**One day of nitrogen starvation reveals the effect of *sigE* and *rre37*
overexpression on the expression of genes related to carbon and
nitrogen metabolism in *Synechocystis* sp. PCC 6803.**

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Running title: Effect on the expression by N starvation in cyano

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1 **Abstract**

2 Acclimation to nitrogen starvation is indispensable for the survival of cyanobacteria
3 under natural environmental conditions. SigE and Rre37 are transcriptional regulators
4 whose transcript and protein levels increase during nitrogen starvation. Previous work
5 has shown that overexpression of either *sigE* or *rre37* in the unicellular cyanobacterium
6 *Synechocystis* sp. PCC 6803 activates the expression of genes related to sugar
7 catabolism and polyhydroxybutyrate (PHB) biosynthesis, although their transcript levels
8 were measured using the cells grown under nitrogen-replete conditions or after 4 h of
9 nitrogen depletion. In this study, the transcript levels were quantified at one day after
10 nitrogen depletion in the wild-type, *sigE*-overexpressing, and *rre37*-overexpressing cells.
11 The results showed decreased expression of sugar catabolic enzymes after one day of
12 nitrogen depletion in the wild-type strain. The levels of transcripts related to glycogen
13 catabolism, glycolysis, and PHB biosynthesis were not increased by either *sigE* or *rre37*
14 overexpression after one day of nitrogen depletion. The expression of genes related to
15 nitrogen assimilation and nitrate/nitrite transport was also reduced by either *sigE* or
16 *rre37* overexpression after nitrogen starvation, suggesting the genetic engineering had a
17 phase-dependent effect on gene expression during nitrogen starvation.

18

1 **Introduction**

2 Transcriptional cascades are important for the acclimation of bacteria to various
3 environmental stresses including nitrogen starvation [1]. Nitrogen starvation triggers
4 bleaching or chlorosis, which is the degradation of phycobilisomes, the photosynthetic
5 apparatus for absorbing light energy [2]. Signal transduction following nitrogen
6 starvation in cyanobacteria has been intensively studied. An increase in 2-oxoglutarate
7 (2-OG) is first detected in nitrogen-starved cells [3]. 2-OG binds to two proteins; which
8 are NtcA, a transcription factor conserved among cyanobacteria, and PII (encoded by
9 *glnB*), a protein that regulates carbon and nitrogen balance [4-6]. 2-OG promotes the
10 binding of NtcA to the promoter region of its target genes [4, 5]. NtcA was first
11 identified as a regulator of glutamine synthetase (encoded by *glnA*) in a unicellular
12 cyanobacterium *Synechococcus* sp. PCC 7942 (hereafter referred to as *Synechococcus*
13 7942) [7, 8]. NtcA in *Synechococcus* 7942 and *Synechocystis* sp. PCC 6803 (hereafter
14 *Synechocystis* 6803) regulates the transcription of genes related to nitrogen assimilation
15 such as *glnN* (encoding type-III glutamine synthetase), *amt1* (encoding an ammonium
16 transporter), *glnB* [9-11], and genes related to carbon metabolism such as *icd* (encoding
17 an isocitrate dehydrogenase) [12].

18 NtcA regulates the transcription of several transcriptional regulators. NtcA
19 activates the gene expression of *ntcB*, which encodes a transcription factor that activates
20 the transcription of genes encoding ABC-type nitrate/nitrite transporter proteins
21 (encoded by *nrtABCD*) [13, 14]. The expression of *sigE*, which encodes an RNA
22 polymerase sigma factor, is induced by nitrogen depletion and NtcA binds to the
23 promoter region of *sigE* [15]. Transcriptome analysis has revealed that *sigE* knockout
24 decreases the expression of enzymes involved in glycogen catabolism, glycolysis, and

1 the oxidative pentose phosphate (OPP) pathway [16]. The expression of these sugar
2 catabolic genes is induced by nitrogen depletion and controlled by light-to-dark
3 transition [17,18]. Nitrogen depletion and transcriptional activation by NtcA induces the
4 expression of *nrrA*, another transcriptional regulator that encodes a response regulator
5 in *Anabaena* sp. PCC 7120 [19]. The expression of *rre37* (sll1330), an ortholog of *nrrA*
6 in *Synechocystis* 6803, is also enhanced by nitrogen depletion [17]. Analyses using
7 *rre37* knockout demonstrated that Rre37 is a positive regulator of genes related to
8 glycogen catabolism and glycolysis [20, 21]. NtcA proteins bind to the promoter region
9 of *rre37* in *Synechocystis* 6803, and this interaction is promoted in the presence of 2-OG
10 [20].

