The Nitrogen-Regulated Response Regulator NrrA Controls Cyanophycin Synthesis and Glycogen Catabolism in the Cyanobacterium Synechocystis sp. PCC 6803

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Running title: NrrA controls cyanophycin synthesis and glycogen metabolism

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Keywords: Cyanobacteria; NrrA; nitrogen starvation; cyanophycin synthesis; sugar catabolism

Background: Cyanobacterial metabolism is extensively regulated in response to nitrogen limitation.

Results: The regulon of transcriptional factor NrrA was reconstructed in the genomes of diverse cyanobacteria and experimentally characterized in Synechocystis.

Conclusion: NrrA controls cyanophycin accumulation and glycogen catabolism in Synechocystis.

Significance: A molecular mechanism coordinately regulating synthesis and degradation of nitrogen and carbon reserves in cyanobacteria is identified.

SUMMARY

The cellular metabolism in cyanobacteria is extensively regulated in response to changes of environmental nitrogen availability. Multiple regulators are involved in this process, including a nitrogen-regulated response regulator NrrA. However, the regulatory role of NrrA in most cyanobacteria remains to be elucidated. In this study, we combined a comparative genomic reconstruction of NrrA regulons in 15 diverse cyanobacterial species with detailed experimental characterization of NrrA-mediated regulation in Synechocystis sp. PCC 6803. The reconstructed NrrA regulons in most species included the genes involved in glycogen catabolism, central carbon metabolism, amino acid biosynthesis, and protein degradation. A predicted NrrA-binding motif consisting of two direct repeats of TG(T/A)CA separated by an 8-bp A/T-rich spacer was verified by in vitro binding assays with purified NrrA protein. The predicted target genes of NrrA in Synechocystis sp. PCC 6803 were experimentally validated by comparing the transcript levels and enzyme activities between the wild-type and nrrA-inactivated mutant strains. The effect of NrrA deficiency on intracellular contents of arginine, cyanophycin, and glycogen was studied. Severe impairments in arginine synthesis and cyanophycin accumulation were observed in the nrrA-inactivated mutant. The nrrA inactivation also resulted in a significantly decreased rate of glycogen degradation. Our results indicate that by directly up-regulating expression of the genes involved in arginine synthesis, glycogen degradation, and glycolysis, NrrA controls cyanophycin accumulation and glycogen catabolism in Synechocystis sp. PCC 6803. It is suggested that NrrA plays a role in coordinating the synthesis and degradation of nitrogen and carbon reserves in cyanobacteria.

Cyanobacteria are a large group of oxygenic photosynthetic prokaryotes that are found in diverse ecological habitats. In many of these habitats, nitrogen is limiting and cyanobacteria are exposed to periods of severe nitrogen starvation (1). To survive under such conditions, they have evolved sophisticated mechanisms to sense and respond to nitrogen limitation, including induction of the systems for high-affinity uptake of nitrogen-containing compounds (2). During
nitrogen starvation, non-diazotrophic cyanobacteria may consume internal stores of nitrogen to prolong their growth. For example, the unicellular *Synechocystis* sp. PCC 6803 uses cyanophycin (multi-L-arginyl-poly-[L-aspartic acid]), a non-ribosomally synthesized peptide consisting of equimolar quantities of arginine and aspartic acid, as a nitrogen source upon nitrogen starvation (3). After cyanophycin is exhausted, cells degrade the phycobilisomes that are light-harvesting antennae composed of rod and core proteins to provide nitrogen, which leads to a color change of cells from blue-green to yellow-green, known as bleaching (4). Upon reintroduction of nitrogen, cyanophycin is synthesized immediately, thus cyanophycin is considered as a dynamic nitrogen reservoir in *Synechocystis* sp. PCC 6803 and many other cyanobacteria (5). Some cyanobacteria are able to fix dinitrogen in the absence of combined nitrogen such as nitrate or ammonium. The filamentous *Anabaena* sp. PCC 7120 produces heterocysts that are specialized cells for nitrogen fixation (6), while the unicellular *Cyanothece* sp. ATCC 51142 fixes nitrogen and accumulates cyanophycin granules under dark conditions (7). Nitrogen depletion also impacts glycogen accumulation in cyanobacteria (8). Previous studies have shown that during nitrogen starvation, glycogen is accumulated in *Synechocystis* sp. PCC 6803, whereas the expression of sugar catabolic genes is widely up-regulated (9).

The NtcA protein is the global nitrogen regulator in cyanobacteria (10). It senses intracellular 2-oxoglutarate levels and regulates many genes including those involved in nitrogen assimilation. In non-diazotrophic *Synechocystis* sp. PCC 6803, NtcA directly regulates transcription of the *nrrA* gene (referred to as *rre37* in (11)), encoding a nitrogen-regulated response regulator of the OmpR family, which has a response regulator domain at the N-terminus and a DNA-binding domain at the C-terminus. NrrA is involved in induction of sugar catabolic genes in *Synechocystis* sp. PCC 6803 during nitrogen starvation (11), however, it remains unclear whether NrrA directly regulates transcription of these genes and other genes up-regulated by nitrogen deprivation. In diazotrophic *Anabaena* sp. PCC 7120, expression of *nrrA* is up-regulated by nitrogen deprivation under the control of NtcA (12), and NrrA is required for the full induction of the *hetR* gene (13), encoding a master regulator of heterocyst differentiation (14). The NrrA-binding site of the *hetR* promoter has been determined by a DNase footprinting assay (13). NrrA also controls glycogen catabolism in *Anabaena* sp. PCC 7120 by directly regulating expression of *glgP* gene encoding a glycogen phosphorylase and *sigE* gene encoding a group 2 σ factor of RNA polymerase (15). Although NrrA seems to be widely distributed in cyanobacteria (12), nothing was known about its function in the species other than *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120.

The main goal of this study was to investigate the regulatory role of NrrA in diverse cyanobacteria. We used a comparative genomic approach (16) to identify NrrA-binding DNA motifs and reconstruct NrrA regulons in 15 diverse cyanobacterial species. The predicted members of NrrA regulons in most species are involved in glycogen catabolism, central carbon metabolism, amino acid biosynthesis, and protein degradation. A combination of *in vivo* and *in vitro* experimental techniques was used to validate the predicted direct target genes of NrrA in *Synechocystis* sp. PCC 6803. Furthermore, the effect of NrrA deficiency on intracellular levels of arginine, cyanophycin, and glycogen was studied. Our results indicate that NrrA controls cyanophycin accumulation and glycogen catabolism in *Synechocystis* sp. PCC 6803.

**EXPERIMENTAL PROCEDURES**

*Bioinformatics Approaches and Tools*—Genome sequences of cyanobacteria analyzed in this study were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Identification of orthologs was performed using the BLASTP tool provided by NCBI (17). Orthologs of the NrrA proteins from *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 were identified with a 50% protein sequence identity threshold. The ClustalX (version 2.1) program (18) was used for protein sequence alignments, and the PhyML 3.0 program (19) for phylogenetic analysis. A phylogenetic tree of NrrA proteins was built using the maximum likelihood method, with calculation of bootstraps from 1000 replications.

Regulon reconstruction was performed using an
established comparative genomics method based on identification of candidate regulator-binding sites in closely related prokaryotic genomes (16). For identification of the conserved DNA-binding motif for NrrA, we started from training sets of known NrrA-regulated genes in *Synechocystis* sp. PCC 6803 (11) and *Anabaena* sp. PCC 7120 (15) and their orthologs in multiple cyanobacterial genomes. An iterative motif detection algorithm implemented in the RegPredict web-server (regpredict.lbl.gov) (20) was used to identify common regulatory DNA motifs in upstream regions of these genes. For each clade of NrrA proteins on the phylogenetic tree, a separate training gene set was used. A positional weight matrix (PWM) was constructed for each identified motif and used to scan the genomes in this clade. Candidate NrrA-binding sites were identified using the RegPredict (20) and GenomeExplorer tools (21). Scores of candidate sites were calculated as the sum of positional nucleotide weights. The score threshold was defined as the lowest score observed in the training set. Genes with candidate upstream binding sites that are high scored and/or conserved in two or more genomes were included in NrrA regulon. Candidate sites associated with new regulon members were added to the training set, and the respective PWM describing a group-specific NrrA motif was rebuilt to improve search accuracy. The NrrA-binding DNA motifs were visualized as sequence logos using WebLogo (22). Functional annotations of the predicted regulon members were based on the Cyanobase (http://genome.microbedb.jp/cyanobase) (23).

**Strains, and Growth Conditions—*Synechocystis* sp. PCC 6803 and its derivative with the *nrrA* gene inactivation were used in this study. *Synechocystis* strains were routinely grown in the BG-11 medium (24) containing 18 mM NaNO₃ under continuous white light (~70 µmol photons m⁻² s⁻¹). Kanamycin (30 µg/ml) was added when needed. The photo-mixotrophic cultures were started with an optical density at 730 nm (OD₇₃₀) of about 0.05, and grown at 30 °C under continuous illumination in triplicates in 300-ml glass flasks with shaking at 30 °C under light condition. After 12 h of nitrogen deprivation, 5 mM NaNO₃ with or without 5 mM arginine was added to the culture, and cells were grown for another 12 h. Aliquots of the culture were harvested in the course of time for metabolite analyses.

