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Free fatty acid production in the cyanobacterium *Synechocystis* sp. PCC 6803 is enhanced by deletion of the cyAbrB2 transcriptional regulator

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ABSTRACT

The cyAbrB2 (Sll0822) transcriptional regulator in *Synechocystis* sp. PCC 6803 is involved in coordination of carbon and nitrogen metabolism and its deletion causes distinct phenotypes such as decreased expression levels of nitrogen-regulated genes and high accumulation of glycogen granules. From the viewpoint of metabolic engineering, the highly accumulated glycogen granules in the Δ*cyabrB2* mutant could be a valuable source for the production of biofuels. Here, by disruption of the *aas* gene (*slr1609*) encoding acyl–acyl carrier protein synthetase and introduction of a gene encoding thioesterase from *Umbellularia californica* (UcTE), we conferred the ability of production and secretion of free fatty acids on the Δ*cyabrB2* mutant. Notable features of the resulting Δ*cyabrB2*Δ*aas*::UcTE strain compared with Δ*cyabrB2* by RNA-seq analysis were decrease in expression levels of genes related to uptake and subsequent metabolism of nitrogen and carbon and increase in the expression level of *sigE* encoding a group 2 sigma factor. These changes in gene expression profile were not observed when the same genetic modification was introduced in the wild-type background. The Δ*cyabrB2*Δ*aas*::UcTE strain showed two-folds higher free fatty acid productivity on a per OD_{730} basis compared with the Δ*aas*::UcTE strain, without expense of the accumulated glycogen granules. This shows the potential of the Δ*cyabrB2* mutant as the platform of biofuel production. The effective utilization of the accumulated glycogen must be the next task to be pursued.

1. Introduction

Biofuel production from renewable sources is a feasible solution to the energy and environmental problems we are facing. The direct conversion of CO_2 into biofuels by photosynthetic microorganisms, especially by microalgae having the ability to accumulate storage lipids such as triacylglycerol, has been regarded as a promising strategy (Suutari et al., 2015). Because of their fast growth and facile genetic manipulation, cyanobacteria have also been vigorously developed as a platform of metabolic engineering. One of the strategies of biofuel production using genetically modified cyanobacteria, secretion of free fatty acids (FFAs) to the culture medium (Liu et al., 2011), is advantageous in that secreted FFAs can be collected at much lower cost than intracellular storage lipids.

Currently, the most popular strategy for FFA production in cyanobacteria is combination of knockout of the *aas* gene encoding acyl–acyl carrier protein (ACP) synthetase and introduction of a bacterial or plant type acyl-ACP thioesterase (TE) gene. In *Synechocystis* sp. PCC 6803 (S.6803) and *Synechococcus elongatus* PCC 7942 (S.7942), acyl-ACP synthetase was reported to work for recycling of FFAs released from cell membranes by catalyzing ATP-dependent esterification of FFAs to the thiol of ACP (Kaczmarzyk and Fulda, 2010). By knockout of the *aas* (*slr1609*) gene, significant amount of FFAs were secreted into the culture medium (Kaczmarzyk and Fulda, 2010). Acyl-ACP TE terminates *de novo* fatty acid (FA) biosynthesis by hydrolyzing the thioester bond between ACP and synthesized FAs. It was revealed that introduction of a bacterial or plant type acyl-ACP TE gene into the *aas* knockout mutant increased the amount of secreted FFAs in S.6803 (Liu et al.,

Abbreviations: S.6803, *Synechocystis* sp. PCC 6803; S.7942, *Synechococcus elongatus* PCC 7942; FA, fatty acid; FFA, free fatty acid; *aas*, acyl–acyl carrier protein synthetase; TE, thioesterase.

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2011). The chain length of secreted FFAs is determined depending on the substrate specificity of the introduced TE. For instance, a truncated TE from *Escherichia coli* (‘*tesA*) caused secretion of mostly C16 or C18 FAs (Ruffing and Jones, 2012), whereas a plant type TE from *Umbellularia californica* (UcTE) caused secretion of C12 FAs (Liu et al., 2011).

