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NtcA from *Microcystis aeruginosa* PCC 7806 Is Autoregulatory and Binds to the Microcystin Promoter[▽]

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NtcA is a transcription factor that has been found in a diverse range of cyanobacteria. This nitrogen-controlled factor was focused on as a key component in the yet-to-be-deciphered regulatory network controlling microcystin production. Adaptor-mediated PCR was utilized to isolate the *ntcA* gene from *Microcystis aeruginosa* PCC 7806. This gene was cloned, and the recombinant (His-tagged) protein was overexpressed and purified for use in mobility shift assays to analyze NtcA binding to putative sites identified in the microcystin *mcyA*/*D* promoter region. Autoregulation of NtcA in *M. aeruginosa* was shown via NtcA binding in the upstream *ntcA* promoter region. The observation of binding of NtcA to the *mcyA*/*D* promoter region has direct relevance for the regulation of microcystin biosynthesis, as transcription of the *mcyABCDEFGHIJ* gene cluster appears to be under direct control of nitrogen.

Microcystis aeruginosa PCC 7806 is a bloom-forming unicellular photosynthetic cyanobacterium that is capable of producing the hepatotoxin microcystin. Investigations of the role and function of microcystin are ongoing, as the true nature of this toxin has not been determined yet. Cyanobacteria have developed adaptive mechanisms that enable them to survive in a vast range of habitats, and their wide distribution and diversity, coupled with the accelerating frequency and intensity of toxic blooms, have sustained interest in this field in recent years. The transcriptional regulation of the microcystin synthetase gene cluster is a key focus of this research and may provide further evidence for existing hypotheses related to microcystin physiology, including siderophore (19, 26, 28), defense (23), and quorum-sensing (3) functions. Factors such as micronutrient levels in the environment provide interesting links to systems of regulation at the transcriptional level. As elements of transcriptional control are elucidated, a clearer role for microcystin may be proposed.

Microcystin has been shown to be highly toxic to humans (20) and animals and displays bioactivity against algae, bacteria, fungi, and mammalian cell lines (2). Toxicity in mammals occurs through an association with hepatocytes due to active transport of the toxin to the liver via bile acid multispecific organic anion transporters (24). The discovery that this toxin was produced nonribosomally (27) complemented a growing number of studies of the transcriptional regulation of cyclic peptide synthesis gene clusters, and there has been particular interest due to the vast array of bioactive compounds that these gene clusters encode (1). Early indications suggested that the light level and wavelength affect transcription of the microcystin synthetase gene cluster (*mcy*) (9). Acclimation responses to nutrient availability can be characterized as either responses that are specific for the nutrient that is limiting (18, 21) or responses that are general (34) and occur under a number of different nutrient limitation conditions (25). The 750-bp promoter region which bidirectionally initiates transcription of *mcyABC* and *mcyDEFGHIJ* is centrally located in the toxin biosynthesis gene cluster and is predicted to be the principal site of regulation. Characterization of the *mcy* promoter may result in a clearer understanding of the factors that affect toxin production.

Nitrogen control and assimilation in cyanobacteria are subject to fine control. The prokaryotic global nitrogen regulator NtcA facilitates regulation of nitrogen-responsive genes and belongs to the CAP family of transcription factors (4, 5, 29–31). In most cases, NtcA is an activator; however, it may also act as a repressor (7). NtcA was first isolated from *Synechococcus* sp. PCC 7942 (29) and has now been characterized for a wide range of cyanobacterial genera. Due to the large number of cyanobacterial genes shown to be regulated by NtcA, it appears that NtcA responds not only to ammonium but also to the C/N ratio in the cell (13). NtcA is produced at a basal level in the presence of ammonium, and the level is elevated under nitrogen stress conditions (10, 16, 22, 31). NtcA possesses the C-terminal helix-turn-helix motif consistent with the DNA binding domain of transcription factors (33) and binds to the consensus region $GTAN_8TAC$, and there is some variation in the length of the spacer between the palindrome and the bases flanking the binding sequences. The consensus is frequently located 22 or 23 bp upstream from an accompanying σ^{70}-like -10 hexamer of TAN_3T (5, 13), while multiple NtcA binding sites have also been shown to occur in a single promoter. NtcA can autoregulate its own transcription, and therefore a binding site homologous to the established consensus sequence is also expected to be present in the promoter region upstream of an *ntcA* gene.

