

Concerted Changes in Gene Expression and Cell Physiology of the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 during Transitions between Nitrogen and Light-Limited Growth^{1[W][OA]}

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Physiological adaptation and genome-wide expression profiles of the cyanobacterium *Synechocystis* sp. strain PCC 6803 in response to gradual transitions between nitrogen-limited and light-limited growth conditions were measured in continuous cultures. Transitions induced changes in pigment composition, light absorption coefficient, photosynthetic electron transport, and specific growth rate. Physiological changes were accompanied by reproducible changes in the expression of several hundred open reading frames, genes with functions in photosynthesis and respiration, carbon and nitrogen assimilation, protein synthesis, phosphorus metabolism, and overall regulation of cell function and proliferation. Cluster analysis of the nearly 1,600 regulated open reading frames identified eight clusters, each showing a different temporal response during the transitions. Two large clusters mirrored each other. One cluster included genes involved in photosynthesis, which were up-regulated during light-limited growth but down-regulated during nitrogen-limited growth. Conversely, genes in the other cluster were down-regulated during light-limited growth but up-regulated during nitrogen-limited growth; this cluster included several genes involved in nitrogen uptake and assimilation. These results demonstrate complementary regulation of gene expression for two major metabolic activities of cyanobacteria. Comparison with batch-culture experiments revealed interesting differences in gene expression between batch and continuous culture and illustrates that continuous-culture experiments can pick up subtle changes in cell physiology and gene expression.

Cyanobacteria utilize inorganic nutrients and light energy to build their cells. Nitrogen compounds acquired by cyanobacteria are converted to ammonium and assimilated for biosynthesis through the Gln synthase/Gln oxoglutarate aminotransferase pathway.

The Gln synthase/Gln oxoglutarate aminotransferase cycle plays a key role in the connection of carbon and nitrogen fluxes. Once ammonium has been incorporated into Gln, it is used as an amino group of many nitrogenous products in the cell, such as amino acids and nucleotides (Muro-Pastor et al., 2005).

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Tight interconnection of nitrogen metabolism with carbon assimilation follows from concomitant regulation of the many biochemical pathways in which carbon and nitrogen metabolism participate (Miller et al., 2002; Palinska et al., 2002; García-Fernández and Diez, 2004; García-Fernández et al., 2004; Flores et al., 2005; Commichau et al., 2006; Osanai et al., 2006, 2007; Su et al., 2006). In cyanobacteria, regulation of carbon and nitrogen assimilation uses 2-oxoglutarate as a metabolic reporter, the signal protein PII as a sensing transducer, and NtcA with PipX as a transcriptional coactivator for the regulation of transcription. This serves to balance gene expression to optimally sustain the enzyme activities needed for growth in nonequibrated carbon and nitrogen supply conditions (Herrero et al., 2001; Fadi Aldehni et al., 2003; Forchhammer, 2004; Flores and Herrero, 2005; Su et al., 2005; Chen et al., 2006; Espinosa et al., 2006; Singh et al., 2008, 2009).

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On the one hand, a limited availability of nitrate and carbon dioxide lowers the light reactions of photosyn-

thesis and restricts the production of photosynthetic pigments in cyanobacteria (Collier and Grossman, 1994; Collier et al., 1994; MacIntyre et al., 2002; Miller et al., 2002; McGinn et al., 2004; Kanervo et al., 2005; Nixon et al., 2005; Schagerl and Müller, 2006). In addition, nitrogen-limited cyanobacteria have evolved specialized uptake systems that permit the usage of very low concentrations of ammonium, nitrite, and nitrate, and many strains also may use other nitrogen resources, including urea and amino acids (Valladares et al., 2002; García-Fernández et al., 2004; Flores and Herrero, 2005). Cyanobacteria exposed to long-term nitrate starvation demonstrate extreme loss of photosynthetic activity and strong bleaching, but the cells remain viable (Sauer et al., 2001). When nitrogen availability changes, cyanobacteria can rebalance the uptake and assimilation of nitrogen (Herrero et al., 2001; Flores and Herrero, 2005; Espinosa et al., 2006) and adapt their overall metabolism, including that for carbon fixation and sugar metabolism (Miller et al., 2002; Curatti et al., 2006; Osanai et al., 2006, 2007).

On the other hand, nutrient-saturated growth conditions may result in the accumulation of large numbers of cyanobacterial cells, to such an extent that shading of the cyanobacterial cells leads to light limitation (Huisman, 1999; Passarge et al., 2006; Kardinaal et al., 2007). Adaptations to light limitation include an overall increase of light-harvesting and photosynthesis capacity and more subtle changes such as state transitions (Van Thor et al., 1998; Ashby and Mullineaux, 1999; Mullineaux and Emlyn-Jones, 2005), changes of photosystem ratio (De Nobel et al., 1998; Miskiewicz et al., 2002; Aurora et al., 2007; Eisenhut et al., 2007; Singh et al., 2008, 2009), and heterotrophic versatility (Walsby and Jüttner, 2006).

Changes in gene expression reported by DNA microarrays offer a powerful tool to analyze how cells utilize their genomic information under different environmental conditions. DNA microarrays in fact account remarkably well for differences in protein synthesis, resulting differences in cellular protein composition, and eventually cellular metabolism (Conway and Schoolnik, 2003; Murata and Suzuki, 2006; Suzuki et al., 2006). Therefore, whole-genome expression profiling with microarrays provides a comprehensive view of the acclimation responses of cells to changing growth environments. Microarrays have been used to analyze the global gene expression responses of cyanobacteria to a number of growth conditions, including nitrogen limitation in batch cultures (Ehira and Ohmori, 2006; Osanai et al., 2006; Su et al., 2006; Tolonen et al., 2006).

However, the potential role of the culture method has received little attention in gene expression studies. In batch culture, cells cannot be maintained in the exponential growth phase for long times. In the end, the growing cyanobacterial population will deplete either the available nutrients or the photon flux, and the population turns into a stationary phase. In contrast, continuous cultures produce a steady state at which cells retain a constant rate of growth (Monod,

1950; Novick and Szilard, 1950; Hoskisson and Hobbs, 2005). Using nitrogen-poor mineral medium, cyanobacterial populations in continuous culture will proliferate, thereby consuming nitrogen, until the residual nitrogen concentration is reduced to such an extent that the specific growth rate of the microorganisms equals the dilution rate. At this point, a steady state has been reached. Hence, in continuous culture, the specific growth rate is under experimental control and never settles at zero. It has been suggested that the controlled conditions provided by continuous cultures enhance the resolution of results that can be obtained from DNA microarray studies (Hayes et al., 2002; Bull, 2010).

In this paper, we study the whole-genome response of the cyanobacterium *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis*) to nitrogen and light limitation. We have grown *Synechocystis* in continuous cultures that were shifted from nitrogen-limited to light-limited conditions and then back to nitrogen-limited conditions. Dynamic changes in gene expression were recorded with genome-wide oligonucleotide microarrays and directly linked to a set of simultaneously measured physiological parameters. Interesting similarities and differences in genome-wide transcript profiling between our experiments in continuous culture and earlier experiments in batch culture (Osanai et al., 2006) will be discussed.

