Biochimica et Biophysica Acta 1861 (2016) 980–987

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Construction of a cyanobacterium synthesizing cyclopropane fatty acids

Shuntaro Machida [a], Yoshihiro Shiraiwa [b,c], Iwane Suzuki [b,*]

[a] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8572, Japan
[b] Faculty of Life and Environmental Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8572, Japan
[c] Japan Science and Technology Agency, CREST

ARTICLE INFO

Article history:
Received 5 January 2016
Received in revised form 17 May 2016
Accepted 24 May 2016
Available online 2 June 2016

Keywords:
Biofuel
Microalgae
Cyclopropane fatty acid
Acyl-lipid desaturase
Synechocystis sp. PCC 6803

ABSTRACT

Microalgae have received much attention as a next-generation source of biomass energy. However, most of the fatty acids (FAs) from microalgae are multiply unsaturated; thus, the biofuels derived from them are fluid, but vulnerable to oxidation. In this study, we attempted to synthesize cyclopropane FAs in the cyanobacterium *Synechocystis* sp. PCC 6803 by expressing the *cfa* gene for cyclopropane FA synthase from *Escherichia coli* with the aim of producing FAs that are fluid and stable in response to oxidization. We successfully synthesized cyclopropane FAs in *Synechocystis* with a yield of ~30% of total FAs. Growth of the transformants was altered, particularly at low temperatures, but photosynthesis and respiration were not significantly affected. C16:1$^{\Delta 9}$ synthesis in the *desA*$^{-}$/*desD*$^{-}$ strain by expression of the *desC2* gene for *sn*-2 specific Δ9 desaturase positively affected growth at low temperatures via promotion of various cellular processes, with the exceptions of photosynthesis and respiration. Estimation of the apparent activities of desaturases suggested that some acyl-lipid desaturases might recognize the lipid side chain.

1. Introduction

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

Some bacteria, such as *Escherichia coli* and *Lactobacillus arabinosus*, synthesize cyclopropane FAs, which contain a cyclopropane ring in the acyl group. For example, 10% of FAs in the total membrane lipids of *E. coli* cells are cyclopropane FAs. A green sulfur bacterium, *Chlorobaculum tepidum*, also produces cyclopropane FAs attached to glycolipids [15]. This suggests that heterotrophic bacteria are not the only producers of cyclopropane FAs. However, oxygenic photosynthetic organisms do not synthesize cyclopropane FAs. Instead, they produce unsaturated FAs to maintain membrane fluidity, suggesting that cyclopropane FAs might negatively affect oxygenic photosynthesis. Cyclopropane FA synthase in *E. coli* catalyzes the modification of acyl chains to their cyclopropane derivatives through methylation of an unsaturated bond. In this reaction, *S*-adenosyl-L-methionine (SAM) is used as a methyl donor. The enzyme acts on the double bond at the Δ9 or Δ11 in FAs attached to lipids in the membrane [23]. Unsaturated FAs exist in the membrane lipids of most organisms and maintain membrane fluidity. Additionally, SAM exists in most organisms as a methyl donor for methylation reactions. Thus, organisms harboring Δ9 or Δ11 unsaturated FAs in their membrane lipids and in which the cyclopropane FA synthase from *E. coli* is expressed heterologously may have the ability to synthesize cyclopropane FAs. However, this has not been evaluated to date.

Cyanobacterial acyl-lipid desaturases introduce double bonds at specific positions in FAs that are esterified to the glycerol backbone of

* Corresponding author.
E-mail address: iwanes6803@biol.tsukuba.ac.jp (I. Suzuki).

http://dx.doi.org/10.1016/j.bbalip.2016.05.012
1388-1981/

the membrane lipid [14]. 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 FAs attached at the *sn*-1 position of the lipids. In *Synechocystis*, a saturated C18 FA, stearic acid (C18:0), is synthesized and incorporated into the *sn*-1 position of membrane lipids, followed by desaturation. DesC introduces a double bond at the Δ9 position in the saturated FA. Then, DesA and DesD unsaturate at the Δ12 and Δ6 positions, respectively, of oleic acid (C18:$1^{\Delta 9}$), and the Δ12 unsaturated FAs are utilized by DesB as substrates to introduce an unsaturated bond at the Δ15 position. The *desA* and *desD* genes were inactivated in the *desA*$^{-}$/*desD*$^{-}$ strain, and this strain accumulated more C18:$1^{\Delta 9}$ than did the wild-type strain [18]. In *Synechocystis*, the endogenous desaturase specifically unsaturates C18 FAs attached at the *sn*-1 position of lipids but cannot unsaturate C16 FAs attached at the *sn*-2 position. On the other hand, DesC2 from *Nostoc* sp. strain 36 can unsaturate the Δ9 position of C16 FAs attached at the *sn*-2 position [4].

In this study, we attempted to synthesize cyclopropane FAs in the cyanobacterium *Synechocystis* sp. PCC 6803 by expressing the cyclopropane FA synthase from *E. coli* to produce FAs that are fluid and stable in response to oxidization. To increase the yield of cyclopropane FAs in vivo, we also expressed the *desC2* gene from *Nostoc* sp. and examined the effects of mutations in *desA* and *desD* of *Synechocystis*. We analyzed the changes in FA composition, cell growth, and photosynthesis, and respiration activities in the *Synechocystis* transformants.