We have identified regions with similarity to the consensus motifs recognized and bound by NtcA in the internal *mcyA*/*D* promoter region in *M. aeruginosa* PCC 7806. The complete gene encoding NtcA in this cyanobacterium was isolated by degenerate and adaptor-mediated PCR. The protein was then overexpressed and used to characterize the interaction and

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant feature or sequence	Sequence	Source or reference
Strains			
M. aeruginosa PCC 7806	Microcystin-producing cyanobacterium		UNSW culture collection
E. coli DH5α	Cloning host		UNSW culture collection
E. coli BL-21(DE3)	Expression host		UNSW culture collection
Plasmids			
pGEM-T Easy	Cloning vector		Promega
pET-30a	Expression vector		Novagen
Primers			
NF	*ntcA* degenerate	CAGTGTTTTTGGGGTGYT	Timothy Downing, University of Port Elizabeth
NR	*ntcA*	GTTTCAATCATCATTTCCGT	Timothy Downing, University of Port Elizabeth
NF/1	*ntcA* pan-handle	CGAGAGTAATTCTACCGGAG	This study
NF/2	*ntcA*	GATCCCTCCGCAATAATCCC	This study
NF/3	*ntcA*	TTTCTACCTGCTCGATCGG	This study
NF/4	*ntcA*	AGTGAACAGATAGCTGTGC	This study
NF/5	*ntcA*	CTGACCAAACGTGAACCCAT	This study
NF/6	*ntcA*	CGAGTGTCACCTCTATTAAACAC	This study
NTCANDE1F	*ntcA* cloning	TCCATATGGACTTATCATTAATACAAGATAAAC	This study
NTCAXHO1R	*ntcA* cloning	TCCTCGAGAGTAAATTGTTGACTGAGAGCG	This study
T7 term	pET-30a	GCTAGTTATTGCTCAGCGGT	Novagen
T7 prom	pET-30a	TAATACGACTCACTATAGGG	Novagen
MPF	pGEM-T Easy	CCCAGTCACGACGTTGTAAAACG	Promega
MPR	pGEM-T Easy	AGCGGATAACAATTTCACACAGG	Promega
T7Pr1	Adaptor primer	CCCCTATCCACCCTTACACCTATC	15
M4F	NtcA binding site in *mcyA*/*D* promoter	CGAATTCTAATGATTTTTACTAATTTATTGGG	This study
M4R	NtcA binding site in *mcyA*/*D* promoter	CCGCCGGCGAATTGTTCTGAGCCTCGACAT	This study
M5F	NtcA binding site in *mcyA*/*D* promoter	CGAATTCCCGTCGGGTTTCCTGT	This study
M5R	NtcA site in *mcyA*/*D* promoter	CCGCCGGCGCATTGCTGTTCTAACTTTTTCC	This study
M6F	*ntcA* promoter	CGAATTCCAATAGCCGACCCCAGCG	This study
M6R	*ntcA* promoter	CCGCCGGCGTTTTTATTATCCAACGAGTGTCA	This study
NtcArealF	*ntcA* reverse transcription-PCR short product	CATTTCCGTTTGCAGAATCC	This study
NtcArealR	*ntcA* reverse transcription-PCR short product	TGTTTTTGGGGTGCTATCCT	This study
RpoC1F	*rpoc1* reverse transcription-PCR short product	CCTCAGCGAAGATCAATGGT	This study
RpoC1R	*rpoc1* reverse transcription-PCR short product	CCGTTTTTGCCCCTTACTTT	This study
McyBrealF	*mcyB* reverse transcription-PCR short product	CTGAGGGGATTACGGATTGA	This study
McyBrealR	*mcyB* reverse transcription-PCR short product	ACCATATAAGCGGGCAGTTG	This study

binding of NtcA to the *mcyA*/*D* promoter *in vitro*. The autoregulation of *ntcA* was also assessed, along with the transcription of *ntcA* and *mcyB* in response to different nitrogen availability conditions.

MATERIALS AND METHODS

Isolation of *ntcA* from *Microcystis*. Total genomic DNA was extracted from mid-exponential-growth-phase cells of *M. aeruginosa* PCC 7806 as described previously (17). Degenerate primers were designed to target regions of cyanobacterial *ntcA* homology to amplify a corresponding gene fragment from *M. aeruginosa* PCC 7806. The unknown genomic regions flanking this amplicon were elucidated by gene walking based on adaptor-mediated PCR (14). Briefly, primers for short adaptor sequences ligated to partially digested genomic DNA and specific outward-facing primers were utilized to amplify and then sequence flanking regions. Automated sequencing was performed using the Prism BigDye cycle sequencing system and an ABI 3730 capillary sequencer (Applied Biosystems, Inc., Foster City, CA).

