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ORIGINAL ARTICLE

Decoupling of ammonium regulation and *ntcA* transcription in the diazotrophic marine cyanobacterium *Trichodesmium* sp. IMS101

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Nitrogen (N) physiology in the marine cyanobacterium *Trichodesmium* IMS101 was studied along with transcript accumulation of the N-regulatory gene *ntcA* and of two of its target genes: *napA* (nitrate assimilation) and *nifH* (N_2 fixation). N_2 fixation was impaired in the presence of nitrite, nitrate and urea. Strain IMS101 was capable of growth on these combined N sources at $<2\,\mu M$ but growth rates declined at elevated concentrations. Assimilation of nitrate and urea was impaired in the presence of ammonium. Whereas ecologically relevant N concentrations (2–20 μM) suppressed growth and assimilation, much higher concentrations were required to affect transcript levels. Transcripts of *nifH* accumulated under nitrogen-fixing conditions; these transcript levels were maintained in the presence of nitrate (100 μM) and ammonium (20 μM). However, *nifH* transcript levels were below detection at ammonium concentrations $>20\,\mu M$. *napA* mRNA was found at low levels in both N_2-fixing and ammonium-utilizing filaments, and it accumulated in filaments grown with nitrate. The positive effect of nitrate on *napA* transcription was abolished by ammonium additions of $>200\,\mu M$. This effect was restored upon addition of the glutamine synthetase inhibitor L-methionin-DL-sulfoximine. Surprisingly, *ntcA* transcript levels remained high in the presence of ammonium, even at elevated concentrations. These findings indicate that ammonium repression is decoupled from transcriptional activation of *ntcA* in *Trichodesmium* IMS101.
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Introduction

Cyanobacteria require nitrogen (N) as an essential macronutrient and they are capable of assimilation of a suite of organic and inorganic N compounds. Ammonium is the preferred combined N source for most if not all cyanobacteria. Cells acquire ammonium by diffusive pathways as well as active transport (Boussiba and Gibson, 1991; Herrero *et al.*, 2001). Once inside, it is rapidly incorporated into essential cell components by the enzymes of the glutamine synthetase/glutamate synthase pathway. In the presence of high concentrations of ammonium, cyanobacteria are neither capable of assimilating other N sources, nor can they engage in N_2 fixation and heterocyst development (Flores and Herrero, 1994). Type I glutamine synthetase activities are repressed by ammonium additions (Merida *et al.*, 1991). Ammonium reversibly inhibits nitrate assimilation (Ohmori *et al.*, 1977; Flores *et al.*, 1980). This involves the C-terminus of NrtC—one of the ATPase components of the nitrate transporter—and its truncation relieves the ammonium inhibition (Kobayashi *et al.*, 1997). Nitrite uptake by marine *Synechococcus* WH7803 was repressed by ammonium additions (Lindell *et al.*, 1998). Ammonium also affects the transcription of N assimilation genes through (de)repression of *ntcA,* a N-regulatory gene. Its gene product, NtcA, is a 24 kD DNA-binding protein that belongs to the cAMP receptor protein (CRP) family of transcriptional activators (Vega-Palas *et al.*, 1992; Flores and Herrero, 1994). Freshwater and marine unicellular, non-N_2-fixing cyanobacteria show a clear, direct relationship between *ntcA* expression and low ammonium levels (Frias *et al.*, 1994; Lee *et al.*, 1999; Sauer *et al.*, 1999; Lindell and Post, 2001; Lindell *et al.*, 2005) Marine *Synechococcus* WH7803 expresses *ntcA* at basic levels when grown on ammonium. These levels increase to intermediate when exposed to alternative N sources and maximum transcript levels are attained under N starvation (Lindell and Post, 2001; Lindell *et al.*, 2005).

