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Cloning, Sequencing, and Regulation of the Global Nitrogen Regulator Gene *ntcA* in the Unicellular Diazotrophic Cyanobacterium *Cyanothece* sp. Strain BH68K

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In cyanobacteria, ammonium represses expression of proteins involved in nitrogen fixation and assimilation. The global nitrogen regulator gene *ntcA* encodes a DNA-binding protein, NtcA, that is a transcriptional activator of genes subject to nitrogen control. We report the cloning and sequencing of the *ntcA* gene from a nitrogen-fixing unicellular cyanobacterium, *Cyanothece* sp. strain BH68K. The gene comprises 678 nucleotides, and the deduced NtcA protein contains 226 amino acids with a predicted molecular weight of 25,026. In addition, *ntcA* mRNA levels were measured in cells grown under different nitrogen regimes. Under nitrogen-fixing conditions, *ntcA* transcripts were weakly expressed. Furthermore, *ntcA* expression was diminished or inversely proportional to *nifHDK* expression. Conversely, *ntcA* expression increased in nitrate-grown cells, and a concentration-dependent increase was seen in ammonium-grown cells up to 1 mM NH_4Cl. These results indicate that *ntcA* is involved more in nitrogen assimilation than in nitrogen fixation and also imply that the rhythmic expression of *ntcA* and *nifHDK* transcription may be under the control of a circadian clock.

In all cyanobacteria, utilization of combined nitrogen is tightly regulated. This regulation is referred to as global nitrogen control (7). Assimilation of nitrogen is stringently regulated to the extent that in the presence of ammonium, these organisms preferentially utilize ammonium over alternative inorganic sources such as nitrate, nitrite, or gaseous nitrogen (5). It has been shown in *Synechococcus* sp. strain PCC 7942 that mRNA levels of genes encoding proteins for nitrate and ammonium assimilation are negatively regulated by ammonium (9). Furthermore, the presence of ammonium results in inhibition of nitrogen fixation and heterocyst development in *Anabaena* sp. strain PCC 7120 (5, 24).

The *ntrB* and *ntrC* genes have been shown to operate nitrogen control in many prokaryotes, and the P_{II} protein has been reported to have a role in the transcriptional control mediated by the Ntr proteins (10). The P_{II} protein has been found in cyanobacteria, and its presence suggests the existence of a cyanobacterial Ntr system (21). However, repeated efforts to isolate *ntrB* and *ntrC* homologs in cyanobacteria have failed, and the precise role of P_{II} in signal transduction in cyanobacteria is unknown (20). The inability to locate *ntrB*- and *ntrC*-type DNA sequences in cyanobacteria suggested that nitrogen control in cyanobacteria is accomplished via a molecular mechanism other than the *ntr* genes. Subsequent studies with pleiotropic mutant strains isolated from *Synechococcus* sp. strain PCC 7942 that were simultaneously impaired in the expression of all genes known to be subject to repression by ammonia led to the identification of a new gene, *ntcA*, a nitrogen control gene, whose product, NtcA, acts as a positive transcriptional activator of genes subject to regulation by ammonia (23).

More recent studies have shown that *ntcA* is widespread in cyanobacteria (6), and the *ntcA* gene has also been cloned and sequenced from the non-nitrogen-fixing unicellular organism *Synechocystis* sp. strain PCC 6803 (6) and the nitrogen-fixing heterocystous filamentous organism *Anabaena* sp. strain PCC 7120 (6, 25). The *ntcA* gene appears to be highly conserved in these organisms. The *ntcA* gene product, NtcA, belongs to the Crp family of bacterial transcriptional regulators (22). NtcA, like other members of this family, contains a characteristic helix-turn-helix motif near its C-terminal end which is believed to facilitate binding of NtcA directly to the promoter regions of nitrogen-regulated genes (22).