Cloning, overexpression, and isolation of recombinant NtcA. The *ntcA* gene was amplified from *M. aeruginosa* PCC 7806 using primers NtcANde1F and NtcAXho1R (Table 1), which incorporated NdeI and XhoI restriction sites at the N and C termini, respectively. The amplified *ntcA* gene was ligated into the pGEM-T Easy vector (Promega) for sequencing, and the results of sequencing were analyzed using Autoassembler software (Applied Biosystems). The *ntcA* gene was excised from pGEM-T Easy by digestion with NdeI and XhoI and subcloned into the corresponding pET-30a (Novagen) restriction sites. *Escherichia coli* BL21(DE3) was transformed with the new plasmid, and transformants were selected on solid LB agar supplemented with 50 μg/ml kanamycin. Plasmid inserts were again confirmed by sequencing. An initial inoculum of *E. coli* BL21(DE3) harboring the plasmid was grown at 30°C for 16 h and used to inoculate 600 ml of LB broth supplemented with 50 μg/ml kanamycin. The expression culture was incubated at 37°C with orbital shaking at 150 rpm to obtain an optical density at 600 nm (OD_{600}) of 0.6, and then expression was induced by addition of 0.02 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Following incubation at 16°C for 2 h, cells were harvested by centrifugation and stored at −20°C. For processing, cell pellets were thawed on ice, resuspended in 6 ml HEPES buffer (50 mM HEPES, 300 mM NaCl; pH 7.4), and sonicated three times at 4°C and 25% amplitude for 30 s with 0.5-s pulses (Branson, Danbury, CT). Following centrifugation at 4°C at 20,000 × *g* for 20 min, the cleared lysate containing the soluble recombinant NtcA was used for purification with a HiTrap nickel-charged affinity column (Amersham Biosciences) utilizing the Bio-Logics-HR apparatus (Bio-Rad). The purity of fractions was analyzed by SDS-PAGE (15% [wt/vol] polyacrylamide), and the columns were stained with Coomassie brilliant blue and visually recorded with LAS-3000 (FujiFilm). Fractions containing the desired protein were pooled and frozen with 10% glycerol in liquid nitrogen for storage at −80°C. Western blotting was performed to verify that the overexpressed protein was indeed recombinant (His-tagged) NtcA. Protein sequences were aligned using CLUSTAL X, and a neighbor-joining tree was produced (see Fig. 3).

Electromobility shift assay. Short DNA probes that were approximately 200 bp long were generated by PCR using a genomic DNA template from *M. aeruginosa* PCC 7806. These probes were designed to incorporate the putative NtcA binding motifs. Primers were used to introduce EcoRI and NotI restriction sites to facilitate radiolabeling. Two probes represented the *mcyA*/*D* promoter region, while a third probe targeted the promoter region of the newly identified *M. aeruginosa ntcA* gene (accession no. EU402445). The probes were digested

FIG. 1. Sequence data for the *mcyA*/*D* promoter region of *M. aeruginosa* PCC 7806, showing putative NtcA binding sites identified in this study. The consensus sequence target for NtcA binding proteins is $GTAN_8TAC$.

with EcoRI and NotI (Promega), and the resultant overhangs were end labeled on both strands using [α-^{32}P]dCTP and [α-^{32}P]dATP (Perkin-Elmer) in addition to unlabeled dGTP and dTTP (Promega) in a 1-h Klenow reaction (Promega) performed at room temperature. The labeled DNA probes were precipitated with ethanol for 12 h at −20°C and then resuspended in sterile water and stored at 4°C. The probes were incubated with various concentrations of partially purified NtcA in order to establish a binding pattern. Control reactions without NtcA were also performed in order to determine the migration of the unbound free probe DNA. Binding reactions were performed in binding buffer [10 mM Tris, 40 mM KCl, 2 mM $MgCl_2$, 100 μM $MnCl_2$, 5% glycerol, 0.5 mg/ml bovine serum albumin (BSA), 0.5 μg poly(dI-dC), 5 mM dithiothreitol (DTT)] for 1 h at room temperature. Samples were then electrophoresed at 4°C through nondenaturing 4% polyacrylamide gels for approximately 4 h at 4°C. The gels were exposed to radiosensitive imaging plates for up to 18 h and visualized using an FLA-5010 phosphoimager (FujiFilm). Analysis of labeled hybridization products was performed utilizing Image Gauge 4.0 software.

Nitrogen-limited growth of *M. aeruginosa* PCC 7806. *M. aeruginosa* PCC 7806 was grown under nitrogen-excess, nitrogen-limited, and nitrogen-starved conditions using defined BG11 medium supplemented with 1.5 g/liter, 0.75 g/liter, and 0 g/liter sodium nitrate, respectively. To investigate the *ntcA* and *mcyB* transcription profile under the new transition conditions, RNA was extracted after 28 days (mid-log phase). To analyze *ntcA* and *mcyB* transcription in cells acclimatized to the different levels of nitrogen, RNA was extracted from the fourth generation of cells, again during the mid-log phase of growth (28 days), following three subcultures under the same conditions.

