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 $\Delta cyabrB2$ 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 $\Delta cyabrB2$ mutant. Notable features of the resulting $\Delta cyabrB2\Delta aas::UcTE$ strain compared with $\Delta cyabrB2$ 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 into WT. The $\Delta cyabrB2\Delta aas::UcTE$ strain showed two-folds higher free fatty acid productivity on a per OD₇₃₀ basis compared with the $\Delta aas::UcTE$ strain, without expense of the accumulated glycogen granules. This shows the potential of the $\Delta cyabrB2$ mutant as the platform of biofuel production. The effective utilization of the accumulated glycogen must be the next task to be pursued.

Highlights

The cyAbrB2 transcription factor works for regulation of N and C metabolism.

Disruption of *cyabrB2* caused two-fold higher productivity of free fatty acids.

Omics analyses were performed to gain insight into the high productivity.

Relocation of fixed carbon would be a key for further increase of the yield.

Key words

Cyanobacterial biofuels, Free fatty acid production, Metabolic engineering, cyAbrB2 transcriptional regulator

Abbreviations

S.6803, *Synechocystis* sp. PCC 6803; S.7942, *Synechococcus elongatus* PCC 7942; FA, fatty

57 acid; FFA, free fatty acid; *aas*, acyl-acyl carrier protein synthetase; TE, thioesterase

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1. Introduction

Biofuel production from renewable sources is a feasible solution to the energy and environmental problems we are facing. The direct conversion of CO₂ 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., 2011). The chain length of secreted FFAs is determined depending on the substrate specificity of the introduced TE. For instance, a truncated TE from *E. 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 ($\Delta cyabrB2$) 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 $\Delta cyabrB2$ 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 $\Delta cyabrB2$ 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 $\Delta cyabrB2$ 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 $\Delta cyabrB2$ mutant and evaluated the effectiveness of *cyabrB2* deletion on FFA production. As a result, more than two-folds improvement of FFA production per OD₇₃₀ 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 $\Delta cyabrB2$ 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 were listed in Supplementary Table 2.

2.1.1. Generation of the $\Delta recJ$ 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 $\Delta recJ::Cm$ fragment. The WT strain was transformed with the $\Delta recJ::Cm$ fragment, and transformants ($\Delta recJ$ mutant) were selected in the presence of Cm.

2.1.2. Generation of the $\Delta recJ \Delta cyabrB2$ mutant

The *cyabrB2* (*slr0822*)-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 $\Delta recJ$ mutant strain to yield the $\Delta recJ \Delta cyabrB2$ mutant (designated as $\Delta cyabrB2$ 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- $\Delta aas::sp$ construct.

The artificially synthesized coding sequence of the thioesterase gene from *Umbellularia carfonica* (UcTE) (Liu et al., 2011) was amplified using the primer set UcTE-F and UcTE/sp-R. The strong promoter *PpsbA2* 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- $\Delta aas::sp$ plasmid was linearized by inverse PCR using the primers *slr1609f*-R and Sp-F to introduce *PpsbA2*-UcTE into just upstream of Sp resistance cassette. Linearized pUC118- $\Delta aas::sp$ construct, *PpsbA2* and UcTE fragments were fused using the In-Fusion Kit described above. By transformation with the resulting pUC118- $\Delta aas::PpsbA2$ -UcTE-sp construct, simultaneous knockout of *aas* and introduction of the UcTE gene to *Synechocystis* genome can be achieved.

pUC118- $\Delta aas::PpsbA2$ -UcTE-sp construct was transformed into the $\Delta recJ$ and $\Delta recJ \Delta cyabrB2$ mutants to generate FFA-producing strains $\Delta recJ \Delta aas::PpsbA2$ -UcTE (designated as $\Delta aas::UcTE$) and $\Delta recJ \Delta cyabrB2 \Delta aas::PpsbA2$ -UcTE (designated as $\Delta cyabrB2 \Delta aas::UcTE$), respectively.

2.2. Culture conditions

The WT strain of S.6803 were grown photoautotrophically at 30°C in BG-11 medium containing 5 mM TES-KOH, pH 8.2, under continuous illumination at 40 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ with bubbling of air. Mutants were grown under the same conditions, except that antibiotics were added to the medium: 25 $\mu\text{g mL}^{-1}$ each of Km and Cm for the $\Delta\text{cyabrB2}$ mutant, Sp and Cm for the $\Delta\text{aas::UcTE}$ mutant, and Km, Cm and Sp for the $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ 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 ($\text{OD}_{730}=1.0$) under the same condition described above was inoculated into 50 mL BG-11 in 100 mL Erlenmeyer flask at initial $\text{OD}_{730}=0.2$ and grown at 30°C under continuous illumination at 40 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ with rotary shaking.

2.3. FFA analysis

Secreted FFAs were extracted from the culture using hexane according to the method described in Liu et al. (2011) with slight modification. 1g of NaH_2PO_4 and an internal standard, 50 μL of 1 mg mL^{-1} C15 FA dissolved in methanol, were added to 50 mL of the culture. Then, 10 mL of hexane was mixed with the culture to extract FFAs. After centrifugation, upper organic layer was collected into the glass tube. The extraction with hexane was repeated twice and collected organic layer was dried with nitrogen gas. 3 mL of 5% hydrochloric acid in methanol solution was added to the dried sample, and methyl esterification of FFAs was performed at 80°C for 3 h. Thereafter, the mixture was added with 3 mL of hexane, sufficiently stirred and left standing for 30 min. The upper layer was collected and concentrated to be analyzed with a gas chromatograph (GC-2014, Shimadzu). The gas chromatography was carried out under the following conditions: [capillary column: ULBON HR-SS-10 25 m x 0.25 mm Φ (Shimadzu), mobile layer: high purity helium, flow rate inside the column: 1.98 mL min^{-1} , temperature rise program: Oven temperature started at 170°C held for 30 min and then increased at rate of 10°C min^{-1} to 220°C and held for 5 min, equilibration time: 3 min, injection port: split injection (split ratio: 10:1), pressure: 40.32 psi, 24.8 mL min^{-1} , amount of injection: 2 μL , vial cleaning: methanol and chloroform, detector temperature: 250°C].

Amounts of fatty acid methyl esters were quantitatively determined based on the peak areas of the waveform data obtained by the above gas chromatographic analysis using an internal standard C15 FA.

