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TRANSCRIPTIONAL ANALYSIS OF THE UNICELLULAR, DIAZOTROPHIC CYANOBACTERIUM *CYANOTHECE* SP. ATCC 51142 GROWN UNDER SHORT DAY/NIGHT CYCLES[1]

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***Cyanothece* sp. strain ATCC 51142 is a unicellular, diazotrophic cyanobacterium that demonstrates extensive metabolic periodicities of photosynthesis, respiration, and nitrogen fixation when grown under N_2-fixing conditions. We have performed a global transcription analysis of this organism using 6 h light:dark (L:D) cycles in order to determine the response of the cell to these conditions and to differentiate between diurnal and circadian-regulated genes. In addition, we used a context-likelihood of relatedness (CLR) analysis with these data and those from 2 d L:D and L:D plus continuous light experiments to better differentiate between diurnal and circadian-regulated genes. *Cyanothece* sp. acclimated in several ways to growth under short L:D conditions. Nitrogen was fixed in every second dark period and only once in each 24 h period. Nitrogen fixation was strongly correlated to the energy status of the cells and glycogen breakdown, and high respiration rates were necessary to provide appropriate energy and anoxic conditions for this process. We conclude that glycogen breakdown is a key regulatory step within these complex processes. Our results demonstrated that the main metabolic genes involved in photosynthesis, respiration, nitrogen fixation, and central carbohydrate metabolism have strong (or total) circadian-regulated components. The short L:D cycles enable us to identify transcriptional differences among the family of *psb*A genes, as well as the differing patterns of the *hup* genes, which follow the same pattern as nitrogenase genes, relative to the *hox* genes, which displayed a diurnal, dark-dependent gene expression.**

***Key index words:* circadian and diurnal behavior; cyanobacteria; glycogen metabolism; light–dark cycles; nitrogen fixation; photosystems**

***Abbreviations:* CLR, context-likelihood of relatedness; DP, dark period; FTIR, Fourier transform infrared; L:D, light:dark; LP, light period; PPP, pentose phosphate pathway; RT-PCR, reverse transcription polymerase chain reaction**

Circadian rhythms have been identified and studied in all classes of organisms including the cyanobacteria. Cyanobacteria are the simplest organisms to have a circadian clock, although the clock components are different than those in higher organisms. One of the more interesting findings in *Synechococcus elongatus* sp. PCC 7942, the main model cyanobacterium for circadian studies, is that all *S. elongatus* genes apparently are expressed in a circadian rhythmic fashion (Liu et al. 1995a,b, Ditty et al. 2003, Golden and Canales 2003). These findings are based on multiple techniques, and it appears evident that actual transcription is globally circadian. However, in practice, it is very likely that only a fraction of the genes will display a perfectly rhythmic pattern of expression. First, mRNAs have different half lives, and the gene transcript level at any time period will be the net sum of new transcription plus the accumulation and degradation of older transcripts. Second, the fundamental circadian rhythms will be regulated by many posttranscriptional processes, which will alter the frequency or amplitude of the pattern of transcription.

Circadian rhythms function to optimize specific physiological mechanisms to the correct time of day (Golden and Canales 2003, Golden 2007). We are particularly interested in the relationship of photosynthesis, which generates O_2, to the O_2-sensitive nitrogen fixation throughout a 24 h diurnal cycle. Transcript levels may be circadian (L:D independent) or be directly affected by light or darkness. Such differences will become manifest when cells are grown under photocycles that differ significantly from the usual 12:12 L:D diurnal cycle (e.g.,

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continuous light, short L:D cycles, etc.). Similar considerations are also critical in plants, and a comprehensive study in *Arabidopsis thaliana* has investigated the impact of both photocycles and thermocycles on circadian rhythms (Michael et al. 2008). This study led to the conclusion that 89% of *Arabidopsis* transcripts cycled under at least one condition and that the expression of most genes peaked at a specific time of day. Furthermore, this peak can shift depending on the environment (Michael et al. 2008). This finding provides the most detailed study to date in a photosynthetic organism on the relationship of the underlying circadian clock to environmental parameters that impact on metabolic rhythms.

We have been involved with related studies of the unicellular diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142, hereafter *Cyanothece* sp. (Reddy et al. 1993, Schneegurt et al. 1994), which performs photosynthesis during the light and fixes nitrogen during the dark (Schneegurt et al. 1997a,b, Sherman et al. 1998). Previous studies with *Cyanothece* sp. showed a strong correlation between activity and transcript level for a subset of genes related to photosynthesis (Meunier et al. 1998) and N_2 fixation (Colón-López et al. 1997) during 12 h L:D cycles. In addition, differential gene expression was reported for the main photosynthetic genes and the nitrogenase genes in the light and dark, respectively (Colón-López et al. 1997, 1999, Colón-López and Sherman 1998). Recently, whole genome microarray experiments were carried out to determine the diurnal gene expression under L:D conditions (12 h L:D) (Stöckel et al. 2008) and under continuous light (Toepel et al. 2008). Both groups determined that ~30% of the ~5,000 genes on the microarray exhibited diurnal oscillations under 12 h L:D conditions, and Toepel et al. (2008) demonstrated that ~10% of the genes demonstrated circadian behavior during growth in free-running (continuous light) conditions. Toepel et al. (2008) also observed that nitrogenase transcript abundance and nitrogenase activity were correlated in *Cyanothece* sp. under continuous light and that N_2 fixation followed a ~24 h rhythm under these conditions, albeit with reduced rates. These results indicate an L:D-independent expression pattern for nitrogenase genes, consistent with the circadian behavior for nitrogenase-related genes suggested by Sherman et al. (1998). This finding is in contrast with results from *Gloeothece* sp., where N_2 fixation appears to be under the control of an undefined endogenous rhythm (Gallon 2001), but one that was not circadian. When grown in continuous light, *Gloeothece* did not display a ~24 h periodicity for N_2 fixation, but reverted to one of 30–40 h (Gallon and Chaplin 1988). In addition, the results of Taniuchi and Ohki (2007) in *Gloeothece* sp. indicated that mRNA abundance and nitrogenase activity were not always connected, and that nitrogenase activity was dependent on the time span between dark periods and occurred 4 h into the dark.

Photosynthetic activity depends upon incident light, although genes encoding photosynthetic proteins display a diurnal or a circadian-dependent expression pattern (Michael et al. 2008, Stöckel et al. 2008). In *Cyanothece* sp., maximum photosynthetic rates in this L:D cycle occurred after 6–8 h light incubation, and photosynthetic capacity decreased strongly during the N_2-fixation period (Meunier et al. 1998, Toepel et al. 2008). Toepel et al. (2008) showed that the photosynthetic rates were lower during growth under continuous light and demonstrated no circadian-related pattern for photosynthesis genes. In the case of growth under continuous light, the glycogen content stayed at high levels and did not decrease until the cells were again placed in darkness (Toepel et al. 2008). Furthermore, these results indicated that nitrogenase transcription and activity were metabolically or energetically regulated via glycogen breakdown and suggested that photosynthesis is light activated, but probably regulated by the internal carbohydrate level.

