Combinatorial deletions of *glgC* and *phaCE* enhance ethanol production in *Synechocystis* sp. PCC 6803

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Abstract

Synechocystis sp. PCC 6803 is an attractive host for bio-ethanol production. In the present study, a nitrogen starvation approach was applied on an ethanol producing strain for inhibiting the growth, since ethanol production competes with the cell growth. The effect of gene deletions in the glycogen and polyhydroxybutyrate (PHB) synthesis pathways was investigated. Measurements of intracellular glycogen and PHB revealed that the glycogen was accumulated under the nitrogen starvation condition and the gene deletion of glycogen synthesis pathway caused the accumulation of PHB. The ethanol producing strain harboring deletions for both the glycogen and the PHB synthesis pathways ($\Delta\text{glgC}\Delta\text{phaCE}/\text{EtOH}$) produced ethanol at the specific rate of $240 \text{ mg g (dry cell weight)}^{-1} \text{ day}^{-1}$ under the nitrogen starvation condition. In a high cell density culture ($\text{OD}_{730} = 50$) using this $\Delta\text{glgC}\Delta\text{phaCE}/\text{EtOH}$ strain, the ethanol production rates were 1.08 and $2.01 \text{ g L}^{-1} \text{ day}^{-1}$ under light conditions of 40 and $80 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$, respectively.

Keywords: ethanol production, nitrogen starvation, *Synechocystis* sp. PCC 6803, glycogen, polyhydroxybutyrate

1. Introduction

Ethanol is the most widely used bio-fuel, and currently 90 billion liters of ethanol is produced from feedstock such as cornstarch (Gombert and Maris, 2015). Since the bio-ethanol production using grain competes with food production, the bioprocess using photosynthetic microorganism, which can convert CO₂ to ethanol, is attracting attention (Deng and Coleman, 1999; Dexter and Fu, 2009; Gao et al., 2012). Cyanobacteria are well studied photosynthetic microorganism, and can be photoautotrophically grown using CO₂ and sunlight. Deng and Coleman (1999) achieved 0.23 g L⁻¹ of ethanol production using *Synechococcus elongates* PCC 7942 (formerly, *Anacystis nidulans* R2) by introducing *pdc* encoding for pyruvate decarboxylase, and *adhII* encoding for alcohol dehydrogenase from *Zymomonas mobilis*. The ethanol production in cyanobacteria was further enhanced to 5.5 g L⁻¹ (212 mg L⁻¹ day⁻¹) by introducing two copies of exogenous *pdc* from *Z. mobilis* and endogenous alcohol dehydrogenase gene (*slr1192*) and by disrupting a polyhydroxybutyrate (PHB) synthesis pathway in *Synechocystis* sp. PCC 6803 (hereafter, PCC 6803) (Gao et al., 2012). The control of the central metabolic pathway should be combined to further enhance the ethanol production. Yoshikawa et al. (2014) suggested that an optimal level of malic enzyme activity is required for ethanol production in PCC 6803. It was also reported that the deletion of the gene encoding glucose-1-phosphate adenylyltransferase (*glgC*) in the glycogen biosynthesis pathway rewired the carbon flow from glycogen storage to isobutanol synthesis in *S. elongates* PCC 7942 (Li et al., 2014).

Growth inhibition is a useful approach for enhancing production of target biomolecule because the biomass synthesis and the target production compete with cell growth in terms of carbon usage in many cases. Starvation of essential nutrient such as nitrogen is commonly used for bio-production in cyanobacteria (Osanai et al., 2013; van der Woude et al., 2014). It has been reported that the nitrogen starvation causes an accumulation of the intracellular glycogen (Allen, 1984; Hickman et al., 2013). Furthermore, a disruption mutant of *glgC* encoding glycogen synthesis pathway enzyme excreted the pyruvate (Pyr) and α -ketoglutarate (AKG) into the culture under the nitrogen starvation (Carrieri et al., 2012; Grundel et al., 2012). van der Woude et al. (2014) successfully produced lactate under the nitrogen starvation conditions by introducing the *ldh* gene of *Lactococcus lactis* in a PCC 6803 Δ *glgC* mutant. The nitrogen starvation approach has not yet been applied to the ethanol production in PCC 6803.

