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## Chapter 4

### **Gene expression of the cyanobacterium *Synechocystis* PCC 6803 in response to nitrogen starvation**

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## ABSTRACT

Acclimation to nitrogen starvation involves major changes in cyanobacterial physiology through regulation of gene expression. In this study, we report a genome-wide expression analysis of the response of the model cyanobacterium *Synechocystis* PCC 6803 after incubation of 12 h under nitrogen-free medium. For this purpose, we utilized an oligonucleotide microarray covering the whole genome of *Synechocystis* PCC 6803. Observed changes in gene expression were largely consistent with previous reports on short-term nitrogen starvation. The full transcriptome picture that was recorded after 12 h of nitrogen-free incubation showed that genes encoding components of the photosynthetic apparatus, including genes for phycobilisome synthesis, were significantly down-regulated. Conversely, phycobilisome degradation proteins were up-regulated. Furthermore, many genes that play key roles in nitrogen metabolism and transport of nitrogenous compounds were up-regulated. Nitrogen starvation caused a reduction of the transcription level of many genes related to carbon fixation. Genes encoding for carbon metabolism enzymes were a major point of regulation as well. We provide a novel interpretation of the carbon-related gene expression changes under nitrogen limitation, where carbon metabolism is prepared for rapid channelling of carbon reserves to nitrogen assimilation in case nitrogen would become available again. The results provide a detailed perspective of the functional changes in whole-cell metabolism of nitrogen-starved cyanobacteria.

## INTRODUCTION

Low nitrogen availability limits the growth rate of cyanobacteria in a wide variety of freshwater and marine ecosystems (Goldman *et al.*, 1990; Vitousek and Howarth, 1991). Most cyanobacteria use nitrate, nitrite or ammonium ions as nitrogen sources, with the preferred source being ammonium (Flores and Herrero, 2005). Some cyanobacteria may utilise urea, cyanate or amino acids, and some strains are also able to fix dinitrogen gas. Cyanobacteria have evolved sophisticated mechanisms to sense and respond to nitrogen limitation. Cyanobacteria typically bleach in response to prolonged nitrogen starvation; a phenomenon that is commonly known as chlorosis (Allen and Smith, 1969; Sauer *et al.*, 2001). A number of genes and their products that participate in the process of chlorosis have been identified (Baier *et al.*, 2001). A regulatory network driven by the transcription regulation factor NtcA (Herrero *et al.*, 2001) and the regulatory protein P<sub>II</sub> (Hisbergues *et al.*, 1999) has been studied in great detail. This regulatory network responds to the nitrogen status of the cell, perceived by sensing the intracellular concentration of 2-oxoglutarate, one of the major metabolites in the glutamine synthetase – glutamate synthase (GS-GOGAT) cycle responsible for ammonium assimilation (Muro-Pastor *et al.*, 2001; Muro-Pastor *et al.*, 2005).

During the past decade, sequence analyses of entire genomes have become available for several cyanobacteria, such as the freshwater cyanobacterium *Synechocystis* PCC 6803. Despite the relatively small genome size of *Synechocystis* (3.6 Mbp with 3264 ORFs), nearly half of its open reading frames (ORFs) still lack an assigned function. The availability of the genome sequence and annotation for *Synechocystis* PCC 6803 (Kaneko *et al.*, 1996) enabled the construction of a DNA-microarray platform for global gene

expression analysis. The DNA-microarray approach has been successfully applied to study the whole-genome response of *Synechocystis* to high light and UV (Hihara *et al.*, 2001; Huang *et al.*, 2002; Tu *et al.*, 2004), phosphate limitation (Suzuki *et al.*, 2004), iron starvation-recovery (Singh *et al.*, 2003), oxidative stress (Kobayashi *et al.*, 2004), high salinity and osmotic shock (Kanesaki *et al.*, 2002; Marin *et al.*, 2003; Marin *et al.*, 2004; Shoumskaya *et al.*, 2005), cold-shock (Suzuki *et al.*, 2001; Mikami *et al.*, 2002), and to survey genes responsive in light-to-dark transitions (Gill *et al.*, 2002).

The whole-genome response of cyanobacteria to nitrogen starvation was studied first for the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120 (Ehira *et al.*, 2003). This relatively early study made use of arrays that consisted of spotted fragments, each containing one to eight ORFs, rather than individually spotted genes. The study highlighted expressed sets of genes related to heterocyst formation and nitrogen assimilation. More recently, studies with oligonucleotide microarrays have focussed on different aspects of heterocyst formation in *Anabaena* sp. PCC 7120 (Ehira and Ohmori, 2006; Suzuki *et al.*, 2006). Other recent studies have investigated the response of non-nitrogen-fixing cyanobacteria to nitrogen starvation (Krasikov *et al.*, 2005; Osanai *et al.*, 2006; Su *et al.*, 2006). For instance, Osanai *et al.* (2006) presented the whole-genome response of *Synechocystis* PCC 6803 after an incubation of 4 h of nitrogen starvation. These authors found an induction of many sugar catabolic genes and a repression of sugar anabolic genes. They also showed the induction of several nitrogen related genes and the repression of photosynthetic genes and ribosomal protein genes. The persistence of these responses under long-term nitrogen starvation is so far unknown. Studies of the whole-genome responses of cyanobacteria to longer nitrogen starvation periods are therefore an exciting prospect in cyanobacterial genomics.

Here, we present the results of a genome-wide transcriptome analysis to further characterize the responses of *Synechocystis* PCC 6803 to nitrogen starvation. We have focussed on the response to nitrogen starvation occurring within one generation (12 h), a time span in which the acclimation process is already very active, but the observed physiological changes are not too dramatic yet.

## MATERIALS AND METHODS

### Strains and growth conditions

*Synechocystis* PCC 6803 was grown in 5 batch cultures at 30°C under continuous illumination ( $50 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) in BG11 medium (Rippka *et al.*, 1979). This nutrient-rich medium contains 17.6 mM inorganic nitrogen in the form of nitrate. All other nutrients are also at saturating concentrations in BG11 medium. Aeration was provided by CO<sub>2</sub>-enriched air (3% on volume basis). Growth was monitored by measuring the optical density at 750 nm (OD<sub>750</sub>) with a double-beam spectrophotometer (Aminco DW2000, USA). To induce nitrogen starvation, each batch culture of *Synechocystis* was grown to an optical density of OD<sub>750</sub> = 0.5 cm<sup>-1</sup>, and subsequently divided into two sub-samples. Each sub-sample was harvested by centrifugation. Thereafter, one sub-sample of each culture was re-suspended in batch culture with the nitrogen-rich BG11 medium to an OD<sub>750</sub> of 0.1 cm<sup>-1</sup> and allowed to grow. These yielded 5 batch cultures, which were referred to as the

controls. The other set of sub-samples was re-suspended in batch culture with BG11<sup>0</sup> medium lacking inorganic nitrogen, washed once in this medium by centrifugation, and next inoculated in fresh BG11<sup>0</sup> to an OD<sub>750</sub> of 0.1 cm<sup>-1</sup> and allowed to grow. This yielded 5 batch cultures, which were referred to as the nitrogen-starvation treatment. After 12 h of growth, all cultures were harvested by centrifugation. Cells pellets were frozen immediately in liquid nitrogen and stored at -80°C, for later RNA isolation.