2.4. Measurement of photosynthetic activities

Culture was diluted to $\text{OD}_{730}=0.5$ before the measurement and a 1 mL aliquot was placed in a Clark-type oxygen electrode chamber and stirred gently at 30°C. Whole-cell photosynthetic

activity was measured as oxygen evolution supported by 2 mM NaHCO₃. Oxygen evolution rate was calculated in terms of μmol of oxygen evolved $\text{OD}_{730}^{-1} \text{ mL}^{-1} \text{ h}^{-1}$.

2.5. Determination of glycogen content

Glycogen content was determined by the method described in Suzuki et al. (2010). Briefly, the cell pellet was resuspended in absolute methanol and kept at -20°C overnight. After centrifugation, the dried pellet was resuspended and incubated at 100°C for 40 min, and then at 40°C for 1 h with glucoamylase. Glycogen content was determined enzymatically by addition of hexokinase and glucose 6-phosphate dehydrogenase. The amount of glucose moieties derived from glycogen was determined as the increase in OD_{340} . Cellular glycogen content was calculated in terms of μg of glucose $\text{mL}^{-1} \text{ OD}_{730}^{-1}$ using the extinction coefficient of NADPH at 340 nm ($6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). In the glycogen-less strain $\Delta\text{recJ}\Delta\text{agp}$ in which the gene encoding glucose-1-phosphate adenylyltransferase (AGPase, Slr1176) was disrupted, glycogen content measured by this method was $1.95 \mu\text{g Glc mL}^{-1} \text{ OD}_{730}^{-1}$.

2.6. Electron microscopy

Electron microscopy was performed as described previously (Nitta et al., 2009; Yamauchi et al., 2011). Cultures grown under FFA-producing conditions for two weeks were used for observation.

2.7. RNA-seq analysis

RNA-seq analysis was carried out using cultures grown under FFA-producing conditions for a week. Isolation of total RNA by the hot phenol method was performed as described previously (Muramatsu and Hihara, 2003). To eliminate genomic DNA from total RNA samples, each sample was added with DNaseI (TaKaRa) and incubated at 37°C for 3 h. The Ribo-Zero Magnetic Kit for Bacteria (Epicentre) was used to remove ribosomal RNA from each sample. Concentration and quality of mRNA samples were examined using an Agilent 2100 Bioanalyzer. cDNA library construction and RNA-seq analysis using Illumina HiSeq 2500 were outsourced to Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan). A total of 290 million reads data were obtained from 9 samples. To quantify expression level of each gene, nucleotide sequences of obtained reads were mapped to the genomic sequence of S.6803 substr. GT-I (Kanesaki et al., 2012) (NC_017038.1; http://www.ncbi.nlm.nih.gov/nuccore/NC_017038).

2.8. Metabolome analysis

Metabolome analysis was carried out using cultures grown under FFA-producing conditions for a week. Cells were harvested by filtration and metabolites were extracted with methanol containing internal standards according to the protocol provided by Human

Metabolome Technologies, Inc. (Tsuruoka, Yamagata, Japan). Culture volume to be harvested (mL) was determined using the following equation. [culture volume (mL) = 20 / OD₇₃₀]. After addition of water and chloroform, the upper aqueous layer was filtered through a Millipore 5-kDa cutoff filter to remove proteins. The filtrate was lyophilized and dissolved in water. Measurement of ionic metabolites using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF/MS) was carried out by Human Metabolome Technologies, Inc.

3. Results

3.1. Genetic engineering of the $\Delta cyabrB2$ mutant for FFAs production

When starting the genetic engineering of the $\Delta cyabrB2$ mutant, its low transformation efficiency turned out to be problematic. It was reported that the transformation efficiency of S.6803 could be improved by about 100-fold by deletion of the *recJ* (*slr1354*) gene encoding single-stranded-DNA-specific exonuclease (Kufryk et al., 2002). We made the $\Delta cyabrB2\Delta recJ$ double mutant and found that the transformation efficiency of the double mutant was more than 100-fold higher than that of the $\Delta cyabrB2$ mutant. Thus, in this study, the $\Delta recJ$ mutant was used for the genetic background of all strains.

The *aas* (*slr1609*) gene encoding acyl-ACP synthetase was disrupted by insertion of the UcTE gene from *Umbellularia californica* together with the Sp resistance gene cassette in WT (the $\Delta recJ$ background strain) and in the $\Delta cyabrB2$ mutant (the $\Delta cyabrB2\Delta recJ$ double mutant) to obtain FFA-producing mutants, $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$, respectively. The UcTE gene was expressed under the control of the strong promoter *PpsbA2*. The promoter activity of *PpsbA2* was shown to be higher than that of *Pspac* under the experimental conditions employed in this study (data not shown). Fully segregated mutants were obtained and constitutive high expression of UcTE gene was observed in these strains (data not shown).

3.2. Growth properties of FFA-producing and -nonproducing strains

We compared the growth rate of the FFA-nonproducing $\Delta cyabrB2$, and FFA-producing strains $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$ in liquid BG-11 medium under FFA-producing conditions (Fig. 1A). No significant difference in growth rate was observed among these strains during 3 weeks of cultivation. The color of 3-weeks culture was almost the same among strains, indicating that bleaching of photosynthetic pigments or cell lysis was not induced by FFA production.

3.3 FFA productivity of FFA-producing strains

Secreted FFAs were extracted with hexane from the entire culture including cyanobacterial cells and FFA productivity was analyzed in $\Delta cyabrB2$, $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$ strains after 1, 2 and 3 weeks of cultivation (Fig. 1B). The FFA concentration in culture was 2.14 mg L⁻¹ (1 w), 6.67 mg L⁻¹ (2 w) and 13.45 mg L⁻¹ (3 w) in the $\Delta aas::UcTE$ strain. On the other hand, the $\Delta cyabrB2\Delta aas::UcTE$ strain secreted 2.95 mg L⁻¹ (1 w), 15.08 mg L⁻¹ (2 w) and 22.58 mg L⁻¹ (3 w). Under the same condition, less than 0.06 mg L⁻¹ (3 w) of FFA was detected in the culture of the FFA-nonproducing $\Delta cyabrB2$ strain. FFA productivity of $\Delta cyabrB2\Delta aas::UcTE$ was 1.37- (1 w), 2.26- (2 w) and 1.68-fold (3 w) higher than that of $\Delta aas::UcTE$. When expressed on a per OD₇₃₀ basis, $\Delta cyabrB2\Delta aas::UcTE$ strains showed 1.51- (1 w), 2.49- (2 w), and 1.85-fold (3 w) higher FFA productivity compared to $\Delta aas::UcTE$ (Fig.1C). These results suggested that deletion of the *cyabrB2* gene causes significant increase of FFA productivity per cell.

