**Metabolomics-based analysis revealing the alteration of primary
carbon metabolism by the genetic manipulation of a hydrogenase HoxH
in *Synechocystis* sp. PCC 6803**

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Running title: HoxH-mediated metabolic alterations

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2

1 **Abstract**

2 Cyanobacterial hydrogenases are important owing to the association between hydrogen
3 metabolism and cell physiology and the production of future renewable energy. Many
4 studies have examined hydrogen productivity, transcriptional regulation of
5 hydrogenases, and the biochemistry of hydrogenases; however the relationship between
6 hydrogen and primary carbon metabolism using metabolomic techniques has not been
7 elucidated. Here, we studied the effect of the genetic manipulation of a hydrogenase on
8 primary carbon metabolism in the model unicellular cyanobacterium *Synechocystis* sp.
9 PCC 6803. Metabolomic analysis revealed that the *hoxH* mutant with reduced *hoxH*
10 transcripts exhibited increased sugar phosphates in dark, anaerobic conditions. Organic
11 acids, lactate, succinate, fumarate, and malate increased substantially by the *hoxH*
12 mutation both inside and outside of cells in dark, anaerobic conditions. Transcriptome
13 analysis revealed higher expression of genes encoding the RNA polymerase sigma
14 factor SigE, which is a positive regulator of sugar catabolism, and 6-phosphogluconate
15 dehydrogenase in the *hoxH* mutant than in the wild-type strain. Immunoblotting results
16 showed that sugar catabolic enzymes and SigE proteins increased in the *hoxH*
17 mutant. These results demonstrate the wide alterations of primary metabolism by the
18 genetic manipulation of a hydrogenase subunit in this cyanobacterium.

19

20 **Keywords:** cyanobacteria, hydrogenase, metabolomics, primary metabolism, sugar
21 catabolism

22

1 **1. Introduction**

2 Cyanobacteria are a group of photosynthetic bacteria that are of particular interest as
3 cell factories capable of producing biomaterials and bioenergy including
4 hydrogen. Many researchers have considered cyanobacteria as a biological chassis for
5 hydrogen production and have tried to increase hydrogenase activity by genetic
6 engineering (Khanna and Lindblad, 2015). Cyanobacteria possess [NiFe]-type
7 hydrogenases, which have bidirectional catalytic properties with a bias towards H₂
8 production (McIntosh *et al.*, 2011). The physiological role of hydrogenases in
9 cyanobacteria is obscure, but it is speculated that they function as electron valves under
10 anaerobic conditions (Appel *et al.*, 2000). The bifunctional hydrogenase consists of five
11 subunits, i.e., HoxEFUYH; HoxEFU constitute a diaphorase moiety and
12 HoxYH constitute a hydrogenase moiety (Schmitz *et al.*, 2002; Tamagnini *et al.*,
13 2007). The maturation of a hydrogenase complex of the unicellular non-nitrogen-fixing
14 cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) starts with
15 the binding of unprocessed HoxH and HoxY (Eckert *et al.*, 2012). The knockout of
16 *hoxH* results in a complete loss of hydrogenase activity; therefore, HoxH is an important
17 subunit for the bidirectional hydrogenase reaction in *Synechocystis* 6803. Proteomic
18 analysis has revealed that the levels of 17 out of 210 detected proteins, which are
19 involved in photosynthesis and respiration, translation, antioxidant defense, energy
20 metabolism, and transcription, are altered by *hoxYH* knockout (Pinto *et al.*, 2012).
21 Cyanobacterial hydrogenases have been intensively investigated in view of their
22 potential in applied and basic sciences; however, the metabolomes of hydrogenase
23 mutants in *Synechocystis* 6803 have not been analyzed to date.

24

1 The discovery of the closed tricarboxylic acid (TCA) cycle in cyanobacteria
2 revealed the importance of this cycle for primary carbon and energy metabolism (Zhang
3 and Bryant 2011). The TCA cycle provides reductants for oxidative phosphorylation and
4 four-carbon precursors, such as 2-oxoglutarate (2-OG), succinate, fumarate, malate, and
5 oxaloacetate. The reversal of the TCA cycle (i.e., the reductive TCA cycle) is observed
6 under anaerobic conditions, which is widely conserved among various bacteria as a
7 “metabolite-producing device” (Aoshima 2007). In cyanobacteria, an increase in 2-OG
8 levels is a signal of nitrogen starvation, leading to transcriptional activation mediated by
9 the NtcA and PII signaling proteins (Muro-Pastor *et al.*, 2001b). In addition, the levels of
10 succinate, fumarate, and malate increase in response to nitrogen depletion, possibly
11 functioning in carbon storage under nitrogen starvation (Osanai *et al.*, 2014b). Organic
12 acids including succinate, lactate, and acetate, are excreted from *Synechocystis* 6803 cells
13 under dark, anaerobic conditions (Osanai *et al.*, 2015b). Hydrogen and organic acid
14 production in anaerobic conditions depend on the carbon and nitrogen content of the
15 media, indicating that there is a close relationship between primary carbon and
16 hydrogen metabolism (Carrier *et al.*, 2010; McNeely *et al.*, 2014). However, the effect of
17 hydrogen metabolism modification on primary carbon metabolism is unclear in this
18 cyanobacterium owing to the lack of metabolomic analyses of hydrogenase mutants.