### RNA isolation and DNA microarray analysis

Total RNA was isolated by hot acid phenol and chloroform extraction, followed by LiCl precipitation and final RNA clean-up with a RNeasy mini kit (Qiagen, Germany). This method routinely removes soluble carbohydrates from RNA samples which improves the spectral characteristics of the RNA preparation. Quality and quantity of the obtained RNA were monitored using Nanodrop spectra (ND-1000, Nanodrop Technologies, USA) and Bioanalyzer assays (Agilent 2100 bioanalyzer, Agilent Technologies, USA). Single-stranded cDNA was prepared in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Biosciences, UK) fluorescent dyes as follows. 10 µg total RNA and 0.5 µg of random hexamer (Amersham Biosciences) were mixed in total volume of 15 µL in water and the mixture was heated at 70°C for 10 min and then chilled on ice. The reverse transcription reaction was performed by addition to a final concentration of 10 mM DTT (Invitrogen, USA), 500 µM each of dATP, dCTP, and dGTP, 100 µM dTTP (dNTP set, Amersham Biosciences), and 65 µM Cy3-dUTP or Cy5-dUTP (Amersham Biosciences) in 30 µL of 1x First-Strand Buffer (Invitrogen). The mixture was incubated for another 10 min at 25°C. Subsequently, 400 units of Superscript II Reverse Transcriptase (Invitrogen) were added and the mixture was incubated for 110 min at 42°C. Next, the reaction was stopped and the RNA was hydrolyzed by addition of 1.5 µL of 1 M NaOH, followed by incubation for 10 min at 70°C. Thereafter, 1.5 µL of 1 M HCl was added for neutralization. Not-incorporated fluorescent nucleotides were removed with a QIAquick PCR purification kit (Qiagen). The reverse transcription and fluorescent dye incorporation efficiency was monitored with Nanodrop spectra. Cy3 and Cy5 labelled cDNA probes from control and nitrogen-starved cells, respectively, were combined, denatured for 2 min at 98°C, and mixed with Control Targets and 2x Hybridization Buffer (Agilent *in situ* hybridization kit-plus, Agilent Technologies). The mixture was then loaded on the glass slide. Hybridization and washing conditions were as described in the “Agilent 60-mer oligo microarray processing protocol, 6-screw chamber, SSPE Wash, version 2.0” (Agilent Technologies). Slides were scanned at 10 micron resolution in an Agilent microarray scanner and the spot intensities profiles thus derived were extracted by Feature Extraction Software version 7.5 (Agilent Technologies). To eliminate artefacts from differences in dye incorporation efficiency between the Cy3 and Cy5 dyes, the dye-swap strategy was applied (Churchill, 2002), and each sample was labelled with each of the dyes. To estimate the reliability of the microarray platform, self-self hybridizations have been performed. One microarray slide presented problems in the hybridization reaction, and was discarded, leaving 4 independent replicate cultures for the subsequent analysis.

## Microarray design and data treatment

In order to investigate the transcriptional status of *Synechocystis*, we designed custom-made 45-65-mer oligonucleotides microarrays, which were printed for us by Agilent in 11K format on a standard 1x3 inch glass slide. Each of the 3264 genes of *Synechocystis* PCC 6803 (the full sequence and annotation is available at CyanoBase – <http://www.kazusa.or.jp/cyano/>) is represented in the array design by 1 to 4 different oligonucleotides dependent on the length of the ORF concerned. The complete design strategy for this oligonucleotide microarray for *Synechocystis* is described in Chapter 2 of this thesis.

## Microarray data analysis

Statistical analysis of the microarray data was based on normalization of the data, followed by tests of the statistical significance of changes in gene expression, and finalized by a control step limiting the number of false positives.

Normalization of the microarray data was conducted without background subtraction, as has been recommended for Agilent microarrays (Zahurak *et al.*, 2007). The data were log transformed, using natural logarithms, to reduce the skewness of the data distribution. To enable comparison of the data from all the microarray series, we utilized a parametric normalization procedure which was designed specifically for data sets with highly expressed genes (Chapter 3 of this thesis). This normalization procedure models the distribution of the microarray data using a Generalized Extreme Value (GEV) distribution, and subsequently converts the microarray data to a common distribution for all microarrays. The normalization procedure also adjusts partially or fully saturated values to make these values comparable between samples (for example, to compare saturated with down-regulated signals). After normalization, the 12 data-series had a common GEV distribution (see Chapter 3 for details).

Next, we proceeded to test for significant differences between the control treatments and the nitrogen-starvation treatments, using a gene by gene four-way Analysis of Variance (ANOVA). The gene by gene ANOVA model was modified from Kerr and Churchill (2001) and Wolfinger *et al.* (2001):

$$r_{gajk} = G_g + (GT)_{ga} + P_b + (GTP)_{gab} + (GA)_{gj} + (GS)_{gk} + \gamma_{gajk}$$

where  $r_{gajk}$  is the normalized intensity for gene  $g$  ( $g=1, \dots, 3264$ ), treatment  $a$  ( $a=0,1$ ), probe  $b$  ( $b=1, \dots, 8091$ ), array  $j$  ( $j=1, \dots, 6$ ) and sample  $k$  ( $k=1, \dots, 6$ ),  $G_g$  is the main effect of each gene,  $GT_{ga}$  is the interaction effect between gene and treatment,  $P_b$  is the main effect of each probe,  $GTP_{gab}$  is the probe-specific treatment effect on each gene,  $GA_{gj}$  is the interaction effect between gene and array, and  $GS_{gk}$  is the interaction effect between gene and sample. The error term  $\gamma_{gajk}$  was assumed to be normally distributed with zero mean and variance  $\sigma_\gamma^2$ . Note that the  $G$  term represents the fact that the model is fitted gene by gene. We included the  $GS$  term because the samples were randomized in the different arrays rather than hybridized in pairs, which allows quantification of the contributions of the different samples to the signal of each gene. The difference in gene expression between

the control treatment and the nitrogen-starvation treatment is determined by the values of the *GT* coefficients. Differences in gene expression between treatment and control samples hybridized to the same microarray are typically expressed as the log ratio (*M*) of the two signals. The *GT* coefficient is essentially equivalent to the log ratio, but takes into account all the microarrays of the experiment. For each of the 3264 genes of *Synechocystis* PCC 6803, the ANOVA produces a p-value indicating the significance of the *GT* term (i.e., the significance of changes in gene expression in response to nitrogen starvation).