The composition of the secreted FFAs from each strain after 2 weeks of cultivation is shown in Figure 2 and Table 1. In the case of the trace amount of FFAs secreted from $\Delta cyabrB2$, C16:0 which is a main component of membrane lipids of S.6803 occupied up to 70% (Fig. 2A). On the other hand, in both $\Delta aas::UcTE$ (Fig. 2B) and $\Delta cyabrB2\Delta aas::UcTE$ (Fig. 2C), C12:0 FFAs to which UcTE shows the highest substrate specificity occupied up to 40% of the secreted FFAs. The rest was FFAs of various chain lengths ranging from C14 to C18. No difference in FFA composition was observed between $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$.

3.4 Photosynthetic activity of FFA-producing and -nonproducing strains

Next, we examined the effect of FFA production on photosynthetic activity. Figure 3 shows the oxygen evolution activity of $\Delta cyabrB2$, $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$ strains after 1, 2, 7, and 14 days of cultivation in the presence of 2 mM NaHCO₃. Both under growth light conditions (40 μ mol photons m⁻²s⁻¹) (Fig. 3A) and saturating light conditions (1500 μ mol photons m⁻²s⁻¹) (Fig. 3B), there was no significant difference in the time course change of photosynthetic activity among strains. The highest photosynthetic activity was recorded at the beginning and then activity gradually decreased with incubation time. There have been reports showing the inhibitory effect of FFA production on photosynthetic activity. For example, Ruffing and Jones (2012) observed decrease in photosynthetic activity in the FFA-producing strains of S.7942 likely caused by the toxicity of produced FFAs and changes in membrane composition. However, in this study, the tendency of decrease in photosynthetic activity was observed both in FFA-nonproducing and FFA-producing strains. This suggests that the gradual decrease in photosynthetic activity was due to the transition of the growth phase from logarithmic to stationary phase rather than the production of FFAs or elevated FFA concentration in culture.

3.5. Accumulation level of glycogen in FFA-producing and -nonproducing strains

The $\Delta cyabrB2$ mutant is known to accumulate much more glycogen granules than WT (Yamauchi et al., 2011). In the $\Delta cyabrB2\Delta aas::UcTE$ strain, high FFA productivity may be attained by usage of accumulated glycogen granules as carbon source for FA synthesis. If so, accumulation level of glycogen should be lowered in the $\Delta cyabrB2\Delta aas::UcTE$ strain than in the $\Delta cyabrB2$ strain during cultivation. To test the possibility, the intracellular glycogen content of the $\Delta cyabrB2$, $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$ strains were examined after 1, 2, 7, and 14 days of cultivation (Fig. 4). The accumulation level of glycogen after 1 day was about six-fold higher in $\Delta cyabrB2$ and $\Delta cyabrB2\Delta aas::UcTE$ than in $\Delta aas::UcTE$, whereas no difference was observed in the accumulation level among the strains after 2 days. The higher glycogen content observed in the $\Delta cyabrB2$ background strains after 1 day may be carried over from the seed culture at late-log phase. Then the active catabolic activity of logarithmically grown cells may cause the decrease of glycogen content to the level comparative to the $\Delta aas::UcTE$ strain after 2 days. During further cultivation, glycogen content continued to increase in $\Delta cyabrB2$ and $\Delta cyabrB2\Delta aas::UcTE$, whereas the increase was much slower in $\Delta aas::UcTE$. There was no clear effect of circadian rhythm on the accumulation level of glycogen. As a result, $\Delta cyabrB2$ and $\Delta cyabrB2\Delta aas::UcTE$ exhibited 2-fold accumulation compared with $\Delta aas::UcTE$ after 14 days. The accumulation level of glycogen was similar between $\Delta cyabrB2$ and $\Delta cyabrB2\Delta aas::UcTE$, showing that FFA production did not cause decrease in glycogen storage.

3.6. Electron microscopic observation of FFA-producing and -nonproducing strains

We carried out the electron microscopic observation of $\Delta cyabrB2$ and $\Delta cyabrB2\Delta aas::UcTE$ after two weeks of cultivation to examine the effect of FFA production on cellular structure. In the FFA-nonproducing $\Delta cyabrB2$ strain, numerous glycogen granules were observed between the thylakoid membrane layers (Fig.5A) as reported in the previous studies (Hanai et al., 2014; Kaniya et al., 2013). The structure of thylakoid layers and accumulation level of glycogen granules in FFA-producing $\Delta cyabrB2\Delta aas::UcTE$ were similar to those in $\Delta cyabrB2$ (Fig.5A). The observation was consistent with the results showing similar levels of photosynthetic activity (Fig. 3) and glycogen content (Fig. 4) between $\Delta cyabrB2$ and $\Delta cyabrB2\Delta aas::UcTE$.

Careful examination of multiple sections revealed that aggregation of the filamentous material with high electron density was often observed inside the cytoplasm and between the thylakoid membrane layers of $\Delta cyabrB2\Delta aas::UcTE$ but not of $\Delta cyabrB2$ (Fig.5B). In addition, release of the filamentous materials from the cell surface was observed in some $\Delta cyabrB2\Delta aas::UcTE$ cells (Fig.5B). Extracellular aggregation of materials with high electron density was also observed in the case of $\Delta cyabrB2\Delta aas::UcTE$ but not of $\Delta cyabrB2$ (Fig.5A). It is known that unsaturated FAs can become electron dense by crosslink of OsO_4 to their double

bonds (Belazi et al., 2009). Since these materials with high electron density were observed only in FFA producing $\Delta cyabrB2\Delta aas::UcTE$, they may be FFAs produced by $\Delta cyabrB2\Delta aas::UcTE$.

3.7. Omics analyses of FFA-producing and -nonproducing strains

In order to examine the effect of FFA production on gene expression profile and cellular metabolism, we performed RNA-seq and metabolome analyses of FFA-producing and -nonproducing strains after a week of cultivation. Firstly, we performed control experiments comparing gene expression profile of WT having the intact *recJ* gene and the $\Delta aas::UcTE$ strain of the $\Delta recJ$ background. The growth property and accumulation level of glycogen of these strains were quite similar during 2 weeks of cultivation under FFA-producing conditions (data not shown). As shown in Supplementary Fig.1 and Supplementary Table 3, gene expression profile after a week was also very similar between the strains.