RESULTS

Experimental Dynamics

At the start of the experiment, four replicate continuous cultures had reached a nitrogen-limited steady state, with an average cell concentration of approximately 20 million cells mL⁻¹ and a light transmission through the culture vessels of approximately 180 μmol quanta m⁻² s⁻¹ (Fig. 1C). After 3 d, we increased the concentration of nitrate in the inflowing mineral medium from 500 to 6,000 μM (Fig. 1A). This nitrogen-enriched mineral medium slowly dripped into the chemostat and thereby gradually increased the total nitrogen concentration in the culture vessel (intracellular nitrogen plus free nitrate) during the next 6 d (Fig. 1B). As a consequence, the average cell concentration increased to about 200 million cells mL⁻¹, and the light transmission through the culture vessel was reduced to less than 1 μmol quanta m⁻² s⁻¹ (Fig. 1C). Stabilization of the cell concentration from day 8 onward and nearly complete extinction of the light supply indicated that growth had ceased to be determined by the nitrate supply and that a light-limited steady state was reached. On day 10, the concentration of nitrate in the inflowing medium was decreased back to 500 μM (Fig. 1A) and the total nitrogen concentration in the culture vessel gradually declined (Fig. 1B). From day 12 onward, the cell concentration started to decline, and about 10 d later, the cell concentration and light transmission through the culture vessel

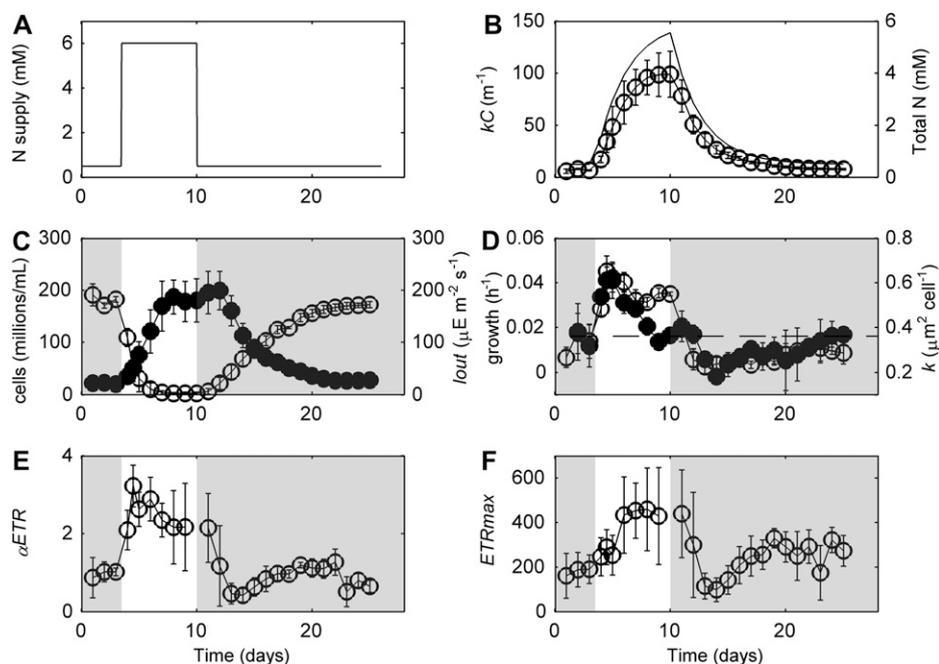


Figure 1. Experimental and physiological parameters. A, Nitrate concentration in the inflowing medium (i.e. nitrogen supply). B, Calculated total nitrogen content in the culture vessel (line) and the product (kC) of the light attenuation coefficient of the cells and the cell concentration (white symbols). C, Cell concentration (black circles) and light transmission through the culture (I_{out} ; white circles). D, Specific growth rate (black circles) and light attenuation coefficient of the cells (white circles). The dilution rate is shown by the horizontal dashed line and has the same units as the specific growth rate. E, Photosynthetic potential (α_{ETR}). F, Maximum electron transport rate (ETR_{max}). Error bars are SDS of four independent continuous cultures.

approached the same values as at the start of the experiment (Fig. 1C).

Consistent with chemostat theory, the specific growth rate was equal to the dilution rate at the initial nitrogen-limited steady state (days 2–3), at the intermediate light-limited steady state (days 8–12), and again at the final nitrogen-limited steady state (days 23–25; Fig. 1D). The specific growth rate increased to 0.04 h^{-1} in the transient period after the nitrate inflow had been raised to $6,000 \mu\text{M}$ and decreased to nearly 0 h^{-1} in the transient period after the nitrate inflow had been lowered to $500 \mu\text{M}$. The light attenuation coefficient (k) was approximately $0.3 \mu\text{m}^2 \text{ cell}^{-1}$ at the initial nitrogen-limited steady state (Fig. 1D). When the nitrate inflow concentration was increased, k increased rapidly to a maximum of $0.65 \mu\text{m}^2 \text{ cell}^{-1}$ and then slightly decreased to settle at approximately $0.55 \mu\text{m}^2 \text{ cell}^{-1}$ at the light-limited steady state. When the nitrate input was again decreased, k decreased to a minimum of $0.2 \mu\text{m}^2 \text{ cell}^{-1}$ and then slowly returned to the initial value of $0.3 \mu\text{m}^2 \text{ cell}^{-1}$ at the nitrogen-limited steady state. The light attenuation coefficient and specific growth rate (μ) followed the same pattern during the entire experiment (Pearson correlation: $\rho = 0.75$, $n = 99$, $P < 0.001$), except at the light-limited steady state, where μ stabilized at relatively lower values than k . The product of the light attenuation coefficient and the cell concentration, which determines the absorbance of the culture, followed precisely the same pattern as the total nitrogen concentration (Fig. 1B; Pearson correlation: $\rho = 0.96$, $n = 104$, $P < 0.001$).

Physiological Measurements

The photosynthetic potential (α_{ETR}) was low under nitrogen-limited conditions, increased rapidly after

nitrate addition, and reached its lowest values in the transient period after the onset of the second transient of low-nitrate conditions (Fig. 1E). We note the similarities between the time course of the specific growth rate and the time course of α_{ETR} (Fig. 1, compare D and E; Pearson correlation: $\rho = 0.71$, $n = 94$, $P < 0.001$). The maximum electron transport rate (ETR_{max}) was also higher under light-limited conditions than under nitrogen-limited conditions (Fig. 1F). However, ETR_{max} increased more slowly after nitrate addition than α_{ETR} .