The p-values obtained from the ANOVA estimate the probability of declaring a gene differentially expressed when it actually is not differentially expressed (error type 1). One needs to choose a critical p-value ( $p_c$ ) below which all genes are considered significantly differentially expressed. Since we tested a total of 3264 hypothesis, we expected many false positives (i.e., many of the p-values might be low merely by chance). For instance, if one investigates 1000 genes, and declares genes as differentially expressed if  $p < 0.05$ , then one may expect 50 false positives. Therefore, we used the q-value method of Storey and Tibshirani (2001) to limit the false discovery rate ( $q$ ), which is an estimate of the proportion of false positives among the genes declared significant. The false discovery rate is a function of  $p_c$ , and can be calculated as

$$q(p_c) = N_f / N_s$$

where  $N_f$  is the expected number of false positives, and  $N_s$  is the number of genes declared significant (significant genes). We choose a critical p-value corresponding to a false discovery rate of  $q=0.01$ , to obtain an expected 1% of false positives among the significant genes.

## RESULTS AND DISCUSSION

### DNA microarray analysis

RNA from 4 nitrate-starved and 4 control *Synechocystis* PCC 6803 cultures was extracted, labelled and hybridized to 45-60-mer oligonucleotide microarray slides. The selected false discovery rate of  $q = 0.01$  corresponded to a critical p-value of  $p < 0.0142$ . In total, 1485 genes (45% of the genome) were differentially expressed at a significance level of  $p < 0.0142$  (Figure 1). Accordingly, the number of false positives was estimated to be about 15 genes. Of the 1485 genes with significantly different expression in the control and treatment samples, 835 genes were up-regulated under nitrogen starvation and 650 genes were down-regulated. Complete information of all significantly regulated genes is listed in Appendix 4A (up-regulated genes) and 4B (down-regulated genes). The results obtained were similar to the results reported by Osanai *et al.* (2006), since the *M* values of both studies were positively correlated with a correlation coefficient of  $\rho=0.7$  (product-moment correlation:  $R^2=0.5$ ,  $n=3264$ ,  $p<0.001$ ), which is very high considering that the studies were performed in different labs and differed in the duration of nitrogen starvation (4 h vs. 12 h).

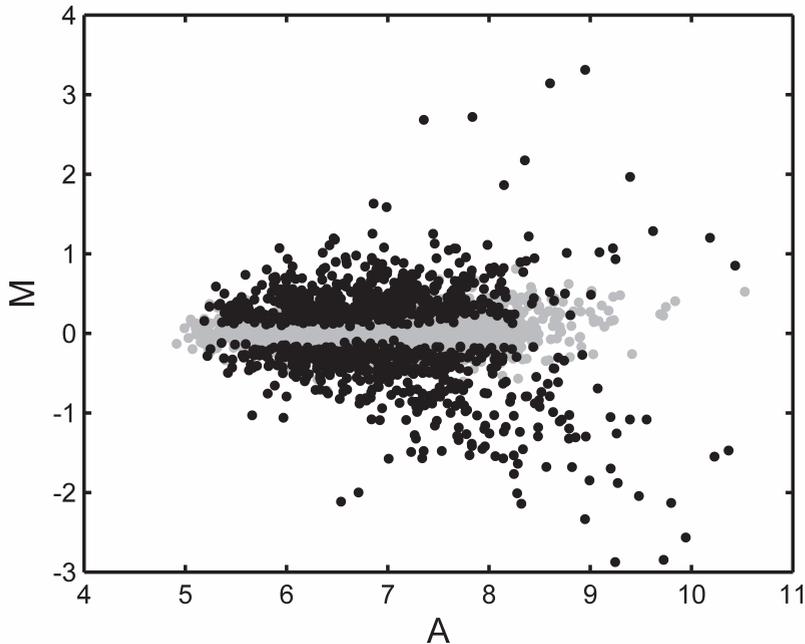


Figure 1. Response (M) of *Synechocystis* PCC 6803 gene expression to 12 h nitrogen starvation as a function of signal intensity (A) on the microarrays. The A values were calculated as the mean normalized signal intensity of all the probes for each gene on all arrays. The M values were estimated using analysis of variance as the gene by treatment interaction coefficients. Light gray points represent genes whose expression was not significantly altered by the treatment, whereas black dots represent the genes significantly responding to the treatment ( $p < 0.0142$ ,  $q = 0.01$ ). Values are in natural logarithmic scale.

### Nitrogen uptake and assimilation genes

Increased efficiency of nitrogen assimilation is crucial for survival under nitrogen-depleted conditions. Accordingly, many genes involved in nitrogen uptake and nitrogen transport were found up-regulated after 12 h of nitrogen starvation (Table 1). In particular, the active uptake system for nitrate/nitrite (*nrtABC*) together with nitrite reductase (*nirA*), genes for glutamine permease (*bgtB*), urea transport genes (*urtD* and *urtA*) and cyanate lyase (*cynS*) were significantly up-regulated. The ammonium permease gene *amt1* was significantly induced, but not *amt2* and *amt3*. The gene *amt1* is generally responsible for 95% of the ammonium uptake at very low ammonium concentrations (Montesinos *et al.*, 1998), which are typical for natural habitats.

Intracellular ammonium, produced from the reduction of nitrate and nitrite, is incorporated into amino acids by sequential action of glutamine synthetase (GS) and glutamate synthase (GOGAT). Significant up-regulation was observed only for the GS type III that is encoded by the *glnN* gene (Table 1), suggesting that in particular the activity of this enzyme is required under nitrogen deficiency, as has been observed before (Reyes *et al.*, 1997).

Table 1. Responses to nitrogen starvation of genes involved in nitrogen metabolism.

