Dynamic changes in gene expression of the cyanobacterium Synechocystis sp. PCC 6803 in response to nitrogen starvation

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Chapter 4

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

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Summary

Acclimation to nitrogen limitation 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 cyanobacterium *Synechocystis* sp. PCC 6803 after 12 h of nitrogen starvation. For this purpose, we designed a new DNA microarray covering the whole genome of *Synechocystis* PCC 6803, using the Agilent custom 60-mer oligonucleotide platform. 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. The results provide a detailed perspective of the functional changes in whole-cell metabolism of nitrogen-starved cyanobacteria.

Introduction

Low nitrogen availability may limit the growth rate of cyanobacteria in a wide variety of freshwater and marine ecosystems (Goldman *et al.*, 1990; Vitousek and Howarth, 1991; Conley *et al.*, 2009). Most cyanobacteria use nitrate, nitrite or ammonium ions as nitrogen sources. 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). 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-II (Hisbergues *et al*., 1999) has been studied in great detail. This regulatory network responds to the nitrogen status of the cell, with sensing perceived by the intracellular concentration of 2-oxoglutarate, one of the major metabolites in the glutamine synthetase – glutamate synthase (GS-GOGAT) cycle (Muro-Pastor *et al*., 2005).

During the past decade, sequence analyses of entire genomes have become available for several cyanobacteria, such as the cyanobacterium *Synechocystis* PCC 6803. Despite the relatively small genome size of *Synechocystis* (3.6 Mbp with 3317 ORFs on its chromosome and 408 ORFs on plasmids), 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), heat shock (Tuominen *et al.*, 2008), carbon limitation (Eisenhut *et al.*, 2007), 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. PCC 7120 (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. 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 results from a genome-wide transcriptome analysis to characterize the responses of *Synechocystis* PCC 6803 to nitrogen starvation from a whole cell perspective. 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* sp. PCC 6803 was grown in 5 batch cultures at 30°C under continuous illumination (50 µmol photons m^{-2} 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_2-enriched air (3% on volume basis). Growth was monitored by measuring the optical density at 750 nm (OD_{750}) 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_{750} = 0.5 cm^{-1}, and subsequently divided into two subsamples. Each subsample was harvested by centrifugation. Thereafter, one subsample was resuspended in batch culture with the nitrogen-rich BG11 medium to an OD_{750} of 0.1 cm^{-1} and allowed to grow. These yielded 5 batch cultures, which were referred to as the controls. The other set of subsamples was resuspended in batch culture with $BG11^0$ medium lacking inorganic nitrogen, washed once in this medium by centrifugation, and next inoculated in fresh $BG11^0$ to an OD_{750} of 0.1 cm^{-1} 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. Cell pellets were frozen immediately in liquid nitrogen and stored -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 assay (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 a 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 in 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, all experiments were performed with use of the dye-swap strategy (Churchill, 2002). To estimate the reliability of the microarray platform two self-self hybridizations have been performed.

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. At that time, in 2005, Cyanobase reported a total of 3264 genes for *Synechocystis* PCC 6803 (the full sequence and annotation is available at CyanoBase – http://www.kazusa.or.jp/cyano/) Each of these genes is represented in the array design by 1 to 4 different oligonucleotides dependent on the length of the ORF concerned. More recently, in 2009, Cyanobase was updated and now reports a total of 3317 genes on the chromosome and 408 genes on the plasmids. The complete design strategy for our oligonucleotide microarray for *Synechocystis*, based on the Cyanobase information of 2005, is described by Aguirre von Wobeser (2010).

Microarray data analysis. Our microarray data analysis was carried out using the limma package (Smyth, 2005) of the Bioconductor project (software for analysis of genomic data; www.bioconductor.org). The limma package is based on the R language (environment for statistical computing; www.r-project.org). It is designed for the analysis of gene expression microarray data, using linear models for the assessment of differential gene expression.

Briefly, raw intensity data of each spot on the array from all biological and technical replicates for all time points were loaded inside the R environment as array-object, including annotation for each spot. Background correction was performed according to the "minimal" method. This method offers a simple approach to avoid negative intensity values after background subtraction. More specifically, any intensity which is zero or negative after background subtraction is set equal to half the minimum of the positive corrected intensities for that array. Background correction was followed by "within array" normalization for each microarray separately using global loess normalization (Cleveland and Devlin, 1983), which is used to make two channels on the single array comparable. After normalization, we calculated the mean log intensity A and the log ratio M of the intensity data, for each spot on the microarray. These quantities are defined as:

