSTAGPEDADYGLHLKSTRGTTYLATAP
R_qingsh    1   -MTTFKDSK----SQD-KLSIAEILETLSDG-MLPLRFTAYDGSTAGPEDADYGLHLKSTRGTTYLATAP
R_ruber     1   -MTTLREPR----TPDGRLTLAQILETVTGC-ELPVRFTAYDGSSAGPTDAPYGLRLTSTRGTTYLATAP
N_donost    1   -MTTFRERSDVFADLGTKLSIAEIFDLIVDG-EIPIRLTAYDGSATGPADSEFALDIRNARGINYLATAP
A_orient    1   ---------------MAETTIVGKIFERLLGP-STEVSITAYDGSTSGPADAPVSIQVRSPLALTYLMSSP
C_urealy    1   ------------------MTVAEIVEAITAP-PLPFHVTGFDGSETGPADARLRLDITSSDALAYIVTAP
```

```
M_chloro   70   GDLGLARAYISGNLEAHGVHPGDPYELLNALTE--KLDFKRPSARVLAQVIRSIG-IEHLKPISPPPQEA
M_tuber    51   GELGLARAYVSGDLQAHGVHPGDPYELLKTLTE--RVDFKRPSARVLANVVRSIG-VEHILPIAPPPQEA
R_eryth    64   GDLGMARAYVSGDLEAEGVHPCNPYEILRIMGD--ELHLKRPSALTLASITRSLG-WDLLRPIAPPPQEH
R_qingsh   64   GDLGMARAYVSGDLEAEGVHPGDPYEILRIMGD--ELHLKRPSALTLASITRSLG-WDLLRPIAPPPQEH
R_ruber    65   GDLGMARAYVSGDLEAEGVHPCNPYELLKALG---DLHFQRPPALTLAQIARSLG-LETLKPIAPPPQEH
N_donost   69   GDLGMARAYISGDMTATGVHPGDPYEILKAMQ---DLKFRRPSALALLTIARSLG-WERLRPVPPPPQET
A_orient   56   GDLGLARAYVAGALDVDGDLYTALRALVAQVDQ--LSTADR------LWLLRELG-PRHLRRVAPPAEEL
C_urealy   52   GDLGLARAYITGALQVSCENPCHPYGMFDALQTFYYESFRKPSAATGARIARSLKHLDAIRPMPIPPQED
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```
M_chloro  137   LPRWRRFAEG--LRHSKTRDAEAIHHHYDVSNTFYEWVLGPSMTYTCACYQHPE----------------
M_tuber   118   RPRWRRMANG--LLHSKTRDAEAIHHHYDVSNNFYEWVLGPSMTYTCAVFPNAE----------------
R_eryth   131   LPRWRRVAEG--LRHSKTRDAEVIHHHYDVSNTFYEYVLGPSMTYTCAAYDNAE----------------
R_qingsh  131   LPRWRRVAEG--LRHSKTRDAEVIHHHYDVSNTFYEYVLGPSMTYTCAAYDNAE----------------
R_ruber   131   LPRWRRFAEG--LRHSRSRDAEAIHHHYDVSNAFYEMVLGPSMTYTCALFDSEA----------------
N_donost  135   LPRWRRIAEG--LRHSKLRDAEAIHHHYDVSNAFYEMVLGPSMTYTCACYGDQD----------------
A_orient  117   PSRAKRVFDG--LRHSKARDSNAISNHYDVSNRFYELVLGPSMAYTCAAYPSAD----------------
C_urealy  122   QPGWKKALFEGLSRHSRERDKEVVQRHYDVGNDLYELFLGDSMTYTCAYYPADESDTPEGWDKSQWAKGT
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```
M_chloro  189   ---ATLEEAQDNKYRLVFEKLRLQPGDRLLDVGCGWGGMVRYAARHG-VTAIGVTLSKQQAEWAQKAIAE
M_tuber   170   ---ASLEQAQENKYRLIFEKLRLEPGDRLLDVGCGWGGMVRYAARRG-VRVIGATLSAEQAKWCQKAVED
R_eryth   183   ---QTLEEAQENKYRLVFEKLGLQKGDRLLDIGCGWGSMVRYAARRG-VKVIGVTLSREQADWAQKAIED
R_qingsh  183   ---QTLEEAQENKYRLVFEKLGLQKGDRLLDIGCGWGSMVRYAARRG-VKVIGVTLSREQADWAQKAIED
R_ruber   183   ---QSLEDAQENKYRLIFEKLRLQPGDRLLDIGCGWGGMVRYAARRG-VKALGVTLSREQAEWAQKTIAD
N_donost  187   ---WTLEQAQENKYRLIFEKLRLSPGDRLLDLGCGWGGMVRYAASRG-VQAIGATLSAEQAEWAQRKIAE
A_orient  169   ---ASLEEAQAHKFDLVCRKLDLKPCMRLLDVGCGWGGMVEHAVEHYG-VEALGVTLSREQAQWAQKDIVT
C_urealy  192   AVKEPLDRAQENKYRLVFDKLRLAEGDRLLDVGCGWGGMVRYAARRG-VKAIGVTLSEEQVAWGNEKIRE
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```
M_chloro  255   EGLGDLAEVRHSDYRDVRESQFDAVSSIGLTEHIGVANYPAYFRFLKSKLRTGGLLLNHCITRHDNRHGA
M_tuber   236   EGLSDLAQVRHSDYRDVAETGFDAVSSIGLTEHIGVKNYPFYFGFLKSKLRTGGLLLNHCITRHDNRSTS
R_eryth   249   EGLADLAQVRFSDYRDVAETGFDAVSSIGLTEHIGVGNYPAYFEFLKGKLREGGRLLNHCITRPDNRSSA
R_qingsh  249   EGLADLAQVRFSDYRDVAETGFDAVSSIGLTEHIGVGNYPAYFEFLKGKLREGGRLLNHCITRPDNRSSA