The *in vivo* light absorption spectra of the *Synechocystis* cultures changed substantially during the transition from nitrogen limitation to light limitation (Fig. 2A). Averaged over the entire photosynthetically active radiation spectrum, light absorption per cell was relatively low during nitrogen-limited conditions and much higher during light-limited conditions, consistent with changes in the light attenuation coefficient (compare Figs. 1D and 2A). Pigments of interest are chlorophyll *a* (absorption peaks at 436 and 686 nm), carotenoids (480–530 nm), and phycocyanin (630 nm). The three-dimensional presentation of daily measured spectra shows several changes in time. Directly after nitrate addition, on day 3, the phycocyanin peak sharply increased, but soon it dipped and then continued to rise again simultaneously with chlorophyll *a*. During this transient, the culture visibly changed from a yellow-brown color to a fresh blue-green color that changed gradually into the familiar darker blue-green complexion of light-limited cells. During the subsequent transition from high-nitrate to low-nitrate conditions, phycocyanin and chlorophyll strongly decreased (Fig. 2B); carotenoids increased during this transition and gave again a yellow-brown color to the culture. These data demonstrate extensive changes in pigment composition and show that cells increased

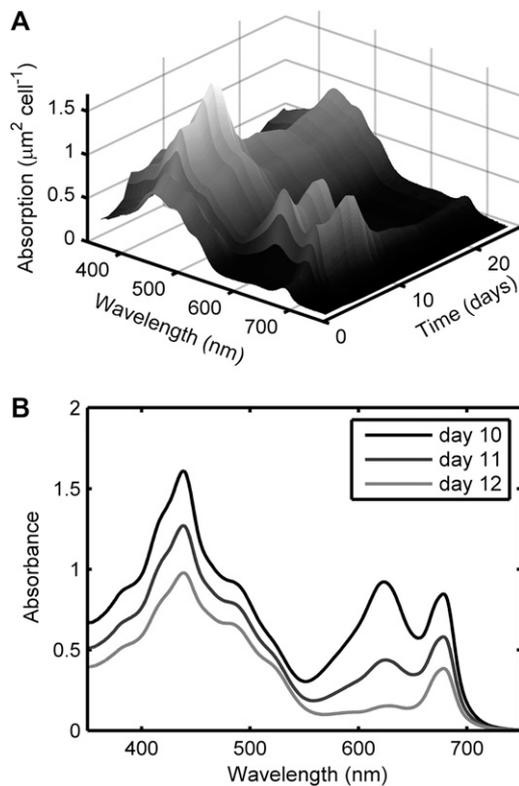


Figure 2. In vivo light absorption spectra of *Synechocystis* sampled from the continuous cultures. A, Light absorption spectra per cell, measured daily during the entire experiment. B, Absorbance normalized to the chlorophyll *a* peak at 680 nm, measured at the light-limited steady state (day 10) and just after the nitrogen supply was reduced (days 11 and 12).

their photosynthetically active pigment content during light limitation.

Dynamic Changes in Gene Expression

ANOVA revealed that, in total, 1,597 of the 3,264 genes analyzed on the microarray displayed significant changes in gene expression during the experiment at a significance level of $P < 0.01$ (Table I). This represents about half of the entire genome of *Synechocystis*. With such a large number of significantly regulated genes, the expected number of false positives remained below 1% of the total number of genes declared significant, such that correction for multiple hypothesis testing was not needed. The presentation of changes in gene expression in this paper differs slightly from the usual presentation of the ratio between gene expression in a treatment versus a control. Here, changes in gene expression were monitored in time. Hence, changes in gene expression were not normalized with respect to a single control but with respect to the entire time series. That is, the time series in Figure 3 display expression signals of genes normalized with respect to the mean and SD of their

expression signals (see "Materials and Methods"). Cluster analysis performed on the time series of all 1,597 regulated open reading frames (ORFs) resulted in seven distinct clusters (Table I). In total, 1,390 of the 1,597 genes produced time series that mimicked the centroid of the cluster to which it was assigned. The remaining 207 genes produced time series deviating strongly from the centroids of these seven clusters and therefore were assigned to an eighth cluster. Figure 3 shows the dynamic changes in gene expression representative for each of the eight clusters. A complete record of observed changes in gene expression and associated P values for all 3,264 ORFs, their cluster assignments, and additional information on EC numbers and linkage to KEGG maps is presented in Supplemental Table S1.

Description of Gene Clusters

Cluster 1 was the largest cluster, containing 307 ORFs (Table I). Gene expression in cluster 1 increased when the nitrogen supply was raised and had a relatively high value at the light-limited steady state (Fig. 3A). When the nitrogen supply was reduced again, expression rapidly decreased to a minimum level, and then it returned to initial levels when the second nitrogen-limited steady state was approached. This cluster contained many genes involved in photosynthesis and respiration (Table I; Supplemental Table S1), including several genes encoding for the major protein complexes of the thylakoid membrane (PS2, PS1, cytb6f complex, ATP synthetase) and for phycocyanin synthesis (e.g. *sll1577* and *sll1578*, shown in Fig. 4A). Several genes involved in central intermediary metabolism, energy metabolism, and amino acid biosynthesis pathways were also present in this group, such as the Gln synthase gene *slr0710* (Fig. 4A).

Genes in cluster 2 at first dropped their level of expression when the nitrogen supply was raised, but only a few days later their gene expression increased beyond initial values (Fig. 3B). When the nitrogen input was reduced again, genes in this cluster reduced their expression as in cluster 1. Cluster 2 contained many genes for phosphate acquisition, like *sll0682*, a homolog of a permease protein of a phosphate transport system, and a few chemotaxis genes, like *sll1533*, a twitching mobility protein (Fig. 4B; Supplemental Table S1).

Genes in cluster 3 did not respond to the increase in nitrogen supply but were transiently repressed when the nitrogen supply was decreased and then recovered again (Fig. 3C). The time point of transient repression coincided with the lowest growth rate, when the population was rapidly declining (Fig. 1, C and D). As shown in Table I, the most conspicuous groups of genes in this cluster were involved in transcription and translation. Examples include the RNA polymerase α -subunit (*sll1818*) and various ribosomal proteins such as *sll1740* (Fig. 4C). FtsH, a cell division protein, was also present in this cluster and was thus repressed

Table 1. Cluster analysis of genes that displayed significant changes in gene expression

The genes are grouped into functional categories according to CyanoBase. The entries present the percentage of genes within a functional category that were assigned to a given cluster (i.e. 20% of all *Synechocystis* genes involved in amino acid biosynthesis were assigned to cluster 1). The temporal changes in gene expression of each cluster are presented in Figure 3. Cluster 8 contains all genes whose temporal variation deviated strongly from the centroids of the other seven clusters. An overview of all ORFs and their cluster assignments is presented in Supplemental Table S1.

Functional Category	Cluster							
	1	2	3	4	5	6	7	8
Amino acid biosynthesis (97 genes)	20	4	6	5	3	4	5	6
Biosynthesis of cofactors (124 genes)	13	2	7	6	2	7	6	10
Cell envelope (67 genes)	8	0	5	5	5	5	12	13
Cellular processes (78 genes)	9	6	5	4	1	9	3	13
Central intermediary metabolism (31 genes)	19	7	0	10	7	10	7	7
Energy metabolism (93 genes)	14	1	2	10	7	4	5	10
Fatty acid metabolism (39 genes)	33	0	3	5	5	3	5	15
Photosynthesis and respiration (142 genes)	43	1	11	4	2	1	7	8
Purines, nucleotides (41 genes)	20	0	7	2	2	5	10	0
Regulatory functions (146 genes)	4	3	3	9	6	19	3	6
DNA replication (60 genes)	2	2	3	5	7	13	8	2
Transcription (30 genes)	10	3	23	10	0	7	13	0
Translation (168 genes)	13	2	31	3	2	4	7	8
Transport and binding proteins (196 genes)	9	7	2	7	1	7	6	9
Other categories (311 genes)	7	2	3	8	10	13	5	5
Hypothetical (1,132 genes)	6	2	3	7	5	9	4	5
Unknown (508 genes)	5	1	1	8	8	8	9	7
Total no. of genes per cluster	307	71	158	227	172	267	188	207

when the growth rate was lowest. Furthermore, some photosynthetic genes were temporarily repressed during these severely growth-limiting conditions but were not repressed at the nitrogen-limited steady states. Interestingly, these included allophycocyanin genes, which are more proximal to the core of the phycobilisomes than phycocyanin and are likely to be used for photosynthesis once growth resumes. Likewise, expression of the core proteins PsaA and PsaB of PSI was temporarily down-regulated during the most severe conditions, but it recovered once growth resumed. A few stress-related genes were also in this cluster, like a probable molecular chaperone (sll1384), an antioxidant protein (slr1198), and a stationary-phase survival protein homolog (SurE homolog; sll1108). Cluster 3 also contained genes for nitrate reduction, like ferredoxin-nitrate reductase (sll1454; Fig. 4C). Hence, these were only temporarily suppressed when nitrate uptake rates were very low.