Although combination of *aas*-knockout and TE-introduction was shown to be effective for increase of FFA secretion, productivity of FFAs in cyanobacteria is still significantly low compared with that in heterotrophic organisms such as engineered *E. coli* (Janssen and Steinbuchel, 2014). Attempts to increase the yield of FFA production in cyanobacteria have been made by various approaches of metabolic engineering. For instance, the *acc* genes encoding acetyl-CoA carboxylase, the rate-limiting enzyme in the fatty acid synthetic pathway, were overexpressed (Ruffing, 2013a). The *sll1951* gene encoding the surface layer protein and the *slr1710* gene encoding the peptidoglycan assembly protein were knocked out to weaken the hydrophilic peptidoglycan layers of the cell wall (Liu et al., 2011). RNA-seq analysis was performed for FFA-producing strain of S.7942 to identify genes encoding transporters involved in the secretion of FFA or proteins working for mitigation of the toxic effect of FFAs (Ruffing, 2013b). However, further technical development is required to improve the yield of FFA production in cyanobacteria to achieve the industrial application.

In this study, we propose the use of the *cyabrB2*-knockout (Δ*cyabrB2*) mutant in S.6803 as a platform of FFA production. cyAbrB2 (Sll0822) is a member of the cyanobacteria-specific transcriptional regulator family belonging to the AbrB superfamily (Ishii and Hihara, 2008). The Δ*cyabrB2* mutant revealed distinct phenotypes such as decreased expression levels of nitrogen-regulated genes (Ishii and Hihara, 2008) and high accumulation of glycogen granules and sugar phosphates (Hanai et al., 2014; Kaniya et al., 2013) under normal growth conditions. It is supposed that the Δ*cyabrB2* mutant has a defect in balancing of carbon and nitrogen metabolism and accumulates fixed carbon as glycogen granules. From the viewpoint of metabolic engineering, the highly accumulated glycogen granules in the Δ*cyabrB2* mutant could be a valuable material for the production of biofuels if we succeed to reallocate these excess carbons to a desired biosynthetic pathway. Here, by disruption of the *aas* gene and introducing UcTE gene, we conferred the ability of production and secretion of FFAs on the Δ*cyabrB2* mutant and evaluated the effectiveness of *cyabrB2* deletion on FFA production. As a result, more than two-folds improvement of FFA production per OD_{730} basis was attained in the mutant after two weeks of cultivation, without expense of the accumulated glycogen granules. This shows the high potential of the Δ*cyabrB2* mutant as the platform of biofuel production. The effective utilization of the accumulated glycogen must be the next task to be pursued.

2. Materials and methods

2.1. Generation of mutant strains

A glucose-tolerant wild-type (WT) strain of S.6803 was used to generate all the mutants described below (Supplementary Table 1). PCR primers used for construction of the plasmids for genetic manipulation are listed in Supplementary Table 2.

2.1.1. Generation of the ΔrecJ mutant

Single-stranded-DNA-specific exonuclease *recJ* (*sll1354*) gene was disrupted by insertion of the chloramphenicol (Cm) resistance cassette into the coding region. The upstream and downstream halves of the *recJ* coding sequence were amplified by PCR from the genomic DNA of the WT strain using the primer sets recJ-F and Cm/recJ-R (for the upstream fragment), and Cm/recJ-F and recJ-R (for the downstream fragment). Cm resistance cassette was PCR amplified from the pTCP2031V plasmid (Muramatsu et al., 2009) using primers Cm-F and Cm-R. The amplified *recJ* fragments and Cm resistance cassette were fused together by Splicing by Overhang Extension (SOE)-PCR with primers recJ-F and recJ-R to generate the Δ*recJ*::Cm fragment. The WT strain was transformed with the Δ*recJ*::Cm fragment, and transformants (Δ*recJ* mutant) were selected in the presence of Cm.