Although NtcA is relatively widespread and appears to be highly conserved in cyanobacteria, to date no systematic studies have been undertaken to document its presence and function in nitrogen-fixing unicellular cyanobacteria. *Cyanothece* sp. strain BH68 (ATCC 51142) is a marine unicellular cyanobacterium capable of carrying out both oxygenic photosynthesis and oxygen-sensitive nitrogen fixation without any apparent morphological differentiation (15). Here we describe the cloning and sequencing of the *ntcA* gene from *Cyanothece* sp. strain BH68K. In addition, *ntcA* mRNA levels in this organism were measured to determine the pattern of *ntcA* expression under both nitrogen-fixing and non-nitrogen-fixing conditions.

Cyanobacterial strain, culture media, and growth conditions. *Cyanothece* sp. strain BH68K is a clonal isolate derived from the original *Cyanothece* sp. strain BH68 (19). Stock cultures of this strain were grown in medium AN^- (16). Cultures used as the inoculum were grown in 250-ml Erlenmeyer flasks containing 100 ml of medium AN^-. These cultures were maintained at 30°C in a Lab-line Environ-Shaker incubator and were shaken continuously at 100 rpm under a light intensity of 20 to 30 microeinsteins/m^2/s. Growth was determined spectrophotometrically by measuring the optical density at 750 nm (A_{750}).

For nitrate- and ammonium-grown cultures, *Cyanothece* sp. strain BH68K cells were grown in 1,500 ml of medium AN^- supplemented with 12 mM $NaNO_3$ and NH_4Cl (0.5, 1.0, or 2.0 mM), respectively. The initial A_{750} value for all cultures was approximately 0.02. These 1,500-ml cultures were grown in 2-liter aspirator bottles to late exponential phase (A_{750} of 0.22)

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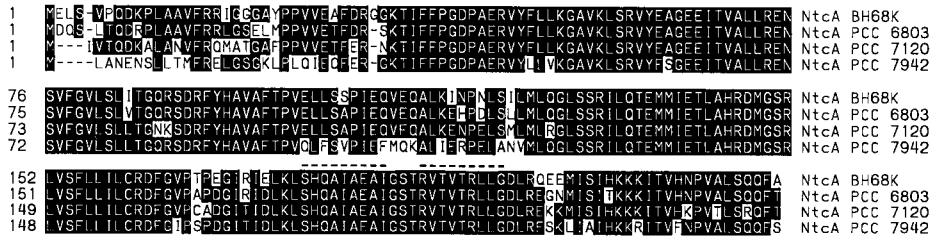

FIG. 1. Alignment of NtcA proteins from *Cyanothece* sp. strain BH68K, *Synechocystis* sp. strain PCC 6803, *Anabaena* sp. strain PCC 7120, and *Synechococcus* sp. strain PCC 7942. Positions at which identical residues are found in two or more proteins are indicated by the areas shaded in black. The putative helix-turn-helix motif is overlined.

at 30°C, under a light intensity of 90 to 100 microeinsteins/m^2/s, while being constantly bubbled with sterile moist air.

General genetic techniques. Plasmid DNA was isolated by the alkaline extraction method described by Birnboim and Doly (2). The pBS^+ vector (Stratagene) was used for all subcloning. DNA fragments used for subcloning into pBS^+ and as probes were purified by electrophoresis on low-melting-point agarose or with the use of the QIAEX Gel Extraction Kit (QIAGEN) as instructed by the manufacturer. All ligations were performed with T4 DNA ligase (GIBCO-BRL). *Escherichia coli* XL1-Blue MRF′ (Stratagene) was used in all transformation assays. DNA sequencing was carried out by the dideoxy chain termination method (17) with a Sequenase version 2 kit (U.S. Biochemical Corp.) and α-^{35}S-dATP (Dupont NEN). Similarity searches were performed with the BLAST program (1), and DNA sequence alignments were performed with the DNASTAR Lasergene program.