19

20 We performed transcriptome and metabolome analyses by using microarray
21 analysis, liquid-chromatography/mass spectrometry (LC-MS), and
22 gas-chromatography/mass spectrometry (GC-MS) with the wild-type strain and *ahoxH*
23 mutant. *Synechocystis* 6803 with a hydrogenase mutation exhibited modifications in
24 sugar catabolism and the TCA cycle.

1

2 **2. Materials and Methods**

3 **2.1. Bacterial strains and culture growth conditions**

4 The glucose-tolerant (GT) strain of *Synechocystis* sp. PCC 6803 (Williams, 1988) was
5 cultivated in modified BG-11 medium, which was BG-11₀ liquid medium (Rippka,
6 1988) supplemented with 5 mM NH₄Cl and buffered with 20 mM HEPES–KOH, pH
7 7.8. The GT-I strain was used in this study (Kanesaki *et al.*, 2012). Liquid cultures were
8 bubbled with 1% (v/v) CO₂ in air and incubated at 30°C under continuous white light
9 (~50–70 μmol photons m⁻² s⁻¹). For the *hoxH* mutant, 0.3 μg/mL gentamycin was added
10 during preculture. Cell densities were measured at A₇₃₀ using a Hitachi U-3310
11 spectrophotometer (Hitachi High-Tech., Tokyo, Japan).

12

13 **2.2. Generation of the *hoxH* mutant**

14 The *hoxH* open reading frame (sll1226) was amplified by PCR using KOD Plus Neo
15 Polymerase (Toyobo, Osaka, Japan) and the specific primers
16 5'-TTCCGCATGCTTACAAGATCCGGCTTTC-3' and
17 5'-TTAAGATATCAGCCCGTTGCCGATATT-3'. The amplified DNA fragments were
18 digested with SphI and EcoRV (Takara Bio, Shiga, Japan) and cloned into the *SphI* and
19 SmaI site of the pUC119 vector (Takara Bio). The region including the gentamycin
20 resistance cassette was amplified using KOD Plus Neo Polymerase and the primers
21 5'-TATTCTGGGCCCTTTGCTTCATCGCTCGAG-3' and
22 5'-TATCAAGGGCCCATCCAATGTGAGGTAAAC-3' using a pTGP2031 vector
23 (Osanai *et al.*, 2014a) as a template. The amplified DNA was cloned into the MscI site
24 of *hoxH* using DNA Ligation Mix (Takara). Transformation was performed by natural

transformation, in which plasmid DNA was mixed with *Synechocystis* cells and spread on a BG-11 plate containing 3 µg/mL gentamycin. After colonies appeared, several colonies were transferred onto a BG-11 plate containing 3 µg/mL gentamycin several times.

2.3. Excretion of organic acids under dark, anaerobic cultivation

Cell cultivation under dark, anaerobic conditions was performed as previously described (Osanai *et al.*, 2015b). Briefly, cells grown in 70 mL of modified BG-11 medium (started at $A_{730} = 0.4$) for three days were collected and re-suspended in 10 mL of HEPES buffer (20 mM HEPES–KOH, pH 7.8) to $A_{730} = 20$ in a GC vial. The vial was sealed using butyl rubber, and N₂ gas was introduced for 1 h by using syringes. After removing the syringes, the vial was wrapped with aluminum foil and shaken at 30°C for one, three, or five days. Cell cultures were transferred to a 15-mL tube and centrifuged at $5,800 \times g$ for 2 min. The supernatant was filtered, and 1 mL of supernatant was freeze-dried for one day. The dried sample was used for the high-performance liquid chromatography (HPLC) analysis.

2.4. Gas chromatography/thermal conductivity detector (GC-TCD) for hydrogen measurement

Hydrogen was measured by GC-TCD as described previously (Osanai *et al.*, 2013). Cells were cultured in modified BG-11 for three days and collected by centrifugation at $10,000 \times g$ for 2 min, followed by concentration in 10 mL of 20 mM HEPES–KOH (pH 7.8) to $A_{730} = 20$ in a 20-mL GC vial (leaving 10 mL of headspace). The GC vial was filled with N₂ gas introduced with a GC syringe for 1 h. After removal of the syringe,

1 the sealed vial was incubated at 30°C in dark conditions with shaking for three days.