$A=(1/2)(^{2}\log R+^{2}\log G)$ and $M=^{2}\log(R/G)$,

where R and G are the intensities measured in the red and green channels, respectively (Yang et al., 2002; Smyth and Speed, 2003). Next, "between array" normalization was performed. This method normalizes data, such that the mean log intensities or log ratios have similar distributions across a series

of arrays. In particular, we applied A-quantile normalization, which ensures that the A-values have the same empirical distribution across arrays leaving the M-values unchanged (Yang and Thorne, 2003). The normalized data were then fitted with a linear model for each probe on the array for each biological replicate for each time point (Smyth, 2004). Limma uses an empirical Bayes method to estimate the standard error of the log ratios (Efron and Tibshirani, 2002). Subsequently, it was tested whether the log ratio of each spot on the microarray differs significantly from zero using a simple t test. The p-values were adjusted for multiple hypothesis testing by controlling the false discovery rate (Hochberg and Benjamini, 1990; Reiner *et al.*, 2003). The false discovery rate (FDR) was set to 0.01 and only probes with adjusted p-values less than this FDR were judged as differentially regulated. Because each gene was presented by several probes on the microarray, the gene was judged to be differentially regulated only if all probes for that gene had adjusted p-values less than 0.01. The p-value and log ratio assigned to each gene were based on the maximum p-value and the mean log ratio of its corresponding probes. Further details of the data treatment are presented in Chapter 3 of this thesis.

Results and Discussion

DNA microarray analysis. RNA from 5 nitrate-starved and 5 control *Synechocystis* PCC 6803 cultures was extracted, labelled and hybridized to 45-60-mer oligonucleotide microarray slides. Dye-swap and self-self hybridization experiments showed reliability of single microarray hybridizations (Data not shown). In total, 4073 probes were differentially expressed at a significance level of $p < 0.01$, where p-values were adjusted for multiple hypothesis testing. For the selection of the differentially expressed genes, we chose a stringent strategy: genes were selected only if all their probes were significantly regulated. This resulted in 1120 differentially expressed genes, of which 390 genes were up-regulated and 356 genes were down-regulated with a more than 1.5-fold change in their expression ratio of the nitrogen-starved versus control treatment. Selecting only those genes with a more than 2-fold change in their expression ratio, a total of 174 genes were up-regulated, while 190 genes were down-regulated in response to nitrogen starvation. Complete information of all significantly regulated genes is listed in the Supplementary Materials (Appendices 4A, B and C).

Effect of starvation on nitrogen assimilation related genes. Increased efficiency of nitrogen assimilation is crucial for survival under nitrogen-limited conditions. Accordingly, many genes involved in nitrogen uptake and nitrogen transport were found up-regulated after 12 h of nitrogen starvation (Table 1). Most prominently, all genes in the active uptake system for nitrate/nitrite (*nrt*ABCD) and nitrite reductase (*nir*A), which constitute an operon on the *Synechocystis* chromosome, were upregulated, with exception of the *nar*B gene (encoding nitrate reductase) on the last position of the operon. It is noteworthy that *nrt*A and *nir*A ranked in the top 10 of differentially expressed genes. These genes are involved in the ferredoxin-dependent reduction of nitrate to nitrite and then to ammonia. In addition, genes for glutamine permease (*bgt*B), cyanate lyase (*cyn*S) and urea transport (*urt*D and *slr*1200) were all up-regulated. Urease encoding genes (*ure*ABCDEFG) were not significantly altered according to our stringent selection criterion, except for the *ure*D gene

Table 1. List of selected genes encoding proteins with a direct function in nitrogen assimilation or a closely related supplementary function.

ORF, gene and functional annotation are as in Cyanobase (genome.kazusa.or.jp/cyanobase). Genes showing significant changes in expression and a more than 1.5-fold change in expression ratio are highlighted in bold. The fold change gives the ratio in gene expression between the nitrogen starvation treatment and the control, where positive values indicate up-regulation while negative values indicate down-regulation. Fold changes are given only for significantly regulated genes with adjusted p-values less than 0.01.