In the present study, the nitrogen starvation approach was applied to the ethanol producing PCC 6803 strain. The results showed that the combinatorial deletions of the

glycogen synthesis pathway and the PHB synthesis pathway enhanced the ethanol production under the nitrogen starvation condition.

2. Material and methods

2.1 Strain and culture conditions

Synechocystis sp. PCC 6803, a glucose-tolerant strain, was used as the candidate strain. PCC 6803 was cultivated in modified BG11 medium (Yoshikawa et al., 2013), the pH of which was adjusted to 7.5 using 1 M KOH (hereafter BG11). Modified BG11 medium without NaNO₃ was used as a nitrogen-free BG11 medium. Culture experiments were performed using flasks at 34°C with shaking using bio-shaker BR-43FL (TAITEC, Saitama, Japan) under continuous light illumination by a light-emitting diode (LED) plate (LC-LED 450W, TAITEC, Saitama, Japan). The flask was capped with a non-porous plug to avoid evaporation of produced ethanol. Carbon source was supplied by addition of NaHCO₃ to the culture medium.

2.2 Culture experiment for ethanol production

Pre-culture was performed in 500-mL Erlenmeyer flasks with 100 mL of BG11 containing 100 mM NaHCO₃ with rotary agitation at 150 rpm under continuous light illumination at 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The pre-cultured cells were centrifuged and washed by nitrogen-free BG11 twice. The cells were inoculated to achieve an optical density of 4 in the culture at 730 nm (OD₇₃₀) in 20 mL of fresh BG11 with or without nitrogen in 100-mL Erlenmeyer flasks. The main culture was performed with rotary agitation at 150 rpm under continuous light illumination at 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The pH was periodically adjusted at pH 7.5, and did not exceed pH 12 at any given time. NaHCO₃ was added to the culture medium at a final concentration of 100 mM every 12 h to supply carbon source.

For high-cell-density ethanol production, pre-culture was performed in 500-mL Erlenmeyer flasks with 150 mL of BG11 containing 10 mM NaHCO₃ with rotary agitation at 150 rpm under continuous light illumination at 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ until the OD₇₃₀ reached approximately 3. The pre-cultured cells were centrifuged and washed by nitrogen free BG11 twice. The cells were inoculated into 20 mL of fresh nitrogen free BG11 in 500-mL Erlenmeyer flasks to achieve an optical density of OD₇₃₀ of 50. The main culture was performed with reciprocal shaking at 200 strokes-per-min under continuous light illumination at 40 or 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$. pH was periodically adjusted at pH 7.5, never exceeding pH 12. NaHCO₃ was added to the culture medium at a final concentration of 100 mM every 12 h to supply carbon source.

2.3 Strain construction

Strains used in this study are summarized in Table 1. The ethanol producing strain of PCC 6803, named 6803/EtOH, was constructed by introduction of *pdh* and *adhII* from *Z. mobilis* under control of the promoter of *nblA* (*ssl0452*) in our previous study (Yoshikawa et al., 2014). It has been reported that a nitrogen starvation leads to *nblA* induction (Richaud et al., 2001). Due to evaluate the ethanol productions under the nitrogen starvation condition, the *nblA* promoter was chose to stably induce the gene expressions of ethanol synthesis pathway under the condition.

The deletion strain of *glgC* (*slr1176*) in the 6803/EtOH, named Δ *glgC*/EtOH, was constructed by replacing *glgC* with kanamycin resistance gene (*Km^r*) as follows. The upstream and downstream regions of *glgC* were amplified by PCR with the primers *glgC_up_F* and *glgC_up_R*, and *glgC_down_F* and *glgC_down_R*, respectively. KOD-plus-NEO (Toyobo, Japan) was used for PCR. The primers used in this study are listed in Supplementary Table S1. *Km^r* was amplified from pHSG298 (TakaraBio, Siga, Japan) using the primers *Km_F* and *Km_R*. *glgC_up_R* and *glgC_down_F* containing overlapping regions for the amplified *Km^r*. The three PCR products were connected using overlap-extension PCR (OE-PCR) with the primers *glgC_up_F* and *glgC_down_R*. The resulting PCR product was introduced into the 6803/EtOH. The transformants were screened on BG11 plates containing 20 $\mu\text{g mL}^{-1}$ of kanamycin, and then restreaked on fresh BG11 plates containing kanamycin. Complete segregation of *glgC* by *Km^r* gene was confirmed by the difference in the size of the PCR products generated using the primers *glgC_check_F* and *glgC_check_R*, and *glgC_checkF* and *glgC_int_F*, respectively (Supplementary Fig. S1).