The main circadian control genes detected in *S. elongatus* are the *kai*-genes that function as internal oscillators (Bell-Pedersen et al. 2005), and a cyclic behavior of gene expression during continuous light growth had been demonstrated. Short dark pulses reset the internal clock and lead to a phase shift of cultures grown under continuous light (Mackey and Golden 2007). Therefore, light or dark signals can synchronize the cell's internal clock with the external environment. Three proteins are described with a receptor function for external signals. The *cik*A (circadian input kinase) (Ivleva et al. 2006) most likely senses the redox state of the plastoquinone (PQ) pool, and *ldp*A (light-dependent period) and *pex* (period extender) determine the period length. The way in which temporal information is converted into global oscillation of transcription remains unsolved, but chromosome compaction (Smith and Williams 2006) and the above signal-transduction pathway may well be involved (Takai et al. 2006). However, the CikA kinase does not have a clear homolog in other cyanobacteria, including *Cyanothece* sp. ATCC 51142, and variations in the circadian mechanism should be considered the norm (Golden 2007, Mackey and Golden 2007).

We are interested in relating cellular energy levels to the nature of circadian and diurnal control of gene expression in *Cyanothece* sp. Cellular processes depend on the cellular energy level, and enzyme activity is often substrate regulated. Therefore, we asked the following questions: How stable is the circadian or diurnal rhythm of the genome if the ambient L:D cycles are permanently different from typical 12 h L:D periods? Can *Cyanothece* sp. grow and fix N_2 under 6 h L:D, N_2-fixing conditions?

Finally, can we use the information from this experiment, in conjunction with the previous transcriptional studies, to better define genes under either circadian or diurnal regulation?

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Cyanothece* sp. ATCC 51142 cultures were grown in an airlift bioreactor (BioFlo 3000, 6-L, New Brunswick Scientific, Edison, NJ, USA) in ASP2 medium without nitrate at 30°C in 12:12 L:D cycles (Toepel et al. 2008). The culture was illuminated by two LED panels using alternating arrays of orange (640 nm) and blue (430 nm) LEDs, yielding an intensity of ~100 μmol photons $\cdot m^{-2} \cdot s^{-1}$ inside the bioreactor (underwater quantum light meter [LI 192; Li-Cor, Lincoln, NE, USA]). Cultures were inoculated in the bioreactor at a cell density of $\sim 1 \times 10^6$ cells $\cdot mL^{-1}$ and were grown for 5–6 d under L:D conditions prior to the experiments. We then applied 6 h L:D cycles for 24 h and took samples for mRNA isolation and measurements of cell number, chl *a* concentration, photosynthetic O_2 evolution, and respiration and glycogen content (50 mL per time point). Cell number and the chl *a* concentration were measured using a Petroff-Hauser cell count chamber and a Perkin-Elmer spectrophotometer (Lambda 40, Shelton, CA, USA), respectively, as described (Schneegurt et al. 1994). Photosynthetic oxygen evolution (using the growth light intensity, ~100 μmol photons $\cdot m^{-2} \cdot s^{-1}$ for illumination) and respiration rates (monitored as O_2 consumption in the dark) were determined using a Clark type electrode (Hansatech, Norfolk, England), without adding bicarbonate. N_2-fixation rates were determined by measuring ethylene on a Hewlett Packard 8460 gas chromatograph (Wilmington, DE, USA) and calculated as described (Colón-López et al. 1997). The rates of photosynthesis, respiration, and N_2 fixation in Figure 1 are plotted on the right axis as relative activity. Photosynthesis is represented as O_2 evolution (positive activity), whereas respiration is represented as O_2 uptake (negative activity). This experimental protocol was repeated three times with essentially identical results.

Microarray platform. The high-density microarray platform consisted of 5,096 open reading frames (ORFs) based upon the rough draft of the *Cyanothece* genome sequence that was obtained by the Washington University Genome Center (Welsh et al. 2008). The ORFs were identified using Critica and Glimmer, and the 60-mers appropriate for each gene were determined by a computer program written by Dr. Rajeev Aurora, St. Louis University, and provided by him to Agilent Inc. (Santa Clara, CA, USA). The microarrays were fabricated by Agilent, provided to MoGene (St. Louis, MO, USA), along with the purified RNA samples for hybridization, scanning, and initial data analysis [see Stöckel et al. (2008) and Toepel et al. (2008) for further details].

RNA isolation. For the microarray experiment, 300 mL samples were taken at each time period (t = 2, 4, 8, 10, 14, 16, 20, and 22 h) over a 24 h period. The cells were centrifuged (Beckman J-21 centrifuge; Beckman-Coulter, Fullerton, CA, USA) at 5,000*g*, resuspended in STET-Buffer (8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8, with DEPC water), and stored at −80°C. The RNA extraction was performed using a slightly modified version of the protocol described in Reddy et al. (1990). The RNA Clean-up Kit-5 columns from Zymo Research Corp. (Orange, CA, USA) were used to remove contamination (e.g., carbohydrates, organic solvents).

Results are for a combination of three biological and two technical replicates. For each microarray, 1 μg RNA was used (0.5 μg sample + 0.5 μg control). Total RNA was labeled with either cyanine-5 or cyanine-3 using ULS, an RNA fluorescent labeling kit from Kreatech Biotechnology (Amsterdam, the Netherlands) according to the manufacturer's protocol. The labeled material was passed through Zymo RNA Clean-up Kit-5 columns (Zymo Research Corp.) to remove unincorporated label and eluted in 15–20 μL of RNase-free water. Concentration of labeled total RNA and label incorporation was determined on a Nanodrop-1000 spectrophotometer (Wilmington, DE, USA). All of the labeling and postlabeling procedures were conducted in an ozone-free enclosure to ensure the integrity of the label. Labeled material was hybridized for 17 h in a rotating oven at 65°C in an ozone-free room. Wash conditions used were as outlined in the Agilent processing manual (Santa Clara, CA, USA) and the arrays were scanned by an Agilent scanner. Analysis was performed using Agilent's Feature Extraction Software version 9.1 and Rosetta Luminator software (Rosetta Biosoftware, Kirkland, WA, USA).