RNA extraction and real-time PCR. RNA was extracted after cell lysis with Trizol (Invitrogen), which was followed by DNase treatment (Promega) in order to remove all traces of DNA, which was confirmed by PCR. cDNA was generated by reverse transcription using a First Strand cDNA synthesis system (Marligen Biosciences). Samples containing 100 ng/μl of cDNA were analyzed in triplicate using a Corbett Rotor-Gene real-time PCR machine (Corbett Life Sciences) with SYBR brilliant green chemistry (Invitrogen). Primers were designed to amplify 200-bp products of *ntcA*, the *rpoC1* RNA polymerase housekeeping gene, and *mcyB* to indicate microcystin synthetase transcription. The primer efficiencies for amplification of *ntcA*, *mcyB*, and *rpoC1* were calculated, which yielded E values of 1.6745, 2.29848, and 2.56774, respectively, where an E value of 2 indicates 100% PCR efficiency. Relative quantification of the *ntcA* and *mcyB* target genes compared with the *rpoC1* reference gene was also performed (11), which yielded a fold change in transcription compared to the results for the control conditions (excess nitrogen).

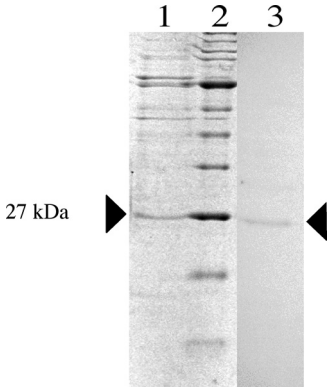

FIG. 2. Partially purified recombinant NtcA electrophoresed through a 15% polyacrylamide gel (lane 1) along with a broad-range NEB ladder (lane 2). Lane 3 shows the corresponding Western blot verifying overexpression of the His-tagged protein.

RESULTS

Identification of NtcA binding sites. Characterization of the *mcyA*/*D* promoter sequence revealed regions that were identified as potential binding sites for the global nitrogen regulator transcription factor NtcA (Fig. 1). Possible binding and regulation by NtcA were suggested by identification of regions with high levels of similarity to the highly conserved sequence $GTAN_8TAC$ (5, 12). Three putative NtcA binding sites were found in the *mcyA*/*D* promoter.

NtcA from *M. aeruginosa* PCC 7806. The 675-bp *ntcA* gene was identified and amplified in its entirety utilizing an adaptor-mediated PCR. Following ligation into the pGEM-T Easy cloning vector and sequencing, the gene was subcloned into the pET-30a expression vector, overexpressed, and purified (Fig. 2). The molecular mass of the recombinant protein was approximately 27 kDa, and the presence of the His tag was confirmed by Western blotting. Figure 3 shows the neighbor-joining phylogenetic tree produced from a Clustal X protein alignment. The *E. coli* K-12 cyclic AMP receptor protein (CRP) protein was used as an outgroup to root the tree. The NtcA protein sequence of *M. aeruginosa* PCC 7806 clustered with that of *Synechocystis* sp. PCC 6803.

Autoregulation of *ntcA*. Genome walking was also used to obtain the nucleotide sequence upstream from the *ntcA* start codon. Autoregulation of *ntcA* would require the *ntcA* promoter in *M. aeruginosa* PCC 7806 to have one or several NtcA binding sites to enable gene activation under nitrogen-depleted conditions. Two putative NtcA binding sites with significant similarity to the $GTAN_8TAC$ consensus sequence were identified. Compared to the palindromic consensus sequence, the upstream NtcA binding site was identical for all six bases, while the other site matched only four bases in the motif.

The physical interaction of the recombinant NtcA protein heterologously expressed from *M. aeruginosa* PCC 7806 and the *ntcA* promoter was assayed by using the electromobility gel shift assay. Probes (200 bp) were created using the *ntcA* promoter sequence containing the two putative NtcA binding