The deletion strain of *phaC* (*slr1830*) and *phaE* (*slr1829*) in the 6803/EtOH, named Δ *phaCE*/EtOH, was constructed by replacing *phaCE* with chloramphenicol resistance gene (*Cm^r*) in a way similar to the construction of the Δ *glgC*/6803. The upstream and downstream regions of *phaCE* were amplified by PCR with the primers *phaCE_up_F* and *phaCE_up_R*, and *phaCE_down_F* and *phaCE_down_R*, respectively. *Cm^r* was amplified from pHSG398 (TakaraBio, Siga, Japan) with the primers *Cm_F* and *Cm_R*. The three PCR products were connected by OE-PCR, and the resulting PCR product was introduced into the 6803/EtOH. The transformants were screened on BG11 plates containing 10-20 $\mu\text{g mL}^{-1}$ of chloramphenicol. Complete segregation of *phaCE* by *Cm^r* gene was confirmed by PCR using the primers *phaCE_check_F* and *phaCE_check_R*, and *phaCE_checkF* and *phaCE_int_F*, respectively (Supplementary Fig. S1). The deletion strain of *glgC* (*slr1176*) in the Δ *phaCE*/EtOH, named

ΔglgCΔphaCE/EtOH, was constructed by the same way as used for constructing the ΔglgC/EtOH

2.4 Analyses of cell, **PBS and Chlorophyll a concentrations**

Cell concentration was estimated by measuring the optical density of the culture at 730 nm using a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan). Whole-cell absorption spectrum between 400-800 nm was measured using a spectrophotometer (DU800, Beckman Coulter, CA, USA). PBS and Chlorophyll a concentration were calculated based on the previous study (Moller et al., 2014).

2.5 Ethanol measurement

Ethanol concentration of the culture broth was measured by a gas chromatograph system with hydrogen flame ionization detector (7890A; Agilent Technologies, CA, USA) and equipped with a Stabilwax column (0.32-mm internal diameter, 60-m length, and 1-μm thickness; Restek, PA, USA). The inlet temperature was set to 250°C. The column oven temperature was initially set at 70°C for 3 min, and then increased to 200°C at 10°C/min. 3-methyl-1-butanol was used as an internal standard for quantification.

2.6 Glycogen measurement

Glycogen was extracted from cell according to the protocol of a previous study (Ernst et al., 1984). Culture broth was centrifuged and cell pellet was washed by 1 mL of water twice. Cell pellet was hydrolyzed by 200 μL of 30% KOH (g/L) at 95°C for 90 min. Sample was cooled on ice and incubated with 600 μL of cooled ethanol for 1 h. The sample was centrifuged and mixed with 600 μL of cooled ethanol twice. The sample was then dried and re-suspended in 100 μL of water. Glycogen concentration in the supernatant of the centrifuged sample was measured by EnzyChrom Glycogen Assay Kit (E2GN-100, BioAssay Systems, CA, USA).

2.7 PHB measurement

Intracellular PHB was measured according to the protocol of a previous study (Karr et al., 1983; Tyo et al., 2006; Taroncher-Oldenburg and Stephanopoulos, 2000). Cells were centrifuged and the cell pellet was washed with cold water. The cell pellet was dried at 80°C for 16 h, and then boiled in 1 mL of concentrated sulfuric acid for 1 h to decompose PHB to crotonic acid. The sample was diluted in 4 mL of 14 mM sulfuric acid. The supernatant of the centrifuged sample was analyzed using a high-pressure

liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (Bio-Rad, CA, USA). The column temperature was kept at 65°C; the mobile phase, 2 mM H₂SO₄, was run at 0.5 mL/min; and the eluent absorbance was detected at 210 nm. The conversion factor 0.86 was used to calculate PHB concentration from crotonic acid (Taroncher-Oldenburg and Stephanopoulos, 2000).

2.8 Organic acids measurement

Organic acids in the culture broth were measured by HPLC equipped with an Aminex HPX-87H column (Bio-Rad, CA, USA). The column temperature was kept at 65°C; the mobile phase, 2 mM H₂SO₄, was run at 0.5 mL/min; and the eluent absorbance was detected at 210 nm.