11 Altering the primary metabolism of *Synechocystis* 6803 is challenging because of
12 the ability of this species to use light energy and carbon dioxides. Overexpression of
13 *sigE* results in the activation of sugar catabolism and the increased production of
14 polyhydroxybutyrate (PHB) and hydrogen [22-24]. PHB is a biodegradable plastic
15 produced during nitrogen starvation [25]. PHB is synthesized from acetyl-CoA by three
16 reactions. Acetoacetyl-CoA is synthesized from acetyl-CoA, which is catalyzed by
17 -thiolase (encoded by *phaA*) and 3-hydroxybutylyl-CoA is synthesized from
18 acetoacetyl-CoA catalyzed by acetoacetyl-CoA reductase (encoded by *phaB*) [26].
19 3-Hydroxybutylyl-CoA is polymerized by PHA synthase, which in *Synechocystis* 6803
20 consists of the PhaC and PhaE subunits [27]. The expression of *phaA* and *phaB* is
21 enhanced by *rre37* overexpression [28], and that of *phaC* and *phaE* is increased by *sigE*
22 overexpression under nitrogen-replete conditions [24]. However, in previous studies,
23 transcript levels were measured using cells grown under nitrogen-replete or -depleted
24 cells (4 h after nitrogen depletion), and the transcript levels following long-term

nitrogen starvation have not been quantified.

Here we measured the levels of transcripts related to PHB biosynthesis, sugar catabolism, and nitrogen metabolism by using the cells starved of nitrogen for one day. Overexpression of either *sigE* or *rre37* did not increase the levels of transcripts involved in PHB biosynthesis after one day of nitrogen depletion, suggesting that transcript levels of PHB biosynthetic enzymes do not correlate with PHB levels. Expression of genes related to sugar catabolism and nitrogen assimilation was reduced by *sigE* or *rre37* overexpression, indicating the differential effect of these genetic modifications on transcription during nitrogen starvation.

Materials and Methods

Bacterial Strains and Culture Conditions.

The glucose-tolerant (GT) strain of *Synechocystis* sp. PCC 6803 was isolated by Williams [29]. The GT-I strain was used in the present study [30]. The *sigE*- and *rre37*-overexpressing strains, generated by Osanai et al. [22, 28], were grown in modified BG-11 medium, which consisted of BG-11₀ liquid medium [31] containing 5 mM NH₄Cl (buffered with 20 mM Hepes-KOH, pH 7.8). The *sigE*- and *rre37*-overexpressing strains were named GOX50 and ROX370, respectively [22, 28]. Liquid cultures were bubbled with 1% (v/v) CO₂ in air and incubated at 30°C under continuous white light (approximately 50–70 μmol photons m⁻² sec⁻¹). Growth and cell densities were measured at 730 nm by using a Hitachi U-3310 spectrophotometer (Hitachi High-Tech., Tokyo, Japan).

RNA Isolation and Quantitative Real-time PCR.

Cells were diluted to $A_{730} = 0.2$ in 70 ml of modified BG-11 medium and grown for one day under nitrogen-replete conditions. Nitrogen-replete cells from 35 ml culture were collected by centrifugation (5,800 g 2 min) and frozen by liquid nitrogen. The cells from the residual 35 ml culture were collected by filtration and resuspended in BG-11₀ medium, followed by growth for one day. Cells were similarly corrected by centrifugation and frozen by liquid nitrogen.

Methods used for RNA isolation, cDNA synthesis, and quantitative real-time PCR were described previously [32]. The cDNA synthesis was performed using SuperScript III RT (Life Technologies Japan, Tokyo, Japan) and each 2 µg of the total RNAs as a template. Amplification was performed using FastSYBR Master Mix (Life Technologies Japan) by two-step cycling with a preheating step of 20 sec at 95°C, 40 cycles at 95°C for 3 sec and 60°C for 30 sec. The relative transcript levels were determined using StepOnePlus software (Life Technologies Japan), which were normalized to the level of *rnpB*. The 2^{-CT} method was used to analyze relative changes in transcripts. Primers used for quantitative real-time PCR are listed in Table S1.

Immunoblotting

Cells were diluted to $A_{730} = 0.2$ in 70 ml of modified BG-11 medium and grown for one day under nitrogen-replete conditions. Cells grown under nitrogen-replete or depleted conditions were similarly collected in case of RNA isolation. Immunoblotting was performed as described previously [32], using 12% SDS-PAGE gels. Proteins were visualized by alkali-phosphatase-conjugated antibodies and 1-Step NBT/BCIP solution (Takara Bio, Shiga, Japan).