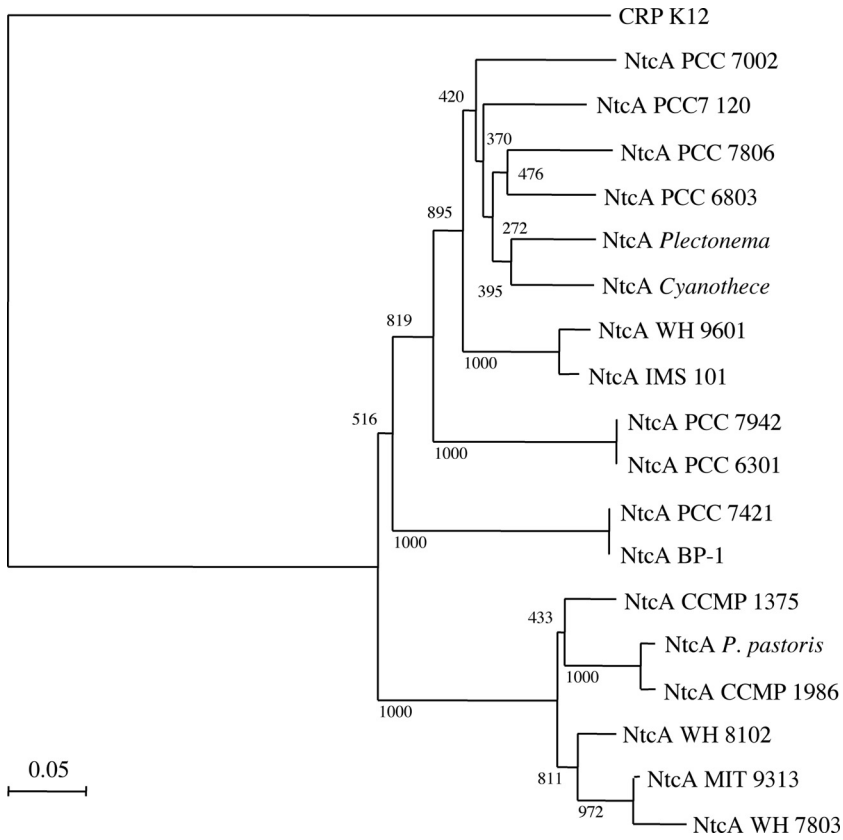

FIG. 3. Neighbor-joining phylogenetic tree of NtcA protein sequences from cyanobacteria identified thus far. Bootstrap values resulting from 1,000 trials are indicated at the nodes. The sequences included in the tree are sequences obtained from the following cyanobacteria: *Synechococcus* sp. PCC 7002 (accession no. AAK49022), *Anabaena* sp. PCC 7120 (BAB76091), *Microcystis aeruginosa* PCC 7806, *Synechocystis* sp. PCC 6803 (BAA18011), *Plectonema boryanum* (AAK26380), *Cyanothece* sp. ATCC 51142-BH68K (AAB62977), *Trichodesmium* sp. WH9601 (AAF63203), *Trichodesmium* sp. IMS 101 (AAD53080), *Synechococcus* sp. PCC 7942 (CAA42755), *Synechococcus* sp. PCC 6301 (YP_172087), *Gloeobacter* sp. PCC 7421 (NP_926231), *Thermosynechococcus* sp. BP-1 (NP_682440), *Prochlorococcus* sp. CCMP 1375 (AAY25557), *Prochlorococcus marinus* subsp. *pastoris* (AAM82249), *Prochlorococcus* sp. CCMP 1986 (AAZ93923), *Synechococcus* sp. WH8102 (AAY25543), *Prochlorococcus* sp. MIT 9313 (AAY25548), and *Synechococcus* sp. WH7803 (AAQ55486). The *E. coli* K-12 CRP protein (AAC76382) was used to root the tree.

sites. Electrophoretic retardation of the DNA probe was observed when it was incubated with NtcA, confirming that the promoter region was bound by NtcA (Fig. 4A). An increase in the shift intensity was observed when the amount of protein was increased, and no retardation was observed for the sample lacking protein. The approximate percentage of the DNA probe retarded in the gel is shown in Fig. 4B. At an NtcA concentration of 500 nM, 18% of the probe was retarded. To investigate the potential redox regulation of DNA binding, the assay was repeated with increasing concentrations of DTT. This reducing agent did not appear to increase binding of NtcA to the probe (data not shown).

NtcA binding to the *mcyA*/*D* promoter. Three putative NtcA binding sites were also identified in the region upstream from the *mcyD* start codon (Fig. 1). Figure 5A shows NtcA binding to the *mcyA*/*D* promoter in the electromobility shift assay, and the estimated percentage of the radiolabeled DNA probe retarded in the gel due to NtcA binding (Fig. 5B) was up to 7%. Increasing the concentration of the reducing agent DTT did not appear to affect binding of NtcA to the *mcyA*/*D* promoter (data not shown).