3.7.1. Comparison between $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$

As described above, the FFA productivity of $\Delta cyabrB2\Delta aas::UcTE$ was much higher than that of $\Delta aas::UcTE$ (Fig.1B and C). By comparison of gene expression profile and metabolite levels of these strains, we may know the reason why deletion of the *cyabrB2* gene leads to the high FFA productivity.

We previously observed that expression levels of a part of the nitrogen-regulated genes such as the *nrt* operon were significantly low in the $\Delta cyabrB2$ mutant (Ishii and Hihara, 2008). Here, in addition to the nitrogen-regulated genes, expression levels of genes related to inorganic carbon uptake and fixation, such as *ndhF3D3* operon, *cmp* operon, *sbtA* and *rbc* operon, were lower in $\Delta cyabrB2\Delta aas::UcTE$ than in $\Delta aas::UcTE$ (Supplementary Table 4). On the other hand, most of the genes whose expression level was higher in $\Delta cyabrB2\Delta aas::UcTE$ were functionally unknown (Supplementary Table 5). Expression levels of the *fab* genes encoding enzymes for fatty acid synthesis were similar between these two strains (Supplementary Table 6). It is striking that expression level of *fabF* (*slr1069*) and *fabH* (*slr1551*) in $\Delta cyabrB2\Delta aas::UcTE$ was much lower than those in $\Delta aas::UcTE$. These results show that high FFA productivity of $\Delta cyabrB2\Delta aas::UcTE$ is attained without up-regulation of *fab* genes.

The difference in levels of metabolites between $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$ (Fig.6A) was similar to that observed between WT and the $\Delta cyabrB2$ mutant in the previous studies (Hanai et al., 2014; Kaniya et al., 2013). Namely, sugar phosphates in early steps of the glycolytic pathway as well as those in the oxidative pentose phosphate (OPP) pathway accumulated to higher levels, whereas the levels of metabolites in later steps of the glycolytic pathway were significantly lower in $\Delta cyabrB2\Delta aas::UcTE$. In addition, the accumulation levels of aconitate, isocitrate and 2-oxoglutarate in tricarboxylic acid (TCA) cycle and several amino acids

were also lower in $\Delta cyabrB2\Delta aas::UcTE$ (Fig.6A).

3.7.2. Comparison between $\Delta cyabrB2$ and $\Delta cyabrB2\Delta aas::UcTE$

Next, gene expression profile and metabolite levels were compared between $\Delta cyabrB2$ and $\Delta cyabrB2\Delta aas::UcTE$ to assess the effect of FFA production on gene expression and metabolism. In contrast to only a small effect of disruption of *aas* and introduction of *UcTE* on gene expression profile of WT (Supplementary Fig.1 and Supplementary Table 3), the same genetic modification significantly affected gene expression of the $\Delta cyabrB2$ mutant as described below.

Conferring the ability of FFA production to the $\Delta cyabrB2$ mutant caused typical responses of nitrogen-regulated genes under nitrogen-replete conditions. Namely, transcript levels of NtcA regulon genes such as the *nrt* operon, *amt1*, *glnN* significantly decreased (Table 2), whereas those of *gifA* and *gifB* genes encoding glutamine synthetase inactivating factor were up-regulated (Table 3). Although lowered expression of the nitrogen-regulated genes is distinct characteristic of the $\Delta cyabrB2$ mutant (Ishii and Hihara, 2008), introduction of $\Delta aas::UcTE$ seems to cause their further decrease. The unexpected finding is that the expression level of *sigE* encoding a group 2 sigma factor was about 7-fold higher in $\Delta cyabrB2\Delta aas::UcTE$ than that in $\Delta cyabrB2$ (Table 3). *sigE* is belonging to the NtcA regulon and is upregulated upon nitrogen deficiency (Muro-Pastor et al., 2001). In the $\Delta cyabrB2$ mutant, expression level of *sigE* is lowered together with other nitrogen-regulated genes (Ishii and Hihara, 2008). In this study, FPKM values of *sigE* in WT, $\Delta cyabrB2$, $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$ were 238.3 ± 20.9 , 31.8 ± 2.6 , 278.4 ± 17.5 and 220.6 ± 18.0 , respectively (Supplementary Tables 3 and 6). This indicates that the low transcript level of *sigE* in the $\Delta cyabrB2$ mutant was recovered to WT level by introduction of $\Delta aas::UcTE$. The induction of *sigE* despite the repression of other nitrogen-regulated genes in $\Delta cyabrB2\Delta aas::UcTE$ may suggest specific requirement of upregulation of *SigE* for FFA production in the $\Delta cyabrB2$ mutant.

In addition to nitrogen-regulated genes, expression levels of genes related to inorganic carbon uptake and fixation, such as *ndhF3D3* operon, *cmp* operon, *sbtA* and *rbc* operon, and *atp* operon encoding subunits of ATP synthase were lower in $\Delta cyabrB2\Delta aas::UcTE$ than in $\Delta cyabrB2$ (Table 2). Decrease in expression levels of these genes in $\Delta cyabrB2\Delta aas::UcTE$ implies that FFA production leads to inactivation of uptake and subsequent metabolism of nitrogen and carbon either directly or indirectly.

Other notable features in gene expression of $\Delta cyabrB2\Delta aas::UcTE$ were induction of *cta* genes encoding subunits of cytochrome c oxidase and induction of *petE* encoding plastocyanin with repression of *petJ* encoding cytochrome c553. The former may indicate activation of respiratory electron transport and the latter may be caused by iron depletion within

$\Delta cyabrB2\Delta aas::UcTE$ cells.

In comparison with large effects on gene expression, the effect of introduction of $\Delta aas::UcTE$ on metabolite levels were limited (Fig.6B). The accumulation level of lactate was about 3.6-fold higher in $\Delta cyabrB2\Delta aas::UcTE$. On the other hand, the amount of most amino acids tended to decrease in $\Delta cyabrB2\Delta aas::UcTE$.

4. Discussion

In this study, we succeeded in improving FFA productivity of the $\Delta aas::UcTE$ strain by deletion of *cyabrB2*. $\Delta cyabrB2\Delta aas::UcTE$ strain showed 2-fold higher FFA productivity compared to $\Delta aas::UcTE$ when expressed on a per OD₇₃₀ basis (Fig.1C). Although introduction of $\Delta aas::UcTE$ did not affect cell size (data not shown), disruption of *cyabrB2* has been reported to cause 1.7-times increase in cell diameter (Yamauchi et al., 2011). The difference in cell size causes different cell number in equal OD₇₃₀. The culture of $\Delta cyabrB2$ contains 3.4-fold less cells than that of WT culture of the same OD₇₃₀ (Yamauchi et al., 2011), which means that the FFA productivity per single cell is more than 6 times higher in $\Delta cyabrB2\Delta aas::UcTE$ than in $\Delta aas::UcTE$.