Cloning and sequencing of the *Cyanothece* sp. strain BH68K *ntcA* gene. The presence of DNA sequences homologous to *ntcA* in the *Cyanothece* sp. strain BH68K chromosome was initially confirmed by Southern blot analysis using a heterologous *ntcA* gene probe. A 471-bp *Bam*HI-*Bgl*II internal fragment encoding the *Synechocystis* sp. strain PCC 6803 *ntcA* gene was labeled to high specific activity with [α-^{32}P]dCTP (Dupont NEN) by using a random primer kit from GIBCO-BRL. *Cyanothece* sp. chromosomal DNA was isolated as described previously (19) and transferred to NitroPlus transfer membranes (MSI) with 0.4 N NaOH–0.6 M NaCl. Membranes were hybridized with the labeled DNA probe under low-stringency conditions at 55°C in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.4])–5× Denhardt's solution (11)–0.5% (wt/vol) sodium dodecyl sulfate (SDS). Membranes were washed twice with 2× SSPE–0.1% SDS for 15 min at 55°C and once with 1× SSPE–0.1% SDS for 15 min at 55°C. This assay indicated that the *ntcA* gene was indeed present within the *Cyanothece* sp. strain BH68K chromosome and that it was present as a single-copy gene (data not shown). Two plasmids, designated pCN1 and pCN2, harboring the *Cyanothece* sp. *ntcA* gene region were subsequently isolated by screening a *Cyanothece* sp. strain BH68K genomic plasmid library (18). The pCN1 insert was 1.7 kb, and pCN2 was 2.1 kb.

Sequencing of the entire *ntcA* gene from *Cyanothece* sp. strain BH68K was accomplished with both pCN1 and pCN2. Eighty-five percent of the *ntcA* gene sequence was obtained from the pCN2 clone, and the remaining 15% was acquired from pCN1. DNA was sequenced in both directions. Sequence analysis revealed an open reading frame of 678 nucleotides. The deduced NtcA protein from *Cyanothece* sp. strain BH68K contains 226 amino acids with a predicted molecular weight of 25,026 and shows 88, 86, and 77% identity with the NtcA proteins from *Synechocystis* sp. strain PCC 6803, *Anabaena* sp. strain PCC 7120, and *Synechococcus* sp. strain PCC 7942, respectively (Fig. 1). Furthermore, the amino acid sequence of the helix-turn-helix motif region of the *Cyanothece* sp. strain BH68K NtcA protein is 100% identical to those present in the other three proteins (Fig. 1). It has been demonstrated in PCC 7942 and PCC 7120 that NtcA binds to the promoter region of and activates genes involved in nitrogen control such as *glnA*, *nir*, and *ntcA* itself (3, 9, 12, 13). Given its similarity to the NtcA proteins from PCC 7942 and PCC 7120, it is possible that the *Cyanothece* NtcA protein may bind similar target sequences.

Induction of aerobic nitrogenase activity in continuous light. Induction of aerobic nitrogenase activity in a *Cyanothece* sp. in continuous light was achieved as follows. A *Cyanothece* sp. strain BH68K culture (A_{750} of 0.4) was used to inoculate 3,000 ml of medium AN^- to an initial A_{750} value of 0.02. This 3,000-ml culture was grown in a 4-liter aspirator bottle to late exponential phase (A_{750} of 0.22) at 30°C under a light intensity of 90 to 100 microeinsteins/m^2/s while being bubbled continuously with sterile moist air. This late-exponential-phase culture was then subjected to 20-h of darkness by wrapping the flask first with black plastic and then with aluminum foil in the growth chamber. At the end of the dark period, the flask was unwrapped, and cell growth was allowed to resume in continuous light. The pattern of aerobic nitrogenase activity was then monitored by the acetylene reduction assay as described previously (16) at 2-h intervals (and at 0.5-h intervals when aerobic nitrogenase activity was high) for 24 h beginning immediately after the cells were returned to continuous light. Figure 2A shows the pattern of expression of aerobic nitrogenase activity following the 20-h dark period. No nitrogenase activity was seen for 12 h after the cells had been returned to continuous light. Aerobic nitrogenase activity then increased rapidly to a high level at 17 h. This activity subsequently declined to zero at 24 h.