ORF	Gene	Fold change*	p-value	Gene function
<i>Regulators of nitrogen metabolism</i>				
slI1423	ntcA	-0.7	0.04	global nitrogen regulator
slr0395	ntcB	1.1	0.82	nitrate assimilation transcriptional activator, LysR family protein
<b>ssl0707</b>	<b>glnB</b>	<b>2.4</b>	<b>9.03X10<sup>-11</sup></b>	<b>nitrogen regulatory protein P-II</b>
slI1771	pphA	1.1	0.48	protein serin-threonin phosphatase
slr2031	rsbU	1.1	0.89	putative PP2C-type protein phosphatase, required to recover from N or S starvation induced stationary phase
<b>ssl0452</b>	<b>nblA1</b>	<b>16.6</b>	<b>9.99X10<sup>-16</sup></b>	<b>phycobilisome degradation protein NblA</b>
<b>ssl0453</b>	<b>nblA2</b>	<b>25.8</b>	<b>2.61X10<sup>-4</sup></b>	<b>phycobilisome degradation protein NblA</b>
<b>slI1515</b>	<b>gifB</b>	<b>-0.3</b>	<b>5.55X10<sup>-5</sup></b>	<b>glutamine synthetase inactivating factor IF17</b>
ssl1911	gifA	-0.4	0.02	glutamine synthetase inactivating factor IF7
<i>Nitrate/Nitrite uptake</i>				
<b>slr0898</b>	<b>nirA</b>	<b>2.5</b>	<b>1.70X10<sup>-11</sup></b>	<b>ferredoxin--nitrite reductase</b>
<b>slI1450</b>	<b>nrtA</b>	<b>6.8</b>	<b>2.89X10<sup>-8</sup></b>	<b>nitrate/nitrite transport system substrate-binding protein</b>
<b>slI1451</b>	<b>nrtB</b>	<b>2.0</b>	<b>2.53X10<sup>-7</sup></b>	<b>nitrate/nitrite transport system permease protein</b>
slI1452	nrtC	1.5	0.02	nitrate/nitrite transport system ATP-binding protein
slI1453	nrtD	1.1	0.58	nitrate/nitrite transport system ATP-binding protein
slI1454	narB	-0.9	0.34	ferredoxin-nitrate reductase
<i>Ammonium uptake</i>				
<b>slI0108</b>	<b>amt1</b>	<b>4.1</b>	<b>2.63X10<sup>-5</sup></b>	<b>ammonium/methylammonium permease</b>
slI0537	amt3	1.2	0.31	ammonium/methylammonium permease
slI1017	amt2	1.7	0.18	ammonium/methylammonium permease
<i>Ammonium assimilation</i>				
<b>slr1289</b>	<b>icd</b>	<b>2.0</b>	<b>5.59X10<sup>-8</sup></b>	<b>isocitrate dehydrogenase (NADP+)</b>
slr1756	glnA	1.2	0.42	glutamate--ammonia ligase
<b>slr0288</b>	<b>glnN</b>	<b>2.2</b>	<b>1.93X10<sup>-4</sup></b>	<b>glutamate--ammonia ligase</b>
slI1499	glsF	-0.8	0.22	ferredoxin-dependent glutamate synthase
slI1502	gltB	1.1	0.73	NADH-dependent glutamate synthase large subunit
slI1027	gltD	1.2	0.45	NADH-dependent glutamate synthase small subunit
slr1145	gltS	1.4	0.54	Monocomponent sodium-dependent glutamate permease GltS
<b>slr0710</b>	<b>gdhA</b>	<b>-0.5</b>	<b>4.13X10<sup>-5</sup></b>	<b>glutamate dehydrogenase (NADP+)</b>
slr1735	bgtA	1.2	0.62	ATP-binding subunit of the ABC-type Bgt permease for basic amino acids and glutamine
<b>slI1270</b>	<b>bgtB</b>	<b>2.0</b>	<b>1.55X10<sup>-6</sup></b>	<b>periplasmic substrate-binding and integral membrane protein of the ABC-type Bgt permease for basic amino acids and glutamine BgtB</b>
slI1102	gtrA	2.2	0.24	integral membrane protein (small) of a TRAP-type permease that mediates sodium-dependent glutamate transport GtrA
<b>slI1103</b>	<b>gtrB</b>	<b>1.4</b>	<b>2.85X10<sup>-4</sup></b>	<b>integral membrane protein (large) of a TRAP-type permease that mediates sodium-dependent glutamate transport GtrB</b>
slI1104	gtrC	1.2	0.81	periplasmic substrate-binding protein of a TRAP-type permease that mediates sodium-dependent glutamate transport GtrC
<i>Urea uptake</i>				
slI0374	urtE	1.5	0.05	urea transport system ATP-binding protein
<b>slI0764</b>	<b>urtD</b>	<b>2.7</b>	<b>2.99X10<sup>-11</sup></b>	<b>urea transport system ATP-binding protein</b>
<b>slr0447</b>	<b>urtA</b>	<b>2.9</b>	<b>1.78X10<sup>-4</sup></b>	<b>periplasmic protein, ABC-type urea transport system substrate-binding protein</b>
<b>slr1200</b>		<b>1.7</b>	<b>7.17X10<sup>-8</sup></b>	<b>urea transport system permease protein</b>
slr1201		1.3	0.70	urea transport system permease protein
slr1256	ureA	1.1	0.23	urease gamma subunit
slI0420	ureB	1.3	0.12	urease beta subunit
slI1750	ureC	1.2	0.35	urease alpha subunit
<b>slI1639</b>	<b>ureD</b>	<b>-0.7</b>	<b>2.65X10<sup>-4</sup></b>	<b>urease accessory protein D</b>
slr1219	ureE	1.3	0.49	urease accessory protein E
slI0643	ureG	1.2	0.25	urease accessory protein G
slr1899	ureF	1.2	0.15	urease accessory protein F
<i>Cyanate uptake</i>				
<b>slr0899</b>	<b>cynS</b>	<b>2.2</b>	<b>1.88X10<sup>-13</sup></b>	<b>cyanate lyase</b>

Notes: \* Genes with positive fold changes were up-regulated in response to nitrogen starvation, while genes with negative fold changes were down-regulated. Genes with significant changes in gene expression ( $p < 0.0142$ ;  $q = 0.01$ ) are printed in bold.

Alternatively, ammonium can be incorporated directly into glutamate by glutamate dehydrogenase (*gdhA*), in a less efficient but less energy costly reaction. Concomitantly with the increased expression of *glnN*, the expression of *gdhA* was repressed (Table 1), showing the need for a high efficiency of this key reaction when energy is abundant. The supply of carbon skeletons for the GS-GOGAT cycle in the form of 2-oxoglutarate is provided by isocitrate dehydrogenase (encoded by the *icd* gene). The transcript level for this gene was significantly increased (Table 1), providing the GS-GOGAT cycle optimally with 2-oxoglutarate obtained from preceding carbon metabolism. Two small proteins, IF7 and IF17 (encoded by *gifA* and *gifB* genes), inhibiting the activity of GS by direct protein-protein interactions (García Domínguez *et al.*, 1999), were strongly down-regulated (Table 1). These genes are in turn under the control of the global nitrogen regulator *NtcA* (García Domínguez *et al.*, 2000). In our study, the *ntcA* gene did not show any significant change of expression level, though many regulated genes are under its direct transcriptional control, including *cynS* (Harano *et al.*, 1997), *icd* (Muro-Pastor *et al.*, 1996), and probably *glnA*, *urtAB*, *nirA*, *glnB*, *amt1*, and *ndh* (Su *et al.*, 2005). Osanai *et al.* (2006) did not find expression changes of *ntcA* after 4 h of N starvation either. *NtcA*-mediated regulation of gene expression is controlled by complex interactions between this regulator and other molecules. For instance, *ntcA* interacts with the signal transduction protein P<sub>II</sub> through the mediation of *pipX* (Espinosa *et al.*, 2006, 2007). The apparent lack of induction of *ntcA* expression suggests that the physiological state of *NtcA* might be more important for its regulation function than the concentration of *NtcA*. Alfonso *et al.* (2001) showed that the *ntcA* gene is transcribed at two different promoters, and one of the products is actually induced by nitrogen starvation. However, the change in the total *ntcA* mRNA concentration is apparently too low to be detected without the discrimination of the two transcripts, as is the case with microarrays. In contrast, the P<sub>II</sub> protein gene *glnB*, which can sense the nitrogen, carbon and energy balance of the cell by binding to 2-oxoglutarate and ATP (Forchhammer, 2004) was up-regulated under nitrogen starvation (Table 1). This is consistent with the study of Osanai *et al.* (2006), and previous reports (García Domínguez and Florencio, 1997).