Cluster 4 responded in an opposite way to cluster 1. The genes had high expression levels at the nitrogen-limited steady state but were repressed when the nitrogen supply was increased (Fig. 3D). When the nitrogen supply was reduced again, these genes were induced above initial levels and then slowly returned to steady state. Many genes had a function in the uptake and assimilation of different forms of nitrogen, including genes for the utilization of urea (urease gene slr1899; urea transport genes sll0374, sll0764, slr0447, slr1200, slr1201), ammonium (sll0537 and sll1017 of an ammonium methylammonium permease; slr0288 of Gln synthase), and nitrate (slr0898 of a ferredoxin-nitrite reductase; slr0900 of a molybdopterin biosyn-

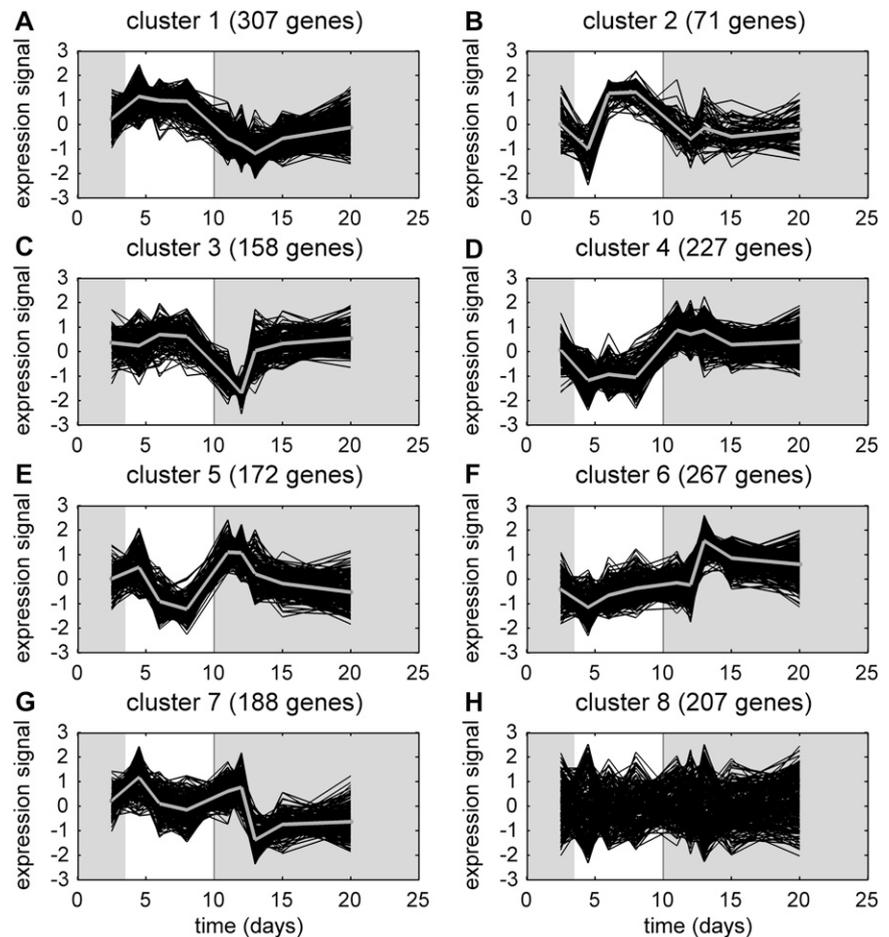
thesis protein; Fig. 4D). This cluster also contained phycobilisome degradation genes ssl0452 and ssl0453 (Fig. 4D).

Cluster 5 genes responded in an opposite way to genes in cluster 2. When the nitrogen supply was raised, genes in cluster 5 at first increased or maintained their expression, but only a few days later they were suppressed beyond initial values (Fig. 3E). When the nitrogen supply was reduced, again they first increased their expression level but then gradually settled down to lower steady-state values. Cluster 5 contained several regulatory proteins, including the nitrogen regulatory protein PII (ssl0707; Fig. 4E). It also contained two urease-related proteins (sll0643 and sll1750; Fig. 4E).

Genes in Cluster 6 did not show substantial changes in expression during the initial transient from low to high nitrogen supply, but they were induced several days after the nitrogen supply was reduced and did not return to the initial expression level within the duration of the experiment (Fig. 3F). This cluster included several genes involved in DNA replication, some genes related to nitrogen metabolism such as cyanate lyase (slr0899; Fig. 4F), and many genes with regulatory functions, including the polymerase σ factor SigC (sll0184; Fig. 4F) and genes involved in two-component signal transduction.

Cluster 7 genes responded in an opposite pattern to those of cluster 6 (Fig. 3G). These genes showed only mild changes in expression levels during the transition from nitrogen to light limitation but were strongly repressed when cells almost ceased growth several days after reduction of the nitrogen supply. This

Figure 3. Dynamic changes in gene expression during the continuous culture experiment for each of the eight clusters introduced in Table I. Thin black lines show the time series of all ORFs within the cluster. Each time series is normalized with respect to its mean and *sd*, such that each time series has a mean of 0 and a *sd* of 1. Thick gray lines indicate the centroids, which show the mean values of all time series within a cluster at each point in time. The number of ORFs per cluster is given in parentheses. Shaded and nonshaded areas correspond to periods with low nitrogen supply and high nitrogen supply, respectively. A complete list of all ORFs is presented in Supplemental Table S1.



cluster included many genes involved in synthesis of the cell envelope and in transcription (Table I). Cluster 7 also contained the RNA polymerase σ factor SigE and an oxidative stress-related protein known as glutathione *S*-transferase (*sll1545*; Fig. 4G).

Cluster 8 genes had a variety of different expression patterns that could not be reliably assigned to any of the other seven clusters (Fig. 3H). Two examples with opposite patterns are *sll1883* and *slr1604* (Fig. 4H).

Physiological Parameters and Gene Clusters

Changes in gene expression were related to changes in physiological parameters. Comparison of the time series showed that several physiological parameters correlated well with the centroids of different gene clusters (Table II). The photosynthetic parameters α_{ETR} and ETR_{max} , the light attenuation coefficient *k*, as well as the specific growth rate μ showed similar temporal variation as cluster 1, which contained many genes related to photosynthesis. The same group of physiological parameters were also positively correlated with cluster 7 and negatively correlated with clusters 4 and 6. The total nitrogen concentration in the culture and ETR_{max} were positively correlated with cluster 2, which contained many genes involved in phosphate

acquisition. The cell concentration was negatively correlated with cluster 3, while light transmission through the cultures was not significantly correlated to any cluster (Table II).