**Mutant Construction**—To construct the *nrrA* gene-inactivated mutant of *Synechocystis* sp. PCC 6803, DNA fragments immediately upstream and downstream of the *nrrA* gene (sll1330) were amplified by PCR using the primers shown in supplemental Table S1. The upstream fragment was cloned between the Sacl and BamHI sites of pBluescript KS⁺ (Agilent Technologies), and the downstream fragment was cloned between the EcoRI and SalI sites. A kanamycin resistance cassette from the plasmid pUC4K (25) was inserted between the upstream and downstream fragments to form the plasmid pKSnrrA. This plasmid was introduced into *Synechocystis* sp. PCC 6803 according to (26). The mutant was selected on BG-11 plates supplemented with kanamycin, and segregation was confirmed by PCR using the primers shown in supplemental Table S1. In the mutant, the region from +95 to +450 with respect to the translation start site of the *nrrA* gene (753 bp long) was replaced with the kanamycin resistance cassette.

**RNA Isolation and Real-time PCR Analysis—*Synechocystis* sp. PCC 6803 cells were harvested by centrifugation, frozen immediately in liquid nitrogen, and ground into powder. RNA was isolated using TRIzol reagent (Invitrogen). Contaminant DNA was removed by DNase I (Takara) digestion. RNA (1 µg) was transcribed into cDNA with random primers using the ReverTra-Plus kit from TOYOBO. The product was checked by melting curve analysis. The reaction mixture (20 µl) contained Power SYBR green PCR master mix (Takara) and 0.3 µM gene-specific primers (as shown in supplemental Table S1). The PCR parameters were 1 cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. The accuracy of the PCR product was checked by melting curve analysis.
The expression level of each gene was normalized with the value for the \textit{rnpB} gene encoding RNase P subunit B, which was used as a reference gene with constitutive expression (27). Data were presented as the average of six measurements from two biological replicates, with the corresponding standard deviation.

\textbf{Protein Overexpression and Purification—}The \textit{nrrA} genes (sll1330 and all4312) were PCR-amplified from the genomic DNA of \textit{Synechocystis} sp. PCC 6803 and \textit{Anabaena} sp. PCC 7120, respectively, using the primers shown in supplemental Table S1. The PCR product was ligated into the expression vector pET28a cleaved by NdeI and BamHI. The resulting plasmid pET28a-nrrA was sequenced to exclude unwanted mutations in the \textit{nrrA} gene and used to produce NrrA protein with an N-terminal hexahistidine tag. For overproduction of NrrA proteins, \textit{E. coli} BL21 (DE3) was transformed with plasmid pET28a-nrrA and cultivated in LB medium at 37 °C to an optical density at 600 nm (OD$_{600}$) of 0.8. Protein expression was induced by the addition of 0.2 mM isopropyl-$\beta$-1-thiogalactopyranoside, and the culture was incubated for another 18 h at 16 °C. After the cells were harvested, purification of NrrA by nickel-nitrilotriacetic acid affinity chromatography was performed as described previously (28). The purified protein was run on a 12% sodium dodecyl sulfate-polyacrylamide gel to monitor its size and purity.

\textbf{Electrophoretic Mobility Shift Assay (EMSA)—}The 200-bp DNA fragments in the promoter regions of individual genes of \textit{Synechocystis} sp. PCC 6803 and \textit{sigE} gene (alr4249) of \textit{Anabaena} sp. PCC 7120 were PCR-amplified using the primers shown in supplemental Table S1. Both forward and reverse primers were Cy5 fluorescence labeled at the 5’-end (Sangong Corp., Shanghai, China), and the PCR products were purified with a PCR purification kit (AXYGEN). Purified NrrA protein was incubated with the fluorescence-labeled DNA fragment (1 nM) in 20 µl of binding buffer containing 20 mM Tris (pH 7.5), 0.25 mM DTT, 10 mM MgCl$_2$, 5% glycerol, 0.8 µg bovine serum albumin (BSA), and 1 µg salmon sperm DNA (non-specific random-sequence competitor). After incubation at room temperature for 20 min, the reaction mixture was electrophoresed at 4°C on a 6% native polyacrylamide gel in 0.5×Tris-borate-EDTA for 1.5 h at 100 V. Fluorescence-labeled DNA on the gel was then detected by the Starion FLA-9000 (FujiFilm, Japan). Specificity of the NrrA-DNA interactions was tested by including a 200-fold excess of unlabeled target DNA (specific competitor) in binding reaction mixtures.

\textbf{Analysis of Enzyme Activities—}Enzyme activities were measured in crude cell extracts from 25-ml culture aliquots. The cell pellets were washed and resuspended in 100 mM Tris-HCl buffer (pH 7.5). After sonication, cell debris was removed by centrifugation, and the supernatant was used for determination of enzyme activities and protein concentration.

Glycogen phosphorylase activity was measured by monitoring the increase in NADPH concentration using phosphoglucomutase and glucose-6-P dehydrogenase as coupling enzymes (29). Briefly, 10 µl of the cell extract was added to 200 µl of 100 mM potassium phosphate buffer (pH 7.5) containing 2.5 mM EDTA, 2.5 mM MgCl$_2$, 2 mM NADP$^+$, 1 unit of phosphoglucomutase, 6 units of glucose-6-P dehydrogenase, and 1 g/l glycogen. The change in NADPH concentration was monitored at 340 nm by using a Beckman DU-800 spectrophotometer.

Glyceraldehyde-3-P dehydrogenase activity was measured by adding 10 µl of the cell extract to 200 µl of 100 mM potassium phosphate buffer (pH 7.5) containing 4 mM glyceraldehyde-3-P, 10 mM EDTA, and 2 mM NAD$^+$. The formation of NADH was monitored spectrophotometrically at 340 nm.

\textit{N}-Acetylornithine aminotransferase activity was determined by adding 10 µl of the cell extract to 200 µl of 100 mM Tris-HCl buffer (pH 8.5) containing 0.5 mM \textit{a}-ketoglutarate, 4 mM NAD$^+$, 20 µM pyridoxal 5’-phosphate, 3 mM acetylcysteine, and 5 units of glutamate dehydrogenase (30). The formation of NADH was monitored spectrophotometrically at 340 nm.

Argininosuccinate synthetase activity was assayed by coupling the formation of AMP to the oxidation of NADH to NAD$^+$ through adenylate kinase, pyruvate kinase, and lactate dehydrogenase (31). Briefly, 10 µl of the cell extract was added to 200 µl of 100 mM Tris-HCl buffer (pH 7.5) containing 1 mM ATP, 5 mM MgCl$_2$, 2 mM KCl, 16 mM phosphoenolpyruvate, 0.2 mM NADH, 7.5 mM citrulline, 7.5 mM aspartate, 10 units of inorganic pyrophosphatase, 10 units of adenylate

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kinase, 4 units of pyruvate kinase, and 4 units of lactate dehydrogenase. The change in NADH absorbance was monitored at 340 nm.

Metabolite Measurements—For analysis of extracellular metabolites, culture samples were harvested by centrifugation at $15,000 \times g$ for 10 min at 4 °C. Glucose concentration was determined with an enzymatic test kit (r-Biopharm, Darmstadt, Germany). Nitrate concentration was measured with a colorimetric assay kit (Roche, Switzerland).

For determination of intracellular arginine concentration, cells were harvested by centrifuging 20 ml of culture broth at 9000 × g and 4 °C for 10 min and resuspended in 10 ml of 80% (vol/vol) ethanol. Norleucine was added as an internal standard. After heating at 65 °C for 3 h, cell debris was removed from extracts by centrifugation at 18000 × g for 15 min. The extracts were dried in a vacuum centrifuge. Arginine in the extracts was derivatized with phenylisothiocyanate (PITC) by incubating with 200 µl of the derivative reagent (Sigma) at room temperature for 45 min (32). The resulting PITC-arginine was quantitated by high pressure liquid chromatography (HPLC) using an Agilent model 1260 instrument equipped with an Ultimate Amino Acid Column (4.6 by 250 mm; Welch, Shanghai, China) and a UV detector (Agilent) operated at 254 nm. The mobile phase solutions were pumped at a flow rate of 1.0 ml/min, and the temperature of the column was kept at 40 °C.

Determination of Glycogen, Cyanophycin, and Phycocyanin Levels—For determination of intracellular glycogen levels, cell pellets were harvested by centrifuging 1 ml of culture aliquots, resuspended in 100 µl of 3.5% (vol/vol) sulfuric acid and boiled for 40 min. The amount of glucose in the hydrolysate was determined by using $\alpha$-toluidine reagent and reading the absorbance at 635 nm (33).

Cyanophycin was isolated and purified using a previously published method with minor modifications (5). Cells were harvested by centrifuging 25 ml of culture aliquots and resuspended in 2 ml of Tris-HCl buffer (pH 7.0). After sonication, the suspension was centrifuged and the supernatant was discarded. The pellet was washed twice with distilled water and extracted by two successive treatments with 0.5 ml of 0.1 M HCl for 30 min at room temperature. The suspension was centrifuged and the supernatant was neutralized with 0.1 M NaOH. Cyanophycin that is insoluble at neutral pH was collected by centrifugation, washed with distilled water, and solubilized in 0.1 ml of 0.1 M HCl. The cyanophycin content was measured with the Bradford reagent using bovine serum albumin as the standard.

The ratio of phycocyanin to chlorophyll levels was used as a measure of the phycocyanin content. Phycocyanin and chlorophyll levels were obtained by spectrophotometry as described previously (34).