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR). RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C, and successful DNase I treatment was confirmed by PCR on each RNA sample. Reverse transcription was performed using Superscript II (Invitrogen) and random primers using the manufacturer's

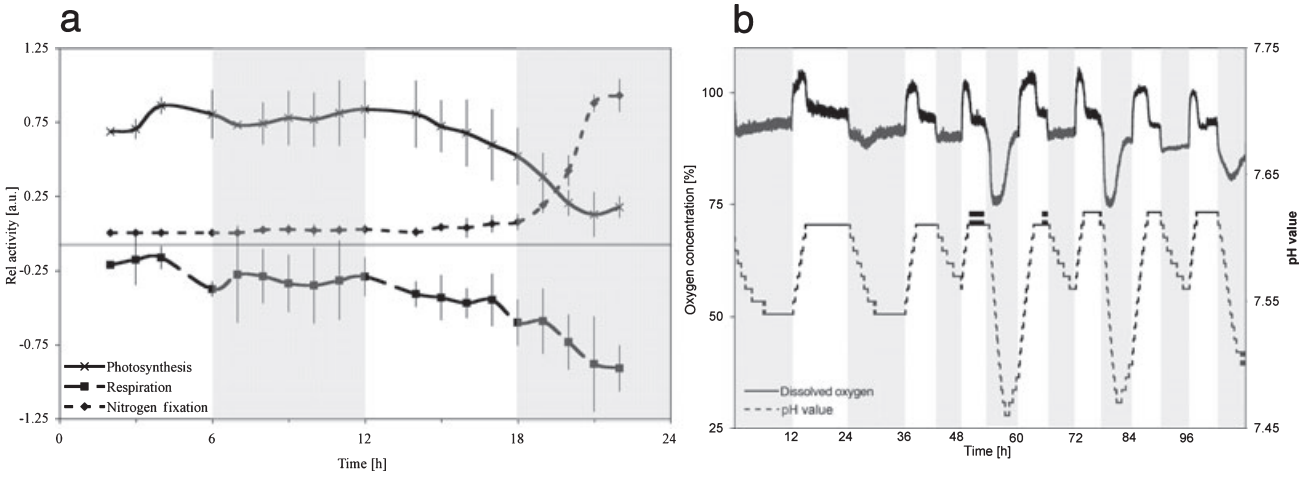


FIG. 1. (a) Photosynthetic rates, respiration rates, and nitrogenase activity (relative activity) of *Cyanothece* sp. during 6 h light:dark (L:D) cycles. Oxygen evolution increases upward, whereas oxygen consumption increases downward. (b) Dissolved oxygen and pH value of *Cyanothece* sp. grown in a bioreactor for 5 d under 12 h L:D and 6 h L:D cycles.

instructions. PCR was carried out at 94°C for 1 min, 30 cycles of 94°C for 30 s, 54°C for 30 s and 68°C for 30 s, to amplify regions of the genes *nif*H, *cox*C1, and *rpn*A. Due to the high transcript level of these genes, amplification using *psb*A and *psb*D primers was performed using 20 cycles of the PCR conditions described above. The *rpn*A transcript abundance was used as a control, since microarray data indicated the transcript level for this gene was unchanged under these growth conditions. The primers and amplified product sizes for the following genes were as used in Toepel et al. (2008): *nif*H, *psb*A1, *cox*C1, *rnp*A, and *psb*D.

Fourier transform infrared (FTIR) spectroscopy. The macromolecular composition of the cells was determined over the 48 h period with an FTIR-spectrometer (Thermo-Electron, Madison, WI, USA). Samples (2 mL) were taken every 6 h over a 24 h time period, washed twice with distilled water, and stored at −80°C. The cells were dried prior to measurements on an IR-slide at 45°C for 4 h. For each sample, 128 spectra × 50 spots were measured and analyzed according to B. Penning (see http://cellwall.genomics.purdue.edu/). The spectra for each sample were baseline corrected, area normalized, and averaged.

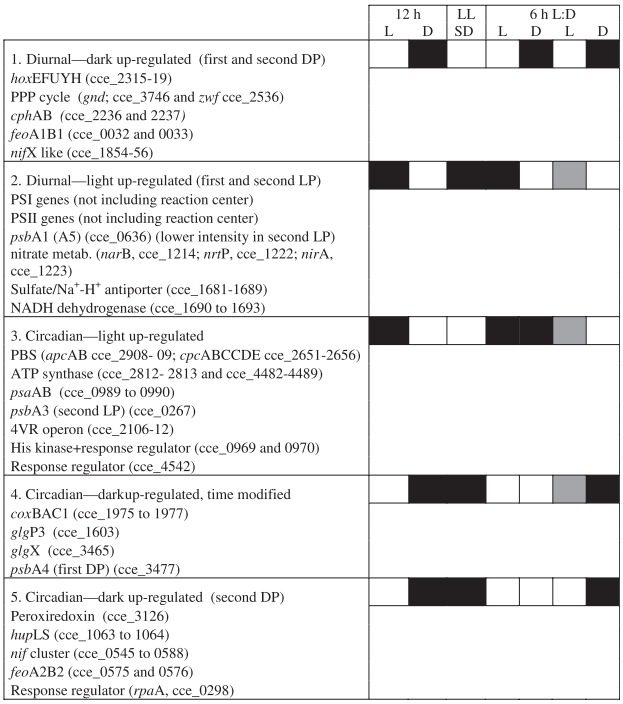
Context-likelihood of relatedness analysis. Transcriptomic profiles were assembled for each gene in *Cyanothece* from experiments for 12 h L:D conditions (Stöckel et al. 2008); continuous light (Toepel et al. 2008); low O_2 conditions for 1, 2, and 6 h (J. Toepel, T. C. Summerfield, and L. A. Sherman, unpublished data); and the 6 h L:D experiment reported here. These profiles were analyzed using the CLR method (Faith et al. 2007) using the SEBINI framework (Taylor et al. 2006). CLR calculates the mutual information (a sensitive measure of similarity) between transcription profiles from all pairs of genes and then applies statistical filters to predict relationships among genes. Filtering these relationships to include only those between transcription factors provides a high-confidence regulatory network if enough individual conditions are used (Faith et al. 2007). We did not filter the genes considered, so the relationships predicted are more generic and represent highly significant similarities between the expression profiles among all of the genes. We used a spline degree of 3 and 8 bins for input parameters to CLR. The resulting network (Fig. 5) was filtered using a CLR zscore of 5.0 and visualized with Cytoscape (Shannon et al. 2003). Grouping of genes in the network into clusters was accomplished using hierarchical clustering with Cluster 3.0 (Eisen et al. 1998), using average pair-wise linkage and the CLR scores as the similarity metric.

RESULTS

Photosynthesis and N_2 fixation in 6 h L:D growth. Growth of *Cyanothece* sp. in 6 h L:D cycles showed a specific cyclic pattern of enzyme activity, as demonstrated by the O_2-evolution capacity, respiration, and N_2 fixation during the 24 h time period (Fig. 1a). The net photosynthesis rate increased during the first light period (LP), and the capacity for photosynthesis remained high during the following dark period; respiration was low in the first LP and in the first dark period (DP). Net photosynthetic rates decreased steadily in the second half of the second LP and then decreased to 20% of the maximum light rate in the second DP. The rate of respiration increased during the second LP and reached maximum values during the second DP. The gross photosynthetic rates remained constant through the first 12 h of the experiment, decreased slightly during the second LP, and decreased further in the second DP (50% decrease, data not shown). The initial N_2-fixation activity rates were very low but increased in the second DP (Fig. 1a). The peak of N_2 fixation was at ~20–22 h, which was a delay of 4–6 h in comparison to the 12 h L:D cycle (Schneegurt et al. 1994). The transition to the 6 h L:D cycle led to a 24 h rhythm of N_2 fixation, which was not influenced by the short L:D rhythms (Fig. 1a). Furthermore, the larger decline in the pH during each second DP correlated with higher rates of respiration (Fig. 1b) during that period and the high level of nitrogenase activity (compare the increase in nitrogenase activity in Fig. 1a with the increase in respiration and the decrease in pH in Fig. 1b).