Profiles of *ntcA* and *mcyB* expression under different nitrogen conditions. The relative levels of transcription of *ntcA* and *mcyB* under nitrogen-excess, nitrogen-limited, and nitrogen-starved culture conditions were determined (Fig. 6). The transcription levels were expressed as the relative levels of expression by using the cycle threshold (C_T) values of *rpoC1* and *ntcA* or *mcyB* in addition to the primer efficiency E value. The nitrogen-excess conditions were designated the control conditions, and therefore the results were normalized to a value of 1. For acclimatized cells (Fig. 6A), *ntcA* transcription increased 4.07-fold under the nitrogen-limited conditions. The level of upregulation in the nitrogen-starved conditions was 2.36-fold. The transcription of *mcyB* increased markedly under the nitrogen-limited conditions to a level that was 14.09 greater than the level under nitrogen-excess conditions. The increase again was smaller under the nitrogen-starved conditions (9.70-fold compared to the control). For the nutrient-shocked cells (Fig. 6B), *ntcA* transcription increased 1.21-fold under the nitrogen-limited conditions and 4.40-fold under the nitrogen-starved conditions. A similar trend was seen for *mcyB* transcription; there was a 2.75-fold increase under the nitrogen-limiting con-

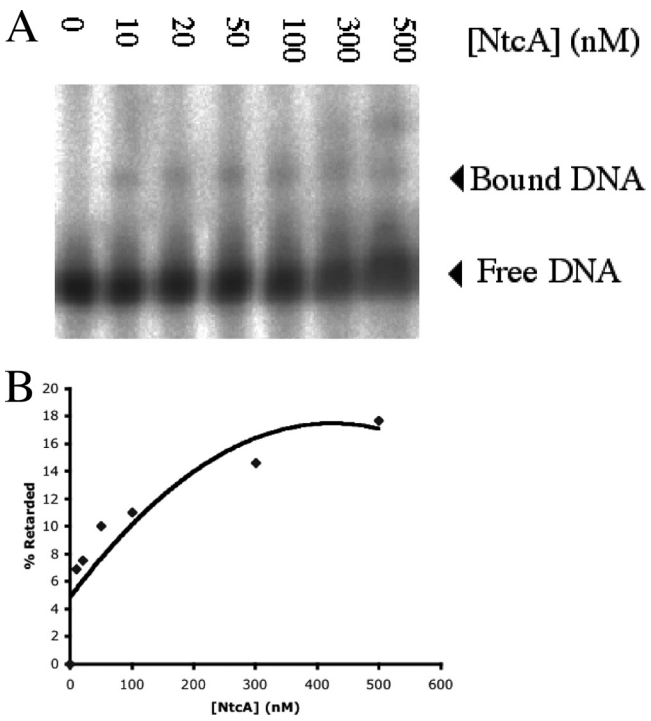

FIG. 4. (A) Electromobility shift assay of recombinant NtcA from *M. aeruginosa* binding to the *ntcA* promoter. Lane 0 contained no protein and shows the migration of unbound free DNA. (B) Approximate percentages of the probe retarded in the gel.

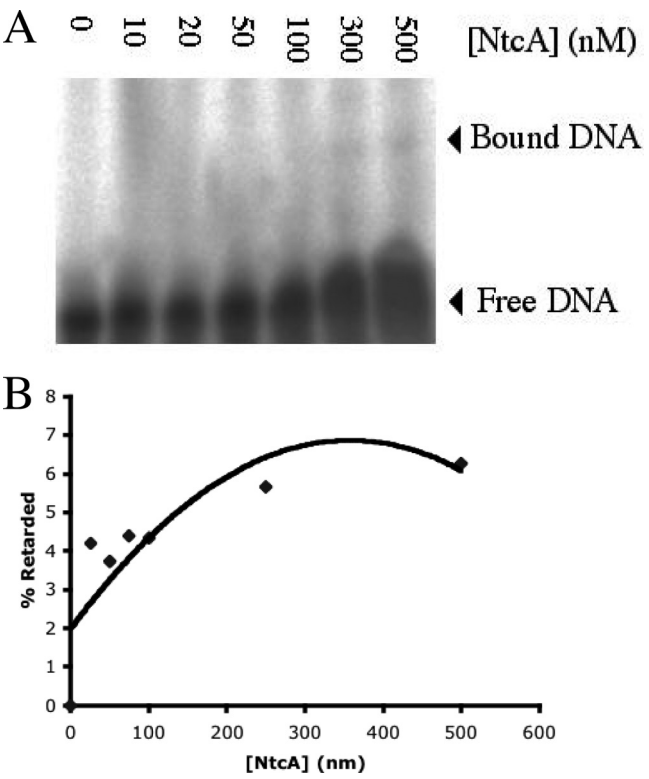

FIG. 5. (A) Electromobility shift assay of recombinant NtcA from *M. aeruginosa* binding to the *mcyA/D* promoter. Lane 0 contained no protein and shows the migration of unbound free DNA. (B) Approximate percentages of the probe retarded in the gel.

ditions, and there was a 4.76-fold increase under the nitrogen-starved conditions.