RESULTS
Genomic Reconstruction of NrrA Regulons in Cyanobacteria

Phylogenetic Distribution of NrrA Proteins—Orthologs of NrrA proteins from Synechocystis sp. PCC 6803 and Anabaena sp. PCC 7120 were identified by BLASTP searches in the reference protein database (refseq-protein). NrrA orthologs were detected in 6 cyanobacteria orders including Chroococcales, Oscillatoriales, Nostocales, Gloeobacterales, Pleurocapsales, and Stigonematales but not in the Prochlorales order (supplemental Table S2). A single copy of nrrA is present in the genomes of 39 unicellular, 4 baecytous, 20 filamentous, 15 heterocystous, and 1 ramified species. Thus NrrA proteins are widely distributed in cyanobacteria, independent of morphology and taxonomy of species. A maximum likelihood phylogenetic tree was constructed for the NrrA proteins identified in cyanobacteria (supplemental Fig. S1 and Fig. 1), which largely coincides with the phylogeny of cyanobacterial species (35). The NrrA proteins from Chroococcales and Pleurocapsales are similar, while they are distantly related to NrrA from Nostocales. The major subclade of NrrA proteins from Oscillatoriales is split on the tree into two separated groups (supplemental Fig. S1), which largely coincides with the phylogeny of cyanobacterial species (35). The NrrA proteins from Chroococcales and Pleurocapsales are similar, while they are distantly related to NrrA from Nostocales. The major subclade of NrrA proteins from Oscillatoriales is split on the tree into two separated groups (supplemental Fig. S1), which may reflect the functional divergence, e.g. by the set of target genes or by the DNA recognition motifs (see next section).

Identification of NrrA-binding Motifs and Regulons—To reconstruct the NrrA regulons in cyanobacteria, we applied the integrative
comparative genomics approach that combines identification of candidate transcription factor-binding sites with cross-genomic comparison of regulons and with the functional context analysis of candidate target genes (16). The analyzed cyanobacteria include Synechocystis sp. PCC 6803, Synechococcus sp. PCC 7002, Leptolyngbya sp. PCC 7376, Microcystis aeruginosa NIES-843, Pleurocapsa sp. PCC 7327, Cyanobacterium staniert PCC 7202, Cyanothece sp. PCC 8801, Cyanothece sp. PCC 7822, Cyanothece sp. PCC 7424, Cyanothece sp. ATCC 51142, Trichodesmium erythraeum IMS101, Nostoc punctiforme PCC 73102, Nodularia spumigena CCY 9414, Nostoc sp. PCC 7107, and Anabaena sp. PCC 7120. These 15 species with complete genome sequences exhibit diverse morphologies (unicellular, baecystous, filamentous, and heterocystous) and metabolisms (e.g. diazotroph and non-diazotroph). For identification of the conserved DNA-binding motif for NrrA, we started from training sets of known NrrA-regulated genes in Synechocystis sp. PCC 6803 and Anabaena sp. PCC 7120 and their orthologs in other genomes. The upstream regions of these genes were analyzed using a motif recognition program to identify conserved NrrA-binding motifs. After construction of a positional weight matrix for each identified motif, we searched for additional NrrA-binding sites in the analyzed genomes and finally performed a cross-species comparison of the predicted sets of potentially co-regulated genes to define the NrrA regulon for each species.

Based on the identified NrrA-binding motifs and regulons, the analyzed cyanobacterial species can be divided into two groups. As shown in Fig. 1A, a highly conserved signal consisting of two direct repeats of TG(T/A)CA separated by an 8-bp A/T-rich spacer was identified as a candidate NrrA-binding motif in the first group including 8 species of Chromococcales, Pleurocapsa sp. PCC 7327 (Pleurocapsales), and Leptolyngbya sp. PCC 7376 (Oscillatoriales). This motif is similar to the pho box, the specific DNA target sequence of E. coli PhoB regulator that also belongs to the OmpR family (36). A slightly different binding motif with consensus TGTCATCNNAANTTNACA was detected for NrrA from the second group including 4 species of Nostocales and T. erythraeum IMS101 (Oscillatoriales). This result is in accordance with the experimentally determined NrrA-binding sequence of the hetR promoter in Anabaena sp. PCC 7120 (13). The obtained NrrA-binding motifs were used to detect candidate members of the NrrA regulons in the 15 cyanobacterial genomes (Table 1). Detailed information about the predicted DNA-binding sites and downstream regulated genes is provided in supplemental Table S3.

**Predicted Members of NrrA Regulons**—The reconstructed NrrA regulons control central carbon metabolism in most of the analyzed cyanobacteria (Fig. 1B). However, the specific content of NrrA regulons is highly variable between different species (Table 1). Most of the predicted members of NrrA regulons in the 10 species of the first group are involved in glycogen catabolism, central carbon metabolism, amino acid biosynthesis, and protein degradation. For example, candidate NrrA-binding sites were identified in the promoter regions of the genes coding for glycogen phosphorylase (glgP) and two glycolytic enzymes (pfkA and gapl) in the Synechocystis sp. PCC 6803 genome, suggesting that these genes are direct targets of NrrA in Synechocystis. A putative NrrA-binding site was also identified to be located upstream of the icfG gene cluster, which encodes a glycogen isoamylase (glgX) and IcfG protein phosphatase participating in the regulation of glucose metabolism in Synechocystis (37). Moreover, the predicted NrrA targets in Synechocystis include genes encoding proteases (prp1, prp2, and pfpl) and the enzymes of arginine biosynthesis (argG and argD) (Table 1). Arginine can be polymerized with aspartic acid to form cyanophycin, a nitrogen reserve present in most cyanobacteria (38).

Similar to that in Synechocystis, the reconstructed NrrA regulon in Cyanothece sp. ATCC 51142 contains genes from glycogen catabolism (glgP), central carbon metabolism (pfkA), arginine biosynthesis (argG), and protein degradation (prpl-prp2, clpS-cc2239) (Table 1). We found that the nrrA gene is preceded by a putative NrrA-binding site in all the four Cyanothece species analyzed (ATCC 51142, PCC 8801, PCC 7822, and PCC 7424), indicating that NrrA could regulate expression of its own gene in these Cyanothece species. Additionally, a candidate NrrA-binding site was identified upstream of the pipX gene in the genomes of Cyanothece sp. PCC 8801 and M. aeruginosa.
NIES-843. The \textit{pipX} gene encodes a nitrogen regulator protein that coactivates NtcA-controlled genes in \textit{Synechococcus} and \textit{Anabaena} under nitrogen starvation (39). To provide a nitrogen source during nitrogen starvation, many cyanobacteria degrade the phycobilisomes (4). The \textit{nblA} gene required for phycobilisome degradation (40) was preceded by a predicted NrrA-binding site in the genomes of \textit{Synechococcus} sp. PCC 7002, \textit{Leptolyngbya} sp. PCC 7376, \textit{M. aeruginosa} NIES-843, and \textit{Pleurocapsa} sp. PCC 7327 (Table 1).

A decrease in the size of the reconstructed NrrA regulons was observed for the 5 species of the second group (Fig. 1B). For instance, the NrrA regulons in \textit{Synechocystis} sp. PCC 6803 and \textit{Cyanothecae} sp. ATCC 51142 constitute 10 and 11 operons, respectively, whereas in \textit{Anabaena} sp. PCC 7120 NrrA is predicted to control only 6 operons. Candidate NrrA-binding sites were identified in the promoter regions of the \textit{glgP} gene encoding glycogen phosphorylase and the \textit{sigE} gene encoding a group 2 \sigma factor of RNA polymerase in the \textit{Anabaena} sp. PCC 7120 genome (Table 1). The identified binding sites were found within the experimentally determined NrrA-binding regions of \textit{glgP} and \textit{sigE} promoters (15). We found that the \textit{hetR} gene encoding a master regulator of heterocyst differentiation (14), is preceded by a candidate NrrA-binding site not only in \textit{Anabaena} sp. PCC 7120 but also in \textit{Nostoc} sp. PCC 7107, \textit{N. punctiforme} PCC 73102, and \textit{T. erythraeum} IMS101 (Table 1). This suggests that NrrA may also directly regulate expression of the \textit{hetR} gene in the latter three diazotrophic species. The presence of a putative NrrA-binding site upstream of the peptidoglycan synthetic genes \textit{murD} and \textit{murE} was detected for multiple genomes including \textit{Anabaena} sp. PCC 7120, \textit{N. spumigena} CCY 9414, and \textit{T. erythraeum} IMS101 (Table 1).

In summary, the comparative genomics analysis allowed us to identify the conserved NrrA-binding motifs and reconstruct the NrrA regulons in 15 diverse cyanobacterial species. Among these species, \textit{Synechocystis} sp. PCC 6803 has one of the largest set of predicted NrrA targets, including 19 genes organized in 10 putative operons that are involved in glycogen catabolism, glycolysis, arginine biosynthesis, and protein degradation. We then performed experimental validation of the predicted NrrA-binding motif and characterization of the NrrA regulon in \textit{Synechocystis} sp. PCC 6803 as described below.

\textbf{Experimental Characterization of NrrA Regulon in \textit{Synechocystis} sp. PCC 6803}

\textbf{NrrA Binds Its Cognate DNA Sites in Vitro}—To validate the predicted NrrA regulon, EMSAs were performed using the recombinant NrrA protein from \textit{Synechocystis} sp. PCC 6803, which was overexpressed in \textit{E. coli} with the N-terminal His\textsubscript{6} tag and purified with a nickel-chelating affinity column. For all predicted NrrA target genes or operons in \textit{Synechocystis} sp. PCC 6803, DNA fragments (200 bp) in the promoter regions containing candidate NrrA-binding sites were tested in EMSAs (Fig. 2). A shifted band was observed upon incubation of NrrA protein with each promoter fragment, and its intensity was enhanced in the presence of increasing amounts of NrrA protein. The promoter fragments of \textit{glgP}, \textit{icfG} operon (slr1852-slr1861), \textit{argG}, \textit{prp1}, \textit{flv3}, \textit{pill}, and slr0185 genes were completely shifted with 500 nM NrrA protein (Fig. 2A). For the upstream fragments of \textit{gap1}, \textit{pfkA}, and \textit{argD}, only incomplete shifts were achieved using the same concentration of NrrA, suggesting that NrrA exhibited a lower affinity for these binding sites (Fig. 2B). No specific shift was observed with the promoter regions of \textit{Synechocystis} \textit{sigE} (sll1689) and \textit{cysA} (slr1455) genes (Fig. 2C). The \textit{sigE} gene of \textit{Synechocystis} lacks a predicted NrrA-binding site in the upstream region, while the \textit{cysA} gene is preceded by a sequence bearing some resemblance to a NrrA-binding site but with one mismatch in the direct repeat. Both \textit{sigE} and \textit{cysA} genes show unaltered mRNA levels in the \textit{nrrA}-inactivated mutant (11). The formation of the NrrA-DNA complex was suppressed in the presence of 200-fold excess unlabeled DNA fragments containing a NrrA-binding site but not in the presence of non-specific competitors (Fig. 2D). These observations confirm that NrrA binds specifically to the promoter regions of predicted targets in \textit{Synechocystis} sp. PCC 6803.