2.9 Calculation of carbon fixation

The amount of carbon fixation was calculated using the concentrations of ethanol, organic acids, glycogen, PHB and OD₇₃₀ at 0 and 48 h. The OD₇₃₀ was converted into dry cell weight (g-DCW) using the measured conversion factor (g-DCW L⁻¹ OD₇₃₀⁻¹). To determine the conversion factor, the cells were harvested from 8 mL of culture broth at 48 h on membrane filter (0.45 µm, ADVANTEC), and dry cell weight was measured after dried up at 60 °C for overnight. The conversion factors for each condition were listed in Supplementary Table S2. Because the amounts of glycogen and PHB were significantly changed among the strains, these carbon storage compounds were not included in the carbon content of biomass. The total carbon content of biomass, glycogen and PHB was estimated from the dry cell weight (gDCW L⁻¹) using a conversion coefficient (0.5 C-g gDCW⁻¹) based on the atomic composition of cyanobacterial cells (Yu et al., 2013). The carbon content of biomass was calculated by subtraction of glycogen and PHB contents from the total carbon content. Molecular weights of glycogen and PHB were assumed to be 666 (number of carbon atoms is 86) and 86 (number of carbon atoms is 4), respectively.

3. Results and discussion

3.1 Ethanol production under nitrogen starvation conditions

The effects of deletions of the glycogen synthesis pathway and the PHB synthesis pathway in the ethanol producing PCC 6803 strains were investigated using 6803/EtOH, ΔglgC/EtOH and ΔglgCΔphaCE/EtOH. Initially, the strains were growth in normal BG11 media. The cells were transferred into fresh BG11 media with or without nitrogen,

and were cultured under continuous light illumination at 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Fig. 1 shows the time-course of cells, ethanol, AKG, and Pyr concentrations of strains 6803/EtOH, ΔglgC /EtOH, and $\Delta\text{glgC}\Delta\text{phaCE}$ /EtOH under each culture condition.

Slight growth inhibition was observed in the ΔglgC /EtOH and $\Delta\text{glgC}\Delta\text{phaCE}$ /EtOH strains in the presence of nitrogen. The ethanol production after 72 h was 210 mg L^{-1} in the 6803/EtOH strain, and increased to 297 and 332 mg L^{-1} in the ΔglgC /EtOH and $\Delta\text{glgC}\Delta\text{phaCE}$ /EtOH strains, respectively. The results indicated that the ΔglgC /EtOH and $\Delta\text{glgC}\Delta\text{phaCE}$ /EtOH strains diverted carbon from cell growth to ethanol production. No by-products, such as AKG and Pyr, were detected by HPLC in this condition. The growth of the ΔglgC /EtOH and the $\Delta\text{glgC}\Delta\text{phaCE}$ /EtOH strains stopped completely under the nitrogen starvation conditions, whereas that of the 6803/EtOH strain continued. It has been reported that cyanobacteria degrade their photosynthetic antenna protein and use it as a nitrogen source for cell growth under such conditions (Carrieri et al., 2012).

The ethanol production rate of the 6803/EtOH strain under the nitrogen starvation condition ($30.9 \pm 0.8 \text{ mg gDCW}^{-1} \text{ day}^{-1}$), was decreased as compared to that in the presence of nitrogen ($47.7 \pm 0.3 \text{ mg gDCW}^{-1} \text{ day}^{-1}$). In contrast, the ethanol production rates of the ΔglgC /EtOH and the $\Delta\text{glgC}\Delta\text{phaCE}$ /EtOH strains increased from 70.5 ± 1.0 and $86.4 \pm 2.1 \text{ mg gDCW}^{-1} \text{ day}^{-1}$ to 192.4 ± 2.4 and $239.6 \pm 2.7 \text{ mg gDCW}^{-1} \text{ day}^{-1}$, respectively under the nitrogen starvation. Gao et al. (2012) reported that the ethanol production rate increased by engineering the expressions of *pdc* and *slr1192*. The $\Delta\text{glgC}\Delta\text{phaCE}$ /EtOH strain has a potential to further enhance the ethanol production by engineering of ethanol synthesis pathway. The secretions of Pyr and AKG were observed in the ΔglgC /EtOH and the $\Delta\text{glgC}\Delta\text{phaCE}$ /EtOH strains under the nitrogen starvation conditions.