Differential gene expression in 6 h L:D cycle. Analysis of the microarrays yielded ~1,400 up-regulated genes (>2-fold) during the 24 h experiment out of 5,096 annotated genes in *Cyanothece* sp. ATCC 51142. We identified 520 up-regulated genes in the first LP, 214 in the second LP, 394 in the first DP, and 275 in the second DP. The expression patterns for some of the genes encoding energy-related functions are summarized in Table 1. This table defines the expression patterns that we have used to categorize circadian and diurnal regulation. This includes data from the 6 h L:D experiment described herein, the 12 h L:D experiment described in Stöckel et al. (2008), and the L:D+LL experiment described in Toepel et al. (2008). The darkened sectors indicate where expression increased the most, and the shaded sector indicates an increase, but not a peak. The results from the 6 h L:D experiment show that some genes, such as those encoding phycobiliproteins or the ATP synthase proteins, demonstrated a light-independent gene expression (e.g., circadian), in that they continued to have expression peaks in the first LP, although there was a smaller peak in the second LP. Other genes, such as *cox*BAC1, peaked in the second DP, but the time of peak expression was modified (from ~t = 12 h in 12 h L:D to t = 16 h in 6 h L:D). A second class of potential circadian genes, with a peak in the second DP, included the *nif* gene cluster, *hup*LS, and *feo*A2B2. At the same time, some genes demonstrated a significant dark regulation and showed increased transcript levels in each DP (e.g., *hox*EFUYH, *cph*AB, and *feo*A1B1). There was a large class of light up-regulated genes, including many of the PSI and PSII genes (not including those encoding the reaction center proteins) and genes encoding nitrate and sulfate transporters. The reaction center genes responded quite differently. The *psa*AB operon followed a circadian pattern, whereas each gene in the *psb*A family demonstrated a unique pattern of transcription. Thus, *psb*A1 (and its identical copy, *psb*A5) peaked in the first LP but had a smaller peak in the second LP. The *psb*A3 gene had a single peak in the second LP, but *psb*A4 demonstrated a distinct peak in the first DP.

TABLE 1. Gene transcription classes in *Cyanothece* sp. ATCC 51142 during growth in 6 h L:D periods[a].



[a]Darkened boxes show that the expression of the respective genes peaked at the indicated time in the specific experiment (top). Gray background indicates a significant increase in expression, but not a peak. Experiments are 12 h L:D, L:D followed by continuous light (peak expression in the subjective dark, SD, is shown), and the experiment reported in this paper, 6 h L:D. Group headings (1–5) summarize the circadian behavior suggested by the expression patterns shown.

DP, dark period; L:D, light:dark; LP, light period.

The majority of the genes encoding PSI and PSII proteins were up-regulated at the beginning of both the first LP and the second LP. Notably, the expression level of all of the photosynthesis-related genes was lower in the second LP in comparison to the first LP, although the expression was still enhanced >2-fold in comparison to the DP. Figure 2 shows the expression pattern of genes involved in several functional processes in *Cyanothece* sp. during the 6 h L:D cycles compared to the patterns in the 12 h L:D experiment (Toepel et al. 2008). The different L:D conditions had little impact on the expression of the genes encoding the RUBISCO subunits and PsaAB in the two experiments. The transcripts for CO_2 fixation (*rbc*LSX) were up-regulated during the first LP in both experiments and again 24 h later. This expression pattern may contribute to the limited photosynthetic capacity during the second LP in the 6 h L:D experiment. The genes encoding the PSI reaction center proteins (PsaAB) demonstrated a maximum expression at $t = 4$ to 8 h in both experiments (Fig. 2a). However, the majority of the photosynthetic genes showed light-dependent gene expression during both experiments. The respiration-related genes (*cox*BAC1) were up-regulated during the first DP and displayed higher expression levels through the rest of the experiment, when the 6 h L:D results were compared to the 12 h L:D results (Fig. 2c). Since nitrogenase requires both energy and a low level of cellular O_2, glycogen breakdown and respiration need to be enhanced during this period.

Nitrogen fixation and nitrogen assimilation. Genes encoding nitrogenase and related proteins were up-regulated during the second DP, corresponding to nitrogenase activity, but with a 4 h time shift compared to the 12 h L:D cycle. As shown in previous experiments (Stöckel et al. 2008, Toepel et al. 2008), several ferredoxins and flavodoxins were up-regulated during the same period. In addition, the gene for peroxiredoxin, a thiol-based peroxide reductase (Dietz et al. 2006) was 78-fold up-regulated in the second DP, indicating a possible function in detoxification of H_2O_2 (Table 1). Some genes encoding nitrate-assimilation-related proteins were also up-regulated at the beginning of the second DP ($t = 14$ h), similar to the 12 h L:D cycle. We found little variation in the expression level of *ntc*A, and the nitrate/nitrate assimilation regulator *ntc*B was up-regulated at $t = 14$, which represents the typical dark period; the same pattern was detectable during 12 h L:D growth.

Light- and dark-dependent and independent gene expression. The comparison between the 12 h L:D cycle experiments and the 6 h L:D cycle experiments allowed us to differentiate genes under control of the L:D signal from genes showing alternative gene expression patterns (e.g., depending on the absolute time of day and response to the energy level in the cell). Table S1 (in the supplementary material) shows the genes with an L:D-dependent gene expression, separated as to the different light and dark period. Analysis of the up-regulated genes during the first DP ($t = 6$ to 12 h) yielded several genes encoding energy-producing enzymes. The rate-limiting enzymes for the pentose phosphate pathway (PPP; *zwf* and *gnd*) showed an up-regulation during the first DP and remained high during the rest of the experiment (Fig. 3c) (Tian et al. 1998). Several fermentative-related genes were up-regulated exclusively during the first DP (e.g., the malate dehydrogenase [pyruvate carboxylation] was 8-fold up-regulated, and the aldehyde dehydrogenase, probably involved in acetate production, was up-regulated 17-fold). No alcohol dehydrogenase gene was up-regulated during the experiment.