To characterize the NrrA-binding DNA motif in \textit{Synechocystis} sp. PCC 6803 and other species of the first group, site-directed mutagenesis was performed on the promoter regions of three \textit{Synechocystis} genes including \textit{glgP}, \textit{argG}, and
piiL gene encoding a sensory transduction histidine kinase. These three genes are involved in
glycogen catabolism, arginine biosynthesis, and
other functions, respectively (Table 1), and their
promoter fragments showed a substantial shift in
the presence of 200 nM Synechocystis NrrA protein
(Fig. 2). Nineteen mutated piiL promoter fragments,
each with one or two nucleotide substitutions upstream, downstream, or within the
two direct repeats, were tested in EMSAs (Fig. 3A).
In the case of fragments M3-M7 and M13-17, the
mutations prevented the binding of NrrA,
indicating that the nucleotides within the direct
repeat region were required for binding (Fig. 3A).
Exchange of the nucleotides between the two
repeats showed that an optimal spacer has to be
A/T rich (Fig. 3A, M8-M12). The mutations
outside of the predicted NrrA-binding sequence
showed no significant effect (Fig. 3A, M1, M2,
M18, and M19). For mutation analysis of the
predicted NrrA-binding sites upstream of glgP and
argG genes, eight DNA fragments with a single
base substitution in the direct repeat region were
amplified by PCR. As shown in Fig. 3B, no
binding of NrrA was observed for the mutated
fragments. These results confirm the predicted
NrrA-binding sites and DNA motif in
Synechocystis sp. PCC 6803.

In addition, to provide support for the identified
NrrA-binding motif in species of the second group,
we performed site-directed mutagenesis on the
upstream region of Anabaena sp. PCC 7120 sigE
gene (alr4249). NrrA directly regulates the sigE
expression in Anabaena sp. PCC 7120 (15),
however, its NrrA-binding site has not been
identified. The binding affinity of Anabaena NrrA
protein was tested by EMSAs for DNA fragments
with a single base substitution on the predicted
NrrA-binding sequence. As shown in Fig. 4,
nucleotide substitutions within the direct repeat
region remarkably reduced binding activity of
NrrA to the fragments (M30-M35, M39, M40,
M42-M44) except for the fragment M41. The
binding of NrrA remained largely unaffected when
nucleotides in the spacer region and outside of the
predicted site were exchanged (M28, M29,
M36-M38, M45, and M46). This result is
consistent with the previously reported
NrrA-binding sequence of the hetR promoter in
Anabaena sp. PCC 7120 (13).

NrrA Positively Regulate Expression of Its

Target Genes in Vivo—To validate the predicted
regulation of NrrA on gene expression in vivo, the
coding region of nrrA gene (sll1330) was partly
deleted from the chromosome of Synechocystis sp.
PCC 6803, resulting in the nrrA-inactivated
mutant (ΔnrrA) (Fig. 5A). Complete segregation of
the mutant was confirmed by PCR (Fig. 5A). The
transcript levels of the predicted NrrA target genes
in the ΔnrrA mutant were compared with those in
the wild-type by using quantitative RT-PCR. The
two strains were cultivated photo-mixotrophically
under continuous illumination in BG-11 liquid
medium supplemented with 10 mM glucose.
Despite a longer lag phase for the ΔnrrA mutant,
both strains grew at a similar growth rate during
the exponential growth phase (Fig. 5B). For
comparison of transcript levels, cells were
harvested in the exponential growth phase at an
OD730 of 1.2 and a growth rate of 0.13 h⁻¹ for both
strains, and total RNA was isolated. Six qRT-PCR
measurements from two independent cultures were
performed. As shown in Table 2, the relative
mRNA levels of 17 genes were decreased more
than 1.8-fold in the ΔnrrA mutant compared with
the wild-type strain. The most prominent effect of
nrrA mutation was observed for the two glycolytic
genes pfkA and gap1, which showed a ≥19-fold
reduced mRNA level in the ΔnrrA mutant. The
genes with a strongly decreased expression also
include the icfG gene cluster, which contains the
glgX, icfG, and pfpI genes. The prp1-prp2 operon,
glgP, argD, argG, and piiL genes showed a
1.8–6-fold decreased transcript level in the ΔnrrA
mutant. Expression of the sll0185 and flv3 genes
was not significantly affected by nrrA mutation,
which may be explained by possible involvement
in their regulation of other still unknown
regulatory mechanisms. Hence, the quantitative
RT-PCR results confirm that NrrA is a positive
regulator of glgP, glgX, pfkA, gap1, icfG, argG,
argD, prp1, prp2, pfpI, and piiL genes involved in
glycogen catabolism, glycolysis, arginine
biosynthesis, and protein degradation in
Synechocystis sp. PCC 6803.

To investigate the effect of NrrA deficiency on
the protein level, enzyme activities were
determined in cell crude extracts of Synechocystis
sp. PCC 6803 wild-type and ΔnrrA mutant strains.
As shown in Table 3, glycogen phosphorylase (GP)
and glyceraldehyde-3-P dehydrogenase (GAPDH)
exhibited approximately 4–6-fold decreased activities in crude extract of the ΔnrrA mutant as compared to the wild-type. Moreover, the activities of two enzymes of arginine biosynthesis, N-acetylornithine aminotransferase and argininosuccinate synthetase, were about 3–4-fold lower in the ΔnrrA mutant than in the wild-type strain.

**NrrA Controls Cyanophycin Accumulation and Glycogen Catabolism in Synechocystis**—Argininosuccinate synthetase (ASS) catalyzes the rate-limiting step in arginine synthesis pathway, and N-acetylornithine aminotransferase (AcOAT) is also a key enzyme in this pathway (30, 41). Arginine can serve as a nitrogen buffer in cyanobacteria, storing excess nitrogen in the form of cyanophycin. A previous study has suggested that cyanophycin accumulation in *Synechocystis* sp. PCC 6803 is controlled by arginine synthesis under conditions of nitrogen excess (42). Based on our observations on the NrrA regulation of ASS and AcOAT levels and the key functions of these two enzymes in arginine synthesis, we hypothesized that NrrA might activate arginine synthesis and play a role in the accumulation of cyanophycin under nitrogen–excess conditions. To test this hypothesis, we investigated the effect of NrrA deficiency on intracellular arginine concentration and cyanophycin accumulation as well as on glycogen catabolism.

*Synechocystis* sp. PCC 6803 wild-type and ΔnrrA mutant strains were cultivated photo-mixotrophically, and the culture samples were harvested in the course of time for analysis of glucose and nitrate consumption (Fig. 6A). Based on the residual nitrate concentration in the medium, five time points during the change from nitrogen-excess to nitrogen-depleted conditions were selected for determination of intracellular arginine and cyanophycin levels. Similarly, seven time points were selected for quantification of glycogen content based on the residual glucose concentration in the medium. As shown in Fig. 6B and 6C, the intracellular arginine concentration and cyanophycin content in the wild-type strain were increased about 5- and 3-fold, respectively, as the extracellular nitrate was consumed and reached the maximum levels when nitrate was depleted in the medium. Then the arginine concentration and cyanophycin content were decreased to low levels within 15 hours after nitrate depletion. By contrast, the intracellular arginine concentration in the ΔnrrA mutant was kept at a very low level and the cyanophycin synthesis before nitrate depletion was almost absent. These results suggest that NrrA activates arginine synthesis and cyanophycin accumulation through up-regulation of ASS and AcOAT levels. On the other hand, quantification of glycogen amounts revealed that the glycogen level in the ΔnrrA mutant was increased almost 2-fold compared to that in the wild-type when glucose was present in the medium (Fig. 6D). After glucose was depleted, the glycogen content was decreased more slowly in the ΔnrrA mutant than in the wild-type.