2 The accumulated H₂ gas was measured with a gas chromatograph (GC-2010 Plus AT;

3 Shimadzu, Kyoto, Japan). N₂ was used as the carrier gas with a flow rate of 10 mL/min.

5 **2.5. Measurement of respiratory activity**

6 Chlorophyll contents were measured as described previously (Iijima *et al.* 2015a).

7 Respiratory activity of the cells was measured with Clark-type oxygen electrode

8 (Hansatech Instruments, King's Lynn, UK) (Osanai *et al.*, 2013). Cells containing 10 µg

9 chlorophyll were resuspended in 1 mL 20 mM HEPES-KOH (pH 7.8), and incubated at

10 30°C within the chamber of the Oxytherm. Cells were incubated for dark conditions

11 with monitoring of oxygen consumption for 7 min. The rate of oxygen consumption in

12 the final 3 min was used to calculate respiration activity.

14 **2.6. LC-MS/MS analysis**

15 Equal amounts of cells (10 mL of cell culture with $A_{730} = 1.0$) were harvested by rapid

16 filtration, and metabolites were extracted using a previously described method (Osanai

17 *et al.*, 2014b).

19 **2.7. GC-MS analysis**

20 Equal amounts of cells (10 mL of cell culture with $A_{730} = 1.0$) were harvested by rapid

21 filtration as well as LC-MS/MS analysis. GC-MS was carried out using a

22 GCMS-QP2010 Ultra, following the protocol described in (Osanai *et al.*, 2015a).

24 **2.8. Measurement of the excreted organic acids by HPLC**

HPLC analysis was performed as described previously (Osanai *et al.*, 2015b). The freeze-dried supernatants were resolved in 100 μ L of filtered 3 mM perchloric acid. The resolved samples were analyzed by HPLC using the LC-2000Plus System (JASCO, Tokyo, Japan). Organic acids were quantified with 0.2 mM bromothymol blue in 15 mM sodium phosphate buffer; peaks were detected at 445 nm.

2.9. Glycogen quantification

Equal amounts of cells (30 mL of cell culture with $A_{730} = 1.0$) were collected by centrifugation ($20,800 \times g$ for 1 min), and the supernatant was removed. Cells were re-suspended in 300~500 μ L of 3.5% sulfuric acid, and incubated at 100°C for 40 min. After centrifugation ($20,800 \times g$ for 1 min), 6.7 μ L of supernatant was mixed with 1 mL of reaction mixture of LabAssay Glucose (Wako, Osaka, Japan), and incubated at 37°C for 5 min. The absorbance was measured at 505 nm.

2.10. RNA isolation

Isolation of RNA from *Synechocystis* cells was described previously (Osanai *et al.*, 2014a). RNAs were resuspended in 20 μ L sterilized water after isopropanol precipitation.

2.11. Microarray analysis

Residual DNA was excluded by reaction with TURBO DNase (Life Technologies Japan, Tokyo, Japan) for 5 h at 37°C. The microarray analysis was performed following the manufacturer's instructions. RNA (100 ng) was used for complementary RNA synthesis using the One-Color Microarray-Based Gene Expression Analysis Kit

(Agilent Technologies Japan, Tokyo, Japan). The Microarray was scanned onto GenePix 4100A scanner (Molecular Devices, Sunnyvale, CA, USA). Signals were quantified using GenePix Pro 7.0 (Molecular Devices). Data were deposited to the Gene Expression Omnibus public database (accession number GSE76625).

2.12. Immunoblotting

Cells were collected by centrifugation ($5,800 \times g$ for 2 min), the supernatant was removed and cells were stored at -30°C . Then, cells were re-suspended in PBS-T and disrupted by sonication as described previously (Osanai *et al.*, 2014a). Immunoblotting was performed as described previously (Osanai *et al.*, 2014a). Antisera against SigE, glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), glycogen phosphorylases (GlgPs), and isoamylases (GlgXs) were generated previously (Osanai *et al.*, 2009 and 2011). Antiserum against Tal was commercially produced by Sigma-Aldrich Japan (Tokyo, Japan) using a synthetic peptide CHAYDLDDGDGFITREEWAG injected into a rabbit.

2.13. Quantitative real-time PCR

Residual DNA in the RNA samples was excluded using TURBO DNase (Thermo Fisher Scientific, Pease, NH, USA), reacted for 5 h at 37°C . cDNA was synthesized from 2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Life Technologies Japan). Quantitative real-time PCR was performed using StepOnePlus (Life Technologies Japan), according to the manufacturer's instructions using primers listed in Table S3. The expression level of *rnpB* (encoding RNaseP subunit B) was used as an internal standard as described previously (Schlebusch and Forchhammer, 2010).