Summarizing, we found gene expression changes in all the major components of nitrogen physiology. The changes consisted mostly of up-regulations, indicating an attempt by the cell to increase its nitrogen acquisition. However, down-regulation of three genes, *gdhA* and *gifAB*, may be as important to achieve that same purpose. Our results indicate that after 12 h of nitrogen starvation *Synechocystis* cells actively invest in the uptake of any nitrogen-containing compounds.

### General stress response genes

The gene that presented the strongest up-regulation in this experiment was *slr0376*. Earlier research has shown that this gene with unknown function responds to various environmental stresses including nitrogen starvation (Singh and Sherman, 2002). The gene is co-transcribed as a 2.4 kb transcript with two other unknown genes, *slr0373* and *slr0374*. These two genes ranked number 6 and 33, respectively, in our list of up-regulated genes in response to nitrogen starvation (Appendix 4A). The three genes are separated by long intergenic spacers. It is known that the full 2.4 kb transcript readily splits into smaller sub-transcripts, and that the relative abundances of these sub-transcripts vary among different stress conditions (Singh and Sherman, 2002). Differential expression of the three co-

transcribed genes therefore suggests differences in transcript stability after the initial transcription of DNA into polycistronic mRNA.

#### Genes encoding terminal respiratory oxidases

After 12 h of nitrogen starvation, there was an increased expression of all genes encoding terminal respiratory oxidases (Appendix 4A). The increased expression of terminal respiratory oxidases could be a means to dissipate reduction equivalents in the thylakoidal membranes, in order to protect photosystem II from photoinhibition.

Table 2. Number of photosynthesis and respiration genes significantly up-regulated or down-regulated after 12 h of nitrogen starvation ( $p < 0.0142$ ;  $q = 0.01$ ).

	Down-regulated	Up-regulated	Total number of genes*
<i>Photosynthesis</i>			
Photosystem I	16	0	16
Photosystem II	22	1	28
cytochrome b6/f	6	2	9
ATPase	10	0	10
NADPH dehydrogenase	6	4	22
Phycobilisomes	14	2	18
Soluble electron carriers	4	4	15
<i>Respiration</i>			
Respiratory terminal oxidases	0	8	9

\*Note: Total number of genes according to the gene categories defined by Cyanobase (<http://www.kazusa.or.jp/cyano/>).

#### Genes involved in photosynthesis and carbon fixation

We observed a major decrease in the expression of all components of the photosynthetic apparatus (Table 2). Almost all genes encoding subunits of Photosystems I and II, components of the phycobilisomes, and all subunits of ATP synthetase were down-regulated in response to nitrogen starvation. Several soluble electron transporters were down-regulated as well, probably anticipating a reduced demand for energy and reducing power in cellular metabolism. Nitrogen starvation leads to reduced growth rates (Richaud *et al.*, 2001), which could be sustained with a reduced photosynthetic activity. Moreover, the photosynthetic apparatus contains a significant fraction of the total nitrogen of cyanobacterial cells under normal growth conditions (MacKenzie *et al.*, 2005). Therefore, a reduction of the photosynthetic apparatus serves two purposes, to decrease the production of photosynthetic energy to match its reduced demand and to reduce allocations of nitrogen to the photosynthetic machinery. This partial arrest of photosynthesis precedes chlorosis (Sauer *et al.*, 2001), which is typically observed later in time than the 12 h period of nitrogen starvation that we focussed on in this study. Chlorosis first involves the controlled degradation of the phycobilisomes and thereafter of the photosynthetic machinery as a whole. Our results show that genes for two small phycobilisome-degradation proteins,