To provide further evidence to NrrA activation of cyanophycin accumulation and glycogen degradation, we performed the nitrogen deprivation and replenishment experiments. *Synechocystis* sp. PCC 6803 wild-type and ΔnrrA mutant strains exponentially grown in BG-11 medium were transferred to the nitrogen-deficient medium. After 12 h nitrate was added to a final concentration of 5 mM and cells were grown for another 12 h. The intracellular arginine concentration, cyanophycin and glycogen contents were measured throughout the nitrogen deprivation and replenishment experiment. The change in the phycocyanin content was also monitored, because *Synechocystis* sp. PCC 6803 uses both cyanophycin and phycobilisome as nitrogen-storage reservoirs (5). As shown in Fig. 7A, the cyanophycin content in the wild-type was decreased 18-fold after nitrogen deprivation, while upon nitrogen replenishment the cyanophycin content was rapidly increased from 0.05% to 2.3% of the total protein. Compared to the wild-type, the cyanophycin amount in the ΔnrrA mutant was reduced by 93% when both strains were exponentially grown under photo-autotrophic conditions (time zero). Although the ΔnrrA mutant also accumulated cyanophycin following nitrate upshift, the formation rate of cyanophycin was decreased by approximately 50% compared to the wild-type. The intracellular arginine concentration was also significantly lower in the ΔnrrA mutant than in the wild-type (Fig. 7B). Quantification of phycocyanin content revealed that the ratio of phycocyanin to chlorophyll levels (PC/Chl) was

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higher in the ΔnrrA mutant than in the wild-type (Fig. 7C). Moreover, the PC/Chl ratio in the ΔnrrA mutant was declined from 7.1 to 6.5 after nitrogen deprivation and then increased to 7.3 upon nitrogen upshift, whereas the wild-type had a relatively stable PC/Chl ratio throughout the experiment. In addition, determination of glycogen content revealed that the ΔnrrA mutant accumulated higher amounts of glycogen than the wild-type during nitrogen starvation (Fig. 7D). Following nitrate replenishment, the glycogen content in the wild-type was rapidly reduced by 70% with 12 h and a notable decrease in the rate of glycogen degradation was observed for the ΔnrrA mutant compared to the wild-type. For comparison of transcript levels of the genes and activities of the enzymes involved in glycogen catabolism and arginine biosynthesis between the wild-type and ΔnrrA mutant, samples were prepared after 4 h of nitrogen starvation and after 4 h following nitrogen replenishment. The quantitative RT-PCR analyses showed that the transcript levels of glgP, glgX, gap1, pfkA, argD, and argG genes were decreased drastically in the ΔnrrA mutant compared with the wild-type (Fig. 7E). Particularly, these genes showed a 4–51-fold reduced mRNA level in the ΔnrrA mutant under nitrogen starvation condition. Determination of enzyme activities revealed that the ΔnrrA mutant exhibited 3–5-fold decreased activities of GP, GAPDH, AcOAT, and ASS compared to the wild-type during the nitrogen deprivation and replenishment experiment (Fig. 7F).

The above results strongly suggest that NrrA activates synthesis of arginine, which then leads to cyanophycin accumulation. To verify that impaired cyanophycin synthesis in the ΔnrrA mutant is indeed due to limiting arginine concentrations and not caused by reduced cyanophycin synthetase levels, nitrate replenishment experiments in the presence of 5 mM arginine were carried out with wild-type and ΔnrrA mutant. As shown in Fig. 8, after arginine and nitrate were added to the medium, the intracellular arginine concentration in wild-type and ΔnrrA mutant was increased to similar levels, and a large amount of cyanophycin was accumulated rapidly in the ΔnrrA mutant. This observation confirms that cyanophycin accumulation in the ΔnrrA mutant is limited by the synthesis of arginine.

**DISCUSSION**

In this work, we performed comparative genomic reconstruction of NrrA regulons in 15 diverse cyanobacterial species by combining the identification of candidate NrrA-binding sites with cross-genomic comparison of regulons. A conserved NrrA-binding motif consisting of two direct repeats of TG(T/A)CA separated by an 8-bp A/T-rich spacer was identified for the ten species of the first group. The combined bioinformatics, *in vitro* and *in vivo* characterization of the NrrA regulon in *Synechocystis* sp. PCC 6803 revealed that NrrA directly regulates the expression of glgP, glgX, pfkA, gap1, argG, argD, prp1, prp2, and prpI genes involved in glycogen catabolism, glycolysis, arginine biosynthesis, and protein degradation (Fig. 9). These NrrA target genes have been shown to be up-regulated under nitrogen depletion (9). Moreover, we demonstrated that NrrA-regulated arginine synthesis controls cyanophycin accumulation, and NrrA also plays a pivotal role in the regulation of glycogen catabolism in *Synechocystis* sp. PCC 6803.

During nitrogen starvation, expression of nrrA gene in *Synechocystis* sp. PCC 6803 is induced (11). The NrrA regulator binds to its operator sites, leading to activation of expression of argG and argD genes coding for two key enzymes (ASS and AcOAT) of arginine synthesis. The availability of arginine appears to limit cyanophycin synthesis in *Synechocystis* (Fig. 8; (43)). Thus, NrrA-mediated up-regulation of argG and argD genes during nitrogen starvation could contribute to the immediate synthesis of arginine and cyanophycin in *Synechocystis* once nitrogen is replenished. In fact, we found that the arginine synthesis and cyanophycin accumulation upon nitrogen upshift were significantly impaired in the ΔnrrA mutant when compared to the wild-type (Fig. 7). According to previous reports (5), cyanophycin serves as a dynamic nitrogen reservoir, while phycobilisomes appear to be the main nitrogen reserve in non-diazotrophic unicellular strains such as *Synechocystis* sp. PCC 6803. Here we noticed that the ΔnrrA mutant exhibited more variable PC/Chl ratio than the wild-type during the nitrogen deprivation and replenishment experiment (Fig. 7), suggesting that the mutant has to degrade and resynthesize phycobilisomes to respond to
transient changes in environmental nitrogen availability.

On the other hand, NrrA directly up-regulates expression of glgP (sll1367), glgX (sll1857), pfkA, and gap1 genes involved in glycolgen degradation and glycolysis in Synechocystis sp. PCC 6803. Glycogen, the carbon sink of most cyanobacteria, is utilized as carbon and energy reserves to cope with transient starvation and stress conditions (44). We found that the ΔnrrA mutant exhibited a high abundance of glycogen during nitrogen depletion and a significantly decreased rate of glycogen degradation after nitrogen replenishment (Fig. 7), indicating that NrrA controls glycolgen catabolism in Synechocystis. Earlier studies have shown that the group 2 σ factor SigE is also involved in the regulation of sugar catabolic genes in Synechocystis (33). It is noteworthy that SigE probably induces expression of pentose phosphate pathway genes and the other copy of glgP and glgX genes (sll1356 and sll0237, respectively) (33). Based on our results (Fig. 2) and previous reports (11), regulation of sigE gene expression is probably independent of NrrA in Synechocystis. Thus, it appears that NrrA and SigE may independently regulate different genes of sugar catabolism in Synechocystis during nitrogen starvation. This is in contrast to the situation in Anabaena sp. PCC 7120, where NrrA directly activates sigE expression and SigE up-regulates the genes of glycolysis and pentose phosphate pathway. Further studies are required to elucidate the contribution of NrrA and SigE to regulation of sugar catabolic genes and metabolic flux responses in Synechocystis. Therefore, our results revealed that by directly regulating expression of the genes involved in glycogen catabolism, glycolysis, and arginine biosynthesis, NrrA may have an important role in coordinating the synthesis and degradation of nitrogen and carbon reserves in Synechocystis.

In addition to nitrogen regulation by NtcA, expression of nrrA gene in Synechocystis is enhanced by glucose and high salt (45, 46). It is tempting to speculate that NrrA may also control cyanophycin accumulation and sugar catabolism under glucose and high salt conditions. In fact, we observed that NrrA deficiency resulted in remarkable changes in intracellular levels of arginine, cyanophycin, and glycogen under the photo-mixotrophic, nitrogen-excess condition (Fig. 6). Further work is needed to clarify the potential role of NrrA in Synechocystis grown under glucose, high salt, and various unbalanced nutrient conditions. Other than transcriptional control, post-translational regulation of NrrA may also occur. NrrA is a response regulator belonging to the OmpR family. The activity of NrrA is probably regulated by phosphorylation, however, the sensory histidine kinase that phosphorylates NrrA has not yet been identified.

Similar to that in Synechocystis, the predicted NrrA regulon in Synechocystis sp. PCC 6803 is composed of genes from glycolgen catabolism (glgP), glycolysis (pfkA), arginine biosynthesis (argG), and protein degradation (prp1-prp2, clps-cce_2239) as well as its own gene nrrA. The unicellular diazotrophic Synechocystis strains perform photosynthesis during the day and nitrogen fixation during the night (7). Accumulation and degradation of glycogen and cyanophycin granules, which occur concomitantly with photosynthesis and nitrogen fixation, are a very important feature of their metabolism, and a strong coordination of correlated processes at the transcriptional level has been proposed (47). Based on the published transcriptome data of Synechocystis sp. ATCC 51142 during dark-light cycles (47), we found that the expression profiles of the nrrA gene and NrrA candidate target genes glgP, pfkA, and argG are very similar and their Pearson correlation coefficients are larger than 0.9. Therefore, the nrrA, glgP, pfkA, and argG genes are coregulated, which is consistent with our prediction. These genes are up-regulated at the beginning of the dark period (47), suggesting that NrrA may be involved in glycogen degradation and cyanophycin accumulation in Synechocystis sp. ATCC 51142 during the night.

Based on identification of the NrrA-binding motif, we predicted the NrrA regulons in the 5 species of the second group. Previous reports have shown that in the filamentous diazotrophic Anabaena sp. PCC 7120, NrrA controls heterocyst differentiation by directly regulating expression of hetR gene (13). Here we predicted a candidate NrrA-binding site located upstream of the hetR gene in the genomes of Nostoc sp. PCC 7107, N. punctiforme PCC 73102, and T. erythraeum IMS101, suggesting that regulation of hetR expression by NrrA may also occur in these three diazotrophic species. HetR is a master regulator of heterocyst development and the basic mechanism.
of heterocyst development seems to be conserved among various heterocystous strains (6). It is worth noting that unlike *Anabaena* sp. PCC 7120, *Nostoc* sp. PCC 7107, and *N. punctiforme* PCC 73102, *T. erythraeum* IMS101 differentiates diazocytes instead of heterocysts for nitrogen fixation, and a possible involvement of HetR in diazocyte differentiation has been implicated (48). Therefore, it remains to be an interesting question whether NrrA may have a general role in the regulation of development of specialized cells for nitrogen fixation in filamentous diazotrophic cyanobacteria.