The expression pattern of the nitrogenase-related genes and both hydrogenase systems (*hox* and *hup* genes) occurred at the same time in the continuous-light experiment (Toepel et al. 2008), whereas *hox* and *hup* genes showed different and distinct expression patterns in the 6 h L:D experiment. The *hox* genes were up-regulated during the first DP (Fig. 3b), whereas the *hup* genes were

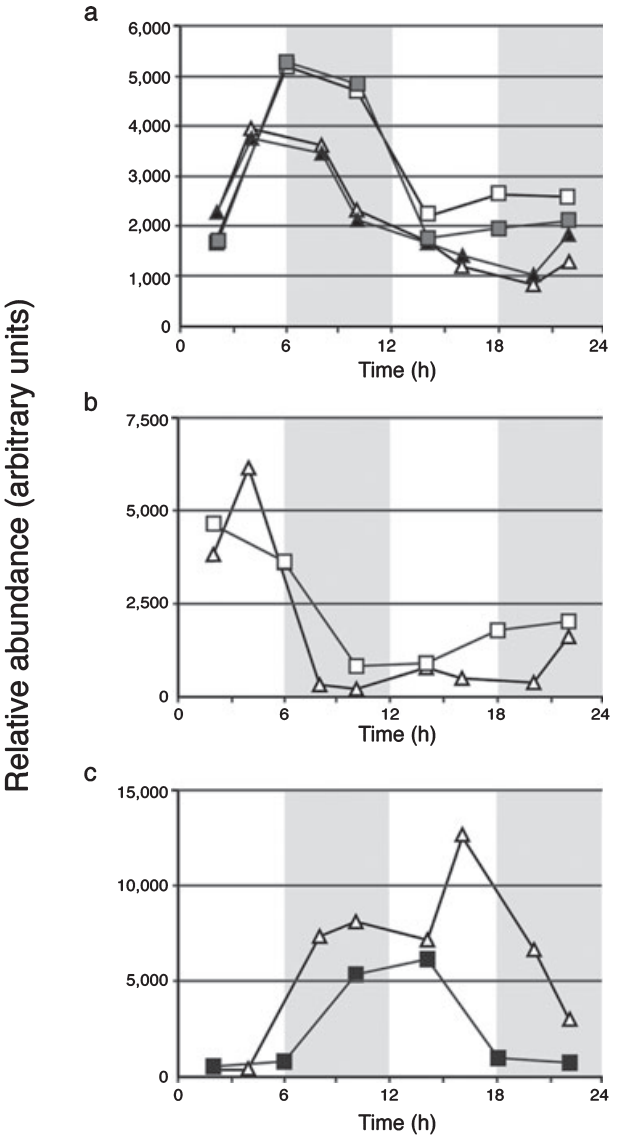


FIG. 2. Comparison of the gene expression pattern (plotted as relative transcriptional abundance vs. time) of the (a) *psa*AB, (b) *rbc*LS, and (c) *cox*BAC1 genes in *Cyanothece* sp. grown under 12 h light:dark (L:D) (squares) or short L:D (6 h) cycles (triangles).

mainly up-regulated during the second DP, similar to the *nif* cluster (Fig. 3a). A similar difference was observed between the two iron-transporter systems *feo*A1B1 (up-regulated in the first and second DP) and *feo*A2B2 (up-regulated only in the second DP). We also identified genes that showed a specific response to the incident light or dark. One operon (cce_1684–cce_1691), with several NADH dehydrogenase subunits, a sulfate-transporter and an Na^+/H^+ antiporter and several unknown genes, was up-regulated exclusively during both light periods. Another gene cluster that encodes proteins with a 4VR domain (cce_2109 and cce_2112), which may be involved in signaling, was up-regulated during the beginning of the first LP, similar to the 12 h L:D experiment (Singh et al. 2004). Finally, the analysis of L:D-dependent gene expression patterns yielded several potential regulator genes responding to the varying L:D conditions (Fig. 3, b and c). A potential L:D receptor, *aph*1, a phytochrome, was up-regulated at the beginning of each DP. In addition, we found several histidine kinases up-regulated: during the first LP (cce_0754 and cce_0755),

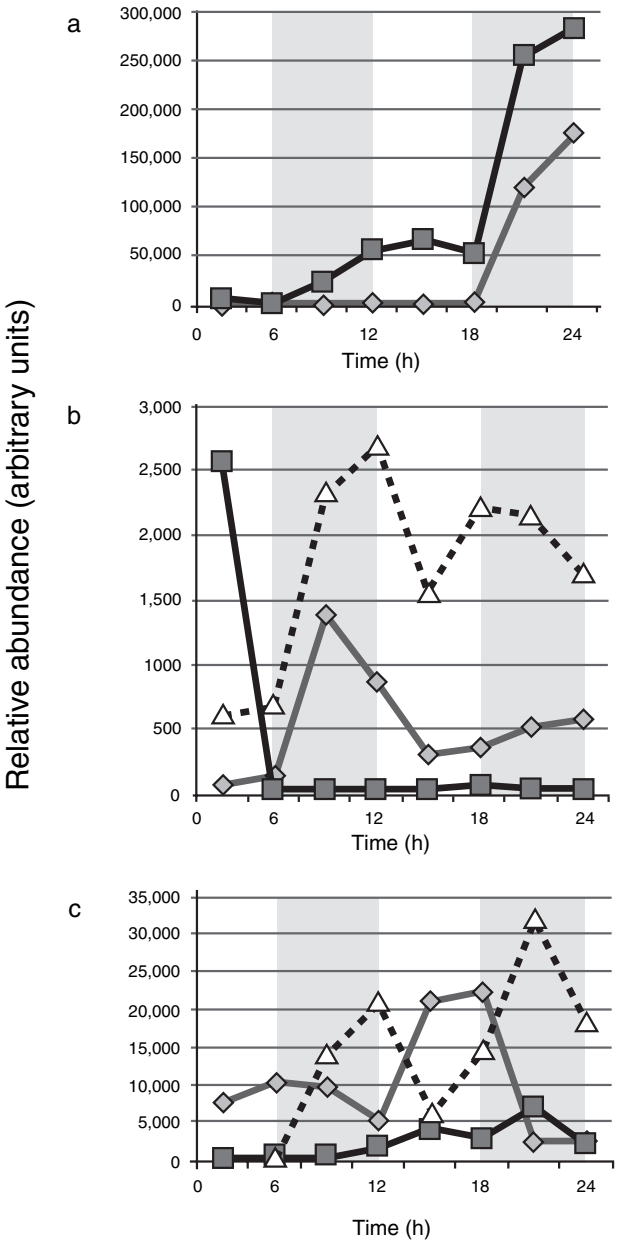


FIG. 3. Transcriptional changes (plotted as relative transcriptional abundance vs. time) in selected genes of *Cyanothece* sp. ATCC 51142 after growth in 6 h light:dark (L:D) cycles. (a) *hup*S (cce_1063) (◆) and peroxiredoxin (cce_3126) (■); (b) *cph*B (cce_2236) (△), *hox*E (cce_2319) (◆), and response regulator (cce_4542) (■); (c) response regulator, *rpa*A, (cce_0298) (◆), response regulator (cce_0970) (■), and *gnd* (cce_3746) (△).

during the second LP (e.g., *rpa*A/B, Fig. 3c), during the first DP (cce_2505, unknown function), during the second DP (cce_2366), and during both DPs (cce_0220). The histidine kinase and response regulator, cce_0969 and cce_0970 (Fig. 3c), were up-regulated during the dark period (time points $t = 16$ to 20 h), identical to the previous 12 h L:D experiments.