2.1.2. Generation of the ΔrecJ ΔcyabrB2 mutant

The *cyabrB2* (*sll0822*)-disruption construct in which the kanamycin (Km) resistance cassette was inserted into the coding region of *cyabrB2* at the StyI site (Ishii and Hihara, 2008) was transformed into the Δ*recJ* mutant strain to yield the Δ*recJ*Δ*cyabrB2* mutant (designated as Δ*cyabrB2* in this study).

2.1.3. Generation of FFA-producing mutants

To produce the deletion construct of the *aas* (*slr1609*) gene encoding Acyl-ACP synthetase, the gene-flanking segment of the *aas* gene was amplified by PCR from genomic DNA of the WT strain using the primer set slr1609f-F and slr1609r-R, and cloned into the HincII site of pUC118 plasmid (TaKaRa). The resulting pUC118-*aas* plasmid was linearized by PCR using the primers slr1609f-R and slr1609r-F. A spectinomycin (Sp) resistance cassette was amplified from pDG1726 plasmid (Guerout-Fleury et al., 1995) using primers slr1609/sp-F and slr1609/sp-R. The amplified linear pUC118-*aas* plasmid and Sp resistance cassette were fused using the In-Fusion HD Cloning Kit (Clontech) according to the manufacturer's instructions to generate the pUC118-Δ*aas*::sp construct.

The artificially synthesized coding sequence of the thioesterase gene from *Umbellularia carfornica* (UcTE) (Liu et al., 2011) was amplified using the primer set UcTE-F and UcTE/sp-R. The strong promoter P*psbA2* of S.6803 was amplified from the genomic DNA of the WT strain using the primer set slr1609/psbA2-F and psbA2/UcTE-R. The pUC118-Δ*aas*::sp plasmid was linearized by inverse PCR using the primers slr1609f-R and Sp-F to introduce P*psbA2*-UcTE into just upstream of Sp resistance cassette. Linearized pUC118-Δ*aas*::sp construct, P*psbA2* and UcTE fragments were fused using the In-Fusion Kit described above. By transformation with the resulting pUC118-Δ*aas*::P*psbA2*-UcTE-sp construct, simultaneous knockout of *aas* and introduction of the UcTE gene to *Synechocystis* genome can be achieved.

pUC118-Δ*aas*::P*psbA2*-UcTE-sp construct was transformed into the Δ*recJ* and Δ*recJ*Δ*cyabrB2* mutants to generate FFA-producing strains Δ*recJ*Δ*aas*::P*psbA2*-UcTE (designated as Δ*aas*::UcTE) and Δ*recJ*Δ*cyabrB2*Δ*aas*::P*psbA2*-UcTE (designated as Δ*cyabrB2*Δ*aas*::UcTE), respectively.

2.2. Culture conditions

The WT strain of S.6803 was grown photoautotrophically at 30 °C in BG-11 medium containing 5 mM TES-KOH, pH 8.2, under continuous illumination at 40 μmol photons m^{-2} s^{-1} with bubbling of air. Mutants were grown under the same conditions, except that antibiotics were added to the medium: 25 μg mL^{-1} each of Km and Cm for the Δ*cyabrB2* mutant, Sp and Cm for the Δ*aas*:UcTE mutant, and Km, Cm and Sp for the Δ*cyabrB2*Δ*aas*::UcTE mutant.

Cell density was estimated by the optical density at 730 nm (OD_{730}) using a spectrophotometer (UV-160A; Shimadzu).

For cultivation to produce FFA, each strain pre-cultured to late-log phase (OD_{730} = 1.0) under the same condition described above was inoculated into 50 mL BG-11 in 100 mL Erlenmeyer flask at initial OD_{730} = 0.2 and grown at 30 °C under continuous illumination at 40 μmol photons m^{-2} s^{-1} with rotary shaking.