ORF	Gene	FC	Function
Regulators of nitrogen metabolism			
sll1423	**ntcA**	-1,56	global nitrogen regulator
slr0395	ntcB		nitrate assimilation transcriptional activator, LysR family protein
ssl0707	**glnB**	3,17	nitrogen regulatory protein P-II
sll1771	pphA		protein serin-threonin phosphatase
slr2031	rsbU		putative PP2C-type protein phosphatase, gene required to recover from the nitrogen or sulfate starvation
sll1515	**gifB**	-6,01	glutamine synthetase inactivating factor IF17
ssl1911	**gifA**	-4,71	glutamine synthetase inactivating factor IF7
Nitrate/Nitrite assimilation			
slr0898	**nirA**	3,83	ferredoxin--nitrite reductase
sll1450	**nrtA**	6,76	nitrate/nitrite transport system substrate-binding protein
sll1451	**nrtB**	2,74	nitrate/nitrite transport system permease protein
sll1452	**nrtC**	2,11	nitrate/nitrite transport system ATP-binding protein
sll1453	**nrtD**	1,61	nitrate/nitrite transport system ATP-binding protein
sll1454	narB		ferredoxin-nitrate reductase
Ammonium asimilation			
sll0108	amt1		ammonium/methylammonium permease
sll1017	**amt2**	1,90	ammonium/methylammonium permease
sll0537	amt3		ammonium/methylammonium permease
Glutamine/glutamate assimilation			
slr1756	glnA		glutamate--ammonia ligase
slr0288	**glnN**	3,23	glutamate--ammonia ligase
sll1499	glsF		ferredoxin-dependent glutamate synthase
sll1502	gltB		NADH-dependent glutamate synthase large subunit
sll1027	gltD		NADH-dependent glutamate synthase small subunit
slr1145	gltS		Monocomponent sodium-dependent glutamate permease GltS
slr0710	**gdhA**	-2,91	glutamate dehydrogenase (NADP+)
slr1735	bgtA		ATP-binding subunit of the ABC-type Bgt permease for basic amino acids and glutamine
sll1270	**bgtB**	2,55	periplasmic substrate-binding and integral membrane protein of the ABC-type Bgt permease for basic amino acids and glutamine BgtB
sll1102	gtrA		integral membrane protein (small) of a TRAP-type permease for sodium-dependent glutamate transport
sll1103	gtrB		integral membrane protein (large) of a TRAP-type permease for sodium-dependent glutamate transport
sll1104	gtrC		periplasmic substrate-binding protein of a TRAP-type permease for sodium-dependent glutamate transport

Table 1 (continued). List of selected genes encoding proteins with a direct function in nitrogen assimilation or a closely related supplementary function.

ORF	Gene	FC	Function
Urea assimilation			
slr0447	**urtA**	1,64	periplasmic protein, ABC-type urea transport system substrate-binding protein
sll0764	**urtD**	4,00	urea transport system ATP-binding protein
sll0374	urtE		urea transport system ATP-binding protein
slr1200		2,06	urea transport system permease protein
slr1201			urea transport system permease protein
slr1256	ureA		urease gamma subunit
sll0420	ureB		urease beta subunit
sll1750	ureC		urease alpha subunit
sll1639	ureD	-2,04	urease accessory protein D
slr1219	ureE		urease accessory protein E
slr1899	ureF		urease accessory protein F
sll0643	ureG		urease accessory protein G
Cyanate assimilation			
slr0899	**cynS**	3,56	cyanate lyase

that was downregulated. However, two out of three probes for each of the urease subunits presented on the microarray, except *ure*D, were significantly upregulated with a fold change above 1.5 (data not shown). Interestingly, of the three ammonium permeases, only the *amt*2 gene was significantly up-regulated. However, two of the three probes for the *amt*1 gene were significantly up-regulated with a 2-fold change in expression ratio (data not shown). The gene *amt*1 is generally held responsible for 95% of the ammonium uptake at very low ammonium concentrations (Montesinos *et al*., 1998), which are typical for natural habitats. All in all, our results indicate that after 12 h of nitrogen starvation *Synechocystis* cells actively invest in the uptake of many nitrogen-containing compounds (Table 1).

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 GS type III that is encoded by the *gln*N gene, suggesting that in particular the activity of this enzyme is required under nitrogen deficiency. A 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) from the tricarboxylic acid cycle (TCA cycle) (Fig. 1). The transcript level for this gene was significantly increased, providing the GS-GOGAT cycle optimally with 2-oxoglutarate obtained from preceding carbon metabolism. Two small proteins, IF7 and IF17 (encoded by *gif*A and *gif*B genes), inhibiting the activity of GS by direct protein-protein interactions were remarkably down-regulated. These genes by themselves appear under the control of the global nitrogen regulator NtcA (García-Domínguez *et al*., 2000). In our study, the *ntc*A gene was observed to be slightly down-regulated (Table 1), despite the fact that many genes related to nitrogen uptake and assimilation including *gln*A and *icd* are under its direct transcriptional control. Another remarkable observation is the increased transcription of the *glnB* gene