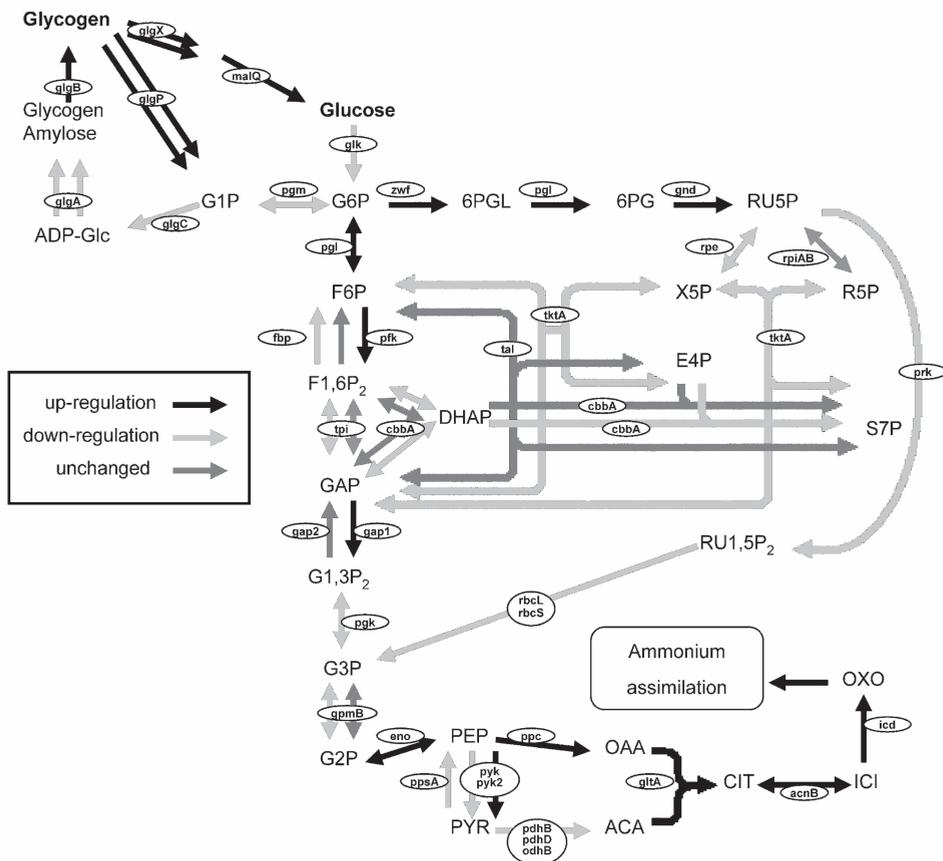


Figure 2. Up- and down-regulation of carbon metabolism gene expression of *Synechocystis* PCC 6803 after 12 h nitrogen starvation displayed on a metabolic pathway map. The pathway map was adapted from Osanai *et al.* (2007) and the Kyoto Encyclopedia of Genes and Genomes (<http://www.genome.jp/kegg/>). Red arrows indicate up-regulation and blue arrows down-regulation. For the gene descriptions and differential expression data, see Table 3. ADP-Glc is ADP-glucose; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; RU5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate; S7P, sedoheptulose-7-phosphate; RU1,5P<sub>2</sub>, ribulose-1,5-biphosphate; F6P, fructose-6-phosphate; F1,6P<sub>2</sub>, fructose-1,6-biphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; G1,3P<sub>2</sub>, 1,3-bisphosphoglycerate; G3P, 3-phosphoglycerate; G2P, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; ACA, acetyl coenzyme A; OAA, oxaloacetate; CIT, citrate; ICI, isocitrate; OXO, 2-oxoglutarate.

*NblA1* and *NblA2*, increased their transcript level more than tenfold (Table 1). Li and Sherman (2002) showed a high accumulation of *nblA* mRNA after a 24 h long nitrogen starvation period. In fact, together with the previously gene encoding a protein of unknown function (*slr0376*), these two phycobilisome-degradation proteins are placed in the top three of the most strongly up-regulated genes in response to nitrogen starvation (Appendix 4A). The presence of these genes is known to be essential for directed phycobilisome

degradation (Baier *et al.*, 2001; Richaud *et al.*, 2001), although the actual proteases involved in this process have not yet been characterized.

Consistent with the reduced expression of many genes of the photosynthetic apparatus, the pathway for photosynthetic carbon fixation, the Calvin cycle, also had many of its genes down-regulated under nitrogen starvation (Table 3; Figure 2). Particularly, the genes encoding Ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), which represents an important nitrogen pool in nitrogen replete cells (MacKenzie *et al.*, 2005), were strongly down-regulated, as has been observed before (Aldehni *et al.* 2003; Osanai *et al.*, 2006). These observations indicate a sharp decline in the demand for carbon skeletons in response to nitrogen starvation. This is consistent with the further observation that genes encoding several components of the carboxysomes and their carbon concentration mechanism proteins were also down-regulated, which could lead to a decreased efficiency of RUBISCO, whose activity depends on a CO<sub>2</sub>-rich microenvironment created by these proteins.

### Genes involved in central carbon metabolism

The central carbon metabolism of *Synechocystis* PCC 6803 was extensively regulated at the gene expression level after 12 h of nitrogen starvation (Table 3). Figure 2 presents the up-regulated and down-regulated genes of the central carbon metabolism of *Synechocystis* PCC 6803 after 12 h of nitrogen starvation. This metabolic map assumes that all metabolites involved are accessible to all enzymes at all times (i.e., the no compartmentation assumption). Our results confirm the finding of Osanai *et al.* (2006) that gene expression of sugar catabolic enzymes is induced under nitrogen starvation, while genes involved in sugar anabolism are repressed (Table 3, Figure 2). While genes acting exclusively in the direction of gluconeogenesis were down-regulated (*fbp* and *gapII*), genes acting exclusively on the glycolytic path were induced (*pfk* and *gapI*). This is in apparent contradiction with the clearly established accumulation of glycogen, a product of sugar anabolism, under nitrogen starvation (Tandeau de Marsac *et al.*, 1980; Osanai *et al.*, 2006). Osanai *et al.* (2006) point out that *glgB*, a gene involved in the synthesis of glycogen, was induced after 4 h nitrogen starvation, an observation confirmed by our results after 12 h nitrogen starvation. However, the induction of *glgB* is not enough to explain the accumulation of glycogen, since several enzymes acting before *glgB* on the glycogen synthesis pathway were down-regulated. Rather, the flow of carbon from the Calvin cycle to glycogen synthesis needs to match the degradation of glycogen in order to maintain a large glycogen pool. Metabolic fluxes cannot be predicted with gene expression data alone (Yang *et al.*, 2002), since they depend strongly on the concentrations of the substrates and products of the individual reactions, as well as on allosteric regulation of enzyme activities. We propose that carbon fixation, although itself repressed at the gene expression level (Table 2, Figure 3), is high enough to push the chemical equilibrium towards sugar anabolism, leading to glycogen accumulation.

The accumulation of 2-oxoglutarate under nitrogen starvation, another well established fact (Muro-Pastor *et al.*, 2001; Espinosa *et al.*, 2006), implies carbon flux in the opposite, catabolic direction. We propose a branching point at 3-phosphoglycerate, where part of this metabolite would follow the sugar anabolic route, following the Calvin cycle/gluconeogenesis, and part would follow the glycolytic route and the tricarboxylic acid

cycle to produce 2-oxoglutarate for ammonium assimilation. The stoichiometry of the Calvin cycle implies that for every 3-phosphoglycerate molecule channeled to ammonium assimilation, 5 molecules would need to return to the Calvin cycle to regenerate the ribulose-5-phosphate pool. Additionally, several 3-phosphoglycerate molecules would need to follow this route to compensate for the use of other Calvin cycle compounds for other biosynthetic needs, like the use of 5-carbon sugars for nucleotide synthesis. Once the concentrations of the metabolites in the system are in equilibrium, the carbon flow to 2-oxoglutarate is probably controlled by the ammonium assimilation rate, limited by the nitrogen availability. The high concentration of 2-oxoglutarate probably makes its synthesis thermodynamically less favourable, and only possible at a rate similar to its consumption.

If more nitrogen would become available, more 3-phosphoglutarate would be pulled towards the Citric Acid cycle and ammonium assimilation, because the consumption of 2-oxoglutarate would favour its synthesis. The carbon skeletons driven away from the central intermediary metabolism would then probably be restituted from the glycogen pool. This would explain the up-regulation of the oxidative part of the pentose-phosphate cycle, which would allow the conversion of glucose-6-phosphate directly into ribulose-5-phosphate, with the generation of NADPH. With a sudden increase of nitrogen supply, the glycolytic pathway could also channel carbon from glycogen to 3-phosphoglutarate until the flow from carbon fixation pushes the reaction in the gluconeogenesis direction again. Under this scheme, the glycogen reserves would act as a buffer to compensate for changes in the carbon demand for ammonium assimilation (and other biosynthetic needs). This could ensure constant levels of the Calvin cycle intermediates, anticipating a rapid increase of Calvin cycle activity when nitrogen would become available again.