1 **Results**

2 **Transcript levels for genes related to glycogen catabolism, glycolysis, and the OPP** 3 **pathway.**

4 *Synechocystis* 6803 contains two glycogen phosphorylases, encoded by *glgP*(sll1356)
5 and *glgP*(slr1367), and two isoamylases, encoded by *glgX*(slr0237) and *glgX*(slr1857),
6 which have major roles in glycogen catabolism. The levels of *glgP*(sll1356) transcript in
7 GT after one day of nitrogen depletion decreased to 60% of those in the cells grown
8 under nitrogen-replete conditions, whereas the levels of *glgP*(slr1367) remained
9 constant (Fig. 1). The levels of *glgX*(slr1857) transcripts in GT decreased to <20%
10 following nitrogen starvation for one day, while those of *glgX*(slr0237) transcripts were
11 maintained at constant levels during nitrogen starvation (Fig. 1). Either *sigE* or *rre37*
12 overexpression reduced the transcript levels for four genes after nitrogen starvation (Fig.
13 1).

14 *Synechocystis* 6803 contains two fructose-1,6-bisphosphate aldolases, encoded by
15 *fbaI* and *fbaII*, two phosphofructokinases, encoded by *pfkA*(sll0745) and *pfkA*(sll1196),
16 and two glyceraldehyde-3-phosphate dehydrogenases, encoded by *gap1* and *gap2*. *sigE*
17 overexpression reduced the levels of *fbaI* transcripts after nitrogen depletion, and
18 reduced the levels of *fbaII* transcripts under both nitrogen-replete and nitrogen-depleted
19 conditions (Fig. 2). *rre37* overexpression decreased the levels of *fbaI*, *fbaII*,
20 *pfkA*(sll0745), *pfkA*(sll1196), and *gap1* transcripts after one day of nitrogen depletion
21 (Fig. 2).

22 Transcript levels were measured for four genes involved in the OPP pathway: *zwf*
23 (encoding a glucose-6-phosphate dehydrogenase, G6PD), *opcA* (encoding an activator
24 of G6PD), *gnd* (encoding a 6-phosphogluconase dehydrogenase, 6PGD), and *tal*

(encoding a transaldolase). The expression of all four genes in GT did not increase after one day of nitrogen starvation, compared to the levels observed under nitrogen-replete conditions (Fig. 3A~D). Although *sigE* overexpression increased the expression of these four genes under nitrogen-replete conditions, the transcript levels were similar between the GT and GOX50 after nitrogen starvation (Fig. 3A~D). *rre37* overexpression decreased the levels of transcripts of these four genes under both nitrogen-replete and nitrogen-depleted conditions (Fig. 3A~D).

The protein levels of G6PD and 6PGD were quantified by immunoblotting. G6PD and 6PGD proteins increased by *sigE* overexpression under nitrogen-replete conditions, while G6PD proteins decreased and 6PGD proteins increased by *rre37* overexpression (Fig. 3E~H). The increase in 6PGD proteins by nitrogen starvation was abolished by either *sigE* or *rre37* overexpression (Fig. 3E~H).

Transcript levels of genes related to PHB biosynthesis, nitrogen assimilation and nitrate/nitrite transport.

Unlike sugar catabolic genes, the levels of transcripts of four PHB biosynthetic genes in GT remained high after one day of nitrogen depletion (Fig. 4). *sigE* overexpression increased the levels of *phaC* and *phaE* transcripts, and *rre37* overexpression increased the levels of *phaA* and *phaB* transcripts under nitrogen-replete conditions. However, the levels of transcripts of all four genes were not increased by either *sigE* or *rre37* overexpression after one day of nitrogen depletion (Fig. 4).

The levels of *glnA* transcripts in GT decreased after one day of nitrogen depletion, while those of the *glnB*, *glnN*, and *amt1* transcripts increased (Fig. 5A~D). *sigE* overexpression decreased the transcript levels for all four genes under conditions of

1 nitrogen starvation (Fig. 5A~D). *rre37* overexpression decreased the transcript levels
2 for *glnB*, *glnN*, and *amt1*, after nitrogen depletion (Fig. 5A~D). The *nrtABCD*
3 transcripts in GT were maintained at higher levels during nitrogen starvation (Fig.
4 5E~H). Either *sigE* or *rre37* overexpression decreased the transcript levels of *nrtABCD*
5 after one day of nitrogen depletion (Fig. 5E~H).