Glycogen production and breakdown. The measurements of relative glycogen content demonstrated that glycogen levels increased during the first LP, remained high during the first DP, and increased further in the second LP (Fig. 4). The glycogen content then decreased during the second DP coincident with N_2 fixation and the increase in respiration. These results corresponded with the gene expression of glycogen production and breakdown enzymes encoding genes. The glycogen synthase gene (*glg*A2) was up-regulated at the beginning of the first LP and at $t = 22$ h. The transcripts encoding the glycogen debranching enzyme (*glg*X) were up-regulated during the first DP and at $t = 16$ h. However, the glycogen phosphorylase gene (*glp*3) was up-regulated only during the second DP, and it seemed to be the initiating step in glycogen breakdown. The cyanophycin encoding genes *cph*A and *cph*B were up-regulated during both dark periods (Table 1).

Identification of circadian genes by CLR analysis. A second approach to the identification of genes that displayed a circadian transcriptional pattern used CLR (Faith et al. 2007) analysis of four separate differential transcription experiments: a 2 d 12 h L:D experiment (Stöckel et al. 2008); a 12 h L:D + LL experiment (Toepel et al. 2008); this 6 h L:D experiment; and an experiment that compared an aerobically grown culture before and after a 1 h, 2 h, and 6 h transition to anaerobic conditions (J. Toepel, T. C. Summerfield, and L. A. Sherman, unpublished data). Figure 5 depicts this analysis as a network of relationships between genes that approximates a 24 h clock. The tightness of a cluster and the large number of contacts indicated groups of genes that are transcribed in a similar fashion. We therefore analyzed the network using a hierarchical clustering approach that identified clusters of genes tightly related by CLR. From this analysis, we identified four large clusters and a number of smaller clusters. We were particularly interested in three clusters that contain many genes displaying circadian behavior and that are circled in Figure 5: a photosynthesis cluster, a nitrogenase cluster, and a respiration cluster.

The photosynthesis cluster contains genes encoding proteins for all of the major photosynthesis structures, especially PSII, cyt b_6f, CO_2 fixation, ATPase, and the phycobilisomes (Table 2). Interestingly, the only PSI genes in this cluster are *psa*FJ, and all of the other PSI genes, including circadian operon *psa*AB, are in the predominantly noncircadian cluster

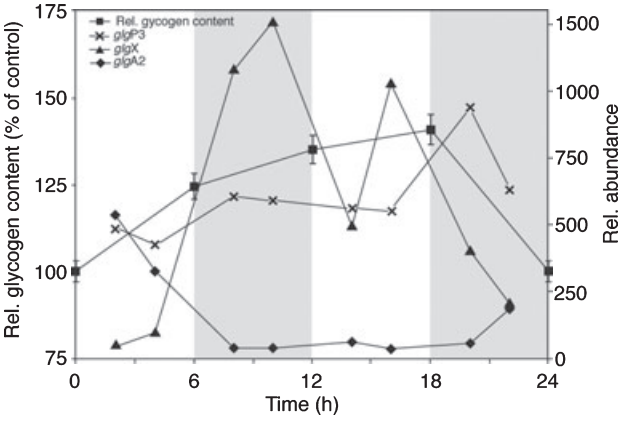


FIG. 4. Variation of the glycogen content and gene expression of glycogen production (synthase; *glg*A2) and breakdown (debranching; *glg*X and phosphorylase; *glg*P3) genes. The glycogen content is displayed as % of the control ($t = 0$), and the error bars represent standard deviation ($n = 3$).

~4–7 o'clock in Figure 5. Other genes in the photosynthesis cluster include glycogen synthetase and sigma factors *sig*G and *sig*H. This cluster is very similar to cluster 3, ''circadian—light up-regulated'' in Table 1.

The nitrogenase cluster at 8 o'clock is the tightest and most cohesive cluster as described in previous publications (Stöckel et al. 2008, Toepel et al. 2008). The CLR analysis determined that virtually every gene between cce_0545 and 0580 was in the cluster (Table 2). In addition, *hup*LS genes were associated very close to this cluster. In particular, *hup*L is just outside the *nif* cluster, but with edges connecting all nitrogenase genes in the cluster. This finding clearly identifies a functional relationship among these genes. In addition, the *rpa*A gene, which has been determined to be involved in circadian regulation in *Synechococcus elongatus* (Takai et al. 2006), is at ~7 o'clock in the CLR diagram (Fig. 5) and is associated with many *nif* genes. This association might represent a regulatory loop in which *rpa*A is transcribed, translated, and then used in the regulation of the *nif* cluster.

The cluster at 11 o'clock includes genes involved in respiration and in central metabolism, such as glycolysis, the pentose phosphate pathway, and glycogen degradation (Table 2). This is a most interesting cluster because it includes some gene sets that are correlated and others that are anticorrelated. Thus, the cluster includes genes that have peaks at the end of the light period and other genes with peaks toward the end of the dark period. This cluster includes circadian-regulated genes that are needed to prepare for the next light or dark phase. Therefore, under these 12 h L:D conditions, the cytochrome oxidase genes have peak transcript levels around L10 to D2.