**Concluding Remarks**—This study provided a comprehensive bioinformatic analysis of the NrrA regulons in 15 diverse cyanobacterial species. By integrating experimental characterization of the predicted NrrA-binding motif and regulon, the regulatory function of NrrA in *Synechocystis* sp. PCC 6803 was elucidated. Moreover, this study gains an insight into the potential regulatory role of NrrA in other species by predicting its candidate targets and provides a framework for further studies of the NrrA-dependent regulation in diverse cyanobacteria.

**REFERENCES**

1. Schwarz, R., and Forchhammer, K. (2005) Acclimation of unicellular cyanobacteria to macronutrient deficiency: emergence of a complex network of cellular responses. *Microbiology.* **151**, 2503-2514
2. Forchhammer, K. (2004) Global carbon/nitrogen control by PII signal transduction in cyanobacteria: from signals to targets. *FEMS Microbiol. Rev.* **28**, 319-333
3. Allen, M. M. (1984) Cyanobacterial cell inclusions. *Annu. Rev. Microbiol.* **38**, 1-25
4. Grossman, A. R., Schaefer, M. R., Chiang, G. G., and Collier, J. L. (1993) The phycobilisome, a light-harvesting complex responsive to environmental conditions. *Microbiol. Rev.* **57**, 725-749
5. Li, H., Sherman, D. M., Bao, S. L., and Sherman, L. A. (2001) Pattern of cyanophycin accumulation in nitrogen-fixing and non-nitrogen-fixing cyanobacteria. *Arch. Microbiol.* **176**, 9-18
6. Zhang, C. C., Laurent, S., Sakr, S., Peng, L., and Bedu, S. (2006) Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. *Mol. Microbiol.* **59**, 1853-1859
7. Ehira, S., and Ohmori, M. (2011) NrrA, a nitrogen-regulated response regulator protein, controls glycogen catabolism in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Biol. Chem.* **286**, 38109-38114
8. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W., and Lipman, D. J. (1997)
Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal W and clustal X version 2.0. *Bioinformatics.* **23**, 2947-2948

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **59**, 307-321

Novichkov, P. S., Rodionov, D. A., Stavrovskaya, E. D., Novichkova, E. S., Kazakov, A. E., Gelfand, M. S., Arkin, A. P., Mironov, A. A., and Dubchak, I. (2010) RegPredict: an integrated system for regulon inference in prokaryotes by comparative genomics approach. *Nucleic Acids Res.* **38**, W299-W307

Mironov, A. A., Vinokurova, N. P., and Gelfand, M. S. (2000) Software for analysis of bacterial genomes. *Mol. Biol. (Mosc.)* **34**, 253-262

Crooks, G. E., Hon, G., Chandonia, J. M., and Brenner, S. E. (2004) WebLogo: A sequence logo generator. *Genome Res.* **14**, 1188-1190

Nakao, M., Okamoto, S., Kohara, M., Fujishiro, T., Fujisawa, T., Sato, S., Tabata, S., Kaneko, T., and Nakamura, Y. (2010) CyanoBase: the cyanobacteria genome database update 2010. *Nucleic Acids Res.* **38**, D379-D381

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **111**, 1-61

Taylor, L. A., and Rose, R. E. (1988) A correction in the nucleotide sequence of the Tn903 kanamycin resistance determinant in pUC4K. *Nucleic Acids Res.* **16**, 358-358

Williams, J. G. K. (1988) Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. *Methods Enzymol.* **167**, 766-778

Vioque, A. (1992) Analysis of the gene encoding the RNA subunit of ribonuclease P from cyanobacteria. *Nucleic Acids Res.* **20**, 6331-6337

Yang, C., Rodionov, D. A., Rodionova, I. A., Li, X., and Osterman, A. L. (2008) Glycerate 2-kinase of *Thermotoga maritima* and genomic reconstruction of related metabolic pathways. *J. Bacteriol.* **190**, 1773-1782

Fu, J., and Xu, X. D. (2006) The functional divergence of two glp homologues in *Synechocystis* sp. PCC 6803. *FEMS Microbiol. Lett.* **260**, 201-209

Rajaram, V., Prasuna, P. R., Savithri, H. S., and Murthy, M. R. N. (2008) Structure of biosynthetic N-acetylornithine aminotransferase from *Salmonella typhimurium*: Studies on substrate specificity and inhibitor binding. *Proteins: Struct. Funct. Bioinform.* **70**, 429-441

Schuegraf, A., Ratner, S., and Warner, R. C. (1960) Free energy changes of the argininosuccinate synthetase reaction and of the hydrolysis of the inner pyrophosphate bond of adenosine triphosphate. *J. Biol. Chem.* **235**, 3597-3602

Mao, H.-m., Wei, W., Xiong, W.-j., Lu, Y., Chen, B.-g., and Liu, Z. (2010) Simultaneous determination of L-citrulline and L-arginine in plasma by high performance liquid chromatography. *Clin. Biochem.* **43**, 1141-1147

Osaanai, T., Oikawa, A., Azuma, M., Tanaka, K., Saito, K., Hirai, M. Y., and Ikeuchi, M. (2011) Genetic engineering of group 2 sigma factor SigE widely activates expressions of sugar catabolic genes in *Synechocystis* species PCC 6803. *J. Biol. Chem.* **286**, 30962-30971

Arnon, D. I., McSwain, B. D., Tsujimot.Hy, and Wada, K. (1974) Photochemical activity and components of membrane preparations from blue-green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. *Biochim. Biophys. Acta.* **357**, 231-245

Shih, P. M., Wu, D., Latifi, A., Axen, S. D., Fewer, D. P., Talla, E., Calteau, A., Cai, F., de Marsac, N. T., Rippka, R., Herdman, M., Sivonen, K., Coursin, T., Laurent, T., Goodwin, L., Nolan, M., Davenport, K. W., Han, C. S., Rubin, E. M., Eisen, J. A., Woyke, T., Gugger, M., and Kerfeld, C. A. (2013) Improving the coverage of the cyanobacterial phyllum using diversity-driven genome sequencing. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 1053-1058

Baek, J. H., and Lee, S. Y. (2006) Novel gene members in the Pho regulon of *Escherichia coli*. *FEMS Microbiol. Lett.* **264**, 104-109

Beuf, L., Bedu, S., Durand, M. C., and Joset, F. (1994) A protein involved in co-ordinated regulation of inorganic carbon and glucose metabolism in the facultative photoautotrophic cyanobacterium *Synechocystis* PCC 6803. *Plant Mol. Biol.* **25**, 855-864
38. Ziegler, K., Diener, A., Herpin, C., Richter, R., Deutzmann, R., and Lockau, W. (1998) Molecular characterization of cyanophycin synthetase, the enzyme catalyzing the biosynthesis of the cyanobacterial reserve material multi-L-arginyl-poly-L-aspartate (cyanophycin). Eur. J. Biochem. 254, 154-159
39. Espinosa, J., Forchhammer, K., Burillo, S., and Contreras, A. (2006) Interaction network in cyanobacterial nitrogen regulation: PipX, a protein that interacts in a 2-oxoglutarate dependent manner with PII and NtcA. Mol. Microbiol. 61, 457-469
40. Karradt, A., Sobanski, J., Mattow, J., Lockau, W., and Baier, K. (2008) NblA, a key protein of phycobilisome degradation, interacts with ClpC, a HSP100 chaperone partner of a cyanobacterial Clp protease. J. Biol. Chem. 283, 32394-32403
41. Haines, R. J., Pendleton, L. C., and Eichler, D. C. (2011) Argininosuccinate synthase: at the center of arginine metabolism. Int. J. Biochem. Mol. Biol. 2, 8-23
42. Aboulmagd, E., Sanio, F. B. O., and Steinbuchel, A. (2001) Purification of Synechocystis sp. strain PCC 6308 cyanophycin synthetase and its characterization with respect to substrate and primer specificity. Appl. Environ. Microbiol. 67, 2176-2182
43. Maheswaran, M., Ziegler, K., Lockau, W., Hagemann, M., and Forchhammer, K. (2006) PII-regulated arginine synthesis controls accumulation of cyanophycin in Synechocystis sp. strain PCC 6803. J. Bacteriol. 188, 2730-2734
44. Gruendel, M., Scheunemann, R., Lockau, W., and Zilliges, Y. (2012) Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium Synechocystis sp. PCC 6803. Microbiology. 158, 3032-3043
45. Lee, S., Ryu, J.-Y., Kim, S. Y., Jeon, J.-H., Song, J. Y., Cho, H.-T., Choi, S.-B., Choi, D., de Marsac, N. T., and Park, Y.-I. (2007) Transcriptional regulation of the respiratory genes in the cyanobacterium Synechocystis sp. PCC 6803 during the early response to glucose feeding. Plant Physiol. 145, 1018-1030
46. Marin, K., Kanesaki, Y., Los, D. A., Murata, N., Suzuki, I., and Hagemann, M. (2004) Gene expression profiling reflects physiological processes in salt acclimation of Synechocystis sp. strain PCC 6803. Plant Physiol. 136, 3290-3300
47. Stockel, J., Welsh, E. A., Liberton, M., Kunnvakkam, R., Aurora, R., and Pakrasi, H. B. (2008) Global transcriptomic analysis of Cyanothece 51142 reveals robust diurnal oscillation of central metabolic processes. Proc. Natl. Acad. Sci. U.S.A. 105, 6156-6161
48. El-Shehawy, R., Lugomela, C., Ernst, A., and Bergman, B. (2003) Diurnal expression of hetR and diazocyte development in the filamentous non-heterocystous cyanobacterium Trichodesmium erythraeum. Microbiology. 149, 1139-1146

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. **Identified NrrA-binding DNA motifs and functional categories of predicted NrrA direct targets in 15 cyanobacterial species.** A, maximum likelihood phylogenetic tree and inferred DNA recognition motifs of the NrrA regulator from the 15 species. The phylogenetic tree was constructed using PhyML 3.0. The numbers indicate the number of bootstrap replications, out of 1000, that support each node on the tree. Branches are color coded according to the taxonomy of species: Chroococcales, black; Pleurocapsales, orange; Oscillatoriales, green; Nostocales, purple. The DNA sequence logos representing the NrrA-binding motifs were constructed using WebLogo. B, functional categories of predicted NrrA-regulated genes or operons. Gene category was defined according to the CyanoBase database.