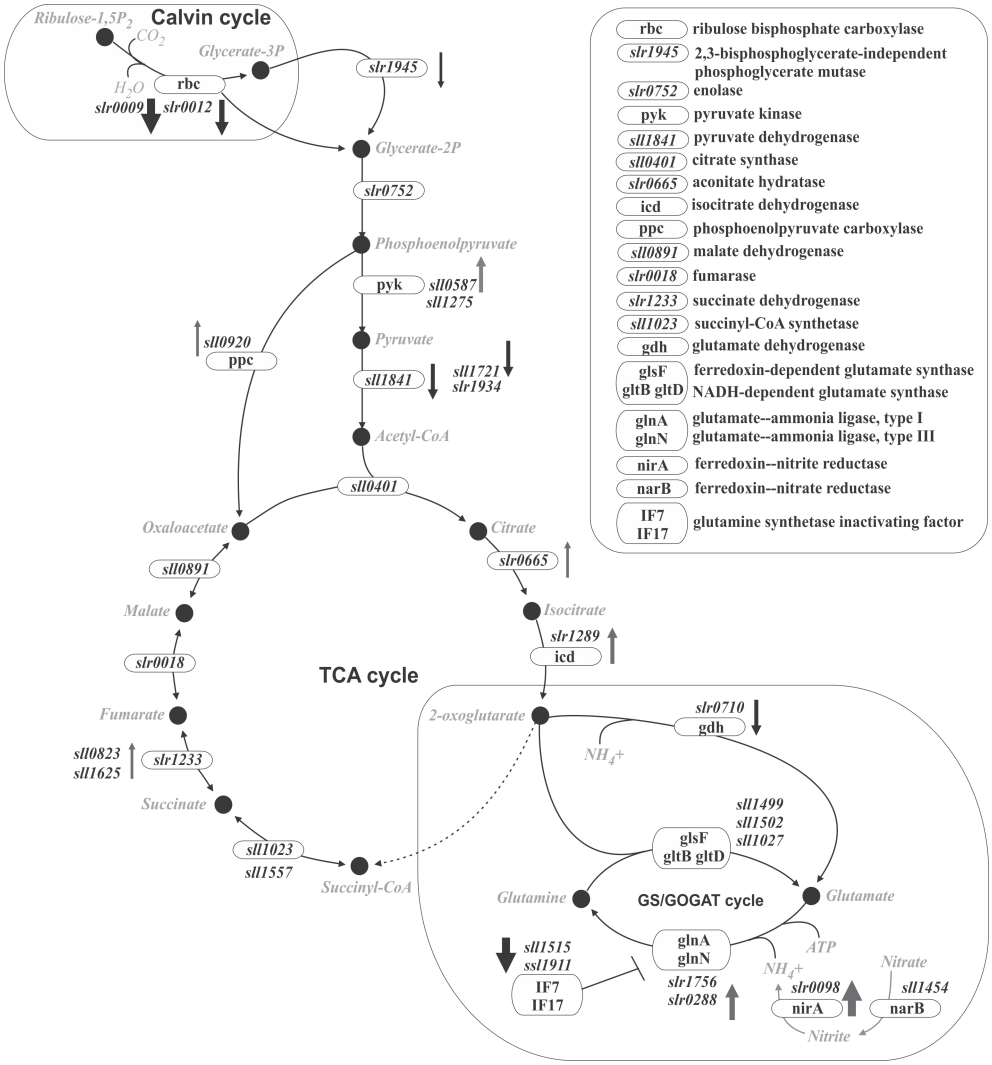

Figure 1. Changes in expression of genes involved in carbon metabolism and nitrogen assimilation. The diagram represents the main enzymes and metabolites of the TCA cycle and GS-GOGAT cycle. Grey arrows pointing upwards indicate significant up-regulation, whereas black arrows pointing downwards indicate significant down-regulation. Arrow thickness is a measure of the relative change in gene expression in response to nitrogen starvation. The broken line represents a metabolic pathway that is lacking in Cyanobacteria.

encoding the P_{II} protein. This result is consistent with the study of Osanai *et al.* (2006), and previous reports (García Domínguez and Florencio, 1997).The P_{II} signal transduction protein has a key function in coordinating the regulation of central metabolic processes. Signals from the carbon, nitrogen and energy status are integrated in different conformational and covalent modification states of the P_{II} protein. Its state of phosphorylation and binding with 2-oxoglutarate and ATP influence the behaviour of various target proteins, which in their turn regulate crucial reactions in nitrogen assimilation (reviewed by Forchhammer 2007). In summary, 2-oxoglutarate is generally believed to be a central intermediate metabolite, which intracellular concentration may act as a sensor of the nitrogen availability status in cyanobacteria (Muro-Pastor *et al*., 2001). We found that many genes of the TCA and GS-GOGAT cycles, which interconnect carbon metabolism and nitrogen assimilation through the 2-oxoglutarate molecule, were regulated in response to nitrogen starvation (Fig. 1).