In order to elucidate the cause of high FFA productivity in $\Delta cyabrB2\Delta aas::UcTE$, we compared transcript and metabolite levels among strains. Notable features of $\Delta cyabrB2\Delta aas::UcTE$ compared with $\Delta cyabrB2$ 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*. These changes in gene expression profile were not observed when the same genetic modification was introduced into WT (Supplementary Table 3). The significant changes in gene expression may lead to the high FFA productivity in $\Delta cyabrB2\Delta aas::UcTE$. For example, upregulation of *sigE* may have a positive effect on FFA production. Osanai et al. (2011) reported that overexpression of *sigE* induces genes for the OPP pathway and glycogen catabolism, leading to increase in sugar catabolic enzymes and decrease in cellular glycogen content. Later, the same group reported that SigE-overexpression is also effective to increase bioplastic production during nitrogen starvation (Osanai et al., 2013a) and hydrogen production under micro-oxic conditions (Osanai et al., 2013b).

Differences in metabolite levels were less evident than those in transcript levels between FFA-producing and -nonproducing strains (Fig. 6B). When metabolite levels were compared between $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$ (Fig. 6A), accumulation of sugar phosphates belonging to the glycolytic and OPP pathways in $\Delta cyabrB2\Delta aas::UcTE$ was evident, as observed in $\Delta cyabrB2$ (Kaniya et al., 2013). Highly accumulated OPP pathway intermediates in the mutant

suggest that NADPH is actively produced via OPP pathway which is one of the major NADPH production pathways (Young et al., 2011). It is possible that higher activity of NADPH production in $\Delta cyabrB2\Delta aas::UcTE$ promotes FA synthesis in which NADPH serves as electron donor in the two reactions involving substrate reduction.

The FFA productivity attained by the $\Delta cyabrB2\Delta aas::UcTE$ strain was 22.6 mg L⁻¹, which is by no means high compared with the productivity reported in the previous studies. In this study, a minimal set of genetic modification for secretion of FFA, that is, deletion of *aas* and introduction of TE, was introduced to examine the effect of presence or absence of *cyAbrB2*. On the other hand, further genetic modification was carried out to increase the productivity in the previous studies. For example, Liu et al. (2011) attained FFA productivity of about 200 mg L⁻¹ in S.6803 by combination of genetic modification such as inhibition of polyhydroxybutyrate biosynthesis, overexpression of acetyl-CoA carboxylase and weakening of the peptidoglycan layer. Ruffing (2014) attained FFA productivity of about 130 mg L⁻¹ in *Synechococcus* sp. PCC 7002 by overexpression of RuBisCO (RbcLS) from S.7942. Moreover, these studies were performed under higher CO₂ concentration compared with this study performed under ambient CO₂ conditions. Thus, direct comparison of FFA productivity among studies should be viewed with caution.

Growth property (Fig. 1A) and photosynthetic activity (Fig. 3) of the FFA-producing strains in this study were similar to those of the FFA-nonproducing strain, although the inhibitory effect of produced FFAs on photosynthetic activity was reported in the FFA-producing strains (Ruffing and Jones, 2012). It is possible that accumulation of FFAs in the $\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$ strains has not reached to the toxic level yet. When higher productivity is achieved by further genetic modification of these strains, use of *UcTE* will be more advantageous than use of other TEs, since *UcTE* mainly produces saturated FFAs that are less toxic than unsaturated ones (Ikawa, 2004). In FFA-producing strains generated by introduction of a truncated TE from *E. coli* (*'tesA*), the proportion of unsaturated FFAs was about 40% of total FFAs (Ruffing and Jones, 2012). In the case of our FFA-producing strains generated by introduction of *UcTE*, the proportion of unsaturated FFAs is about 18% of total FFAs (Fig. 2 and Table 1), which can lower the risk of inhibition of photosynthetic activity during FFA production.

Although we expected consumption of accumulated glycogen supports active FFA production of the $\Delta cyabrB2\Delta aas::UcTE$ strain, glycogen content was similar between $\Delta cyabrB2$ and $\Delta cyabrB2\Delta aas::UcTE$ after two weeks of cultivation and still two-times higher than that in $\Delta aas::UcTE$ (Fig. 4). If these accumulated glycogen granules can be used as carbon source for FFA production, further increase of secreted FFAs is expected. Lennen and Pflieger (2012) calculated that theoretically maximum efficiency of FFA production in *E. coli* is about 0.35 g FFAs from 1 g of glucose. If all of glycogen granules accumulated in $\Delta cyabrB2\Delta aas::UcTE$ are

degraded to glucose and used for FA synthesis, it will be possible to obtain five-folds FFAs compared to the current productivity.

Excessively fixed carbon in $\Delta cyabrB2$ might be efficiently utilized for FFA production by suppressing glycogen synthesis. For this purpose, we attempted to disrupt the *agp* gene encoding glucose-1-phosphate adenylyltransferase (AGPase, Slr1176) catalyzing the synthesis of ADP-glucose, the substrate for glycogen synthesis. The complete blockage of glycogen synthesis by deletion of the *agp* gene has been reported in the WT strains of S.6803 (Miao et al., 2003) and S.7942 (Suzuki et al., 2010). We could obtain the completely disrupted mutant of the *agp* gene in $\Delta aas::UcTE$ background but not in $\Delta cyabrB2\Delta aas::UcTE$ background (data not shown). This suggests that glycogen synthesis is essential for growth of $\Delta cyabrB2$ -background mutants. An alternative strategy for utilization of highly accumulated glycogen as carbon source for FFA synthesis may be promotion of glycogen degradation. Overexpression of glycogenolysis enzymes or transcription factors regulating their expression may be effective for degradation of accumulated glycogen. For instance, Rre37, an OmpR-type response regulator of two-component regulatory system, works for activation of genes encoding glycogenolysis enzymes (Azuma et al., 2011; Tabei et al., 2007) and its overexpression actually decreases the accumulation level of glycogen in S.6803 (Osanai et al., 2014). FFAs are useful not only as biodiesel precursor, but also as precursor materials for green chemicals such as surfactants. We are now trying to introduce further genetic modifications into the $\Delta cyabrB2\Delta aas::UcTE$ strain to establish a highly efficient host for FFA production.