DISCUSSION

Physiological Responses

Our results show concerted changes in physiology and gene expression of *Synechocystis* in response to the transition from nitrogen to light limitation. Before highlighting the changes in gene expression during this transition, we first set the stage by briefly discussing the major physiological changes. These were largely consistent with the existing cyanobacterial literature (Collier et al., 1994; Herrero et al., 2001; MacIntyre et al., 2002; Miller et al., 2002; Commichau et al., 2006; Singh et al., 2008). When the nitrogen supply was raised, cells increased the size of their photosynthetic antennae to harvest more light energy. That is, cells produced more photosynthetic pigments, especially phycocyanin (Fig. 2). The increased pigment content resulted in a higher light attenuation coefficient of the cells (higher *k*; Fig. 1D), which was accom-

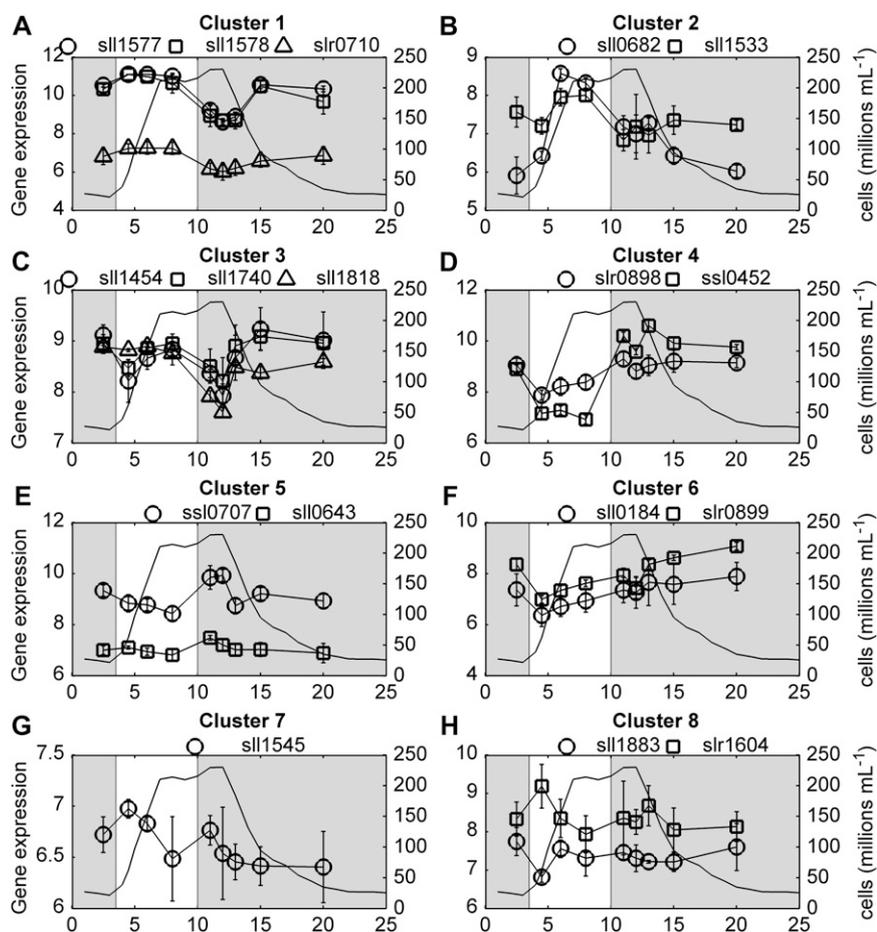


Figure 4. Dynamic changes in gene expression of specific genes during the continuous culture experiment. For each cluster, a few representative ORFs are shown. A, Cluster 1: sll1577, sll1578, slr0710. B, Cluster 2: sll0682, sll1533. C, Cluster 3: sll1454, sll1740, sll1818. D, Cluster 4: slr0898, ssl0452. E, Cluster 5: ssl0707, sll0643. F, Cluster 6: sll0184, slr0899. G, Cluster 7: sll1545. H, Cluster 8: sll1883, slr1604. The y axis for gene expression shows normalized log intensities on a log₂ scale. Error bars indicate sd of replicate probes for the corresponding gene. Shaded and nonshaded areas are as in Figure 3. The thin solid line in each panel is the mean cell concentration.

panied by a higher photosynthetic potential (higher α_{ETR} ; Fig. 1E). The rapid increase of photosynthetic potential was accompanied by an increased specific growth rate (higher μ ; Fig. 1D), which resulted in a 10-fold increase in cell concentration (Fig. 1C). The increased cell concentration, in combination with the higher pigment content per cell, absorbed almost all available light, thus creating light-limited conditions (Huisman, 1999).

Interestingly, the light attenuation coefficient k reached its peak value rapidly after the nitrogen supply was raised (day 5 in Fig. 1D), and then k slightly declined before it settled at its steady-state value. This resembles earlier observations of Allen and Hutchison (1980). It is known that, in addition to its role in photosynthesis, phycocyanin also plays a key role in the nitrogen balance of cyanobacteria. Removal of nitrogen from growth media triggers the degradation of phycocyanin, as phycocyanin is a nitrogen-rich pigment (Allen, 1984; Van de Waal et al., 2009). Conversely, nitrogen addition initiates the rapid synthesis of phycocyanin and also of the polypeptide cyanophycin (Allen and Hutchison, 1980; Stephan et al., 2000; Maheswaran et al., 2006; Van de Waal et al., 2010). Allen and Hutchison (1980) reported that phycocyanin recovery from nitrogen starvation began 3 h

after addition of the nitrogen source, while cyanophycin reached peak levels about 8 to 12 h after nitrogen addition. This was followed by a subsequent rapid decrease in cyanophycin, with its level remaining low even in the light-limited stationary state (Allen and Hutchison, 1980). These observations were very well reproduced for phycocyanin in experiments here, suggesting that phycocyanin acted as a temporary nitrogen store shortly after the shift to high-nitrate medium. Interestingly, allophycocyanin, which is more proximal to the core of the phycobilisomes than phycocyanin, responded less dynamically to changes in nitrogen availability.

Linking Physiology with Gene Expression

In total, we found 1,597 ORFs that changed their expression in a reproducible way during the transition from nitrogen limitation to light limitation and back to nitrogen limitation again. Cluster analysis enabled the classification of these ORFs into clusters with distinctly different patterns of expression. Many ORFs represented genes that had functions consistent with the physiological parameters with which they clustered. For instance, cluster 1 contained many genes involved in photosynthesis, and its pattern was posi-

Table II. Correlations between physiological parameters and gene clusters

The entries show the Pearson correlation coefficients between the values of the physiological parameters and the cluster centroids; only significant correlations ($P < 0.05$) are shown. An overview of all ORFs and their cluster assignments is presented in Supplemental Table S1.