Effect of starvation on photosynthesis, carbon uptake, electron transport and energy metabolism. Nitrogen starvation severely affects photosynthetic carbon fixation. This general expectation was confirmed by a reduction of the expression of most genes in the Calvin cycle (Fig. 2). In particular, 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 also observed in several earlier studies (Aldehni et al. 2003; Osanai et al., 2006). In addition, four genes encoding the carbon-concentrating mechanism protein CcmK, the *ccm*M gene, and the gene encoding carbonic anhydrase in the carboxysomes were down-regulated, reflecting a strong suppression of the carbon-concentrating mechanism (Table 2). Carboxysomes are specialised microcompartments in which the carbon dioxide concentration is enhanced to overcome the low CO_2 affinity of the RuBisCo enzyme. The carbon fixation activity of RuBisCo is generally the rate-limiting step in the Calvin cycle. These observations indicate a substantial decrease in carbon fixation, and a sharp decline in the demand for carbon skeletons in response to nitrogen starvation.

The photosynthetic light-reactions provide energy and reducing power to both carbon assimilation and nitrogen assimilation. Almost all genes encoding subunits of Photosystems I and II, components of the phycobilisomes, and all subunits of ATP synthase were down-regulated in response to nitrogen starvation, probably anticipating a reduced demand for energy and reducing power in cellular metabolism (Table 2). 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 phycobilisomes, which is a very nitrogen-rich component of the cyanobacterial cell, and thereafter of the photosynthetic machinery as a whole. Interestingly, our results show that genes encoding two small phycobilisome-degradation proteins, NblA1 and NblA2, increased their transcript level 20-fold and 10-fold, respectively (Table 2). In fact, together with a gene encoding a protein of unknown function (*sll*0783), these two proteins are placed in the top three of most strongly up-regulated genes in response to

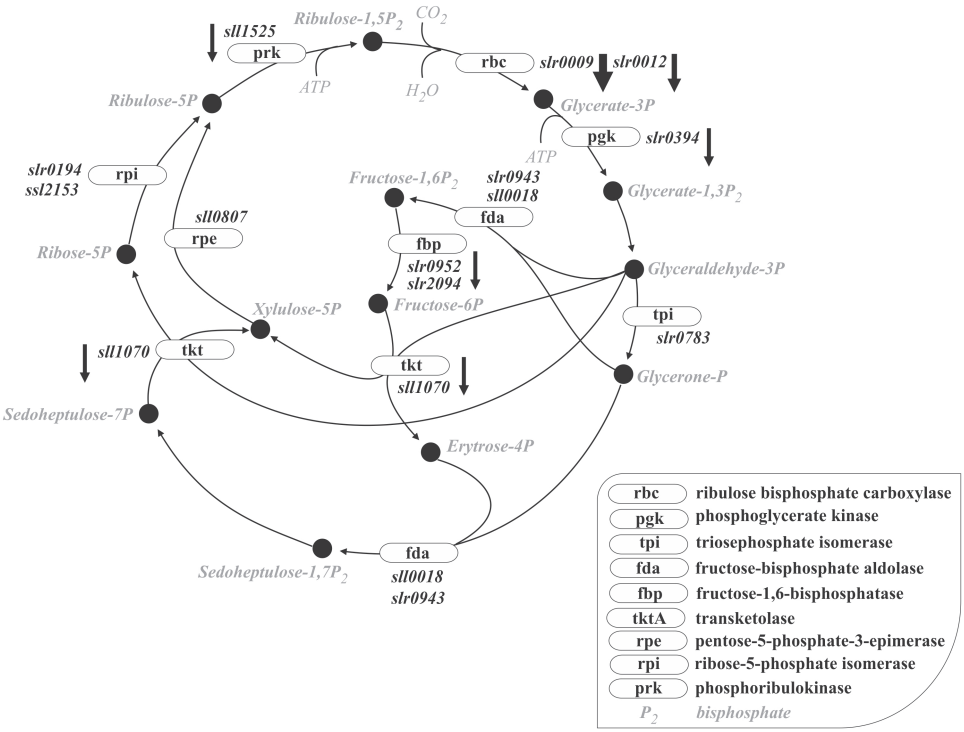

Figure 2. Changes in expression of genes involved in carbon fixation. The diagram represents the main enzymes and metabolites of the Calvin cycle. Black arrows pointing downwards indicate significant down-regulation. Arrow thickness is a measure of the relative change in gene expression in response to nitrogen starvation.

nitrogen starvation (Appendix 4C). The presence of these genes is known to be essential for directed phycobilisome degradation (Baier *et al.,* 2001), and it was recently discovered that NblA proteins bind directly to the phycobilisomes and at the same time serve as an adaptor to the ClpC-ClpP protease complex (Karradt *et al*., 2008). Respiratory terminal oxidases and hydrogenases were remarkably up-regulated after 12 hours of nitrogen starvation (Table 2). They might serve as sinks for the excess of electrons produced in the photosynthetic light reaction, as the phycobilisomes that serve as external antennae for the photosynthetic machinery are not yet degraded at this relatively early stage of nitrogen starvation.