DISCUSSION

Inspection of the *mcyA/D* promoter revealed putative binding sites for the transcription factor NtcA, a global transcription regulator for nitrogen control in cyanobacteria. NtcA binding and regulation sequences in promoter regions are identified by motifs that exhibit significant similarity to the highly conserved sequence $GTAN_8TAC$ (5, 12). Initially identified in *Synechococcus* sp. PCC 7942 as an activator of genes repressed by ammonium (29), NtcA was subsequently identified in many cyanobacteria, including filamentous, unicellular, and heterocyst-forming species (5). Here, an *ntcA* gene fragment was amplified from *M. aeruginosa* PCC 7806 utilizing degenerate primers targeting a highly conserved region of the gene corresponding to the β-roll and α-helices in the protein. Following amplification of the complete *ntcA* gene sequence by adaptor-mediated PCR, the translated NtcA protein was characterized. The NtcA protein was cloned and overexpressed in *E. coli* (Fig. 2). A phylogenetic tree (Fig. 3) indicated that NtcA from *M. aeruginosa* PCC 7806 was closely related to NtcA from *Synechocystis* sp. PCC 6803, a unicellular, non-nitrogen-fixing cyanobacterium. However, *Synechocystis* sp. PCC 6803 is nontoxic and therefore fundamentally distinct from *M. aeruginosa* PCC 7806. It is noteworthy that in a 16S rRNA sequence tree, *M. aeruginosa* PCC 7806 and *Synechocystis* sp. PCC 6803 are also closely related.

In addition to the complete gene sequence for *ntcA*, the upstream promoter region was also obtained by adaptor-mediated PCR. It was hypothesized that a canonical NtcA binding motif would be found in this region. Two putative sites were identified, and their sequences were GTATAATAGGGATAC and GTTGTGTTTAATAG. It has previously been reported that multiple NtcA binding sites may occur in a single promoter, enabling more sensitive attenuation (12). Separated by approximately 60 bp, the NtcA binding motifs presumably are on the same face of the DNA helix. In addition, motifs homologous to a σ^{70}-type *E. coli* promoter were observed, and the short 7-bp spacer region between the −10 (GATAAT) and −35 (GTGACA) regions suggested that the NtcA activator site, which is only 2 bases upstream from the −35 sequence, permits increased affinity for the σ factor of RNA polymerase (33). The stronger NtcA binding consensus sequence (GTAT AATAGGGATAC) was identified at position −52 from the start codon and could therefore provide sensitive NtcA autoregulation.

Weak NtcA binding was observed in electromobility shift assays after incubation of the *ntcA* promoter region with partially purified NtcA (Fig. 4A). Monomeric and dimeric binding was observed and is proposed to occur at the $GTAN_9TAC$ NtcA binding motif identified. Quantification of retarded gel electrophoresis bands revealed that up to 18% of the DNA probe was retarded in the gel (Fig. 4B). This suggested that *ntcA* is autoregulated in *M. aeruginosa* PCC 7806. In particular, analysis of the redox attenuation of NtcA DNA binding affinity has focused on the thiol groups of cysteine residues (7). Ad-

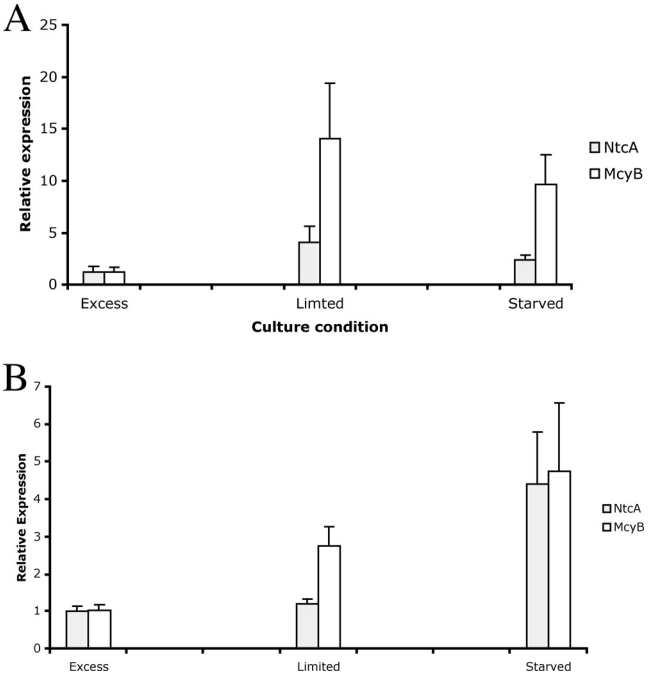

FIG. 6. Relative expression of *ntcA* and *mcyB* under nitrogen-excess, nitrogen-limited, and nitrogen-starved conditions after addition of 1.5 g/liter, 0.75 g/liter, and 0 g/liter sodium nitrate, respectively, to defined BG11 medium. (A) Real-time PCR results for nitrogen-acclimatized cells. (B) Results for nitrogen-shocked cells (see text for details).