R_ruber   249   EGLTDLAEVRFSDYRDVPETGFDAISSIGLTEHIGVQNYPAYFRRLHDKLREGGLLLNHCITRPVNRGPV
N_donost  253   EGLSDLAEVRHCDYRDVPESEFDAVSSIGLTEHIGVHNYPYYFEFIKGKLREGGLFLNHCITRPDNTRGT
A_orient  236   KGLADRAEVRHLDYRDVTETGFDAVSSIGLTEHIGARNLPSYFRFLAGKLKPRGRLLNHCITNPDTTVPH
C_urealy  261   QGLEEFAEIRLMDYRDVPETGFDAISAIGVLEHVGRRQYEEFFQLMYGRLREGGRMLNHCITRPTNERIN
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```
M_chloro  325   AAGGFIDRYVFPDGELTGSGRIITEVQDVGLEVVHEENLRNHYAMTLRDWNRNLVEHWDEAVAEVGLATA
M_tuber   306   FAGGFTDRYVFPDGELTGSGRIITEIQQVGLEVLHEENFRHHYAMTLRDWCGNLVEHWDDAVAEVGLPTA
R_eryth   319   KAGGFIDRYVFPDGELTGSGRIISEIQNVGLEVRHEENLREHYALTLAGWCQNLVDNWDACVAEVGEGTA
R_qingsh  319   KAGGFIDRYVFPDGELTGSGRIISEIQNVGLEVRHEENLREHYALTLAGWCQNLVDNWDACVAEVGEGTA
R_ruber   319   KAGGFIDRYVFPDGELTGSGRIITEVQDVGLEVLHEENLRQHYALTLKGWCENLVANWDACVAEVGEGTA
N_donost  323   KAGDFIDRYVFPDGELIGSGRIISDIQNVGLEVLHEENLREHYALTLAEWCKNLVDNWDACVAEAGEGTA
A_orient  306   RSRGFIDRYVFPDGELESVCEIATAMHDSGLEVRHSENLREHYATTLGAWCANLDENWDAAVAEAGACRS
C_urealy  331   -AGKFIGRYIFPDGELTGSCTIVTRMQDVGFDVHHEENLRFHYQRTLHDWCELLAENWEEAVRIVGEGTA
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```
M_chloro  395   KVWGLYMAGSRVGFEQNAIQLHQVLAVKLDERGRDGGLPLRPWWTA
M_tuber   376   KVWGLYMAASRVAFERNNLQLHHVLATKVDPRG-DDSLPLRPWWQP
R_eryth   389   RVWGLYMAGSRLGFERNVVQLHQVLAVKLGPKG-EAHVPLRPWWKA
R_qingsh  389   RVWGLYMAGSRLGFERNVVQLHQVLAVKLGPKG-EAHVPLRPWWKA
R_ruber   389   RVWGLYMAGSRLGFERNVVQLHQVLAVKLGPDQ-EPNVPLRPWWIA
N_donost  393   KVWGLYMAGSRLGFQRNVVQLHQVLGVKLGVNG-LPSVPLRPWWQP
A_orient  376   RVWALYMAACRLAFERREIELHQVLGVKVGPDC-DSGMPLRPDWGV
C_urealy  400   RLFGLYMAGSEWGFEHNIVQLHQVLAVKSYADG-STGLPVRQWWES
```

Figure 14. Alignments of amino acid sequences of homologous proteins of FAD-binding oxidoreductase WP_048472120 and SAM-dependent methyltransferase WP_048472121

A. Amino acid sequences of homologous proteins of FAD-binding oxidoreductase WP_048472120. R_eryth, WP_019746543 of *Rhodococcus erythropolis* R138; R_qingsh, WP_007731548 of *Rhodococcus qingshengii* BKS 20-40; R_ruber, WP_003935378 of *Rhodococcus ruber* BKS 20-38; N_donost, WP_077118793 of *Nocardia donostiensis* X 1654; M_chloro, WP_048472120 of *Mycobacterium chlorophenolicum* JCM 7439; M_tuber, WP_003917236 of *Mycobacterium tuberculosis* H37Rv; C_urealy, WP_012359407 of *Corynebacterium urealyticum* ATCC 43042; A_orient, WP_037303122 of *Amycolatopsis orientalis* DSM 40040. B. Amino acid sequences of homologous proteins of SAM-dependent methyltransferase WP_048472121. M_chloro, WP_048472121 of *Mycobacterium chlorophenolicum* JCM 7439; M_tuber, WP_003420415 of *Mycobacterium tuberculosis* H37Rv; R_eryth, WP_019746542 of *Rhodococcus erythropolis* R138; R_qingsh, WP_003943932 of *Rhodococcus qingshengii* BKS 20-40; R_ruber, WP_003935379 of *Rhodococcus ruber* BKS 20-38; N_donost, WP_077118797 of *Nocardia donostiensis* X 1654; A_orient, WP_037303124 of *Amycolatopsis orientalis* DSM 40040; C_urealy, WP_012359408 of *Corynebacterium urealyticum* ATCC 43042. Inserted sequences in *C. urealyticum* ATCC 43042 were indicated by red underlines.