In conclusion, our results clearly demonstrate down-regulation of genes encoding proteins that are involved in photosynthesis, cell growth, and the synthesis of nitrogen-rich biomolecules. Conversely, genes encoding proteins involved in the assimilation of nitrogen compounds like nitrite, nitrate, ammonium and urea were significantly up-regulated. The overall protein expression levels were apparently reduced, since most 35S and 50S ribosomal proteins (*rps* and *rpl* genes) and several constitutive subunits of RNA polymerase (*rpo* genes) were down regulated. This is an obvious prelude to the resting state that cells may reach after

Table 2. List of selected genes encoding proteins with a direct function in carbon uptake, photosynthesis, electron transport and energy metabolism or a closely related supplementary function.

ORF, gene and functional annotation are as in Cyanobase (genome.kazusa.or.jp/cyanobase). Genes showing significant changes in expression and a more than 1.5-fold change in expression ratio are highlighted in bold. The fold change gives the ratio in gene expression between the nitrogen starvation treatment and the control, where positive values indicate up-regulation while negative values indicate down-regulation. Fold changes are given only for significantly regulated genes with adjusted p-values less than 0.01.

ORF	Gene	FC	Function
CO_2 fixation			
sll0934	ccmA		carboxysome formation protein CcmA
sll1029	**ccmK1**	-4,54	carbon dioxide concentrating mechanism protein CcmK
sll1028	**ccmK2**	-6,20	carbon dioxide concentrating mechanism protein CcmK
slr1838	**ccmK3**	-2,45	carbon dioxide concentrating mechanism protein CcmK homolog 3
slr1839	**ccmK4**	-3,75	carbon dioxide concentrating mechanism protein CcmK homolog 4
sll1030	ccmL		carbon dioxide concentrating mechanism protein CcmL, putative carboxysome assembly protein
sll1031	**ccmM**	-4,15	carbon dioxide concentrating mechanism protein CcmM, putative carboxysome structural protein
sll1032	ccmN		carbon dioxide concentrating mechanism protein CcmN, putative carboxysome assembly protein
slr0436	ccmO		carbon dioxide concentrating mechanism protein CcmO
slr1347		-1,56	beta-type carbonic anhydrase localized in the carboxysome
slr0051	ecaB		periplasmic beta-type carbonic anhydrase
sll1342	**gap2**	-1,90	NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenase
slr1347		-1,56	beta-type carbonic anhydrase localized in the carboxysome
sll1525	**prk**	-2,66	phosphoribulokinase
slr0783	tpi		triosephosphate isomerase
slr0009	**rbcL**	-8,73	ribulose bisphosphate carboxylase large subunit
slr0012	**rbcS**	-4,38	ribulose bisphosphate carboxylase small subunit
slr0011	**rbcX**	-3,95	possible Rubisco chaperonin
Photosystem I			
slr1834	**psaA**	-1,50	P700 apoprotein subunit Ia
slr1835	psaB		P700 apoprotein subunit Ib
ssl0563	**psaC**	-3,50	photosystem I subunit VII
slr0737	**psaD**	-2,34	photosystem I subunit II
ssr2831	**psaE**	-4,91	photosystem I subunit IV
sll0819	**psaF**	-1,89	photosystem I reaction center subunit III precursor (PSI-F), plastocyanin (cyt c553) docking protein
smr0004	**psaI**	-3,18	photosystem I subunit VIII
sml0008	**psaJ**	-2,91	photosystem I subunit IX
ssr0390	**psaK1**	-2,74	photosystem I reaction center subunit X
sll0629	**psaK2**	-2,96	alternative photosystem I reaction center subunit X
slr1655	**psaL**	-2,85	photosystem I subunit XI
smr0005	**psaM**	-3,30	photosystem I subunit XII
Photosystem II			
slr1645	**psb27**	-1,96	photosystem II 11 kD protein
sll1398	psb28		photosystem II reaction center 13 kDa protein
slr1739	psb28-2		photosystem II 13 kDa protein homolog
slr1181	psbA1		photosystem II D1 protein
slr1311	psbA2		photosystem II D1 protein
sll1867	psbA3		photosystem II D1 protein
slr0906	**psbB**	-2,70	photosystem II core light harvesting protein

Table 2 (continued). List of selected genes encoding proteins with a direct function in carbon uptake, photosynthesis, electron transport and energy metabolism or a closely related supplementary function.