Parameter	Cluster							
	1	2	3	4	5	6	7	8
α_{ETR}	0.77			-0.72		-0.74	0.58	
ETR_{max}	0.51	0.62				-0.49		
Specific growth rate (μ)	0.68			-0.68		-0.79	0.73	
Light attenuation coefficient (k)	0.89			-0.87		-0.78	0.57	
Cell concentration (C)			-0.58					
Total nitrogen		0.60						
Light transmission (I_{out})								

tively correlated to several photosynthetic parameters. The genes and photosynthetic parameters associated with this cluster were up-regulated during light-limited growth but down-regulated during nitrogen-limited growth (Tables I and II). Cluster 4 included several genes involved in nitrogen assimilation. These genes showed the opposite pattern; they were down-regulated during light-limited growth but up-regulated during nitrogen-limited conditions. Cluster 1 and its mirror image cluster 4 thus accounted for the most conspicuous responses to nitrogen limitation, including well-known phenomena such as the repression of the photosynthetic apparatus and the induction of nitrogen-acquisition mechanisms (Sauer et al., 2001; Forchhammer, 2004; Flores and Herrero, 2005) and some sugar catabolic genes (Osanai et al., 2006). In addition, the cluster analysis identified well-defined responses of a large number of ORFs encoding hypothetical and unknown proteins. This indicates that these ORFs have a clear functional potential that in the longer term may become revealed from deletion mutants and comparison of regulation under different culture conditions.

In terms of gene expression, the clustering of genes belonging to the NtcA regulon (the largest group of nitrogen-responsive genes in cyanobacteria) is interesting. While most of these genes, including *nirA* (slr0898), are grouped into cluster 4, *glnB* (ssl0707) and *narB* (sll1454) are grouped into clusters 5 and 3, respectively (Fig. 4). Indeed, unlike in most other cyanobacteria, the functionally related genes *nirA* (for nitrite reductase) and *narB* (for nitrate reductase) are located on separate transcription units in *Synechocystis* (compare with CyanoBase). Also, *glnB*, encoding the PII protein, appears to have two promoters (García-Domínguez and Florencio, 1997) and therefore may show complex regulation of its transcription. Our cluster results seem to reflect these characteristics.

We further observed that phycobilisome synthesis resorted in cluster 1 while phycobilisome breakdown resorted in cluster 4, which is consistent with expectation. However, we also noticed that gene product slr2002, encoding cyanophycin synthase, resorted in cluster 4. This seems a strange result. Why would high-nitrate conditions give rise to the apparent repression of a storage polymer for nitrogen? However,

it has been reported before that the regulation of cyanophycin synthesis depends on a complex interrelation between cyanophycin synthesis, L-Arg catabolism, and photosynthesis (Ziegler et al., 1998; Stephan et al., 2000; Maheswaran et al., 2006; Schriek et al., 2007). It might be that cyanophycin synthetase acts as a cyanophycin lyase as well. This would fit with the observed regulation of carbamoyl phosphate synthase (*sll1498*), an enzyme in Arg metabolism, in cluster 4.

Control at the metabolite and enzyme levels likely plays a role in more processes. The glycogen synthase gene *sll0945*, responsible for the synthesis of this carbon reserve, tended to be less expressed under nitrogen-limited conditions than under light-limited conditions. Glycogen is known to accumulate in *Synechocystis* and other cyanobacteria under nitrogen-limited conditions (Lehmann and Wöber, 1978; Tandeau de Marsac et al., 1980; Osanai et al., 2007). Our results suggest that the accumulation of glycogen under nitrogen-limited conditions is driven by the concentration of substrates and products rather than by the amount of glycogen synthase produced. Only a few genes encoding enzymes involved in amino acid synthesis had lower expression levels under nitrogen-limited conditions. Instead, genes of these pathways were present in all clusters. Many enzymes of those highly branched patterns are posttranscriptionally regulated by their products or substrates (Umbarger, 1978; Keseler et al., 2009). Therefore, our data indicate that posttranscriptional regulation seems more important in the amino acid biosynthesis pathways than regulation at the level of gene expression.

Finally, the induction of phosphate uptake genes under light-limited conditions observed in cluster 2 suggests that polyphosphate may accumulate in light-limited cultures. In nature, polyphosphate accumulation is well known to occur in cyanobacteria (Morohoshi et al., 2002).

Comparison between Batch Culture and Continuous Culture

Our continuous-culture experiment comprised three different steady states connected by two transient states. One of these transients from a light-

limited to a nitrogen-limited state permits comparison with existing data from other laboratories. We observed that our data on that particular transition had many features in common with earlier batch-culture experiments under nitrogen-limited conditions (Ehira and Ohmori, 2006; Osanai et al., 2006; Tolonen et al., 2006), but we also found several interesting differences that are probably related to differences in growth conditions between the two culture techniques. In batch culture, the concentration of free nitrate and ammonium in the mineral medium is completely exhausted. Cells become nitrogen starved and ultimately cease growth in the stationary phase. In contrast, in continuous culture, cell growth is sustained through a low but continuous supply of nitrate into the culture vessel. The cell concentration reaches steady-state values, at which the specific growth rate equals the dilution rate (Fig. 1, C and D). Hence, genes involved in cell growth will remain active in continuous culture. Another important difference with batch-culture experiments is that the experimental growth conditions in continuous culture can be more carefully defined and controlled (Huisman et al., 2002; Hoskisson and Hobbs, 2005; Bull, 2010), which enhances the level of precision that can be achieved in microarray data from continuous-culture experiments.

The batch-culture experiment of Osanai et al. (2006) identified 512 genes with a more than 2-fold change in gene expression in response to nitrogen limitation, of which 390 genes (76%) showed a significant response in our continuous-culture experiment as well. The directions of the changes in gene expression were largely consistent between the Osanai et al. (2006) batch-culture experiment and our continuous-culture experiment. Most down-regulated genes in response to nitrogen limitation in the batch-culture experiment were assigned to clusters 1 and 3 in our continuous-culture experiment (Fig. 5A). However, the continuous-culture experiment distinguished between genes with persistent down-regulation in cluster 1 and those with transient down-regulation in cluster 3. Genes in cluster 3 were repressed only temporarily (Fig. 3C), when population growth almost ceased shortly after the reduction in nitrogen supply (Fig. 1D), but the expression of these genes recovered when cell growth resumed. Indeed, many genes in cluster 3 are essential for cell growth, such as genes encoding RNA polymerase, ribosomal proteins, the core phycobilisome chromophore allophycocyanin, the PSI core subunits PsaA and PsaB, and the cell division protein FtsH.

Most up-regulated genes in the batch-culture experiment of Osanai et al. (2006) were assigned to clusters 4 to 6 in our continuous-culture experiment (Fig. 5B). Again, the continuous-culture experiment distinguished between genes with persistent up-regulation in cluster 4 and those with transient up-regulation in clusters 5 and 6. The latter two clusters contained several genes involved in regulatory functions, which signified the near cessation of cell growth shortly after reduction of the nitrogen supply (Fig. 3, E and F).