dition of the reducing agent DTT to the binding reaction with NtcA and the *ntcA* promoter probe was investigated; however, this agent did not appear to increase binding of the promoter and ligand. Thiol groups on cysteine residues are necessary for DNA binding due to their inactivation when they are incubated with diamide (7), even though a double cysteine mutant strain of *Anabaena* sp. PCC 7120 had increased binding affinity after addition of DTT (32). The translated sequence of NtcA from *M. aeruginosa* PCC 7806 contained only one cysteine; hence, the observed absence of redox regulation of binding affinity may be sequence specific. In fact, it was anticipated that addition of DTT would lead to increased binding, despite the fact that a single cysteine residue was present and the fact that low binding affinity was expected due to the presence of a noncanonical motif.

An additional gel shift product was observed with 300 and 500 nM NtcA (Fig. 4A), which suggested that there was formation and binding of a protein dimer. DNA binding proteins that possess a C-terminal helix-turn-helix motif, such as NtcA, are likely to form dimers or even polymers (12). NtcA also bound weakly to the *mcyA*/*D* $GTAN_9TAC$ motif (Fig. 5A). Approximately 7% of the total DNA probe was retarded in the electromobility gel shift assay by NtcA binding activity (Fig. 5B). This result suggested that the transcription of microcystin is regulated in part by NtcA. Addition of DTT also had no effect on the binding of NtcA and the *mcyA*/*D* promoter. As discussed above, this could be attributed to the lack of multiple cysteine residues in the protein; hence, the redox regulation of the binding of NtcA to the *mcyA*/*D* promoter would be limited.

Although the presence of the $GTAN_9TAC$ NtcA binding motif identified in the *ntcA* and *mcyA*/*D* promoters was confirmed by NtcA binding in electromobility gel shift assays, the observed level of binding was low. NtcA binding to promoters with motifs that differ from the established $GTAN_8TAC$ sequence has been reported (8), and the more divergent the binding sequences compared with the consensus sequence, the lower the NtcA affinity. A weakly conserved sequence central to the palindromic loci was also proposed to result in differential NtcA binding under various physiological conditions (8). It is apparent that the promoter sequences putatively recognized by NtcA from *M. aeruginosa* PCC 7806 include a spacer region consisting of nine bases, and this divergence from the classical consensus explains the weak binding observed.

The classical pattern of transcription expected for an activator regulatory protein such as NtcA includes an increase in transcription under dynamic conditions. Subsequently, as production of the activator protein increases, the transcription of the target protein is also upregulated. The transcription of *mcyB* under nitrogen stress conditions was mirrored by the increase in *ntcA* transcription, which strongly suggested that nitrogen levels affect microcystin production rates (Fig. 6). Multiple transcripts of *ntcA* have been found (22), and this finding could be linked to the theory that changes in the weakly conserved N_8 sequence in the $GTAN_8TAC$ motif may modulate NtcA binding under changing physiological conditions (8). The method employed in this study was unable to determine if multiple transcripts were synthesized from a single promoter; however, it would be beneficial to elucidate if multiple transcripts of *ntcA* or *mcyB* were produced. Future research in this area could also include a variety of nitrogen sources.

It has been suggested that NtcA may also act as a repressor, as reported for *gor* in *Anabaena* sp. PCC 7120 (6), under general stress conditions and in response to nitrogen availability. Several NtcA binding sites were identified, one of which overlaps the σ^{70}-type *E. coli* promoter. This organization may result in the production of multiple transcripts under cellular conditions, as needed (6). This promoter arrangement echoes that of the *mcyA*/*D* promoter, and therefore it is possible that NtcA also acts as a repressor of microcystin synthetase transcription. In this case, however, the level of the *mcyB* transcript would be expected to decline as the level of the *ntcA* transcript increased.

In summary, the cloning and overexpression of NtcA from *M. aeruginosa* PCC 7806 in this study revealed the predicted autoregulation of *ntcA*, and additional results reflected the role of NtcA as a transcriptional activator. Binding of NtcA to the *mcyA*/*D* promoter from *M. aeruginosa* PCC 7806 suggests that the regulation of microcystin synthetase gene transcription is responsive to nitrogen.

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