Table 3. Responses to nitrogen starvation of genes involved in carbon metabolism.\*

ORF	Gene	Fold change	p-value	
<i>Calvin cycle</i>				
slI1028	ccmK2	-3.58772	<1X10 <sup>-16</sup>	carbon dioxide concentrating mechanism protein CcmK
slI1029	ccmK1	-3.23913	<1X10 <sup>-16</sup>	carbon dioxide concentrating mechanism protein CcmK
slr1839	ccmK4	-2.55852	<1X10 <sup>-16</sup>	carbon dioxide concentrating mechanism protein CcmK
slr0009	rbcL	-5.84948	7.77X10 <sup>-16</sup>	ribulose biphosphate carboxylase large subunit
slI1031	ccmM	-2.4129	1.65X10 <sup>-13</sup>	carbon dioxide concentrating mechanism protein CcmM
slr0012	rbcS	-5.36463	1.5X10 <sup>-12</sup>	ribulose biphosphate carboxylase small subunit
slr1838	ccmK3	-1.85192	1.67X10 <sup>-11</sup>	carbon dioxide concentrating mechanism protein CcmK
slr1347	icfA	-1.41546	6.45X10 <sup>-97</sup>	beta-type carbonic anhydrase localized in the carboxysome
slI1525	prk	-2.41091	0.000119	phosphoribulokinase
slr0051	ecaB	1.262831	0.000185	periplasmic beta-type carbonic anhydrase
slI1030	ccmL	-1.41535	0.037607	carbon dioxide concentrating mechanism protein CcmL
slr0436	ccmO	1.181517	0.155448	carbon dioxide concentrating mechanism protein CcmO
slI0934	ccmA	1.069244	0.237757	carboxysome formation protein CcmA
<i>Calvin cycle and glycolysis/gluconeogenesis</i>				
slr0394	pgk	-2.16666	3.67X10 <sup>-13</sup>	phosphoglycerate kinase
slr2094	fbpI	-2.20979	4.22X10 <sup>-11</sup>	fructose-1,6-/sedoheptulose-1,7-bisphosphatase
slI0018	chbA	-1.35063	1.08X10 <sup>-8</sup>	fructose-bisphosphate aldolase, class II
	(class II)			
slI1342	gap2	-1.58593	1.84X10 <sup>-6</sup>	NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenase
slr0783	tpi	1.260804	0.001288	triosephosphate isomerase
slr0952	fbpII	1.175966	0.048053	fructose-1,6-bisphosphatase
slr0943	chbA	-1.04935	0.693025	fructose-bisphosphate aldolase, class I
	(class I)			

*Glycoysis/Gluconeogenesis*

slr1945	<b>pgm</b>	<b>-1.44804</b>	<b>9.99X10<sup>-11</sup></b>	<b>2,3-bisphosphoglycerate-independent phosphoglycerate mutase</b>
slr0301	<b>ppsA</b>	<b>-1.84634</b>	<b>8.22X10<sup>-11</sup></b>	<b>phosphoenolpyruvate synthase</b>
slr0884	<b>gap1</b>	<b>1.92705</b>	<b>1.52X10<sup>-10</sup></b>	<b>glyceraldehyde 3-phosphate dehydrogenase 1 (NAD+)</b>
sl0587	<b>pyk</b>	<b>1.683134</b>	<b>2.84X10<sup>-9</sup></b>	<b>pyruvate kinase</b>
sl1841	<b>odhB</b>	<b>-</b>	<b>4.05X10<sup>-8</sup></b>	<b>pyruvate dehydrogenase dihydrolipoamide acetyltransferase component (E2)</b>
sl1721	<b>pdhB</b>	<b>-</b>	<b>3.27X10<sup>-7</sup></b>	<b>pyruvate dehydrogenase E1 component, beta subunit</b>
sl10920	<b>ppc</b>	<b>1.551811</b>	<b>4.16X10<sup>-7</sup></b>	<b>phosphoenolpyruvate carboxylase</b>
sl1196	<b>pfkA</b>	<b>1.434632</b>	<b>3.39X10<sup>-6</sup></b>	<b>phosphofructokinase</b>
slr0752	<b>eno</b>	<b>1.23512</b>	<b>0.00011</b>	<b>enolase</b>
slr1124	<b>gpmB</b>	<b>-1.48576</b>	<b>0.000251</b>	<b>phosphoglycerate mutase</b>
sl1275	<b>pyk2</b>	<b>-1.19337</b>	<b>0.000551</b>	<b>pyruvate kinase 2</b>
slr1349	<b>pgi</b>	<b>1.126223</b>	<b>0.002693</b>	<b>glucose-6-phosphate isomerase</b>
sl0593	<b>glk</b>	<b>-1.27304</b>	<b>0.006133</b>	<b>glucokinase</b>
slr1096	<b>phdD</b>	<b>-1.13330</b>	<b>0.002031</b>	<b>pyruvate dehydrogenase dihydrolipoamide dehydrogenase (E3)</b>
sl0745	<b>pfkA</b>	<b>1.30439</b>	<b>0.054343</b>	<b>phosphofructokinase</b>
sl0395	<b>gpmB</b>	<b>1.059862</b>	<b>0.547259</b>	<b>phosphoglycerate mutase</b>
slr1934		<b>-1.0852</b>	<b>0.59</b>	<b>pyruvate dehydrogenase E1 component, alpha subunit</b>

*Calvin cycle and Pentose Phosphate cycle*

sl1070	<b>tktA</b>	<b>-1.55334</b>	<b>1.29X10<sup>-11</sup></b>	<b>transketolase</b>
sl0807	<b>rpe</b>	<b>-1.63384</b>	<b>1.37X10<sup>-8</sup></b>	<b>pentose-5-phosphate-3-epimerase</b>
ssl2153	<b>rpiB</b>	<b>1.041954</b>	<b>0.374137</b>	<b>probable ribose phosphate isomerase B</b>
slr0194	<b>rpiA</b>	<b>1.084116</b>	<b>0.538663</b>	<b>ribose 5-phosphate isomerase</b>

*Pentose phosphate cycle*

slr1843	<b>zwf</b>	<b>1.394768</b>	<b>2.77X10<sup>-10</sup></b>	<b>glucose 6-phosphate dehydrogenase</b>
sl0329	<b>gnd</b>	<b>1.631945</b>	<b>4.38X10<sup>-9</sup></b>	<b>6-phosphogluconate dehydrogenase</b>
sl1479	<b>pgl</b>	<b>1.197916</b>	<b>0.000557</b>	<b>6-phosphogluconolactonase</b>
slr1734	<b>opcA</b>	<b>1.519312</b>	<b>0.001734</b>	<b>glucose 6-phosphate dehydrogenase assembly protein</b>
slr1793	<b>tal</b>	<b>1.269939</b>	<b>0.252354</b>	<b>transaldolase</b>