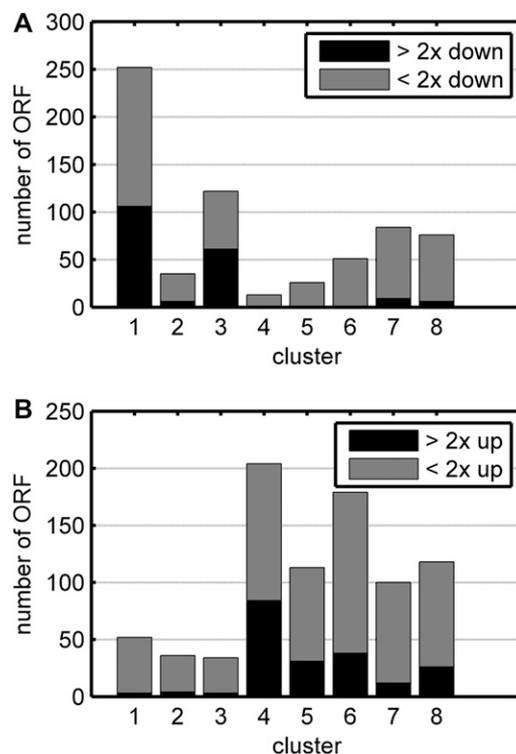


Figure 5. Comparison of responsive genes in our continuous-culture experiment and the batch-culture experiment of Osanai et al. (2006). A, Distribution of ORFs down-regulated in response to nitrogen limitation in the experiment of Osanai et al. (2006) over the eight clusters distinguished in our experiment. Black bars show ORFs down-regulated more than 2-fold; gray bars show ORFs down-regulated less than 2-fold in Osanai et al. (2006). B, Distribution of ORFs up-regulated in response to nitrogen limitation in the experiment of Osanai et al. (2006) over the eight clusters distinguished in our experiment. Black bars show ORFs up-regulated more than 2-fold; gray bars show ORFs up-regulated less than 2-fold in Osanai et al. (2006). A full record of all responsive ORFs in our experiment and the experiment of Osanai et al. (2006) is provided in Supplemental Table S1.

Examples include the nitrogen regulatory protein PII and the induction of *hox* genes that are likely active in the release of excess reductive power.

Many genes with a significant response in our continuous-culture experiment showed a less than 2-fold change in the batch-culture experiments of Osanai et al. (2006; Fig. 5). This can be attributed to at least partly to the broader range of conditions investigated in our continuous-culture experiments, as we exposed cells to both light-limited and nitrogen-limited conditions, while Osanai et al. (2006) investigated nitrogen-limited conditions only. Also, the data analysis method differed between our study and Osanai et al. (2006), and our statistical analysis may have contributed to the detection of a large number of responsive genes. Finally, it has often been argued that the highly controlled conditions provided by continuous-culture experiments may pick up more subtle changes in gene expression than batch-culture experiments (Hayes et al., 2002; Hoskisson and Hobbs, 2005; Bull, 2010).

In conclusion, we found clear effects of the transitions between nitrogen limitation and light limitation on photosynthesis, specific growth rate, and cell concentration. The changes in these physiological parameters were mimicked by changes in gene expression of many genes. Interestingly, some genes displayed only transient up- or down-regulation in response to the imposed changes in growth conditions, whereas other genes displayed persistent changes in gene expression. Our results illustrate that gene expression patterns regulated at the molecular scale can be related to physiological properties observed macroscopically. The biological integration of cellular physiology could thus be traced back to the genomic level, since observed changes in cellular physiology were tightly linked to changes in expression patterns of entire clusters of genes involved in interconnected cellular functions.

MATERIALS AND METHODS

Continuous Cultures

Synechocystis sp. strain PCC 6803 was grown in laboratory-built continuous cultures specially tailored to study phototrophic microorganisms (Matthijs et al., 1996; Huisman et al., 1999, 2002). Each continuous culture consisted of a flat culture vessel with inner dimensions of 30 cm height, 18 cm width, and a depth of 5 cm in the direction of the light path (z). The effective working volume of the vessels was 1,970 mL. The culture vessels were heat sterilized for 1 h at 121°C. During the experiments, the temperature of the culture vessels was maintained at 28°C by a stainless steel “finger” for heat exchange. The latter was submerged in the culture and connected to a thermocryostat (Colora). Homogeneous mixing and a sufficient supply of inorganic carbon were ensured by aerating the culture vessel at a rate of 60 L h⁻¹ with compressed air enriched with 3,000 μL L⁻¹ CO₂.

Light was supplied by four white fluorescent tubes (Philips PLL 24W/840/4P) placed at one side of the culture vessel. Photosynthetically active radiation (400–700 nm) was measured using a LI-190SA quantum sensor attached to a LI-250 light meter (LI-COR). To account for spatial variation, the incident irradiance (I_{in}) and the irradiance transmitted through the culture (I_{out}) were measured at 10 regularly spaced positions on the front and back surface of the culture vessel, respectively. The incident irradiance was set at a constant value of $I_{in} = 569 \pm 26 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. The irradiance transmitted through the vessel, filled with medium in the absence of *Synechocystis* cells, was $I_{out} = 255 \pm 9 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$, which corresponds to a background light absorption by the culture vessel of $K_{bg} = \ln(I_{in}/I_{out})/z = 0.16 \text{ cm}^{-1}$ (Huisman et al., 2002).

Mineral medium was pumped from 3-L bottles into the culture vessel by a peristaltic pump (Watson Marlow 101U/R MkII) at a dilution rate of $D = 0.016 \text{ h}^{-1}$. An outlet of the culture vessel allowed the outflow of medium and cultured cyanobacteria. The mineral medium was based on the Oscillatoria 2 (O2) medium (Van Liere and Mur, 1978; Van de Waal et al., 2009). O2 medium contains a rich supply of all nutrients essential for cyanobacterial growth, thus avoiding limitation by any other nutrient than nitrogen. We used full O2 medium with a nitrogen concentration of 6,000 μM NaNO₃ to induce light-limited conditions and reduced the nitrogen concentration in the medium to 500 μM NaNO₃ to induce nitrogen-limited conditions.

Experimental Setup

We ran four replicate continuous cultures. At first, the cultures were grown under nitrogen-limited conditions until a steady state was reached. This provided the starting point for our experiment. The nitrogen-limited steady state was sampled for 3 d. On day 3, after sampling, the mineral medium was replaced by full O2 medium (Fig. 1A), gradually raising the total nitrogen concentration in the culture vessel toward 6,000 μM (Fig. 1B). A new nitrogen-replete but light-limited steady state ensued, which was reached on day 8. On day 10, the mineral medium was changed back to low-nitrogen concentra-

tions. This resulted in a gradual decrease of the total nitrogen concentration in the culture vessel, returning the continuous cultures to their original nitrogen-limited state (Fig. 1, A and B). The experiment was ended on day 25.

During the experiment, the four continuous cultures were sampled daily around 9 AM for physiological measurements. In addition, two of the four continuous cultures were sampled on days 2, 4, 6, 8, 11, 12, 13, 15, and 20 for transcriptomics. In total, this yielded 18 samples for microarray analysis.

Cell Concentrations and Light Absorption

The cell concentration in the cultures was monitored using a CASY 1 cell counter (Schärfe). The specific growth rate (μ) was calculated as:

$$\mu = \frac{\ln(C_2) - \ln(C_1)}{t_2 - t_1} + D \quad (1)$$

where C_1 and C_2 are the cell concentrations at time t_1 and t_2 , respectively, and D is the dilution rate.