1

2 **3. Results**

3 **3.1. Generation of the *hoxH* mutant and measurement of growth**

4 We attempted to generate a knockout mutant of *hoxH* in *Synechocystis* 6803. Although
5 some studies have shown that the hydrogenase genes in this cyanobacterium are not
6 essential (Appel *et al.*, 2000; Pinto *et al.*, 2012; Eckert *et al.*, 2012), we were unable
7 to obtain the complete segregation of *hoxH* mutants after replating ten times, and their
8 expression levels remained at half that of the wild-type strain (Fig. 1A). Since a genetic
9 analysis of *Synechocystis* 6803 can be performed using the incomplete mutant with
10 reduced expression of the genes of interest (Wilde *et al.*, 2004; Osanai *et al.*, 2006), we
11 analyzed this strain to study the function of the hydrogenase in primary metabolism. The
12 growth of the wild-type strain (i.e., the glucose-tolerant strain, GT) and the *hoxH* mutant
13 were similar under photoautotrophic and photomixotrophic conditions (Fig. 1B). The
14 growth of the *hoxH* mutant under light-activated heterotrophic conditions
15 (supplemented with glucose under continuous dark conditions except 15 min of light
16 exposure per day) increased slightly after one day of cultivation (Fig. 1B).

17

18 **3.2. Metabolomic analysis revealed increased sugar phosphates and organic acids** 19 **in the *hoxH* mutant**

20 *Synechocystis* 6803 cells degrade glycogen and produce hydrogen and organic acids in
21 dark, anaerobic conditions (Osanai *et al.*, 2015b). Similarly, the GT and *hoxH* mutant
22 strains were grown under dark, anaerobic conditions, and the accumulated hydrogen
23 levels were 5.0×10^4 and 4.3×10^4 ppm, respectively, indicating a 14% decrease in
24 hydrogen production by the *hoxH* mutation. The respiratory activity of GT and the *hoxH*

1 mutant was 5.47 ± 0.90 and 4.85 ± 1.06 $\mu\text{mol O}_2/\text{chla mg/h}$, respectively.

2 The levels of 32 metabolites were quantified by LC-MS/MS and GC-MS before
3 and after dark, anaerobic cultivation (Table S1). The levels of 10 or 14 metabolites were
4 statistically different between in the GT and *hoxH* mutant before or after dark, anaerobic
5 cultivation, respectively (*P*-values calculated from Student's *t*-test of less than 0.05)
6 (Table S1). Overall, the metabolite levels of the upper half of glycolysis were similar
7 between the GT and *hoxH* mutant strains, except for some sugar phosphates (Fig. 2).
8 The levels of glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, and
9 sedoheptulose-7-phosphate increased after dark, anaerobic cultivation in the *hoxH*
10 mutant, but not in the GT strain (Fig. 2). The lower half of glycolysis, pyruvate
11 metabolism, and the TCA cycle were, on the other hand, extensively altered by the *hoxH*
12 mutation (Fig. 3). Phosphoenolpyruvate decreased during dark, anaerobic cultivation,
13 and the decrease was enhanced in the *hoxH* mutant (Fig. 3). Acetyl-CoA levels were
14 lower in the *hoxH* mutant than in the GT strain under aerobic conditions, but higher in
15 the *hoxH* mutant than in the GT strain under anaerobic conditions (Fig. 3). Organic
16 acids, such as lactate, succinate, malate, and fumarate were strikingly higher in the
17 *hoxH* mutant than the GT strain during dark, anaerobic conditions (Fig. 3). Succinate,
18 malate, and fumarate in the *hoxH* mutant under dark, anaerobic conditions were 20-, 36-,
19 and 78-fold greater compared to the GT strain under aerobic conditions, respectively
20 (Fig. 3 and Table S1). The levels of reductants and nucleotide phosphates did not
21 increase in the *hoxH* mutant (Table S1). Glycogen levels in the *hoxH* mutant were 60%
22 of the GT strain before dark, anaerobic conditions (Table 1). The amount of glycogen
23 consumed during dark, anaerobic cultivation was similar between GT and the *hoxH*
24 mutant (Table 1).

1 The organic acids excreted from the cells cultivated for one to five days under
2 dark, anaerobic conditions were then quantified. In the GT strain, acetates were the most
3 abundant organic acids excreted from the cells (Fig. 4A and B). Acetates were produced
4 from the GT cells at more than 300 mg/L after three or five days of dark, anaerobic
5 cultivation (Fig. 4A). The acetate levels decreased to one-third that of the GT cells by
6 *hoxH* mutation (Fig. 4A). In contrast, succinate and lactate increased by *hoxH* mutation
7 (Fig. 4A and B). After three days of dark, anaerobic cultivation, succinate and lactate in
8 the *hoxH* mutant reached 106 mg/L and 306 mg/L, which were 5-fold and 13-fold
9 greater than the concentrations observed in the GT strain, respectively (Fig. 4A). The
10 ratio of excreted organic acids was altered by *hoxH*; lactate was the most abundant
11 organic acid excreted from the *hoxH* mutant during dark, anaerobic cultivation (Fig. 4B).