FIGURE 2. **EMSAs with purified NrrA protein and DNA fragments from the promoter regions of predicted target genes in Synechocystis sp. PCC 6803.** A, EMSAs were performed in the absence (lane 1) and in the presence of 50, 100, 200, and 500 nM of Synechocystis NrrA protein (lanes 2 to 5). B, EMSAs were performed in the absence (lane 1) and in the presence of 100, 200, 500, and 1000 nM of Synechocystis NrrA protein (lanes 2 to 5). C, the promoter regions of Synechocystis sigE (sll1689) and cysA (slr1455) genes, which lack a putative NrrA binding site, were used as negative controls. A sequence upstream of the cysA gene, which bears some resemblance to the NrrA-binding motif but with one mismatch in the direct repeat (underlined), is shown. D, specificity of the NrrA-DNA interactions was tested by competition with 10-, 100- or 200-fold of non-labeled target DNA (specific comp). The DNA fragment from the upstream region of the rplW gene (sll1801), which lacks a predicted NrrA binding site, was added at the same concentrations as a non-specific competitor (non-specific comp).

FIGURE 3. **Verification of NrrA-binding sites and DNA motif in Synechocystis sp. PCC 6803.** A, site-directed mutagenesis of the candidate NrrA-binding site in the promoter region of pilL gene (slr0073). The direct repeat region of the NrrA-binding site is indicated by boxes. The mutations M1-M19 are shown below the wild-type sequence. The corresponding DNA fragments were analyzed by EMSAs with purified Synechocystis NrrA protein. B, mutational analysis of the predicted NrrA-binding sites in the promoter regions of glgP and argG genes. Binding of NrrA to the mutated fragments M20-M27 was tested by EMSAs.

FIGURE 4. **Verification of the NrrA-binding site in the promoter region of the sigE gene in Anabaena sp. PCC 7120.** The mutations M28-M46 were introduced by PCR into the candidate NrrA-binding site in the promoter region of Anabaena sigE gene (alr4249) and are shown below the wild-type sequence. The direct repeat region of the NrrA-binding sequence is indicated by boxes. The mutated DNA fragments were analyzed by EMSAs with purified Anabaena NrrA protein.

FIGURE 5. **Inactivation of nrrA in Synechocystis sp. PCC 6803 (A) and growth of the ΔnrrA mutant and wild-type strains under photo-mixotrophic condition (B).** The nrrA gene (sll1330) was inactivated by replacing part of the coding region (~0.4 kbp) with a Km resistance (Km^r) cassette (~1.2 kbp). The complete segregation of the mutant chromosome was confirmed by PCR using the oligonucleotide pairs P1/P2 and P3/P4. Both strains were cultured photo-mixotrophically under continuous illumination in BG-11 medium. Cell growth was monitored spectrophotometrically at 730 nm (OD_{730}). The data points and error bars represent means and standard deviations of three independent cultures. Arrows indicate the sampling for quantitative RT-PCR analysis and enzyme activity measurements.

FIGURE 6. **Glucose and nitrate consumption (A), intracellular arginine concentration (B), cyanophycin (C) and glycogen contents (D) of Synechocystis sp. PCC 6803 ΔnrrA mutant in comparison to the wild-type strain under photo-mixotrophic condition.** Both strains were cultured photo-mixotrophically under continuous illumination in BG-11 medium. The data points and error bars represent means and standard deviations of three independent cultures.
FIGURE 7. Intracellular cyanophycin (A), arginine (B), phycocyanin (C), glycogen (D) contents, gene expression levels (E) and enzyme activities (F) of Synechocystis sp. PCC 6803 wild-type and ΔnrrA mutant strains during the nitrogen deprivation and replenishment experiment. Both strains were cultured photo-autotrophically in BG-11 medium to exponential growth phase and then transferred to nitrogen-deficient medium (time zero). After 12 h nitrate was added to a final concentration of 5 mM and cells were grown for another 12 h. The intracellular cyanophycin, arginine, phycocyanin, and glycogen contents were measured at different time points as indicated throughout the experiment. The transcript levels of the genes and the activities of the enzymes involved in glycogen catabolism and arginine biosynthesis were determined after 4 h of nitrogen starvation (4 h) and after 4 h following nitrogen replenishment (16 h). The data points and error bars represent means and standard deviations of three independent cultures.

FIGURE 8. Intracellular cyanophycin content (A) and arginine concentration (B) of Synechocystis sp. PCC 6803 wild-type and ΔnrrA mutant strains in nitrate starvation medium followed by replenishment with nitrate and arginine. Both strains were grown for 12 h after nitrogen deprivation, then 5 mM nitrate with 5 mM arginine was added and cells were grown for another 12 h. The data points and error bars represent means and standard deviations of three independent cultures.

FIGURE 9. Overview of the NrrA regulon in Synechocystis sp. PCC 6803. The NrrA regulon members characterized in this study are marked by red.
### TABLE 1

NrrA regulons in 15 species of cyanobacteria.