Figure legends

Fig.1. Growth property and FFA productivity of $\Delta cyabrB2$ and FFA-producing strains ($\Delta aas::UcTE$ and $\Delta cyabrB2\Delta aas::UcTE$) under FFA-producing conditions. (A) Growth curve. (B) FFA concentration in the entire culture including cyanobacterial cells. (C) FFA concentration on a per OD₇₃₀ basis. $\Delta cyabrB2$ (open circle), $\Delta aas::UcTE$ (open diamond) and $\Delta cyabrB2\Delta aas::UcTE$ (closed square). Data are means $\pm$ SD from biological triplicates (n=3).

Fig.2. FFA profiles produced by (A) $\Delta cyabrB2$, (B) $\Delta aas::UcTE$ and (C) $\Delta cyabrB2\Delta aas::UcTE$ after two weeks of cultivation. FFAs were extracted from the entire culture including cyanobacterial cells. The relative amounts of FFA species were shown based on results of GC analysis obtained from biological triplicates (n=3).

Fig.3. Effect of FFA production on photosynthetic activity after 1, 2, 7, and 14 days of cultivation.

Photosynthetic O₂-evolving activity was measured with CO₂ as the electron acceptor under (A) growth light conditions (40 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) or (B) saturating light conditions (1500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) and shown on a per OD₇₃₀ basis. $\Delta\text{cyabrB2}$ (open circle), $\Delta\text{aas::UcTE}$ (open diamond) and $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ (closed square). Data are means $\pm$ SD from biological triplicates (n=3).

Fig.4. Effect of FFA production on intracellular **glycogen content** after 1, 2, 7, and 14 days of cultivation. $\Delta\text{cyabrB2}$ (open circle), $\Delta\text{aas::UcTE}$ (open diamond) and $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ (closed square). Data are means $\pm$ SD from biological triplicates (n=3).

Fig.5. Electron micrographs of ultrathin sections of the FFA non-producing $\Delta\text{cyabrB2}$ mutant and the FFA producing $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ mutants after two weeks of cultivation. (A) Low magnification images of $\Delta\text{cyabrB2}$ and $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ cells. (B) Magnified images of filamentous materials with high electron density observed in $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ cells.

Fig.6. Effect of FFA production on metabolite levels after a week of cultivation. (A) Comparison between $\Delta\text{aas::UcTE}$ and $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ cells. (B) Comparison between $\Delta\text{cyabrB2}$ and $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ cells. Intracellular metabolites were extracted and analyzed by CE-TOF/MS. Data are means $\pm$ SD from biological triplicates (n=3). Increased (more than 1.2-fold) and decreased (less than 0.8-fold) metabolite levels are indicated in red and blue, respectively.

Table 1 Amounts of produced FFAs after 2 weeks of cultivation. **FFAs were extracted from the entire culture including cyanobacterial cells.** Data are means $\pm$ SD from biological triplicates (n=3).

Table 2 Top 50 genes whose expression levels were lower in $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ than in $\Delta\text{cyabrB2}$.

The FPKM values are means $\pm$ SD from biological triplicates of RNA-seq analysis. The ratio of the FPKM values of $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ to those of $\Delta\text{cyabrB2}$ is also shown.

Table 3 Top 50 genes whose expression levels were higher in $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ than in $\Delta\text{cyabrB2}$.

The FPKM values are means $\pm$ SD from biological triplicates of RNA-seq analysis. The ratio of the FPKM values of $\Delta\text{cyabrB2}\Delta\text{aas::UcTE}$ to those of $\Delta\text{cyabrB2}$ is also shown.

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Table 1
Amounts of produced FFA after 2 weeks of cultivation. Data are means $\pm$ SD from biological triplicates (n=3).

Strain	Fatty acid content ($\mu\text{mol/L}$)								Total
	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	
<i>$\Delta cyabrB2$</i>	0	0	0.13	0	0.04 $\pm$ 0.1	0.01 $\pm$ 0.1	0	0	0.18 $\pm$ 0.1
<i>$\Delta aas::\text{UcTE}$</i>	14.99 $\pm$ 2.73	1.94 $\pm$ 0.49	6.63 $\pm$ 3.62	2.06 $\pm$ 0.93	3.52 $\pm$ 0.86	0.87 $\pm$ 0.75	2.95 $\pm$ 1.11	0.13 $\pm$ 0.04	34.09 $\pm$ 8.22
<i>$\Delta cyabrB2\Delta aas::\text{UcTE}$</i>	26.5 $\pm$ 3.41	3.95 $\pm$ 0.23	11.97 $\pm$ 3.79	4.17 $\pm$ 0.64	6.45 $\pm$ 1.03	3.33 $\pm$ 0.4	3.58 $\pm$ 0.46	0.21 $\pm$ 0.03	61.18 $\pm$ 3.78

Table 2
Top 50 genes whose expression levels were lower in $\Delta cyabrB2\Delta aas::UcTE$ than in $\Delta cyabrB2$.
The FPKM values are means $\pm$ SD from biological triplicates of RNA-seq analysis. The ratio of
the FPKM values of $\Delta cyabrB2\Delta aas::UcTE$ to those of $\Delta cyabrB2$ is also shown.