13 **3.3. Changes in the expression of sugar catabolic enzymes and the RNA** 14 **polymerase sigma factor SigE in the *hoxH* mutant**

15 To elucidate the cause of the changes in primary metabolism in the *hoxH* mutant, a
16 transcriptome analysis was performed using the cells grown under continuous light
17 conditions for one day. The expression levels of 23 or 34 genes increased or decreased
18 more than two times in the *hoxH* mutant compared to the GT strain, with *P*-values
19 calculated from Student's *t*-test of less than 0.05 (Tables 2, 3 and S2). Expression was
20 induced for hypothetical genes and genes encoding the RNA polymerase sigma factor
21 SigE (sll1689), a transcriptional regulator (sll0782), 6-phosphogluconate dehydrogenase
22 (*gnd*, sll0329), and a hydrogenase expression/formation protein HypB (sll1432) by the
23 *hoxH* mutation (Table 2). Expression was repressed for hypothetical genes including
24 *ycf* genes (hypothetical chloroplast open reading frame) and genes encoding a

1 peptidyl-prolylcis-trans isomerase (*cyp*, slr1251), Photosystem II subunit (*psbI*,
2 sml0001), a subunit of nitrate/nitrite transporter (*nrtB*, slr1451), a nitrite reductase (*nirA*,
3 slr0898), and a UDP-glucose-4-epimerase (*galE*, slr1067) by the *hoxH* mutation (Table
4 3).

5 SigE is one of nine RNA polymerase sigma factors in *Synechocystis* 6803. It
6 activates sugar catabolism, and in particular, enhances the expression of genes
7 encoding the enzymes of glycogen catabolism and the oxidative pentosephosphate
8 (OPP) pathway (Osanai *et al.*, 2011, 2015b). Quantitative real-time PCR confirmed the
9 increased expression of *sigE* in the *hoxH* mutant (Fig. 5A). The transcripts of other
10 sigma factor genes, *sigA*, *sigB*, *sigC*, and *sigD*, were decreased in the *hoxH* mutant (Fig.
11 6). The expression levels of four genes encoding OPP pathway enzymes
12 [glucose-6-phosphate dehydrogenase (G6PD, encoded by *zwf*), OpcA (a positive
13 regulator of G6PD), 6-phosphogluconate dehydrogenase (6PGD, encoded by *gnd*), and
14 transaldolase (Tal)] were upregulated in the *hoxH* mutant under light conditions (Fig. S1).
15 In terms of glycogen phosphorylase (GlgP) and two isoamylase (GlgX), the expression
16 of *glgX*(slr0237) was slightly increased, but that of *glgX*(slr1857) was downregulated by
17 the *hoxH* mutation (Fig. S1).

18 Immunoblotting showed that SigE proteins in the *hoxH* mutant increased by 2.5
19 times compared with the GT strains under light conditions (Fig. 5B). Different from
20 transcript levels, the protein expression levels of five sugar catabolic enzymes, G6PD,
21 6PGD, Tal, GlgP(slr1356), and GlgX(slr0237) in the *hoxH* mutant were higher than
22 those in the GT strain (Fig. 5B). GlgP(slr1356) and GlgX(slr1857) were similar between
23 the GT and *hoxH* strain (Fig. 5B). We also performed an immunoblotting analysis using
24 the *hoxYH* knockout mutant (Δ *hoxYH*) generated by Pinto *et al.* (2012), but SigE and

1 sugar catabolic enzymes were not upregulated in this mutant except GlgP(slr1367) (Fig.
2 S2).

3

4 **4. Discussion**

5 In this study, we first performed a metabolomic analysis using hydrogenase mutants in
6 cyanobacteria. Since HoxH is not a transcriptional regulator, the altered gene expression
7 must be an indirect effect. Our genetic analysis using a metabolomic approach clarified
8 the metabolic alterations caused a hydrogenase gene mutation in cyanobacteria. In
9 particular, sugar catabolism and organic acid production were accelerated by the *hoxH*
10 mutation via the upregulation of genes encoding an RNA polymerase and sugar catabolic
11 enzymes.

12

13 There are several *hoxH* mutants of *Synechocystis* 6803, and they exhibit
14 inconsistent phenotypes (Appel *et al.*, 2000; Pinto *et al.*, 2012; Eckert *et al.*, 2012).
15 Knockout *hoxH* mutants grow slowly (Appel *et al.*, 2000), whereas Δ *hoxYH* cells grow as
16 fast as wild-type cells (Pinto *et al.*, 2012). In addition, studies of electron transport rate
17 have shown inconsistencies among *hox* mutants (Eckert *et al.*, 2000). In our case,
18 *hoxH* was not completely knocked out nevertheless prolonged cultivation. Thus, the
19 phenotypes of *hox* mutants in *Synechocystis* 6803 depend on the wild-type background
20 and experimental growth conditions. Despite phenotypic differences, the upregulation
21 of sugar catabolic enzymes was observed in both Δ *hoxYH* and our *hoxH* mutant (Fig. 5
22 and S1) (Pinto *et al.*, 2012). These results confirmed the close relationship between
23 hydrogenases and primary sugar catabolism. The downregulation of hydrogenase
24 activity may require other route(s) to dissipate excess reducing power. The *hoxH* mutant

