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Concerted Changes in Gene Expression and Cell Physiology of the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 during Transitions between Nitrogen and Light-Limited Growth1[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).

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 nonequilibrated 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).

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

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thesis and restricts the production of photosynthetic pigments in cyanobacteria (Collier and Grossman, 1994; Collier et al., 1994; McIntyre et al., 2002; Miller et al., 2002; McGinn et al., 2004; Kanervo et al., 2005; Nixon et al., 2005; Schagerl and Muller, 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; Garcia-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 Emlen-Jones, 2005), changes of photosystem ratio (De Nobl 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 Jütter, 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 mMol 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 mM (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 mMol 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 mM (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
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 μM and decreased to nearly 0 h⁻¹ in the transient period after the nitrate inflow had been lowered to 500 μM. The light attenuation coefficient (k) was approximately 0.3 μm² 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 μm² cell⁻¹ and then slightly decreased to settle at approximately 0.55 μm² cell⁻¹ at the light-limited steady state. When the nitrate input was again decreased, k decreased to a minimum of 0.2 μm² cell⁻¹ and then slowly returned to the initial value of 0.3 μm² 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: ρ = 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: ρ = 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: ρ = 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
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 sllr0710 (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 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).

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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.

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 biosynthesis 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

<table>
<thead>
<tr>
<th>Functional Category</th>
<th>Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid biosynthesis (97 genes)</td>
<td>20</td>
</tr>
<tr>
<td>Biosynthesis of cofactors (124 genes)</td>
<td>13</td>
</tr>
<tr>
<td>Cell envelope (67 genes)</td>
<td>8</td>
</tr>
<tr>
<td>Cellular processes (78 genes)</td>
<td>9</td>
</tr>
<tr>
<td>Central intermediary metabolism (31 genes)</td>
<td>19</td>
</tr>
<tr>
<td>Energy metabolism (93 genes)</td>
<td>14</td>
</tr>
<tr>
<td>Fatty acid metabolism (39 genes)</td>
<td>33</td>
</tr>
<tr>
<td>Photosynthesis and respiration (142 genes)</td>
<td>43</td>
</tr>
<tr>
<td>Purines, nucleotides (41 genes)</td>
<td>20</td>
</tr>
<tr>
<td>Regulatory functions (146 genes)</td>
<td>4</td>
</tr>
<tr>
<td>DNA replication (60 genes)</td>
<td>2</td>
</tr>
<tr>
<td>Transcription (30 genes)</td>
<td>10</td>
</tr>
<tr>
<td>Translation (168 genes)</td>
<td>13</td>
</tr>
<tr>
<td>Transport and binding proteins (196 genes)</td>
<td>9</td>
</tr>
<tr>
<td>Other categories (311 genes)</td>
<td>7</td>
</tr>
<tr>
<td>Hypothetical (1,132 genes)</td>
<td>6</td>
</tr>
<tr>
<td>Unknown (508 genes)</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total no. of genes per cluster</strong></td>
<td>310</td>
</tr>
</tbody>
</table>
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. 3G). 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 \( \alpha_{ETR} \) and \( ETR_{\text{max}} \), the light attenuation coefficient \( k \), as well as the specific growth rate \( \mu \) 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 \( ETR_{\text{max}} \) 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).
panied by a higher photosynthetic potential (higher \( \alpha_{ETR} \); Fig. 1E). The rapid increase of photosynthetic potential was accompanied by an increased specific growth rate (higher \( \mu \); 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-
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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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$\alpha_{TR}$</td>
<td>0.77</td>
</tr>
<tr>
<td>ETR$_{\text{max}}$</td>
<td>0.51</td>
</tr>
<tr>
<td>Specific growth rate ($\mu$)</td>
<td>0.68</td>
</tr>
<tr>
<td>Light attenuation coefficient ($k$)</td>
<td>0.89</td>
</tr>
<tr>
<td>Cell concentration ($C$)</td>
<td></td>
</tr>
<tr>
<td>Total nitrogen</td>
<td></td>
</tr>
<tr>
<td>Light transmission ($I_{\text{sat}}$)</td>
<td></td>
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</tbody>
</table>

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 (sll0898), are grouped into cluster 4, glnB (sll0707) 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 sll2002, 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, 1-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 *Synecochystis* 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 (Ümbarger, 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).

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 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\(^{-1}\) (Van Liere and Mur, 1978; Van de Waal et al., 2009). O\(_2\) medium (Synechocystis, 2002).

Light was supplied by four white fluorescent tubes (Philips PLL 24W/480/4P) 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 was measured using a LI-190SA quantum sensor attached to a peristaltic pump (Watson Marlow 101U/R MkII) at a dilution rate of \(2 \times 10^{-2}\) s\(^{-1}\). 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\(^{-1}\) CO\(_2\).

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.

Cell Concentrations and Light Absorption

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

\[
\mu = \frac{\ln(C_t) - \ln(C_i)}{t_2 - t_1} + D
\]

where \(C_t\) and \(C_i\) 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 (\(I_0\)), the irradiance transmitted through the culture vessel (\(I_{\text{trans}}\)), the background turbidity (\(K_b\)), and the cell concentration (C Huisman et al., 2002):

\[
k = \frac{1}{z} \left( \ln\left(\frac{I_0/I_{\text{trans}}}{z} - K_b \right) \right)
\]

Photosynthesis

Photosynthetic activity was analyzed using a Phytopam PM-101/P fluorometer (Walz). The quantum yield of PSI electron transport (\(F_{\text{v}}/F_{\text{m}}\)) was estimated according to Genty et al. (1989). Cells were dark incubated for 5 min prior to analysis of \(F_{\text{v}}/F_{\text{m}}\). 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 rates were measured using the light sensor of the Phytopam fluorometer. At the end of each irradiance step, \(F_{\text{o}}/F_{\text{m}}\) was measured. The electron transport rate per cell was calculated as the product of \(F_{\text{v}}/F_{\text{m}}\) the irradiance, the light attenuation coefficient of Synechocystis cells (\(k\)), and a factor of 0.5 accounting for electron excitation by both PSI and PSII (Maxwell and Johnson, 2000). The ETRs measured at the different irradiances were fitted to the photosynthesis model of Webb et al. (1974):

\[
\text{ETR} = \text{ETR}_{\text{max}} \left( 1 - \exp\left( -\frac{\alpha_{\text{ETR}}}{\text{ETR}_{\text{max}}} \right) \right)
\]

where \(\alpha_{\text{ETR}}\) is the slope of the ETR irradiance curve at limiting irradiance and \(\text{ETR}_{\text{max}}\) 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., 2002; 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; Wollinger et al., 2001). The ANOVA model was:

\[
Y_{gtb} = G_t + (GT)_{gt} + P_b + e_{gtb}
\]

(4)

where \(Y_{gtb}\) is the normalized intensity for gene \(g = 1, \ldots, 3,264\), time point \(t = 1, \ldots, 9\), and probe \(b = 1, \ldots, 8,091\). \(G_t\) 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 \(e_{gtb}\) is assumed to be normally distributed with zero mean and variance \(\sigma^2\).

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 false-positive rate due to the thousands of hypotheses (i.e. thousands of 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 \(x = (x - \mu) / \sigma\), where \(x\) is the original data point, \(\mu\) is the mean of the time series, and \(\sigma\) 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 the clusters where the similarity of the time series). The total number of clusters is user defined. The K-means algorithm assigns 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 \(r > 0.7\) 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 parameters 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 CyanolBase (http://genome.kazusa.or.jp/cyanbase/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.genome.ad.jp/kanehsa and Coito, 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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