3.2 Degradations of phycobilisome and chlorophyll

Cell bleaching was observed in the 6803/EtOH strain under the nitrogen starvation condition (Supplementary Fig. S2). The bleaching is caused by the degradation of phycobilisome (Sato et al., 2008). It has been suggested that the degradation of phycobilisome is important to satisfy the cellular nitrogen demand under nitrogen-starved condition in cyanobacteria (Grossman et al., 1993). The relative contents of phycobilisome and chlorophyll in the cells are shown in Fig. 2. Although the contents of phycobilisome and chlorophyll rapidly decreased in 6803/EtOH during the nitrogen starvation, these reductions were alleviated in the ΔglgC /EtOH and $\Delta\text{glgC}\Delta\text{phaCE}$ /EtOH strains. It has been reported that the photosystem II centers in a

glgC mutant in *Synechococcus* sp. strain PCC 7002 were protected from degradation because they entered a quiescent under nitrogen starvation (Jackson et al., 2015). The contents of phycobilisome and chlorophyll were almost constant in the all strains in the presence of nitrogen.

3.3 Intracellular contents of glycogen and PHB

Glycogen and PHB are major storage components for carbon and energy in PCC 6803. The contents of glycogen and PHB in the cells at 48 h were measured in the presence or absence of nitrogen. T

he 6803/EtOH strain under the nitrogen starvation condition contained 4.9 times more glycogen than in the presence of nitrogen (Fig. 3). It has been reported that PCC 6803 produces glycogen and stores it for survival under the starvation condition (Grundel et al., 2012). As expected, the glycogen content of the Δ *glgC*/EtOH and the Δ *glgC* Δ *phaCE*/EtOH strains, which do not possess the glycogen synthesis pathway, significantly decreased under both conditions. PHB was not detected in the 6803/EtOH strain under the nitrogen starvation condition. There is a report that the PCC 6803 accumulates the PHB under a nitrogen starvation condition (Schlebusch and Forchhammer, 2010). The difference might be owing to differences of culture conditions, such as temperature, light intensity and cell density. In the Δ *glgC*/EtOH strain, approximately 36 mg gDCW⁻¹ of PHB was accumulated in the cells. As expected, PHB was not detected in the Δ *glgC* Δ *phaCE*/EtOH strain. No PHB production was detected in all of the strains in the presence of nitrogen.

It has been reported that the deletion of the PHB synthesis pathway does not contribute to the enhanced ethanol production in PCC 6803 (Gao et al., 2012). In the present study, PHB production was not observed in the 6803/EtOH, whereas it was in the Δ *glgC*/EtOH strain. The result suggests that the deletion of PHB synthesis pathway only contributes to ethanol production in glycogen synthesis pathway deletion strain, and is consistent with the culture results shown in Fig. 1.

3.4 Amount of carbon fixation

The amount of carbon fixation was calculated using the concentrations of ethanol, organic acids, glycogen, PHB, and cells at 0 and 48 h (Fig. 4). The carbon content of the cells was calculated based on the atomic composition of cyanobacterial cells (Yu et al., 2013). Because the amounts of glycogen and PHB were significantly changed among the strains (Fig. 3), these carbon storage compounds were not included in the carbon

content of biomass. The total amount of carbon fixation in the absence of nitrogen was lower than that in the presence of nitrogen in all strains. Deletion of the glycogen synthesis pathway caused further decrease in the total amount of carbon fixation under the nitrogen starvation condition, whereas it significantly improved the yield of ethanol production by repression of the cell growth and glycogen production. Furthermore, deletion of the PHB synthesis pathway boosted the yield of ethanol production by repression of PHB production. However, still approximately 20% of fixed carbon was secreted as AKG in the Δ glgC Δ phaCE/EtOH strain. The excess carbons must overflow into the TCA cycle in the mutant because the strain lost the routes for carbon storages as glycogen and PHB. Cyanobacteria have an incomplete TCA cycle due to a lack of AKG dehydrogenase which converts AKG to succinyl-CoA (Pearce et al., 1969). Although the existence of the bypass pathway (γ -aminobutyrate shunt) has been recently suggested by ^{13}C -stable isotope analysis in PCC 6803 (Xiong et al., 2014), the carbons must be discarded into the culture broth as AKG because this pathway cannot afford to through the large flux. The repression of flux from pyruvate to AKG must be effective to further enhance the ethanol production. Because this route is essential, a switching system of between the ethanol formation and the TCA cycle would be effective (Soma et al., 2014) to achieve the flux change without lethal damage by downregulating the TCA cycle enzyme expression after entering the nitrogen starvation condition. Furthermore, introduction of a heterologous pathway from acetyl-CoA to ethanol via acetaldehyde might be effective. For example, AdhE of *E. coli* is a bifunctional enzyme for acetaldehyde dehydrogenase and alcohol dehydrogenase.