1 exhibited both reduced hydrogen production and increased G6PD and 6PGD enzymes.
2 This seems to result in excess reducing power because the OPP pathway
3 enzymes function in NADPH-generation coupled to primary carbon metabolism
4 (Spaans *et al.*, 2015). However, the metabolome analysis showed that the NADH and
5 NADPH levels did not increase in the *hoxH* mutant (Table S1). It is possible that the
6 increase in succinate and lactate biosynthesis and excretion in the *hoxH* mutant is a
7 mechanism for the dissipation of excess reducing powers (Fig. 3 and 4). Therefore, our
8 analysis demonstrates a negative correlation between hydrogen and organic acid
9 production; the suppression of hydrogenase expression increases succinate and lactate
10 biosynthesis in this cyanobacterium. A positive correlation between succinate and
11 lactate, and negative correlation between acetate and succinate/lactate was observed in
12 the *hoxH* mutant (Fig. 4), which is consistent with the previous results (Osanai *et al.*,
13 2015b).

14
15 Metabolic flux of primary carbon metabolism must change in response to
16 environmental signals and growth conditions. Under heterotrophic conditions, the
17 carbon flux from phosphoenolpyruvate to oxaloacetate is relatively stronger than that
18 from phosphoenolpyruvate to pyruvate in *Synechocystis* 6803, and carbon flux from
19 2-OG to succinate is weak (You *et al.*, 2015). Therefore, the reductive TCA cycle is
20 important under non-photosynthetic growth conditions in this cyanobacterium, in
21 agreement with the results of our study. Flux analyses also indicate that glucose is
22 mainly catabolized via the OPP pathway under heterotrophic conditions (Nakajima *et al.*,
23 2014; You *et al.*, 2015). The OPP pathway plays pivotal roles in the provision of
24 reductants under heterotrophic conditions (Summers *et al.*, 1995; Min and Golden,

2000); hence, the integrated regulation of the OPP pathway and TCA cycle must be indispensable in dark, anaerobic conditions. Sugar catabolism of *Synechocystis* 6803 is widely regulated by the transcriptional regulators SigE and Rre37 (Osanai *et al.*, 2014). SigE is under the control of the two-component system SasA/Hik8 and RpaA, possibly transducing light signals (Iijima *et al.*, 2015b). SasA and RpaA in *Synechococcus* are directly regulated by KaiABC circadian clocks, which integrate light and nutrient signals (Diamond *et al.*, 2015; Pattanayak *et al.*, 2015). In this way, these transcriptional cascades determine the metabolic flux of primary carbon metabolism, and studies of transcriptional regulators and circadian clocks are necessary to understand metabolic flux in unicellular cyanobacteria.

Transcript and protein analyses revealed the upregulation of SigE in the *hoxH* mutant (Table 2 and Fig. 5). The correlation deduced from the omics data in this study was summarized in Table S4. It was found that transcripts, proteins and metabolites of glycogen catabolism were positively correlated but the metabolites of the TCA cycle were not correlated with the transcripts of the enzymes in the TCA cycle (Table S4). The non-correlation of sugar catabolism and the TCA cycle may be explained by post-transcriptional regulation, which has been demonstrated by other groups (Hagen and Meeks 2001; Lindahl and Kieselbach, 2009). SigE is also regulated at both the transcriptional and post-transcriptional levels; the *sigE* expression is controlled by light/dark signals, the day/night cycle, nitrogen status, and growth phase (Muro-Pastor *et al.*, 2001a; Kucho *et al.*, 2005; Imamura *et al.*, 2006; Osanai *et al.*, 2009). Transcriptional regulators, such as the nitrogen-related transcription factor NtcA and the sigma factors SigB and SigC, also control the transcription of *sigE* (Muro-Pastor,

2001a; Imamura et al. 2006). Thus, multiple signals are transduced to SigE via several transcriptional regulators. The signals controlling SigE levels in the *hoxH* mutant are unclear at present; however the downregulation of hydrogen production may result in the upregulation of *sigE* expression in order to maintain a balance of reducing power and primary carbon metabolism. It is noteworthy that SigE activates the expression of the *hox* operon (Osanai *et al.*, 2013), and thus, our genetic analysis revealed the mutual regulation of SigE and hydrogenase in this cyanobacterium. The signal transduction between SigE/sugar catabolism and hydrogenases in this cyanobacterium require further investigation.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan, by a grant to T.O. from ALCA (Project name “Production of cyanobacterial succinate by the genetic engineering of transcriptional regulators and circadian clocks”) from the Japan Science and Technology Agency. We have no conflict of interest to declare.

SUPPORTING INFORMATION

Figure S1. Quantitative real-time PCR measurements of the transcript levels of sugar catabolic enzymes.

Figure S2. Protein levels of sugar catabolic enzyme in the glucose-tolerant (GT) strain, *hoxH* mutant, and $\Delta hoxYH$ mutant.

Table S1. Metabolite levels in the GT strain and the *hoxH* mutant analyzed by LC-MS/MS and GC-MS.

1 **Table S2.**Comparison of microarray analysis results between the GT strain and *hoxH*
2 mutant.