6 7 **Discussion**

8 In this study, we examined the levels of transcripts of genes related to sugar catabolism,
9 PHB biosynthesis, nitrogen assimilation, and nitrate/nitrite transport (Fig 6). Although
10 the expression of these genes is transiently induced during nitrogen starvation, the
11 quantification of these transcripts after one day of nitrogen depletion revealed a
12 different aspect of transcript control during prolonged nutrient limitation.

13 The distinct roles of multiple enzymes involved in glycogen catabolism and
14 glycolysis in *Synechocystis* 6803 remain obscure. Koksharova et al. have demonstrated
15 that Gap1 catalyzes the catabolic reaction involving the conversion of
16 glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate while Gap2 catalyzes the
17 anabolic reaction involving the conversion of 1,3-bisphosphoglycerate to
18 glyceraldehyde-3-phosphate [33]. Fu and Xu have shown that *glgP*(sll1356) is essential
19 for the growth at high temperatures, and *glgP*(slr1367) is important during
20 carbon-limited conditions [34]. Rre37 activates the expression of *glgP*(slr1367) by
21 binding to its promoter region [28], while SigE preferentially increases the expression
22 of *glgP*(sll1356) [22], suggesting the different roles and distinct regulatory mechanisms
23 of two glycogen phosphorylases. Recent analysis has revealed that two GlgPs plays
24 pivotal roles in supply of sugar phosphates and maintenance of respiratory activity [35].

1 Our analysis has shown that *sigE* overexpression alters the photosynthetic and
2 respiratory activities in response to light conditions [23], and therefore, the elucidation
3 of the regulatory mechanism underlying sugar metabolism may be useful for
4 understanding photosynthetic electron transport in cyanobacteria.

5 The transcript levels of *zwf* and *gnd* increased by *sigE* overexpression and
6 decreased by *rre37* overexpression (Fig. 3A~D), although the protein levels of 6PGD
7 were not correlated with the transcript levels (Fig. 3E~H). These results imply the
8 post-transcriptional regulation of the metabolic enzymes including 6PGD in this
9 cyanobacterium. Previous proteomic analysis showed that 6PGD is phosphorylated in
10 *Synechocystis* 6803 [36], indicating the existence for post-transcriptional control of
11 6PGD in this cyanobacterium. Redox regulation is also important for glycogen
12 metabolism of *Synechocystis* 6803 in response to changes in nutrient conditions [37].
13 Thus, analysis of protein levels, in addition to transcript levels, is indispensable for
14 understating the primary metabolism in this cyanobacterium.

15 Previous studies demonstrated that PHB levels are increased by *sigE* or *rre37*
16 overexpression during nitrogen starvation [24, 28]. The levels of *phaABCE* transcripts,
17 however, were not increased by either *sigE* or *rre37* overexpression after one day of
18 nitrogen depletion (Fig. 4). The protein levels for PhaA, PhaC, and PhaE in ROX370
19 remained at higher levels than those detected in GT after three days of nitrogen
20 depletion [28]. Thus, the transcript and protein levels of *phaABCE* do not correlate,
21 suggesting the existence for posttranscriptional control of PHB biosynthetic enzymes,
22 such as antisense RNAs which have been intensively studied [38]. The activity of
23 cyanobacterial PHA synthase is activated by acetyl-phosphate [39]; this shows the PHB
24 biosynthesis is post-translationally regulated. PhaC and PhaE of *Synechocystis* 6803

1 were detected within insoluble fractions, indicating that PHA synthase is inserted into
2 the PHB supercomplex [23]. Elucidation of the mechanism for inserting PHA synthase
3 into PHB supercomplex may lead to the regulation of amounts and molecular weights of
4 PHB in cyanobacteria.

5 Promoter analyses of nitrogen-related genes have been intensively performed as
6 mentioned above. All nitrogen-related genes tested in this study are regulated by NtcA.
7 Among the genes tested, the expression pattern of *glnA* was different from the others
8 (Fig. 5). The expression of *glnV* remained high after one day of nitrogen depletion,
9 indicating that GlnN may have an important role in nitrogen assimilation after nitrogen
10 depletion. Here, we showed that sugar catabolic regulators altered the transcript levels
11 of nitrogen-related genes (Fig. 5). Recently, *ntcA* overexpression was shown to alter
12 sugar metabolism in *Synechocystis* 6803 [40]; thus, mutual regulation between sugar
13 and nitrogen metabolism may affect the gene expression of nitrogen-related gene in
14 GOX50 and ROX370. In addition to NtcA, the transcription of nitrogen-related genes is
15 regulated by multiple transcriptional regulators associated with growth phase and
16 environmental conditions [41]. Thus, various transcriptional regulators need to be
17 considered to understand the implication of these genetically modified strains of
18 cyanobacteria.