The light attenuation coefficient (k) of *Synechocystis* cells was estimated directly from the continuous cultures, using measurements of the incident irradiance (I_{in}), the irradiance transmitted through the culture vessel (I_{out}), the background turbidity (K_{bg}), and the cell concentration (C ; Huisman et al., 2002):

$$k = \frac{1}{C} \left(\frac{\ln(I_{in}/I_{out})}{z} - K_{bg} \right) \quad (2)$$

Light absorption spectra (350–700 nm) of the *Synechocystis* cultures were measured using a Perkin-Elmer Lambda 800 UV/VIS spectrophotometer equipped with an integrating sphere.

Photosynthesis

Photosynthetic activity was analyzed using a Phytopam PM-101/P fluorometer (Walz). The quantum yield of PSII electron transport (F_v/F'_m) was estimated according to Genty et al. (1989). Cells were dark incubated for 5 min prior to analysis of F_v/F'_m . Then, the cells were exposed to stepwise increasing irradiances up to 1,200 μmol quanta m⁻² s⁻¹. Each irradiance level was maintained for 30 s. The irradiance values were measured using the light sensor of the Phytopam fluorometer. At the end of each irradiance step, F_v/F'_m was measured. The electron transport rate per cell was calculated as the product of F_v/F'_m , the irradiance, the light attenuation coefficient of *Synechocystis* cells (k), and a factor of 0.5 accounting for electron excitation by both PSII and PSI (Maxwell and Johnson, 2000). The ETRs measured at the different irradiances were fitted to the photosynthesis model of Webb et al. (1974):

$$ETR = ETR_{max} \left(1 - \exp\left(\frac{-\alpha_{ETR} I}{ETR_{max}}\right) \right) \quad (3)$$

where α_{ETR} is the slope of the ETR irradiance curve at limiting irradiance and ETR_{max} is the maximum electron transport rate at saturating irradiance.

Microarray Analysis

Oligonucleotide probes were designed using Array Designer 2.0 (Premier Biosoft International) as described by Aguirre von Wobeser (2010). The design consisted of one to four probes per gene, depending on the availability of unique sequences within each gene from the *Synechocystis* genome (Kaneko et al., 1996). Microarray analysis was performed using laboratory-designed DNA microarray probes that were custom printed (Agilent). Each sample of our time series experiment was brought on a separate microarray, using the sample of day 8 as a common reference. That is, we contrasted the sample of day 8 (labeled with Cy3 dye) against the samples taken at the other time points (labeled with Cy5 dye). RNA isolation, cDNA preparation with reverse transcriptase, Cy3/Cy5 dye labeling, hybridization, washing, and reading of the arrays were performed essentially as described before (Eisenhut et al., 2007; Tuominen et al., 2008).

Statistical Analysis

The time series consisted of 16 microarray measurements (two continuous cultures, each with nine time points, where the one at day 8 was used as a

common reference) as well as daily measurements of several physiological parameters. The raw signal intensities of the spots on the microarrays were log transformed (using log-base 2) to reduce the skewness of the data distribution. In the raw data, on a log₂ scale, the background signal intensity was 6 to 7 and the foreground signal intensity was 10 to 14. After background subtraction, the log intensities obtained from different arrays were normalized by adjusting the distributions of all arrays to the same distribution (Workman et al., 2002). For each gene, we then calculated the average of the normalized log intensities across the probes corresponding to that gene. These normalized log intensities were used as a relative measure of gene expression.

Our expectation is that the expression of many genes will respond to the experimental transition from nitrogen limitation to light limitation and back. In order to detect significant differences in gene expression between time points, we performed an ANOVA for each gene (Kerr and Churchill, 2001; Wolfinger et al., 2001). The ANOVA model was:

$$Y_{gtb} = G_g + (GT)_{gt} + P_b + \varepsilon_{gtb} \quad (4)$$

where Y_{gtb} is the normalized intensity for gene g ($g = 1, \dots, 3,264$), time point t ($t = 1, \dots, 9$), and probe b ($b = 1, \dots, 8,091$). G_g is the main effect of each gene, P_b is the main effect of each probe, and $(GT)_{gt}$ is the interaction effect between each gene and the time points of measurement. The error term ε_{gtb} is assumed to be normally distributed with zero mean and variance σ_ε^2 . Since we used day 8 as a common reference on all microarrays, we obtained many replicate measurements for day 8. All these replicates were highly correlated. To avoid pseudoreplication, therefore, we used only one of the day-8 data sets in the ANOVA model. We did not detect significant effects of arrays or dyes on the normalized log intensities (data not shown); hence, array or dye effects were not included in the ANOVA model. The term of interest in the model is $(GT)_{gt}$, which shows whether the expression of a gene changed significantly in time. The significance level of the analysis was set at $P < 0.01$. In addition, to control for the probability of obtaining false-positive results due to the thousands of hypotheses (i.e. thousands of genes) being tested, the false discovery rate was calculated (Storey and Tibshirani, 2003). We set the false discovery rate at $q < 0.01$, meaning that less than 1% of the genes declared significant are expected to be false positives.

Interpretation of Temporal Changes in Gene Expression

Cluster analysis was performed to identify groups of genes that showed similar changes in gene expression during the experimental transitions. Only genes with significant changes in gene expression during the experiment, as assessed by the ANOVA, were considered for the cluster analysis. Since we were interested in relative changes in gene expression rather than the exact amplitude of the signal, the time series of each gene was normalized by the transformation $(x - \mu)/\sigma$, where x is the original data point, μ is the mean of the time series, and σ is its SD. Thus, we obtained normalized time series with a mean of 0 and SD of 1.

Cluster analysis was performed using the K-means algorithm (Hartigan, 1975; Tavazoie et al., 1999). K-means clustering distributes the time series over a number of clusters so as to minimize the within-cluster variation. We used the Pearson correlation coefficient as our distance measure (i.e. as a measure of the similarity of the time series). The total number of clusters is user defined. To determine an adequate number of clusters, we increased the number of clusters one by one, until two or more clusters became "too similar" to each other. To assess the similarity between clusters, we calculated the mean of each time point over all genes within a cluster. Subsequently, the Pearson correlation coefficients (ρ) between the mean vectors (called centroids) of different clusters were calculated. A correlation coefficient of $\rho > 0.7$ was considered too similar. This procedure resulted in a total of seven clusters, each of which showed distinct temporal patterns of gene expression.

The K-means algorithm assigned the time series of each gene to one of the seven clusters, even if this time series showed a rather unique pattern deviating from other time series within that cluster. To overcome this limitation of the method, we calculated the correlation coefficient between each time series and the centroid of the cluster to which this time series was assigned. Time series with a correlation coefficient of $\rho < 0.7$ were reassigned to a separate new cluster (cluster 8).

To relate gene expression patterns with the physiological and experimental parameters measured during the experiment, we calculated the Pearson correlation coefficients between the time courses of the parameters and the cluster centroids. Temporal changes in the physiological param-

eters could thus be compared with temporal changes in the gene expression data.

Interpretation of Gene Function

In addition to specific literature references, we made extensive use of the Web database CyanoBase (<http://genome.kazusa.or.jp/cyanobase/> *Synechocystis*), which contains the annotated nucleotide sequence of the *Synechocystis* genome (Kaneko et al., 1996; Nakao et al., 2010). From the gene identifiers, a list of enzymes and associated metabolic reactions was assembled using the KEGG database (<http://www.kegg.jp/>; Kanehisa and Goto, 2000).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Overview of changes in gene expression of all 3,264 ORFs of *Synechocystis* during the continuous-culture experiment (Excel file).

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