3 **Table S3.**List of primers.

4 **Table S4.** Summary of transcript, protein, and metabolite analyses in this study.

5

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1 **TABLES and FIGURE LEGENDS**

2 **Table 1.** Relative glycogen levels in GT and the *hoxH* mutant.

3 **Table 2.** Selected microarray results for genes that were upregulated in the *hoxH* mutant.

4 **Table 3.** Selected microarray results for genes that were downregulated in the *hoxH*
5 mutant.

6

7 **Figure 1.**(A) Transcript levels of *hoxH* in the wild-type strain (GT) and *hoxH*
8 mutant. Data represent means $\pm$ SD from biologically independent experiments ($n = 3$).

9 Transcript levels were calibrated relative to those of the corresponding transcripts in GT
10 (set at 100%). (B) Growth curves of GT and *hoxH* mutant strains. Left; cell growth under
11 photoautotrophic (PA) and photomixotrophic (PM, grown with 1 mM glucose under
12 continuous illumination) conditions. Right; cell growth under light-activated
13 heterotrophic conditions (LAHG, grown with 1 mM glucose under continuous dark
14 conditions except for 15 min of light per day). Data represent means $\pm$ SD from
15 four independent experiments. Asterisks indicate statistically significant differences
16 between GT and the *hoxH* mutant (Student's *t*-test; $*P < 0.05$).

17

18 **Figure 2.** Levels of metabolites in sugar metabolism of the wild-type strain (GT) and
19 *hoxH* mutant. Data represent means $\pm$ SD from six independent experiments. Metabolite
20 levels were calibrated relative to those corresponding metabolites in GT under aerobic
21 conditions (set at 100%). P designates phosphate. Asterisks indicate statistically
22 significant differences between GT and the *hoxH* mutant (Student's *t*-test; $*P < 0.05$,
23 $**P < 0.005$). Solid and dashed bars indicate before and after dark anaerobic cultivation,
24 respectively.

1

2 **Figure 3.** Levels of metabolites in pyruvate metabolism and the TCA cycle of the
3 wild-type strain (GT) and *hoxH* mutant. Data represent means $\pm$ SD from six
4 independent experiments. Metabolite levels were calibrated relative to that of
5 corresponding metabolites in GT under aerobic conditions (set at 100%). P designates
6 phosphate. Asterisks indicate statistically significant differences between GT and the
7 *hoxH* mutant (Student's *t*-test; **P* < 0.05, ***P* < 0.005). Solid and dashed bars indicate
8 before and after dark anaerobic cultivation, respectively.

9

10 **Figure 4.** Levels of the excreted organic acids. (A) Organic acids excreted from the cells
11 were cultivated for three days in dark, anaerobic conditions and quantified by HPLC.
12 Data represent means $\pm$ SD from three or four independent experiments. Asterisks
13 indicate statistically significant differences between the wild-type strain (GT) and *hoxH*
14 mutant (Student's *t*-test; **P* < 0.05, ***P* < 0.005). (B) Pie charts depicting the relative
15 amounts of succinate, lactate, and acetate. To obtain the values, each organic acid was
16 divided by the total amount of succinate, lactate, and acetate.

17

18 **Figure 5.** Expression of genes encoding SigE and sugar catabolic enzymes. (A)
19 Quantitative real-time PCR analysis of *sigE* transcript levels in the wild-type strain (GT)
20 and *hoxH* mutant. Data represent means $\pm$ SD from biologically independent
21 experiments (*n* = 4). Transcript levels were calibrated relative to the corresponding
22 transcript levels in GT (set at 100%). (B) Immunoblotting analysis of protein levels in
23 GT and the *hoxH* mutant. Relative protein levels of SigE and sugar catabolic enzymes
24 are shown. Data represent means $\pm$ SD from biologically independent experiments (*n* =

1 4). Protein levels were calibrated relative to the corresponding transcript levels in GT
2 (set at 100%). Asterisks indicate statistically significant differences between GT and the
3 *hoxH* mutant (Student's *t*-test; * $P < 0.05$).

4

5 **Figure 6.** Expression of genes encoding four sigma factors (SigA~D). Data represent
6 means $\pm$ SD from biologically independent experiments ($n = 4$). Transcript levels were
7 calibrated relative to those of the corresponding transcripts in GT (set at
8 100%). Asterisks indicate statistically significant differences between GT and the *hoxH*
9 mutant (Student's *t*-test; * $P < 0.05$).

10

1 **Table 1.**Relative glycogen levels in GT and the *hoxH* mutant.

Strain	Aerobic	Anaerobic
GT	100 ± 31.4	37.1± 28.6
<i>hoxH</i>	60.0 ± 17.1*	8.6± 2.9

2 Data represent means ± SD results from four independent experiments. Glycogen levels
3 were calibrated relative to that in GT under light, aerobic conditions (set at 100%).
4 Anaerobic designates glycogen amounts of the cells cultivated for three days under dark,
5 anaerobic conditions. Asterisks indicate statistically significant differences between GT
6 and the *hoxH* mutant (Student's *t*-test; **P* < 0.05).