Gene No.	Gene symbol	Definition	$\Delta cyabrB2\Delta aas::UcTE$ / $\Delta cyabrB2$	$\Delta cyabrB2\Delta aas::UcTE$		$\Delta cyabrB2$	
			FPKM ratio	FPKM	SD	FPKM	SD
<i>slr0915</i>		endonuclease	0.05	227.06	317.41	4335.67	2140.86
<i>sll1796</i>	<i>petJ</i>	cytochrome c553	0.11	90.25	13.22	838.50	180.22
<i>sll1450</i>	<i>nrtA</i>	ABC-type nitrate/nitrite transport system substrate-binding protein	0.13	28.22	1.63	209.60	113.82
<i>sll1451</i>	<i>nrtB</i>	ABC-type nitrate/nitrite transport system permease protein	0.15	34.72	1.58	235.26	135.83
<i>slr0898</i>	<i>nirA</i>	ferredoxin--nitrite reductase	0.15	63.30	5.73	421.91	196.58
<i>sll1452</i>	<i>nrtC</i>	ABC-type nitrate/nitrite transport system ATP-binding protein	0.15	44.53	1.42	288.67	141.08
<i>slr1512</i>	<i>sbtA</i>	sodium-dependent bicarbonate transporter	0.18	38.00	2.23	214.30	193.53
<i>slr0601</i>		hypothetical protein	0.18	198.53	40.46	1103.43	332.57
<i>sll1453</i>	<i>nrtD</i>	ABC-type nitrate/nitrite transport system ATP-binding protein	0.20	42.27	1.94	211.59	79.26
<i>slr0041</i>	<i>cmpB</i>	ABC-type bicarbonate transport system permease protein	0.23	7.39	0.59	32.70	22.14
<i>slr0009</i>	<i>rbcL</i>	ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	0.24	1598.72	66.56	6676.71	2161.66
<i>sll0783</i>		hypothetical protein	0.25	48.45	1.74	190.68	29.41
<i>smr0003</i>	<i>petM</i>	cytochrome b6-f complex subunit PetM	0.26	1141.24	954.34	4342.48	1561.35
<i>sll1621</i>		glutathion-dependent peroxidase, type II peroxiredoxin	0.27	663.35	3.27	2500.11	916.08
<i>sll0219</i>	<i>flv2</i>	potential FMN-protein	0.28	23.98	1.27	85.10	58.25
<i>slr0011</i>	<i>rbcX</i>	possible Rubisco chaperonin	0.28	1364.97	68.07	4832.52	1577.96
<i>sll0108</i>	<i>amt1</i>	ammonium/methylammonium permease	0.28	484.65	45.82	1707.46	672.95
<i>slr0899</i>	<i>cynS</i>	cyanate lyase	0.29	63.39	3.67	221.90	73.60
<i>slr0288</i>	<i>glnN</i>	glutamate-ammonia ligase, glutamine synthetase type III	0.29	75.91	2.80	264.08	41.35
<i>sll1733</i>	<i>ndhD3</i>	NADH dehydrogenase I chain M, involved in inducible high-affinity CO ₂ uptake	0.29	55.94	1.49	194.38	158.76
<i>slr0012</i>	<i>rbcS</i>	ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit	0.29	1820.76	64.94	6235.87	2257.49
<i>slr1513</i>	<i>sbtB</i>	plasma membrane protein of unknown function	0.30	129.16	4.75	435.09	336.27
<i>sll0218</i>		hypothetical protein	0.30	16.04	0.50	53.92	39.60
<i>ssl0020</i>	<i>petF</i>	ferredoxin I, essential for growth, expression is induced by light	0.31	3855.26	110.63	12567.53	364.36
<i>sll1600</i>	<i>mntB</i>	ABC-type manganese transport system permease protein	0.31	23.08	3.50	73.38	17.31
<i>slr1593</i>		hypothetical protein	0.33	39.67	2.53	120.32	5.47
<i>sll1734</i>	<i>cupA</i>	involved in inducible high-affinity CO ₂ uptake.	0.33	63.35	0.66	191.36	140.97
<i>ssl2542</i>	<i>hliA</i>	high light inducible protein	0.34	39.92	8.36	118.40	25.74
<i>sll1327</i>	<i>atpC</i>	ATP synthase gamma chain	0.34	367.40	10.54	1068.24	140.89
<i>slr0701</i>	<i>merR</i>	mercuric resistance operon regulatory protein	0.35	11.38	1.82	32.66	1.42
<i>sll0384</i>		ABC-type cobalt transport system permease protein	0.35	54.00	2.58	154.00	5.18
<i>sll1471</i>	<i>cpcG2</i>	phycobilisome rod-core linker polypeptide Lrc, C-phycocyanin associated	0.36	161.42	10.13	444.56	275.21
<i>slr1330</i>	<i>atpE</i>	ATP synthase epsilon chain of CF(1)	0.36	453.73	18.03	1246.14	223.47
<i>ssr2153</i>		hypothetical protein	0.37	28679.43	3362.84	78396.37	25273
<i>sll1119</i>		hypothetical protein	0.38	64.83	5.21	172.82	19.07
<i>sll1696</i>		hypothetical protein	0.38	144.06	15.19	380.63	157.31
<i>sll1599</i>	<i>mntA</i>	ABC-type manganese transport system ATP-binding protein	0.38	45.12	15.73	118.97	18.02
<i>sll1454</i>	<i>narB</i>	ferredoxin--nitrate reductase	0.38	19.53	0.33	51.09	9.91
<i>sll0944</i>		hypothetical protein	0.38	86.71	5.51	225.85	38.30
<i>sll1638</i>	<i>psbQ</i>	photosystem II extrinsic protein PsbQ	0.38	669.83	33.29	1740.90	705.59
<i>sll1483</i>		periplasmic protein, similar to transforming growth factor induced protein	0.39	165.48	19.15	428.70	79.09
<i>ssl2084</i>	<i>acpP</i>	acyl carrier protein	0.39	409.05	7.72	1047.58	191.99
<i>slr1761</i>	<i>ytfC</i>	FKBP-type peptidyl-prolyl cis-trans isomerase, periplasmic protein	0.39	210.62	4.22	534.68	117.42
<i>slr1600</i>		hypothetical protein	0.40	167.15	4.74	418.74	13.61
<i>slr2043</i>	<i>zntC</i>	ABC-type zinc transport system substrate-binding protein	0.40	5.49	0.35	13.63	0.90
<i>sll0330</i>	<i>fabG</i>	sepiapterine reductase	0.40	143.68	7.66	356.64	195.07
<i>slr1289</i>	<i>icd</i>	isocitrate dehydrogenase (NADP)	0.41	47.53	5.70	116.57	7.40
<i>sll1326</i>	<i>atpA</i>	ATP synthase alpha chain of F1	0.41	513.09	9.22	1257.92	279.70
<i>sll0385</i>		ABC-type cobalt transport system ATP-binding protein	0.41	32.56	1.39	78.97	7.12
<i>sll0335</i>		hypothetical protein	0.41	36.26	0.85	87.55	1.97

Table 3
Top 50 genes whose expression levels were higher in $\Delta cyabrB2\Delta aas::UcTE$ than in $\Delta cyabrB2$.
The FPKM values are means $\pm$ SD from biological triplicates of RNA-seq analysis. The ratio of the FPKM values of $\Delta cyabrB2\Delta aas::UcTE$ to those of $\Delta cyabrB2$ is also shown.