| Target operon | Functional role | Predicted site | FC<sup>a</sup> |
|---------------|----------------|----------------|--------------|
| **Synechocystis sp. PCC 6803** | | | |
| glgP         | Glycogen phosphorylase | -171          | G            |
| pfkB         | Phosphofructokinase    | -100          | C            |
| gapl         | Glyceraldehyde-3-P dehydrogenase | -229      | C            |
| slr1852--1856-glgX-slr1859-icfG-1861 | Carbon metabolism regulator, glycogen debranching enzyme | -278      | C            |
| argG         | Argininosuccinate synthetase | -166      | A            |
| argD         | N-Acetylornithine aminotransferase | -159      | A            |
| prpl-prp2    | Processing protease | -82           | P            |
| flv3         | Flavoprotein | -85           | O            |
| pilL         | Type IV pili sensor histidine kinase | -167      | O            |
| sll0185      | Hypothetic protein | -298          | O            |
| **Synechococcus sp. PCC 7002** | | | |
| argA         | Phosphoshikimate carboxyvinyltransferase | -197    | A            |
| trpC         | Indole-3-glycerol-P synthase | -240    | A            |
| nblA         | Phycobilisome degradation protein | -100    | P            |
| clpS-SYNPCC7002_A2282 | ATP dependent protease protein | -214    | P            |
| SYNPPCC7002_A0832 | Acetyltransferase | -358    | O            |
| SYNPPCC7002_A0168 | Hypothetic protein | -141    | O            |
| **Leptolyngbya sp. PCC 7376** | | | |
| pfkB         | Phosphofructokinase    | -165          | C            |
| argG         | Argininosuccinate synthetase | -114    | A            |
| argD         | N-Acetylornithine aminotransferase | -181    | A            |
| aroA         | Phosphoshikimate carboxyvinyltransferase | -197    | A            |
| nblA         | Phycobilisome degradation protein | -128    | P            |
| Lepto7376_0096 | Hypothetic protein | -139    | O            |
| **Microcystis aeruginosa NIES-843** | | | |
| pgmB         | Phosphoglycerate mutase III | -253    | C            |
| pipX         | PII interaction protein X | -170    | A            |
| prpl-prp2    | Processing protease | -97           | P            |
| nblA         | Phycobilisome degradation protein | -92     | O            |
| flv3         | Flavoprotein | -96           | O            |
| nrrA         | Transcriptional regulator | -75     | O            |
| MAE_08900    | Acetyltransferase | -145          | O            |
| MAE_06000    | Hypothetic protein | -207          | O            |
| **Pleurocapsa sp. PCC 7327** | | | |
| pyk          | Pyruvate kinase | -168          | C            |
| nblA         | Phycobilisome degradation protein | -166    | P            |
| nrrA         | Transcriptional regulator | -68     | O            |
| Ple7327_2658 | Acetyltransferase | -224    | O            |
| Ple7327_3261 | Hypothetic protein | -206          | O            |
| **Cyanobacterium stanieri PCC 7202** | | | |
| argG         | Argininosuccinate synthetase | -201    | A            |
| argD         | N-Acetylornithine aminotransferase | -120    | A            |
| ilvB         | Acetolactate synthase | -23           | A            |
| nrrA         | Transcriptional regulator | -74     | O            |
| Cyast_0196   | Acetyltransferase | -191    | O            |
| Cyast_2384   | Hypothetic protein | -221          | O            |
| **Cyanothecae sp. PCC 8801** | | | |
| glgP         | Glycogen phosphorylase | -149    | G            |
| trpC         | Indole-3-glycerol-P synthase | -56     | A            |
| pipX         | PII interaction protein X | -146    | A            |
| prpl         | Processing protease | -99           | P            |
| Target operon          | Functional role                                          | Predicted site | FC |
|-----------------------|----------------------------------------------------------|----------------|----|
| psbD-psbC             | Photosystem II D2 and CP43 protein                       | -341           | O  |
| nrrA                  | Transcriptional regulator                               | -67            | O  |
| PCC8801_2632          | Acetyltransferase                                       | -159           | O  |
| PCC8801_1293          | Hypothetic protein                                      | -39            | O  |
| PCC8801_0483          | Hypothetic protein                                      | -383           | O  |
| *Cyanobacterium* sp.  |                                                          |                |    |
| PCC7822               |                                                          |                |    |
| pgmB                  | Phosphoglycerate mutase III                             | -145           | C  |
| pyk                   | Pyruvate kinase                                          | -206           | C  |
| ilvB                  | Acetolactate synthase                                   | -252           | A  |
| prp1-prp2             | Processing protease                                      | -95            | P  |
| nrrA                  | Transcriptional regulator                               | -68            | O  |
| Cyan7822_1485         | Acetyltransferase                                       | -222           | O  |
| Cyan7822_5553         | Hypothetic protein                                      | -220           | O  |
| Cyan7822_5278         | Hypothetic protein                                      | -400           | O  |
| *Cyanobacterium* sp.  |                                                          |                |    |
| PCC7424               |                                                          |                |    |
| pgmB                  | Phosphoglycerate mutase III                             | -147           | C  |
| ilvB                  | Acetolactate synthase                                   | -252           | A  |
| prp1-prp2             | Processing protease                                      | -96            | P  |
| nrrA                  | Transcriptional regulator                               | -69            | O  |
| PCC7424_1966          | Hypothetic protein                                      | -396           | O  |
| *Cyanobacterium* sp.  |                                                          |                |    |
| ATCC 51142            |                                                          |                |    |
| glgP                  | Glycogen phosphorylase                                  | -219           | G  |
| pfkA                  | Phosphofructokinase                                     | -173           | C  |
| argG                  | Argininosuccinate synthetase                            | -88            | A  |
| prp1-prp2             | Processing protease                                      | -88            | P  |
| cclS-ccc_2239         | ATP dependent protease protein                           | -316           | P  |
| *psbD-ccc*            | Photosystem II D2 and CP43 protein                       | -41            | O  |
| nrrA                  | Transcriptional regulator                               | -67            | O  |
| pilL                  | Type IV pili sensor histidine kinase                    | -189           | O  |
| cce_4205              | Acetyltransferase                                       | -164           | O  |
| cce_4491              | Hypothetic protein                                      | -120           | O  |
| cce_3721              | Hypothetic protein                                      | -233           | O  |
| *Anabaena* sp. PCC 7120|                                                          |                |    |
| glgP                  | Glycogen phosphorylase                                  | -97            | G  |
| sigE                  | Group 2 σ factor of RNA polymerase                      | -32            | C  |
| hetR                  | Master regulator of heterocyst differentiation           | -838           | H  |
| asl1664-murE          | UDP-Acetylmuramoylalanyl-glutamate-diaminopimelate ligase| -52            | H  |
| asr0064               | Hypothetic protein                                      | -182           | O  |
| all2705               | Hypothetic protein                                      | -377           | O  |
| *Nostoc* sp. PCC 7107 |                                                          |                |    |
| glgP                  | Glycogen phosphorylase                                  | -96            | G  |
| hetR                  | Master regulator of heterocyst differentiation           | -810           | H  |
| fdxB                  | 4Fe-4S Ferredoxin                                       | -121           | O  |
| Nos7107_4324          | Hypothetic protein                                      | -181           | O  |
| Nos7107_5361          | Hypothetic protein                                      | -287           | O  |
| Nos7107_4780          | Hypothetic protein                                      | -333           | O  |
| *Nodularia spumigena* |                                                          |                |    |
| CCY 9414              |                                                          |                |    |
| glgP                  | Glycogen phosphorylase                                  | -108           | G  |
| murD                  | UDP-Acetylmuramoyl-alanyl-glutamate synthetase           | -275           | H  |
| fdxB                  | 4Fe-4S Ferredoxin                                       | -110           | O  |
| Target operon | Functional role | Predicted site<sup>a</sup> | FC<sup>b</sup> |
|---------------|----------------|---------------------------|--------------|
| N9414_21811   | Hypothetic protein | -181 | O |
| N9414_16574   | Hypothetic protein | -391 | O |
| *Nostoc punctiforme* PCC 73102 |  |  |  |
| glgP          | Glycogen phosphorylase | -97 | G |
| hetR          | Master regulator of heterocyst differentiation | -804 | H |
| fdxB          | 4Fe-4S Ferredoxin | -117 | O |
| Npun_F4928    | Hypothetic protein | -321 | O |
| Npun_F2904    | Hypothetic protein | -385 | O |
| *Trichodesmium erythraeum* IMS101 |  |  |  |
| hetR          | Master regulator of heterocyst differentiation | -134 | H |
| glyS-murD     | UDP-Acetylmuramoyl-alanyl-glutamate synthetase | -392 | H |
| Tery_4152-murE | UDP-Acetylmuramoylalanyl-glutamate-diaminopimelate ligase | -144 | H |

<sup>a</sup> The distance of the 5' end of predicted NrrA-binding site from the translational start is given.

<sup>b</sup> Abbreviations for the functional categories: G, glycogen metabolism; C, central carbon metabolism; A, amino acid metabolism; P, protein degradation; H, heterocyst differentiation or cell wall biosynthesis; O, others.
TABLE 2
Comparison of mRNA levels in *Synechocystis* sp. PCC 6803 wild-type and Δ*nrrA* mutant using quantitative RT-PCR. Data represent means ± S.D. of values from six measurements starting from two independent cultures. The strains were cultivated photo-mixotrophically under continuous illumination in BG-11G medium, and total RNA was isolated in the exponential growth phase at an OD730 of 1.2.

| Gene ID | Gene | Gene function | mRNA ratio (wild-type/Δ*nrrA* mutant) |
|---------|------|---------------|---------------------------------------|
| sll1196 | *pfkA* | Phosphofructokinase | 25.07 ± 0.14 |
| slr0884 | *gap1* | Glyceraldehyde-3-P dehydrogenase | 18.97 ± 0.14 |
| slr1852 | Hypothetic protein | 12.20 ± 0.15 |
| slr1853 | Hypothetic protein | 12.00 ± 0.15 |
| slr1854 | *pfpI* | Intracellular protease | 11.17 ± 0.14 |
| slr1855 | Hypothetic protein | 9.61 ± 0.14 |
| slr1857 | *glgX* | Glycogen isoamylase | 8.27 ± 0.12 |
| slr1856 | Anti-sigma B factor antagonist | 8.18 ± 0.13 |
| slr1859 | Anti-sigma B factor antagonist | 7.72 ± 0.14 |
| slr1860 | *icfG* | Phosphoprotein phosphatase | 7.68 ± 0.14 |
| slr1861 | Ser/Thr protein kinase | 6.40 ± 0.13 |
| sll2008 | *prp1* | Processing protease 1 | 5.72 ± 0.15 |
| slr1367 | *glgP* | Glycogen phosphorylase | 3.49 ± 0.17 |
| sll2009 | *prp2* | Processing protease 2 | 3.31 ± 0.16 |
| slr1022 | *argD* | N-Acetylornithine aminotransferase | 2.93 ± 0.16 |
| slr0585 | *argG* | Argininosuccinate synthetase | 2.06 ± 0.19 |
| slr0073 | *pilL* | Type IV pili sensor histidine kinase | 1.83 ± 0.19 |
| sll0185 | Hypothetic protein | 0.73 ± 0.15 |
| sll0550 | *flv3* | Flavoprotein | 0.69 ± 0.15 |
**TABLE 3**  
Enzyme activities of *Synechocystis* sp. PCC 6803 Δ*nrrA* mutant in comparison to the wild-type strain under photo-mixotrophic condition. Enzyme activities were measured spectrophotometrically in crude cell extracts from photo-mixotrophic cultures. One unit of activity was defined as the conversion or formation of 1 μmol NAD(P)H per min based on the protein contents of the crude extracts. Data represents means ± S.D. of values from three independent experiments.

| Enzyme                        | Specific activity (unit/mg protein) |
|-------------------------------|------------------------------------|
|                               | wild-type                          | Δ*nrrA* mutant                    |
| Glycogen phosphorylase        | 0.181 ± 0.020                      | 0.044 ± 0.011                     |
| Glyceraldehyde-3-P dehydrogenase | 0.647 ± 0.072                | 0.116 ± 0.024                     |
| N-Acetylornithine aminotransferase | 0.375 ± 0.024                | 0.101 ± 0.021                     |
| Argininosuccinate synthetase  | 0.186 ± 0.027                      | 0.073 ± 0.026                     |
FIGURE 1

A

B

Synechocystis sp. PCC 6803
Synechococcus sp. PCC 7002
Leptolyngbya sp. PCC 7376
Microcystis aeruginosa NIES-843
Pleurocapsa sp. PCC 7327
Cyanobacterium stanieri PCC 7202
Cyanobacterium sp. PCC 8801
Cyanobacterium sp. PCC 7822
Cyanobacterium sp. PCC 7424
Cyanobacterium sp. ATCC 51142
Trichodesmium erythraeum IMS101
Nostoc punctiforme PCC 73102
Nodularia spumigena CCY 9414
Nostoc sp. PCC 7107
Anabaena sp. PCC 7120

0 2 4 6 8 10 12
Glycogen metabolism
Central carbon metabolism
Amino acid metabolism
Protein degradation
Heterocyst differentiation/
Cell wall formation
Others
FIGURE 2
FIGURE 3
FIGURE 5
FIGURE 6
FIGURE 7
FIGURE 8

A

B

Cyanophycin (mg/g total protein) vs. Time (h)

Arginine (µmol/g cell dry weight) vs. Time (h)

wild-type

ΔnrrA mutant
FIGURE 9
The Nitrogen-Regulated Response Regulator NrrA Controls Cyanophycin Synthesis and Glycogen Catabolism in the Cyanobacterium Synechocystis sp. PCC 6803

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