To examine *ntcA* regulation under nitrogen-fixing conditions, the pattern of *ntcA* expression during this induction of aerobic nitrogenase activity in continuous light was determined by Northern (RNA) analysis. Total RNA from *Cyanothece* sp. strain BH68K cells was isolated essentially as described by Reddy and Gilman (14). Twenty-microgram samples of total RNA per lane were run in 1.2% formaldehyde–agarose denaturing gels and transferred to NitroPlus transfer membranes in 20× SSC (3 M NaCl plus 0.3 M sodium citrate · $2H_2O$). Denaturing gels were run at 75 V for 3.5 h and were washed twice with 10× SSC prior to setting up transfer in 20× SSC. A random primer-labeled 293-bp *Sau*3AI fragment internal to *ntcA* from *Cyanothece* sp. strain BH68K was used to probe RNA isolated at specific intervals during the induction. Hybridization and washing conditions used for Northern analysis were identical to those used for Southern analysis, with the

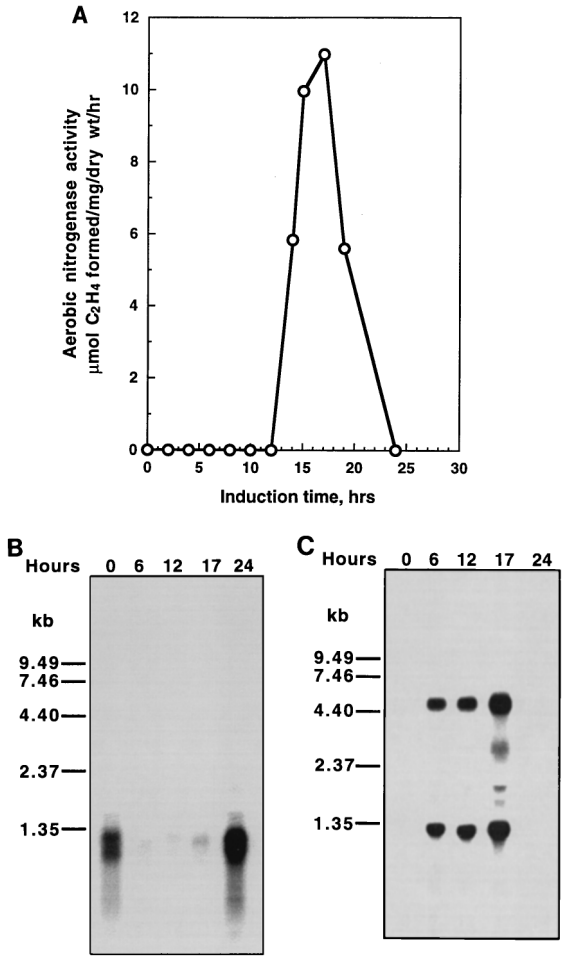

FIG. 2. *ntcA* and *nifHDK* expression during induction of aerobic nitrogenase activity in *Cyanothece* sp. strain BH68K in continuous light. (A) Induction of aerobic nitrogenase activity in continuous light. To induce aerobic nitrogenase activity, the culture was subjected to a 20-h dark period before being switched to continuous light. Aerobic nitrogenase activity was then monitored at the indicated intervals by the acetylene reduction assay. (B) Autoradiogram showing a Northern blot of *ntcA* during the induction assay. Total RNA was isolated at the indicated times (hours) beginning immediately after the 20-h dark period. The probe used was a 293-bp *Sau*3AI fragment internal to *ntcA* from *Cyanothece* sp. strain BH68K. (C) Autoradiogram showing a Northern blot of *nifHDK* transcripts during the induction assay. Total RNA was isolated from *Cyanothece* sp. strain BH68K cells collected at the indicated times (hours) beginning at the end of the 20-h dark period. A homologous DNA fragment containing most of the *nifHDK* operon was used as a probe. The larger 5-kb band represents a contiguous *nifHDK* transcript, and the smaller 1.3-kb band represents *nifH*.