3.5 Ethanol production by High-cell-density culture

One of the challenges in cyanobacterial ethanol production is that it generally requires long fermentation period. To overcome this problem, a high-cell-density culture ($\text{OD}_{730} = 50$) was demonstrated using the engineering strain, Δ glgC Δ phaCE/EtOH. To evaluate the effect of light intensity on the ethanol production, 40 and 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of light conditions were examined. The time-courses of OD_{730} and ethanol concentration are shown in Fig. 5. The reason for the decreasing OD_{730} is dilution by additions of NaHCO_3 for carbon source supply and HCl for neutralization. The strain produced 2.57 ± 0.02 and $2.96 \pm 0.07 \text{ g L}^{-1}$ of ethanol at light conditions of 40 and 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively in 72 h. The ethanol production rates during the first 24 h were 1.08 and 2.01 $\text{g L}^{-1} \text{day}^{-1}$ at 40 and 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. As expected, it was increased 2-fold by raising the light intensity from 40 to 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Since the inhibition of ethanol production rate at 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was observed after 24 h, more strong light

condition was not examined. Although ethanol affects the cell growth of PCC 6803 at the concentrations of up to 10 g L⁻¹ (Dexter and Fu, 2009; Kämäräinen et al., 2012), the value is higher than the maximum ethanol concentration in this study. It would be caused by accumulation of other toxic metabolites which were produced in the high cell density condition. Addressing this problem is an important challenge in the future to enhance the ethanol production rate at the higher light intensity and to continue the ethanol production over a long period.

4. Conclusions

Cyanobacteria fix carbon from CO₂ by using the photosystem and use it for cell growth. Since ethanol production competes with the cell growth, we aimed to enhance ethanol productivity by inhibiting the growth by nitrogen starvation. The culture results suggested that the growth inhibition alone is not enough, and deletions of the glycogen and PHB synthesis pathways were additionally required to enhance the ethanol production. The ethanol production rate of ΔglgCΔphaCE/EtOH was 2.01 g L⁻¹ day⁻¹ under light condition of 80 μmol m⁻² s⁻¹ in the high cell density culture.

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References

- Allen, M.M., 1984. Cyanobacterial cell inclusions. *Annu. Rev. Microbiol.* 38, 1-25.
- Carrieri, D., Paddock, T., Maness, P.C., Seibert, M., Yu, J., 2012. Photo-catalytic conversion of carbon dioxide to organic acids by a recombinant cyanobacterium incapable of glycogen storage. *Energy Environ. Sci.* 5, 9457-9461.
- Deng, M.D., Coleman, J.R., 1999. Ethanol synthesis by genetic engineering in cyanobacteria. *Appl. Environ. Microbiol.* 65, 523-528.
- Dexter, J., Fu, P.C., 2009. Metabolic engineering of cyanobacteria for ethanol production. *Energy Environ. Sci.* 2, 857-864.
- Ernst, A., Kirschenlohr, H., Diez, J., Boger, P., 1984. Glycogen content and nitrogenase activity in *Anabaena variabilis*. *Arch. Microbiol.* 140, 120-125.
- Gao, Z.X., Zhao, H., Li, Z.M., Tan, X.M., Lu, X.F., 2012. Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria. *Energ. Environ. Sci.* 5, 9857-9865.
- Gombert, A.K., van Maris, A.J., 2015. Improving conversion yield of fermentable sugars into fuel ethanol in 1st generation yeast-based production processes. *Curr. Opin. Biotechnol.* 33C, 81-86.
- Grossman, A.R., Schaefer, M.R., Chiang, G.G., Collier, J.L., 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. *Microbiol. Rev.* 57(3), 725-749.
- Grundel, M., Scheunemann, R., Lockau, W., Zilliges, Y., 2012. Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium *Synechocystis* sp. PCC 6803. *Microbiology* 158, 3032-3043.
- Hickman, J.W., Kotovic, K.M., Miller, C., Warrenner, P., Kaiser, B., Jurista, T., Budde, M., Cross, F., Roberts, J.M., Carleton, M., 2013. Glycogen synthesis is a required component of the nitrogen stress response in *Synechococcus elongatus* PCC 7942. *Algal Research.* 2(2), 98-106.
- Jackson, S.A., Eaton-Rye, J.J., Bryant, D.A., Posewitz, M.C., Davies, F.K., 2015. Dynamics of Photosynthesis in a Glycogen-Deficient *glgC* Mutant of *Synechococcus* sp. Strain PCC 7002. *Appl. Environ. Microbiol.* 81(18), 6210-6222.
- Kämäräinen, J., Knoop, H., Stanford, N.J., Guerrero, F., Akhtar, M.K., Aro, E.M., Steuer, R., Jones, P.R., 2012. Physiological tolerance and stoichiometric potential of cyanobacteria for hydrocarbon fuel production. *J. Biotechnol.* 162(1), 67-74.
- Karr, D.B., Waters, J.K., Emerich, D.W., 1983. Analysis of Poly-beta-Hydroxybutyrate in *Rhizobium japonicum* Bacteroids by Ion-Exclusion High-Pressure Liquid