2. Materials and methods

2.1. Organisms and culture conditions

The *Synechocystis* sp. PCC 6803 glucose-tolerant strain [24] was used as the wild type in this study. *Synechocystis* cells were grown in BG11 [17] buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–NaOH (pH 7.5) at 34 °C or 26 °C under continuous illumination by white fluorescent lamps at 70 μmol photons m^{-2} s^{-1} and aerated with 1% (v/v) CO_2-enriched air [21]. All transformants were maintained in BG11 medium solidified with 1.5% (*w*/*v*) Bacto-agar (BD Biosciences Japan, Tokyo) in the presence of 25 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 [25] was grown in 1.5 mL of LB medium [1] at 37 °C with shaking at 200 rpm. All transformants were maintained in LB medium solidified with 1.5% (*w*/*v*) Bacto-agar (BD Biosciences Japan) in the presence of 50 μg/mL sodium ampicillin or 50 μg/mL spectinomycin dihydrochloride pentahydrate, depending on the selection markers used. To supply exogenous FAs, C18:$1^{\Delta 9}$, linoleic acid (C18:$2^{\Delta 9,12}$), γ-linolenic acid (C18:$3^{\Delta 6,9,12}$), and α-linolenic acid (C18:$3^{\Delta 9,12,15}$), to the *E. coli* cells, we cultivated the cells in liquid LB medium [1] containing 1 mM sodium salt of C18:$1^{\Delta 9}$ (Tokyo chemical industry, Japan), C18:$2^{\Delta 9,12}$ (Funakoshi, Tokyo, Japan), C18:$3^{\Delta 6,9,12}$ (Sigma Aldrich, Missouri, USA), or C18:$3^{\Delta 9,12,15}$ (Funakoshi).

2.2. Plasmid construction and transformation

For heterologous expression of the *cfa* and *desC2* genes in *Synechocystis* and overexpression of the *cfa* gene in *E. coli*, we constructed four plasmids—pTHT-cfaSp, pTHT-Sp, pTHT-desC2Sp, and pTHT-cfadesC2Sp—which are derivatives of an expression vector, pTCHT2031V, for this cyanobacterium [9]. Fig. S1 shows the construction scheme for these plasmids, and Table S1 shows the primers used. The plasmid pTCHT2031V contains five DNA fragments in the following order: the sequence upstream of the *slr2031* gene (2031up), a chloramphenicol resistance gene cassette (Cm-r), the *trc* promoter sequence (trc), the sequence downstream of the *slr2031* gene (2031dn), and the plasmid backbone of the pUC vector [9]. The derived plasmids had fragments introduced between 2031up and 2031dn into the *Synechocystis* chromosome through homologous recombination with the coding sequence of *slr2031*, a non-essential gene. At first, to replace the selection marker from the chloramphenicol resistance gene cassette with the spectinomycin resistance gene cassette, we constructed a plasmid lacking the chloramphenicol resistance gene cassette, pTHT2031V, from pTCHT2031V by PCR amplification and using an In-Fusion HD Cloning Kit (Takara Bio, Ohtsu, Japan). The *cfa* gene was amplified by PCR by using *E. coli* chromosomal DNA as the template. The amplified DNA fragment was subcloned into a T-vector pMD19 vector (Takara Bio) to obtain the pMD-cfa plasmid, which was confirmed by DNA sequencing. We next amplified a DNA fragment including the spectinomycin resistance gene (Sp-r) using pAM1146 [20] as a template. The Sp-r fragment, digested with *Bam*HI and *Bgl*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 [4] was artificially synthesized (Life Technologies Japan, Tokyo) and optimized for the codon usage of *Synechocystis*. Finally, we inserted the *desC2* gene into pTHT-cfaSp and amplified the fragment with two primer sets using an In-Fusion HD Cloning Kit (Takara Bio) to obtain pTHT-desC2Sp and pTHT-cfadesC2Sp.

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

2.3. FA analysis

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

To identify and quantify FAMEs, we 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. Most FAMEs were provisionally identified based on retention time and confirmed using commercial FAME standards (Nu-Chek Prep, Elysian, MN). To identify *cis*-9,10-methylenehexadecanoic acid (C17:1cyclo$^{\Delta 9}$) and *cis*-9,10-methyleneoctadecanoic acid (C19:1cyclo$^{\Delta 9}$), we used a gas chromatograph, GC-2010, equipped with a mass spectrometer, QP-2010 (Shimadzu). Conditions of GC were identical to those used for the FAME quantification, as described above. We confirmed the retention times and mass spectrums of C17:1cyclo$^{\Delta 9}$ (Santa Cruz Biotechnology, Dallas, TX) and C19:1cyclo$^{\Delta 9}$(Santa Cruz Biotechnology). *cis*-9,10-methylene-*cis*-12-octadecenoic acid (C19:$2^{\Delta 12}$cyclo$^{\Delta 9}$) and *cis*-9,10-methylene-*cis*-12,15-octadecadienoic acid (C19:$3^{\Delta 12,15}$cyclo$^{\Delta 9}$) were