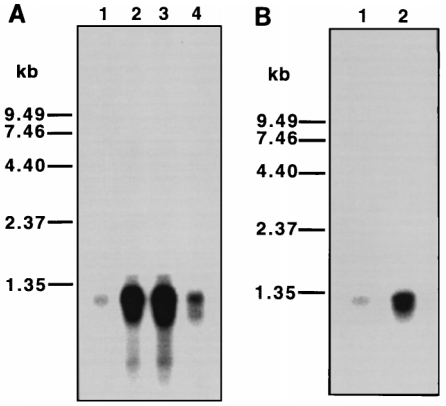

FIG. 3. (A) Autoradiogram showing Northern analysis of *ntcA* from *Cyanothece* sp. strain BH68K cells grown in the presence of increasing concentrations of ammonium. Total RNA was isolated from cells grown in the absence of combined nitrogen (lane 1) and in the presence of the NH_4Cl at 0.5 mM (lane 2), 1 mM (lane 3), and 2 mM (lane 4). (B) Autoradiogram showing Northern analysis of *ntcA* from *Cyanothece* sp. strain BH68K cells grown in the absence (lane 1) and presence (lane 2) of 12 mM $NaNO_3$. Total RNA was probed with the homologous *ntcA* fragment from *Cyanothece* sp. strain BH68K.

exception that the hybridization and wash temperatures were increased to 65°C. The results of this assay are depicted in Fig. 2B. A 1.3-kb transcript and a 1-kb transcript were prominently visible at 0 h, which corresponds to the point in the induction assay when the cells had just been shifted to continuous light after the 20-h dark period. A decline in the levels of these transcripts was subsequently observed. Hybridization signals were barely detectable at 6 and 12 h, respectively. The 1.3-kb transcript subsequently reappeared to some extent at 17 h, the time of high aerobic nitrogenase activity, and reached its highest level at 24 h when nitrogenase activity had completely disappeared.

The blot used to study regulation of *ntcA* was stripped and reprobed with a homologous DNA fragment containing most of the *nifHDK* operon from *Cyanothece* sp. strain BH68 (4). As shown in Fig. 2C, *nifHDK* transcripts were present in cells collected at 6, 12, and 17 h. No hybridization signals were seen at 0 h, and the transcripts present up to 17 h had completely disappeared by 24 h. A larger 5-kb transcript representing a contiguous *nifHDK* transcript and a smaller 1.3-kb transcript corresponding to *nifH* (4) were present at these times. The relative amount of both these transcripts was greatest at 17 h, the time of high aerobic nitrogenase activity. The pattern of *ntcA* expression observed during nitrogenase induction and the inverse relationship between *ntcA* and *nifHDK* expression suggest that in *Cyanothece* sp. strain BH68K, *ntcA* is more involved in nitrogen assimilation than in nitrogen fixation. Alternatively, the expression of these two genes may be under circadian control because the method used to induce nitrogenase activity (i.e., treatment of cells with a 20-h dark period) might have entrained the cells and caused the rhythmic gene expression. A recent study involving *Synechococcus* sp. strain PCC 7942 demonstrated that gene expression by circadian control is more widespread than initially thought and that certain genes are expressed at different phases of the circadian cycle (8).