- Chromatography and UV Detection. Appl. Environ. Microbiol. 46, 1339-1344.
- Li, X., Shen, C.R., Liao, J.C., 2014. Isobutanol production as an alternative metabolic sink to rescue the growth deficiency of the glycogen mutant of *Synechococcus elongatus* PCC 7942. Photosynth. Res. 120(3), 301-310.
- Osanai, T., Numata, K., Oikawa, A., Kuwahara, A., Iijima, H., Doi, Y., Tanaka, K., Saito, K., Hirai, M.Y., 2013. Increased bioplastic production with an RNA polymerase sigma factor SigE during nitrogen starvation in *Synechocystis* sp. PCC 6803. DNA Res. 20(6), 525-535.
- Pearce, J., Leach, C.K., Carr, N.G., 1969. The incomplete tricarboxylic acid cycle in the blue-green alga *Anabaena variabilis*. J. Gen. Microbiol. 55(3), 371-378.
- Richaud, C., Zabulon, G., Joder, A., Thomas, J.C., 2001. Nitrogen or sulfur starvation differentially affects phycobilisome degradation and expression of the *nblA* gene in *Synechocystis* strain PCC 6803. J. Bacteriol. 183(10), 2989-2994.
- Sato, H., Fujimori, T., Sonoike, K., 2008. *sll1961* is a novel regulator of phycobilisome degradation during nitrogen starvation in the cyanobacterium *Synechocystis* sp. PCC 6803. FEBS Lett. 582(7), 1093-1096.
- Schlebusch, M., and Forchhammer, K., 2010. Requirement of the nitrogen starvation-induced protein Sll0783 for polyhydroxybutyrate accumulation in *Synechocystis* sp. strain PCC 6803. Appl. Environ. Microbiol. 76(18), 6101-6107.
- Soma, Y., Tsuruno, K., Wada, M., Yokota, A., Hanai, T., 2014. Metabolic flux redirection from a central metabolic pathway toward a synthetic pathway using a metabolic toggle switch. Metab. Eng. 23, 175-184.
- Takahashi, T., Nakai, N., Muramatsu, M., Hihara, Y., 2010. Role of multiple HLR1 sequences in the regulation of the dual promoters of the *psaAB* genes in *Synechocystis* sp. PCC 6803. J. Bacteriol. 192, 4031-4036.
- Taroncher-Oldenburg, G., Stephanopoulos, G., 2000. Targeted, PCR-based gene disruption in cyanobacteria: inactivation of the polyhydroxyalkanoic acid synthase genes in *Synechocystis* sp. PCC6803. Appl. Microbiol. Biotechnol. 54, 677-680.
- Tyo, K.E., Zhou, H., Stephanopoulos, G.N., 2006. High-throughput screen for poly-3-hydroxybutyrate in *Escherichia coli* and *Synechocystis* sp. strain PCC6803. Appl. Environ. Microbiol. 72, 3412-3417.
- van der Woude, A.D., Angermayr, S.A., Puthan Veetil, V., Osnato, A., Hellingwerf, K.J., 2014. Carbon sink removal: Increased photosynthetic production of lactic acid by *Synechocystis* sp. PCC6803 in a glycogen storage mutant. J. Biotechnol. 184, 100-102.
- Yoshikawa, K., Hirasawa, T., Ogawa, K., Hidaka, Y., Nakajima, T., Furusawa, C.,