7

8

1 **Table 2.** Selected microarray results for genes that were upregulated in the *hoxH*
2 mutant.

GenBank ID	Gene Name	Relative gene expression in GT	Relative gene expression in <i>hoxH</i>
NP_442537.1	sll0597	100 ± 36.9	385.8 ± 119.3
NP_442767.1	sll0499	100 ± 6.1	301.2 ± 35.2
NP_442040.1	slr0388	100 ± 5.0	287.1 ± 22.3
NP_441077.1	sll1689, <i>sigE (rpoD)</i>	100 ± 17.0	254.1 ± 27.1
NP_441266.1	sll0249	100 ± 4.9	249.6 ± 49.9
NP_442649.1	sll0782, transcriptional regulator	100 ± 21.8	242.0 ± 18.9
NP_441262.1	slr0264	100 ± 16.5	226.7 ± 34.7
NP_442004.1	sll0157	100 ± 21.6	219.3 ± 37.8
NP_440004.1	slr0734	100 ± 2.9	217.2 ± 21.6
NP_439932.1	slr1493	100 ± 7.1	211.1 ± 35.9
NP_440355.1	slr1050	100 ± 0.0	196.5 ± 21.3
NP_441828.1	slr0286	100 ± 5.2	196.3 ± 24.6
NP_442035.1	sll0329, <i>gnd</i>	100 ± 20.4	193.6 ± 26.3
NP_442445.1	ssr1407	100 ± 23.0	192.6 ± 46.4
NP_442572.1	sll0098	100 ± 13.6	189.3 ± 11.6
NP_440897.1	slr1363	100 ± 10.4	175.6 ± 40.0
NP_441748.1	sll1541, <i>syc2</i>	100 ± 5.4	163.5 ± 18.2

NP_441632.1	sll1432, <i>hypB</i>	100 ± 3.3	162.3 ± 4.5
NP_442643.1	sll0787	100 ± 8.1	160.7 ± 21.4
NP_441814.1	sll0271, <i>nusB</i>	100 ± 7.6	160.7 ± 10.7

- 1 Data represent means ± SD results from three independent experiments. Expression
- 2 levels were calibrated relative to that of the GT strain (set at 100%). For all genes listed
- 3 in this table, *P*-values for the difference between the wild-type and the *hoxH*
- 4 mutant strains were less than 0.05, calculated by Student's *t*-tests.
- 5

1 **Table 3.** Selected microarray results for genes that were downregulated in the *hoxH*
2 mutant.

GenBank ID	Gene Name	Relative gene expression in GT	Relative gene expression in <i>hoxH</i>
NP_440534.1	slr1396	100 ± 3.0	32.6 ± 22.2
NP_442689.1	sll0661, <i>ycf35</i>	100 ± 1.5	32.7 ± 6.1
NP_440225.1	slr1611	100 ± 3.7	32.8 ± 17.3
NP_441883.1	slr0157	100 ± 0.8	35.6 ± 24.3
NP_442684.1	slr0709	100 ± 18.8	37.6 ± 11.2
NP_441401.1	slr1327	100 ± 1.0	38.6 ± 22.6
NP_441161.1	slr1251, <i>cyp</i>	100 ± 4.0	38.9 ± 17.0
NP_440381.1	slr1763	100 ± 3.7	39.2 ± 23.8
NP_442015.1	sml0001, <i>psbI</i>	100 ± 13.2	39.7 ± 25.4
NP_441606.1	slr1282	100 ± 1.9	39.8 ± 20.7
NP_440164.1	ssl2384	100 ± 1.7	41.5 ± 21.0
NP_441069.1	sll1696	100 ± 2.8	42.0 ± 18.3
NP_441422.1	sll1218, <i>ycf39</i>	100 ± 11.8	42.5 ± 26.8
NP_440238.1	slr1066	100 ± 22.9	42.5 ± 28.2
NP_440811.1	sll1451, <i>nrtB</i>	100 ± 18.6	43.8 ± 9.1
NP_440239.1	slr1067, <i>galE</i>	100 ± 0.4	44.1 ± 20.0
NP_442935.1	sll0733	100 ± 11.8	44.7 ± 19.6
NP_441100.1	slr1740	100 ± 1.8	44.8 ± 12.9

NP_440457.1	sll1835	100 ± 16.0	45.2 ± 16.3
NP_442378.1	slr0898, <i>nirA</i>	100 ± 15.3	45.3 ± 14.3

1 Data represent means ± SD results from three independent experiments. Expression
2 levels were calibrated relative to that of the GT strain (set at 100%). For all genes listed
3 in this table, *P*-values for the difference between the wild-type and the *hoxH* mutant
4 strains were less than 0.05, calculated by Student's *t*-tests.

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6

Figure 1

Figure 2

Figure 3

Figure 4

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Figure 6