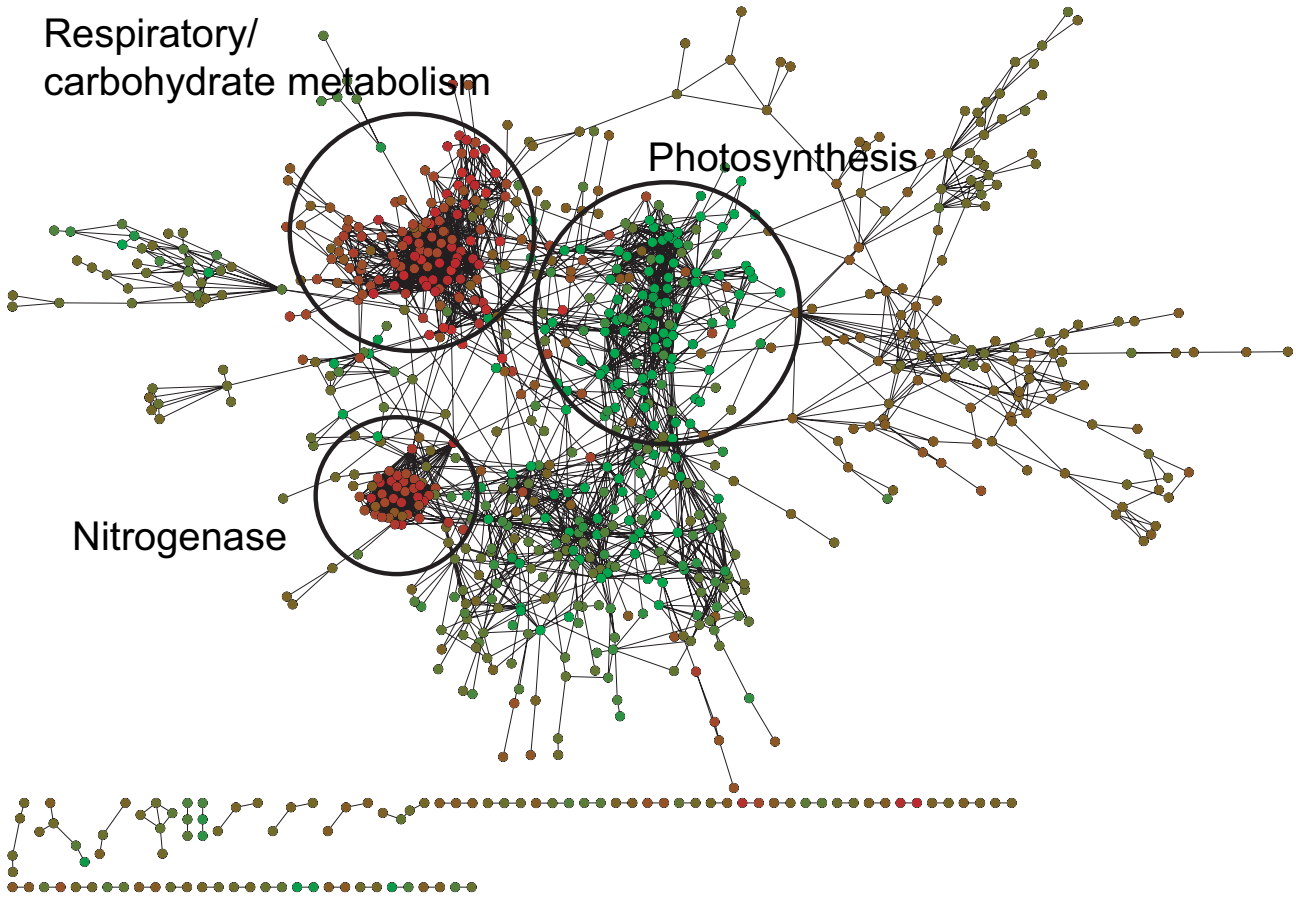


FIG. 5. Inferred network of diurnal and circadian associations. The context-likelihood of relatedness (CLR) algorithm was used to infer statistically significant gene to gene associations based on the combined expression profiles from experiments: growth in 12 h light:dark (L:D), growth in 12 h L:D followed by continuous light, the current experiment, and growth following transition into anaerobic conditions. In the network, circles represent genes, and edges between them an association with a CLR zscore of 5.0 or higher. The genes are colored according to the transcriptional levels at 5 h dark (red, high; green, low). Three clusters of interest are labeled and correspond to the groups described in Table 2. The majority of genes with inferred associations are included in the large network, but there are a number of smaller unconnected networks (including isolated pairs of genes) displayed at the bottom of the figure.

RT-PCR validation of microarray results. We used RT-PCR to validate the results of the microarray experiments. Figure 6 shows a comparison between the microarray results (top) to the RT-PCR results (bottom) for a select set of genes that demonstrated different types of temporal expression. This set included expression of *nif*H that was dramatically up-regulated in the second DP and two PSII genes (*psb*A1 and *psb*D) that were up-regulated in the light, although to a lesser extent in the second LP; note that the microarray data are plotted on a logarithmic scale because the *nifH* gene is transcribed at very high levels in the second DP, as described in Toepel et al. (2008). We also compared the results for *cox*C1, which was transcribed at high levels during the DPs and the second LP. It can be seen that there is a very close correspondence between the microarray data and the RT-PCR data for all three types of regulation. In addition, the results for *rnp*A demonstrated similar variation in both the microarray and the RT-PCR experiments. Based on these results, we concluded that the microarray results were a fair representation of differential gene expression in this organism during the 24 h experiment.

DISCUSSION

We analyzed growth in short L:D periods, along with a comprehensive analysis of growth under different L:D conditions, to develop a fuller understanding of circadian versus diurnal regulation of gene transcription in *Cyanothece* sp. The results in Tables 1 and 2 indicate that the main metabolic genes involved in photosynthesis, respiration, nitrogen fixation, and central carbohydrate metabolism have strong (or total) circadian-regulated components. The transcription of these gene sets was carefully maintained at the appropriate light or dark period, even during 6 h L:D growth, so that the cell could generate sufficient energy and an appropriate anoxic environment, when required. The photosynthate from fixed carbon was placed in glycogen granules, and the degradation of this glycogen was used as a

TABLE 2. Context-likelihood of relatedness (CLR) analysis of *Cyanothece* sp. ATCC 51142 transcription for four microarray experiments.

Function	Genes
Photosynthesis cluster	
PSII	Phycobilisomes (*apc* & *cpc*)
	Oxygen-evolving complex (*psb*O, *psb*U, *psb*V)
	*psb*A4
	*psb*EFJ, *psb*28
PSI	*psa*FJ
$Cytb_6f$	*pet*ACl, *pet*F3F4
ATPase	*atp operons*
CO_2 fixation	*rbc*LXS
	*ccm*MLK1K2
Glycogen	*glg*A2
Sigma factors	*sig*G and *sig*H
Nitrogen cluster	
Nitrogenase	cce_0545–cce_0580 (including *nif*HDK)
Uptake hydrogenase	*hup*LS
Regulatory	cce_0969 + 0970
Respiratory/carbohydrate metabolism cluster	
Regulatory	*kai*B3
	Hybrid sensor and phytochrome (cce_1982–1983)
Sigma factors	*sig*E
Cytochrome oxidase	*cox*BAC1
NADH DH	*ndh*LEID1F1
Pentose phosphate pathway	*gnd, zwf, opc*A
Carbohydrate metabolism	*glp*P1, *glp*X, *ppc, tal*AB *gap, pgl*