*Citric acid cycle*

slr0665	<b>acnB</b>	<b>1.805104</b>	<b>2.22X10<sup>-16</sup></b>	<b>aconitate hydratase</b>
slr1289	<b>icd</b>	<b>1.950823</b>	<b>2.91X10<sup>-14</sup></b>	<b>isocitrate dehydrogenase (NADP+)</b>
sl0401	<b>glcA, gluT</b>	<b>1.360651</b>	<b>3.08X10<sup>-9</sup></b>	<b>citrate synthase</b>
slr1233	<b>frdA</b>	<b>1.310954</b>	<b>1.5X10<sup>-8</sup></b>	<b>succinate dehydrogenase flavoprotein subunit</b>
sl0891	<b>citH, ldh</b>	<b>-1.25475</b>	<b>0.00033</b>	<b>malate dehydrogenase</b>
sl1557	<b>sucD</b>	<b>-1.18632</b>	<b>0.000864</b>	<b>succinyl-CoA synthetase alpha chain</b>
slr0018	<b>fumC</b>	<b>1.188981</b>	<b>0.024007</b>	<b>fumarase</b>
sl0823	<b>sdhB</b>	<b>1.154418</b>	<b>0.025585</b>	<b>probable succinate dehydrogenase iron-sulfur protein</b>
sl1625	<b>sdhB</b>	<b>1.044988</b>	<b>0.076864</b>	<b>succinate dehydrogenase iron-sulphur protein subunit</b>
sl1023	<b>sucC</b>	<b>-1.04068</b>	<b>0.313163</b>	<b>succinyl-CoA synthetase beta chain</b>

*Glycogen catabolism and anabolism*

sl1393	<b>glgA</b>	<b>-1.24565</b>	<b>4.52X10<sup>-5</sup></b>	<b>glycogen (starch) synthase</b>
sl0945	<b>glgA</b>	<b>-1.541</b>	<b>0.000567</b>	<b>glycogen synthase</b>
sl0158	<b>glgB</b>	<b>1.519001</b>	<b>1.05X10<sup>-7</sup></b>	<b>1,4-alpha-glucan branching enzyme</b>
slr1176	<b>glgC</b>	<b>-2.68343</b>	<b>1.82X10<sup>-14</sup></b>	<b>glucose-1-phosphate adenylyltransferase</b>
slr1367	<b>glgP</b>	<b>2.087603</b>	<b>7.42X10<sup>-11</sup></b>	<b>glycogen phosphorylase</b>
sl1356	<b>glgP</b>	<b>1.607764</b>	<b>0.00199</b>	<b>glycogen phosphorylase</b>
slr0237	<b>glgX</b>	<b>1.580152</b>	<b>4.12X10<sup>-8</sup></b>	<b>glycogen operon protein GlgX homolog</b>
slr1857	<b>glgX</b>	<b>1.187306</b>	<b>1.68X10<sup>-5</sup></b>	<b>isoamylase</b>
sl1676	<b>malQ</b>	<b>1.359493</b>	<b>1.87X10<sup>-9</sup></b>	<b>4-alpha-glucanotransferase</b>
sl0726	<b>pgm</b>	<b>-1.14825</b>	<b>0.013064</b>	<b>phosphoglucomutase</b>

*Polysaccharides and glycoproteins*

slr1830	<b>phaC, phbC</b>	<b>2.082989</b>	<b>8.04X10<sup>-12</sup></b>	<b>poly(3-hydroxyalkanoate) synthase</b>
sl1566	<b>ggpS</b>	<b>1.243036</b>	<b>0.000159</b>	<b>glucosylglycerolphosphate synthase</b>
slr1334	<b>abfB</b>	<b>-1.25286</b>	<b>0.000619</b>	<b>phosphoglucomutase/phosphomannomutase</b>
slr0518	<b>abfB</b>	<b>1.220699</b>	<b>0.003636</b>	<b>similar to alpha-L-arabinofuranosidase B</b>
sl1540	<b>dpml, sed3</b>	<b>-1.11624</b>	<b>0.005108</b>	<b>dolichyl-phosphate-mannose synthase</b>
slr1943		<b>-1.11342</b>	<b>0.036227</b>	<b>probable glycosyltransferase</b>
slr0820		<b>-1.15052</b>	<b>0.038349</b>	<b>probable glycosyltransferase</b>
slr0897		<b>1.32225</b>	<b>0.07383</b>	<b>probable endoglucoanase</b>
sl0842	<b>npIT</b>	<b>-1.10417</b>	<b>0.333241</b>	<b>neopullulanase</b>
slr0323	<b>amsI</b>	<b>-1.0221</b>	<b>0.835081</b>	<b>putative alpha-mannosidase</b>

\*Note: Genes with significant changes in gene expression ( $p < 0.0142$ ;  $q = 0.01$ ) are printed in bold.

In summary, the accumulation of 2-oxoglutarate and glycogen under nitrogen starvation suggests that the gene expression changes in central carbon metabolism are preparations for a fast response to increases in nitrogen availability. As an exception, down-regulation of the Calvin cycle is probably exerted to match the reduced photosynthetic activity and reduced demand for carbon skeletons for ammonium assimilation during nitrogen starvation.

## CONCLUSION

In conclusion, nitrogen starvation triggers a general increase of expression of genes involved in nitrogen uptake and assimilation, reflecting an effort of the cells to increase the acquisition of nitrogen. Our results also show an increase in the enzymes leading to the production of 2-oxoglutarate, which probably assures the supply of carbon skeletons for ammonium assimilation, and maintains the nitrogen sensory systems based on the concentration of 2-oxoglutarate in an active state. Our results clearly demonstrate down-regulation of genes encoding proteins that are involved in photosynthesis, cell growth, and the synthesis of nitrogen-rich biomolecules. The overall protein expression levels were apparently reduced, since genes encoding all 53 ribosomal proteins and several constitutive subunits of RNA polymerase were down-regulated (Table S2). This is an obvious prelude to the resting state that cells may reach after prolonged nitrogen starvation (Sauer *et al.*, 2001). The expression changes observed in genes involved in carbon metabolism suggest an anticipation of an increase in the nitrogen availability by an increased expression of genes for the degradation of glycogen, to support a constant carbon supply for ammonium assimilation and carbon fixation. We also found many examples of genes encoding unknown and hypothetical proteins that were either up- or down-regulated in response to nitrogen starvation (Appendix 4A and 4B), reflecting the fact that about 50% of the genes in the *Synechocystis* genome still lack a functional annotation. These unknown and hypothetical proteins offer a source of curiosity, where the construction of deletion mutants may help to render new insight into the molecular biology and physiology of cyanobacteria.

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