ORF	Gene	FC	Function
Photosystem II			
sll0851	**psbC**	-2,17	photosystem II CP43 protein
sll0849	**psbD**	-1,90	photosystem II reaction center D2 protein
slr0927	**psbD2**	-1,67	photosystem II reaction center D2 protein
ssr3451	**psbE**	-2,58	cytochrome b559 alpha subunit
smr0006	**psbF**	-3,19	cytochrome b559 b subunit
ssl2598	**psbH**	-2,52	photosystem II PsbH protein
sml0001	psbI		photosystem II reaction center PsbI protein
smr0008	**psbJ**	-3,31	photosystem II PsbJ protein
sml0005	**psbK**	-2,43	photosystem II PsbK protein
smr0007	**psbL**	-2,96	photosystem II PsbL protein
sml0003	**psbM**	-6,34	photosystem II reaction center M protein
smr0009	psbN		photosystem II PsbN protein
sll0427	**psbO**	-10,71	photosystem II manganese-stabilizing polypeptide
sll1418	psbP2		photosystem II oxygen-evolving complex 23K protein PsbP homolog
smr0001	**psbT**	-1,72	photosystem II PsbT protein
sll1194	**psbU**	-2,17	photosystem II 12 kDa extrinsic protein
sll0258	**psbV**	-3,37	cytochrome c550
sml0002	**psbX**	-1,72	photosystem II PsbX protein
sml0007	**psbY**	-1,69	photosystem II protein Y
sll1281	**psbZ**	-5,04	photosystem II PsbZ protein
ATP synthase			
sll1326	**atpA**	-5,26	ATP synthase alpha chain
slr1329	**atpB**	-3,31	ATP synthase beta subunit
sll1327	**atpC**	-5,46	ATP synthase gamma chain
sll1325	atpD		ATP synthase delta chain of CF(1)
slr1330	**atpE**	-2,42	ATP synthase epsilon chain of CF(1)
sll1324	atpF		ATP synthase B chain (subunit I) of CF(0)
sll1323	**atpG**	-4,39	ATP synthase subunit b' of CF(0)
ssl2615	**atpH**	-3,87	ATP synthase C chain of CF(0)
sll1322	**atpI**	-5,46	ATP synthase A chain of CF(0)
Phycobilisome related			
ssl0452	**nblA1**	19,40	phycobilisome degradation protein NblA
ssl0453	**nblA2**	9,30	phycobilisome degradation protein NblA
slr2067	**apcA**	-2,04	allophycocyanin alpha subunit
slr1986	**apcB**	-2,97	allophycocyanin beta subunit
ssr3383	**apcC**	-5,76	phycobilisome small core linker polypeptide
sll0928	**apcD**	-2,03	allophycocyanin-B
slr0335	apcE	-6,66	phycobilisome core-membrane linker polypeptide
slr1459	apcF		phycobilisome core component
sll1578	**cpcA**	-2,96	phycocyanin alpha subunit
sll1577	**cpcB**	-3,81	phycocyanin beta subunit
sll1580	**cpcC1**	-6,81	phycobilisome rod linker polypeptide
sll1579	**cpcC2**	-7,27	phycobilisome rod linker polypeptide
ssl3093	**cpcD**	-8,07	phycobilisome small rod linker polypeptide
slr1878	cpcE		phycocyanin alpha-subunit phycocyanobilin lyase
sll1051	cpcF		phycocyanin alpha-subunit phycocyanobilin lyase

Table 2 (continued). List of selected genes encoding proteins with a direct function in carbon uptake, photosynthesis, electron transport and energy metabolism or a closely related supplementary function.