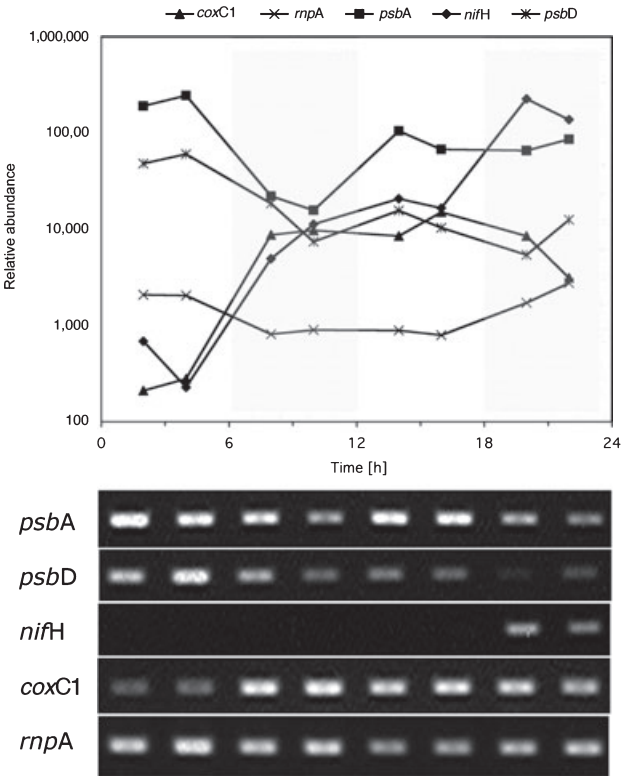


FIG. 6. Validation of the microarray results by comparison of the microarray results (top panel) with those from RT-PCR experiments (bottom panel). The genes chosen were examples of different categories of differential expression, including *nif*H (◆), highly expressed in the second dark; *cox*C1 (▲), highly expressed during both dark periods and the second light period; *psb*A (■) and *psb*D (*), highly expressed in the light periods; and *rnp*A (×), little change in expression throughout the experiment. The microarray data are plotted on a logarithmic scale.

substrate for the massive respiratory burst that occurred prior to and concomitant with N_2 fixation. Coordinating these metabolic properties in a circadian manner appears to be one of the key regulatory functions of the cell. It is interesting that many PSI and PSII genes are transcribed in a diurnal fashion, whereas the reaction center genes and a few genes encoding assembly type proteins are transcribed in a circadian manner. These findings might have important ramifications as to the assembly and specific functions of the two photosystems. We have also identified some specific regulatory molecules that may be involved in the intricate timing of gene transcription from light to dark transitions and vice versa (Tables 1 and 2). We will complete a more detailed computational analysis of these data to determine if we can predict specific interrelationships between regulatory and structural proteins that can then be tested via genetics and physiology.

It was evident that *Cyanothece* undergoes several adaptation processes in order to develop the appropriate environment for N_2 fixation. Nitrogenase activity and *nif* gene expression were in good correspondence. Our results indicated that one 6 h light period is not sufficient for induction of the nitrogenase-related genes, a feature that also was demonstrated in *Gloeobacter* (Taniuchi and Ohki 2007). This finding is consistent with our hypothesis that N_2 fixation is dependent upon the energy status of the cells (Toepel et al. 2008). Our results demonstrated that breakdown of stored glycogen and high respiration rates were connected to the nitrogenase activity and that both processes are necessary to provide sufficient energy equivalents for N_2 fixation. Furthermore, our data suggested that regulation of photosynthesis is independent of incident light during the first 18 h and the decline of photosynthesis is coordinated with the increase in respiration and the concomitant breakdown of glycogen. Our results not only support the hypothesis of diurnal control of the major processes in *Cyanothece* sp. (Stöckel et al. 2008) but also suggest a strong feedback regulation regarding the energy status of the cells. In addition, we demonstrated that respiratory enzyme activity and gene expression patterns did not always correspond, suggesting post-transcriptional, energy-dependent activation of respiration and a tight coregulation with N_2 fixation.

Comparison of experiments in which cells were grown in L:D + LL (Toepel et al. 2008) and this study demonstrated that gene expression for glycogen and cyanophycin production/breakdown was

L:D dependent. In both experiments, glycogen breakdown was clearly dark dependent, and glycogen phosphorylase appeared to be the initializing enzyme and one that most likely required a dark signal for expression. Comparison between the L:D + LL experiments and the 6 h L:D experiment allows us to better differentiate between L:D-dependent and L:D-independent expression and identify circadian-controlled genes.

Two gene sets, encoding proteins for hydrogenases and photosynthesis, were of particular interest to us. Toepel et al. (2008) showed that the uptake hydrogenase (*hup*) and the bidirectional hydrogenase (*hox*), found in *Cyanothece* sp., were cotranscribed with the N_2-fixation genes (*nif*-genes) and probably consumed the produced hydrogen. The expression pattern of the uptake hydrogenase-encoding *hup* genes in *Cyanothece* sp. was in good accordance with previous studies, such that the expression of these genes was closely related to the *nif* genes. In contrast, the hydrogenase-encoding *hox*-genes were mostly up-regulated during the first DP and probably acted as an electron valve during photosynthesis (for review, see Tamagnini et al. 2007). Since the fermentative-related genes for acetate and lactate production and genes for the PPP cycle (especially *gnd*) showed elevated expression during the first DP, it is likely that *Cyanothece* sp. could produce reducing power and energy equivalents from fermentation and the PPP cycle during this DP.

The photosynthesis genes demonstrated rather important behaviors. The majority of the PSI and PSII structural genes were diurnally expressed and had peaks in both light periods when grown in 6 h L:D periods. At the same time, the most highly expressed *psb*A gene, *psb*A1/*psb*A5, also demonstrated a diurnal pattern, although the intensity was lower in the second DP. The expression pattern of the *psb*A1 gene was in good agreement with previous results (Colón-López et al. 1998, Stöckel et al. 2008, Toepel et al. 2008). At the same time, *psa*AB demonstrated a circadian behavior, and the *psa*AB transcript level peak was always near the end of the light period (see also Colón-López and Sherman 1998). In addition, two members of the *psb*A family, *psb*A3 and *psb*A4, also demonstrated a circadian transcriptional pattern, but with peaks at different times (Table 1). It is possible that the encoded D1 proteins are produced at specific times specifically to change the properties of PSII, similar to the situation in *S. elongatus* (Schaefer and Golden 1989a,b). In fact, the transcriptional results for *psb*A3 and *psb*A4 correlate nicely with the protein results using the *S. elongatus* D1 Form 2 antibody as described in Colón-López and Sherman (1998).

In conclusion, we have obtained answers to the three questions that we posed at the outset of this project. The metabolic rhythms in *Cyanothece* sp. ATCC 51142 were extremely stable and persisted even during 6 h L:D growth conditions. In addition, nitrogen fixation continued under these conditions and reached a maximum every 24 h. Finally, we used growth under short day–night cycles to help determine the circadian versus diurnal regulation of genes encoding important nitrogen-fixation and energy-producing functions. We used these data, in conjunction with experiments using other L:D patterns, to develop a comprehensive model of circadian and diurnal associations. Importantly, both approaches resulted in very similar results, thus demonstrating the value of the CLR analysis. Our results clearly indicated that the nitrogenase gene cluster is under very tight circadian regulation, but other energy-producing functions must be modified to ensure that the cell can provide the appropriate energetic and oxygen environment to permit N_2 fixation under such anoxic conditions. This is a key property of *Cyanothece* sp. ATCC 51142 and one that may provide directions for the synchrony of other important metabolic processes, including photosynthetically driven hydrogen production.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. All genes that were up- or down-regulated by at least 2-fold during the course of the experiment. The values for the different time points are the relative abundance, and the column labeled ''Fold change'' represents the maximum relative abundance divided by the minimum relative abundance for the eight samples taken during the 24 h period.

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