***ntcA* expression in ammonium-grown cells.** Regulation of *ntcA* was also studied in *Cyanothece* sp. strain BH68K cells grown in the absence and presence of increasing concentrations of ammonium. RNA isolated from cells grown in the absence and presence of 0.5, 1, and 2 mM ammonium chloride was probed with the *Cyanothece ntcA* probe. As shown in Fig. 3A, in the absence of ammonia (i.e., nitrogen-fixing conditions), there was low-level expression of the 1.3-kb *ntcA* transcript. Much stronger hybridization signals were seen in RNA isolated from cells grown in the presence of 0.5 and 1 mM ammonium chloride. However, cells grown in the presence of 2 mM ammonium chloride exhibited a significant reduction in expression of *ntcA*. At this concentration, ammonium appeared to repress *ntcA* RNA levels. Previous studies with *Synechococcus* sp. strain PCC 7942 have shown that ammonia represses genes involved in the assimilation of inorganic nitrogen sources, including ammonia in this organism (5). However, the mechanism by which ammonia represses genes involved in nitrogen assimilation remains cryptic. The inhibitory effects of 2 mM ammonium chloride on *ntcA* expression in *Cyanothece*

was not due to a change in pH as the growth medium was buffered with 5 mM TAPS [*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid] (Sigma). Furthermore, the growth rate of the culture grown with 2 mM ammonium chloride was similar to that of nitrate-grown cultures.

This blot was subsequently stripped and reprobed with the *nifHDK* probe. For cells grown in the absence of ammonium (i.e., nitrogen-fixing conditions), as expected, strong hybridization signals corresponding to the larger 5-kb transcript representing a contiguous *nifHDK* transcript and the smaller 1.3-kb *nifH* transcript described earlier were present (data not shown). Conversely, no transcripts were detected in RNA isolated from ammonium-grown cells at any concentration of ammonium tested.

***ntcA* expression in nitrate-grown cells.** Expression of *ntcA* was also studied in cells grown in the absence (i.e., nitrogen-fixing conditions) and presence of 12 mM $NaNO_3$. Hybridization signals were detected in RNA isolated from *Cyanothece* sp. strain BH68K cells grown in both the absence and the presence of 12 mM $NaNO_3$ (Fig. 3B). However, hybridization was much stronger in RNA from nitrate-grown cells than in RNA from cells grown in the absence of nitrate. The low level of *ntcA* expression under nitrogen-fixing conditions is somewhat puzzling since fixed nitrogen must also be accumulated by the cells during this time. An explanation for this may be that the ammonia that was present at this time had not yet accumulated to levels high enough to warrant abundant *ntcA* transcription.

This blot was subsequently reprobed with the *nifHDK* probe described earlier (data not shown). In the absence of nitrate (i.e., nitrogen-fixing conditions), both the contiguous *nifHDK* and *nifH* transcripts were strongly expressed. However, in the presence of 12 mM $NaNO_3$, these transcripts were completely repressed.

Conclusions. The results presented here further corroborate the widespread nature of the global nitrogen regulator gene *ntcA* in cyanobacteria by documenting its presence in a nitrogen-fixing unicellular cyanobacterium, *Cyanothece* sp. strain BH68K. The *Cyanothece* sp. *ntcA* gene shows a high degree of similarity to previously reported *ntcA* genes from other cyanobacteria. In addition, the pattern of expression of *ntcA* seen during nitrogenase induction and the abundance of the *ntcA* transcript in the presence of $NaNO_3$ and 1 mM NH_4Cl indicate that *ntcA* is transcriptionally regulated in *Cyanothece* and suggest that *ntcA* is more involved in nitrogen assimilation than in nitrogen fixation. Development of a functional gene transfer system in *Cyanothece* would facilitate the creation of an *ntcA* mutant to determine if *ntcA* has any role in nitrogen fixation in this organism. Furthermore, information on specific mechanisms by which *ntcA* regulates nitrogen assimilation in *Cyanothece* requires further study. Since the unicellular cyanobacteria perform nitrogen fixation and assimilation within a single cell, the underlying molecular mechanisms that govern nitrogen assimilation in *Cyanothece* may ultimately prove to be unique in some respects compared to those of heterocystous filamentous and non-nitrogen-fixing unicellular cyanobacteria.

Nucleotide sequence accession number. The sequence of the *ntcA* gene identified in this study has been assigned GenBank accession number U80855.

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