Gene No.	Gene symbol	Definition	$\Delta cyabrB2\Delta aas::UcTE$ / $\Delta cyabrB2$	$\Delta cyabrB2\Delta aas::UcTE$		$\Delta cyabrB2$	
			FPKM ratio	FPKM	SD	FPKM	SD
<i>slr0450</i>	<i>norB</i>	cytochrome b subunit of nitric oxide reductase	11.58	2645.74	112.27	228.49	23.47
<i>ssr2062</i>		hypothetical protein	9.34	5284.97	295.08	565.81	68.17
<i>slr1898</i>		hypothetical protein	7.72	860.26	28.45	111.40	18.30
<i>slr1515</i>	<i>gltB</i>	glutamine synthetase inactivating factor IF17	7.56	2793.71	348.54	369.71	174.38
<i>slr0451</i>		hypothetical protein	7.32	268.51	9.73	36.70	4.62
<i>slr0804</i>	<i>dacB</i>	D-alanyl-D-alanine carboxypeptidase	7.17	151.65	16.49	21.16	2.30
<i>slr0334</i>		hypothetical protein	7.00	1037.63	15.34	148.21	25.82
<i>slr1689</i>	<i>sigE</i>	RNA polymerase sigma factor, group 2, nitrogen-regulated	6.95	220.64	18.03	31.76	2.64
<i>slr0333</i>		hypothetical protein	6.68	1498.45	50.79	224.33	29.74
<i>slr0556</i>		hypothetical protein	6.59	308.39	8.00	46.78	4.07
<i>slr0108</i>		hypothetical protein	5.36	121.05	5.66	22.60	2.44
<i>ssr1256</i>		hypothetical protein	5.26	741.07	37.85	140.90	5.05
<i>slr0301</i>	<i>ppsA</i>	phosphoenolpyruvate synthase	4.83	365.68	17.83	75.71	22.77
<i>slr0622</i>	<i>nadA</i>	quinolinate synthetase	4.81	245.46	5.40	51.08	11.34
<i>slr1127</i>	<i>menB</i>	1,4-dihydroxy-2-naphthoate synthase	4.33	662.83	40.12	153.15	15.44
<i>slr1495</i>		hypothetical protein	4.10	218.60	13.29	53.38	3.85
<i>ssr1911</i>	<i>gltA</i>	glutamine synthetase inactivating factor IF7	3.97	8579.28	732.64	2158.73	602.30
<i>slr1851</i>		hypothetical protein	3.87	104.72	10.45	27.05	3.18
<i>slr0253</i>		hypothetical protein	3.84	1204.99	34.58	313.96	127.65
<i>slr1899</i>	<i>ctaB</i>	protoheme IX farnesyltransferase	3.72	353.01	19.08	94.94	9.25
<i>ssr2227</i>		putative transposase of unclassified IS	3.60	179.75	17.61	49.91	9.68
<i>slr1251</i>		hypothetical protein	3.44	80.64	3.77	23.46	2.79
<i>slr1667</i>		hypothetical protein	3.39	419.90	29.07	123.69	9.19
<i>slr0572</i>		hypothetical protein	3.35	296.44	6.42	88.62	23.82
<i>slr1634</i>		hypothetical protein	3.34	54722.07	1603.52	16366.70	15003
<i>slr2057</i>	<i>apqZ</i>	water channel protein	3.27	627.47	34.43	191.61	64.15
<i>slr0321</i>	<i>era</i>	GTP-binding protein Era	3.25	38.23	1.01	11.75	1.63
<i>slr0242</i>		hypothetical protein	3.19	53.66	1.04	16.80	3.64
<i>slr0374</i>		putative cell division cycle protein	3.18	21831.30	971.25	6870.10	362.94
<i>slr1864</i>		chloride channel protein	3.14	150.22	18.56	47.83	4.54
<i>slr0813</i>	<i>ctaC</i>	cytochrome c oxidase subunit II	3.13	536.18	13.60	171.49	18.75
<i>slr0185</i>		hypothetical protein	3.07	1323.75	60.24	431.78	126.93
<i>slr1915</i>		hypothetical protein	3.03	1026.84	212.41	338.68	43.14
<i>slr0787</i>		nicotinamide-nucleotide adenyllyltransferase / ADP compounds hydrolase	2.95	54.91	0.81	18.60	6.21
<i>ssr1038</i>		hypothetical protein	2.89	1393.64	119.59	481.40	63.49
<i>slr0373</i>		hypothetical protein	2.89	59130.40	3003.24	20459.30	623.55
<i>ssr0296</i>		putative transposase gene of IS5 family insertion sequence ISY523p	2.88	1.12	1.02	0.39	0.67
<i>slr0109</i>		hypothetical protein	2.80	51.69	0.73	18.48	0.93
<i>ssr1562</i>		hypothetical protein	2.79	3987.62	245.72	1429.20	282.89
<i>slr2082</i>	<i>ctaD</i>	cytochrome c oxidase subunit I	2.76	422.69	8.13	153.11	22.25
<i>slr0199</i>	<i>petE</i>	plastocyanin	2.74	6510.87	589.74	2372.79	340.10
<i>slr0853</i>	<i>rimI</i>	ribosomal-protein-alanine acetyltransferase	2.72	138.64	14.85	50.91	3.10
<i>slr0272</i>		hypothetical protein	2.68	16.10	0.56	6.02	0.70
<i>slr0990</i>		formaldehyde dehydrogenase (glutathione) / alcohol dehydrogenase	2.66	301.50	3.12	113.38	12.53
<i>slr0797</i>	<i>coaT</i>	cobalt-transporting P-type ATPase CoaT or CorT (cobalt efflux pump) involved in cobalt tolerance	2.65	563.62	8.46	212.80	63.93
<i>slr1258</i>		hypothetical protein	2.64	348.02	34.40	131.97	18.98
<i>ssr3129</i>		hypothetical protein	2.61	1181.37	143.20	451.82	51.08
<i>slr0443</i>		hypothetical protein	2.60	385.61	20.17	148.34	15.33
<i>slr1851</i>		hypothetical protein	2.59	2267.45	241.73	875.20	130.90
<i>slr1285</i>	<i>hik34</i>	two-component sensor histidine kinase	2.57	61.20	6.22	23.81	2.04

Figure 1

Figure 2

$\Delta cyabrB2$

$\Delta aas::UcTE$

$\Delta cyabrB2\Delta aas::UcTE$

Figure 3

(A)

(B)

Figure 4

Figure 5

(A)

$\Delta cyabrB2$

$\Delta cyabrB2\Delta 1609::BTE$

(B)

Figure 6

(A)

(B)