ORF	Gene	FC	Function
Phycobilisome related			
slr2051	**cpcG1**	-2,46	phycobilisome rod-core linker polypeptide
sll1471	**cpcG2**	-3,96	phycobilisome rod-core linker polypeptide
Cytochrome b6/f complex and soluble electron carriers			
sll1317	**petA**	-2,62	apocytochrome f, component of cytochrome b6/f complex
slr0342	petB		cytochrome b6
sll1316	**petC1**	-2,76	cytochrome b6-f complex iron-sulfur subunit (Rieske iron sulfur protein)
slr1185	petC2		cytochrome b6-f complex alternative iron-sulfur subunit (Rieske iron sulfur protein)
sll1182	petC3		cytochrome b6-f complex alternative iron-sulfur subunit (Rieske iron sulfur protein)
slr0343	petD		cytochrome b6-f complex subunit 4
sll0199	petE		plastocyanin
ssl0020	petF		ferredoxin I, essential for growth
slr0150	petF		ferredoxin, petF-like protein
slr1828	petF		ferredoxin, petF-like protein
sll1382	**petF**	1,50	ferredoxin, petF-like protein
smr0010	**petG**	-1,50	cytochrome b6-f complex subunit 5
slr1643	petH		ferredoxin-NADP oxidoreductase
sll1796	**petJ**	-1,66	cytochrome c553
smr0003	**petM**	-5,58	cytochrome b6-f complex subunit PetM
sml0004	**petN**	-2,84	cytochrome b6-f complex subunit VIII
NADH dehydrogenase			
slr0851	**ndbA**	2,05	type 2 NADH dehydrogenase
slr1743	ndbB		type 2 NADH dehydrogenase NdbB
sll1484	ndbC		type 2 NADH dehydrogenase
sll0519	ndhA		NADH dehydrogenase subunit 1
sll0223	ndhB		NADH dehydrogenase subunit 2
slr1279	ndhC		NADH dehydrogenase subunit 3
slr0331	ndhD1		NADH dehydrogenase subunit 4 (involved in photosystem-1 cyclic electron flow)
slr1291	ndhD2		NADH dehydrogenase subunit 4
sll1733	**ndhD3**	-1,84	NADH dehydrogenase subunit 4 (involved in low CO2-inducible, high affinity CO2 uptake)
sll0027	**ndhD4**	-1,66	NADH dehydrogenase subunit 4 (involved in constitutive, low affinity CO2 uptake)
slr2007	**ndhD5**	-2,15	NADH dehydrogenase subunit 4
slr2009	**ndhD6**	-1,57	NADH dehydrogenase subunit 4
sll0522	ndhE		NADH dehydrogenase subunit 4L
slr0844	ndhF1		NADH dehydrogenase subunit 5
sll1732	**ndhF3**	-1,50	NADH dehydrogenase subunit 5 (involved in low CO2-inducible, high affinity CO2 uptake)
sll0026	ndhF4		NADH dehydrogenase subunit 5 (involved in constitutive, low affinity CO2 uptake)
sll0521	ndhG		NADH dehydrogenase subunit 6
slr0261	ndhH		NADH dehydrogenase subunit 7
sll0520	ndhI		NADH dehydrogenase subunit NdhI
slr1281	ndhJ		NADH dehydrogenase subunit I
slr1280	ndhK		NADH dehydrogenase subunit NdhK
ssr1386	ndhL		NADH dehydrogenase subunit NdhL
sll1594	ndhR		ndhF3 operon transcriptional regulator, LysR family protein

Table 2 (continued). List of selected genes encoding proteins with a direct function in carbon uptake, photosynthesis, electron transport and energy metabolism or a closely related supplementary function.

ORF	Gene	FC	Function
Respiratory terminal oxidases			
sll1899	**ctaB**	1,75	cytochrome c oxidase folding protein
sll0813	ctaC		cytochrome c oxidase subunit II
slr1136	**ctaCI**	3,90	cytochrome c oxidase subunit II
slr1137	**ctaDI**	2,47	cytochrome c oxidase subunit I
slr2082	ctaDII		cytochrome c oxidase subunit I
slr1138	ctaEI		cytochrome c oxidase subunit III
slr2083	ctaEII		cytochrome c oxidase subunit III
slr1379	**cydA**	2,30	quinol oxidase subunit I
slr1380	**cydB**	2,15	quinol oxidase subunit II
Hydrogenases			
sll1220	**hoxE**	1,81	putative diaphorase subunit of the bidirectional hydrogenase
sll1221	**hoxF**	1,56	diaphorase subunit of the bidirectional hydrogenase
sll1226	**hoxH**	2,55	hydrogenase subunit of the bidirectional hydrogenase
sll1223	**hoxU**	3,22	diaphorase subunit of the bidirectional hydrogenase
sll1224	hoxY		hydrogenase subunit of the bidirectional hydrogenase
slr1675	hypA1		putative hydrogenase expression/formation protein HypA1
sll1078	hypA2		putative hydrogenase expression/formation protein HypA
sll1432	**hypB**	1,89	putative hydrogenase expression/formation protein HypB
sll1079	**hypB**	1,68	putative hydrogenase expression/formation protein HypB
ssl3580	**hypC**	1,58	putative hydrogenase expression/formation protein HypC
slr1498	**hypD**	1,61	putative hydrogenase expression/formation protein HypD
sll1462	hypE		putative hydrogenase expression/formation protein HypE
sll0322	hypF		putative hydrogenase expression/formation protein HypF

prolonged nitrogen starvation (Sauer *et al*., 2001). We also found many examples of genes encoding unknown and hypothetical proteins that were regulated in response to nitrogen starvation (Appendices 4A and B). 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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List of supplemental data

Appendix 4A. List of functional categories of *Synechocystis* genes.

Appendix 4B. List of significantly up-regulated genes.

Appendix 4C. List of significantly down-regulated genes.

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