19 In this study, we have shown that analysis of strains overexpressing transcriptional
20 regulators altered the levels of carbon and nitrogen metabolism (Fig. 6), which
21 potentially contributes to the elucidation of the mechanisms of transcription and
22 metabolism in *Synechocystis* 6803.

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2

1 **FIGURE LEGENDS**

2 **Figure 1.** Quantitative real-time PCR analysis for four genes involved in the glycogen
3 catabolism. Relative transcript levels are described. Data represent the mean $\pm$ SD
4 values from 5–6 independent experiments. Transcript levels were calibrated relative to
5 that of corresponding levels in GT under nitrogen-replete conditions (set at 100%).
6 Asterisks indicate statistically significant differences between GT and GOX50 or
7 ROX370 (Student's *t*-test; **P*<0.05, ***P*<0.005). A, *glgP*(sll1356); B, *glgP*(slr1367); C,
8 *glgX*(slr0237); D, *glgX*(slr1857).

9
10 **Figure 2.** Quantitative real-time PCR analysis for six genes involved in the glycolysis.
11 Relative transcript levels are described. Data represent the mean $\pm$ SD values from 5–6
12 independent experiments. Transcript levels were calibrated relative to that of
13 corresponding levels in GT under nitrogen-replete conditions (set at 100%). Asterisks
14 indicate statistically significant differences between GT and GOX50 or ROX370
15 (Student's *t*-test; **P*<0.05, ***P*<0.005). A, *fbaI*; B, *fbaII*; C, *pfkA*(sll0745); D,
16 *pfkA*(sll1196); E, *gapI*; F, *gap2*.

17
18 **Figure 3.** (A~D) Quantitative real-time PCR analysis for four genes involved in the
19 OPP pathway. Relative transcript levels are described. Data represent the mean $\pm$ SD
20 values from 4–7 independent experiments. Transcript levels were calibrated relative to
21 that of corresponding levels in GT under nitrogen-replete conditions (set at 100%).
22 Asterisks indicate statistically significant differences between GT and GOX50 or
23 ROX370 (Student's *t*-test; **P*<0.05, ***P*<0.005). A, *zwf*; B, *opcA*; C, *gnd*; D, *tal*. (E~H)

1 Protein levels of G6PD and 6PGD in GOX50 (E) and ROX370 (F) during nitrogen
2 starvation. Proteins extracted from the cells under nitrogen-replete or -depleted
3 conditions were subjected to SDS-PAGE. G6PD and 6PGD proteins were detected by
4 antisera previously generated [22]. Relative protein levels of G6PD (G) and 6PGD (H)
5 are described. Data represent the mean $\pm$ SD values from three independent experiments.
6 Protein levels were calibrated relative to that of corresponding levels in GT under
7 nitrogen-replete conditions (set at 100%).

8
9 **Figure 4.** Quantitative real-time PCR analysis for four genes involved in PHB
10 biosynthesis. Relative transcript levels are described. Data represent the mean $\pm$ SD
11 values from 5–6 independent experiments. Transcript levels were calibrated relative to
12 that of corresponding levels in GT under nitrogen-replete conditions (set at 100%).
13 Asterisks indicate statistically significant differences between GT and GOX50 or
14 ROX370 (Student's *t*-test; **P*<0.05, ***P*<0.005). A, *phaA*; B, *phaB*; C, *phaC*; D, *phaE*.

15
16 **Figure 5.** Quantitative real-time PCR analysis for eight genes involved in nitrogen
17 assimilation and transport. Relative transcript levels are described. Data represent the
18 mean $\pm$ SD values from 4–5 independent experiments. Transcript levels were calibrated
19 relative to that of corresponding levels in GT under nitrogen-replete conditions (set at
20 100%). Asterisks indicate statistically significant differences between GT and GOX50
21 or ROX370 (Student's *t*-test; **P*<0.05, ***P*<0.005). A, *glnA*; B, *glnB*; C, *glnN*; D, *amt1*.
22 E, *nrtA*; F, *nrtB*; G, *nrtC*; H, *nrtD*.

23
24 **Figure 6.** Metabolic map of *Synechocystis* 6803, related to glycogen and glucose

- 1 catabolism, PHB biosynthesis, and nitrogen assimilation. The reaction catalyzed by
- 2 transaldolase was designated by dashed line, in order to distinguish from other reactions.
- 3 A phosphate moiety is designated as P.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6