- 416 Shimizu, H., 2013. Integrated transcriptomic and metabolomic analysis of the
417 central metabolism of *Synechocystis* sp. PCC 6803 under different trophic
418 conditions. *Biotechnol. J.* 8, 571-580.
- 419 Yoshikawa, K., Hirasawa, T., Shimizu, H., 2014. Effect of malic enzyme on ethanol
420 production by *Synechocystis* sp. PCC 6803. *J. Biosci. Bioeng.* 119(1), 82-84.
- 421 Yu, Y., You, L., Liu, D., Hollinshead, W., Tang, Y.J., Zhang, F., 2013. Development of
422 *Synechocystis* sp. PCC 6803 as a phototrophic cell factory. *Mar. Drugs.* 11(8),
423 2894-2916.
- 424 Williams, J.G.K., 1988. Construction of Specific Mutations in PhotosystemII
425 Photosynthetic Reaction Center by Genetic Engineering Methods in *Synechocystis*
426 6803. *Method Enzymol.* 167, 766-778.
- 427 Xiong, W., Brune, D., Vermaas, W.F., 2014. The γ -aminobutyric acid shunt contributes
428 to closing the tricarboxylic acid cycle in *Synechocystis* sp. PCC 6803. *Mol.*
429 *Microbiol.* 93(4), 786-796.

Figure legends

Figure 1 Cell growth and the extracellular metabolite production with (A) and without nitrogen (B). Circles, triangles and squares represent the 6803/EtOH, Δ glgC/EtOH, and Δ glgC Δ phaCE/EtOH strains, respectively. Error bars indicate standard deviation from triplicate experiments.

Figure 2 Relative amount of phycobilisome and chlorophyll a per cell mass. The graph (A) and (B) show the conditions with and without nitrogen, respectively. Circles, triangles and squares represent the 6803/EtOH, Δ glgC/EtOH, and Δ glgC Δ phaCE/EtOH strains, respectively. Error bars indicate standard deviation from triplicate experiments.

Figure 3 Glycogen and PHB contents in the cells at 48 h.

Figure 4 Carbon fixations amount, estimated by ethanol, organic acids, glycogen, PHB, and biomass production. The amounts were calculated using the culture results at 48 h.

Figure 5 Ethanol production of Δ glgC Δ phaCE/EtOH under the high cell density condition. (A) OD₇₃₀ and (B) ethanol concentration in the cultures. Open and closed symbols represent the light conditions at 40 and 80 μ mol/m².s, respectively. Error bars indicate standard deviation from triplicate experiments. Although PHB and glycogen are included in the biomass components, these carbon storage compounds were not included in the carbon content of biomass. There is no overlap between biomass and the carbon storage compounds.

1 **Table 1 Strains used in this study**

Strain	Relevant genotype*1	
PCC6803	<i>Synechocystis</i> sp. PCC 6803 Glucose-tolerant	Williams et al., 1988
6803/EtOH	NS1::P _{nblA} - <i>pdc-adh</i> , Am ^r	Yoshikawa et al., 2014
Δ <i>glgC</i> /EtOH	6803/EtOH, <i>glgC</i> ::Km ^r	This study
Δ <i>phaCE</i> /EtOH	6803/EtOH, <i>phaCE</i> ::Cm ^r	This study
Δ <i>glgC</i> Δ <i>phaCE</i> /EtOH	6803/EtOH, <i>phaCE</i> ::Cm ^r , <i>glgC</i> ::Km ^r	This study

2 *1 NS1, neutral site downstream from *ndhB* (Takahashi et al., 2010); Km^r, kanamycin
3 resistance gene; Am^r, ampicillin resistance gene; Cm^r, chloramphenicol resistance gene.

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Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Graphical Abstract