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Marine diazotrophic cyanobacteria like *Cyanothece*, UCYN-A, *Richellia* and *Trichodesmium* can overcome N starvation by means of N_2 fixation (Capone *et al.*, 1997; Zehr *et al.*, 2001, 2008; Tripp *et al.*, 2010). Rates of N_2 fixation by *Trichodesmium* were not affected on the short term by $\leqslant 1\,\mu M$ ammonium or nitrate, but these rates decreased when these compounds were added at $\geqslant 10\,\mu M$ (Mulholland *et al.*, 2001). *Anabaena* PCC 7120 and *Trichodesmium* NIBB 11067 are capable of utilizing combined N compounds (Ohki *et al.*, 1991; Muro-Pastor *et al.*, 2002). Uptake rates of nitrate and urea were low in comparison with those of ammonium, glutamate and mixed amino acids (Mulholland and Capone, 1999; Mulholland *et al.*, 2001). A later report mentioned that N_2-fixing filaments of the non-heterocystous *Trichodesmium* IMS101 release ammonium to the surrounding medium (Mulholland *et al.*, 2001). Uptake rates of ammonium often exceed rates of N_2 fixation (Mulholland *et al.*, 2001). Although both organisms thrive in N-depleted environments, N assimilation is thus not as stringently controlled in *Trichodesmium* as was found in marine *Synechococcus* (Lindell and Post 2001; Lindell *et al.*, 1998, 2005). This led us to hypothesize that NtcA control over N assimilation may be principally different in the non-heterocystous filaments of *Trichodesmium* spp.

The role of *ntcA* in N assimilation by diazotrophic cyanobacteria is far less understood. Two putative binding sites were identified upstream of *ntcA* in *Anabaena* PCC 7120 (Muro-Pastor *et al.*, 2002), but they were not positioned at 40.5 nt as in most NtcA-activated promoters (Luque *et al.*, 1994; Herrero *et al.*, 2001). NtcA is required for heterocyst formation but its requirement for expression of the *nif* genes is not well established (Frias *et al.*, 1994; Ramasubramanian *et al.*, 1994; Muro-Pastor *et al.*, 1999). Studies of the *nifHDK* operon transcription in *Anabaena* PCC 7120 and *Trichodesmium* IMS101 do not identify NtcA recognition sequences (Ramasubramanian *et al.*, 1994; Chen *et al.*, 1998). NtcA binds only weakly to these promoter regions (Ramasubramanian *et al.*, 1994), but it is required for *nif* gene expression in *Anabaena (*Frias *et al.*, 1994). Transcript levels of *ntcA* and *nif* genes were inversely proportional in the unicellular diazotroph *Cyanothece* sp. (Bradley and Reddy, 1997). Nitrate and urea at 20 and 2 mM, respectively, did not inhibit *nifHDK* transcript accumulation in *Trichodesmium* (Dominic *et al.*, 1998). The nitrate transport in *Trichodesmium* IMS101 involves a permease of the Major Facilitator Superfamily (Wang *et al.*, 2000), different from the *nrtABCD*-encoded transporter in *Anabaena* (Frias *et al.*, 1997; Flores *et al.*, 2005). The *nirA* operon of the latter has distinct promoter elements but lacks a clearly defined NtcA-binding motif (Wang *et al.*, 2000). Here we report on the cloning, sequencing and transcriptional regulation of the *ntcA* gene from *Trichodesmium* IMS101 as well as its role in the utilization of N sources other than ammonium.

Materials and methods

Cultivation
Cultures of *Trichodesmium* sp. strain IMS101 were maintained in 50 and 150 ml Nalgene bottles (Rochester, NY, USA) using the seawater-based TMV medium that lacks a combined N source (Prufert-Bebout *et al.*, 1993). Cultures were maintained at 24 °C and a 12/12 h light/dark cycle with light provided by 'warm-white' fluorescent tubes at an incident irradiance of $20\,\mu mol\,quanta\,m^{-2}\,s^{-1}$. Culture bottles were swirled twice a day to avoid adhesion of filaments to the walls. Growth experiments were carried out in basic TMV medium as well as in TMV medium amended with one of the following N sources: $NaNO_3$, $NaNO_2$, NH_4Cl or urea (Merck Chemicals, Darmstadt, Germany; highest purity grade). Growth was monitored from both chlorophyll *a* concentrations and filament counts (Stihl *et al.*, 2001).

Nitrate uptake
To 250 ml cultures at mid-exponential phase treated with 0, 2, 20 or 200 μM of NH_4Cl, $NaNO_3$ was added to a final concentration of 10 μM. Samples (1 ml) were taken at discrete intervals over a 6 h timespan, filtered over a 0.2 μm membrane filter (Poretics Inc., Livermore, CA, USA) and diluted in 9 ml 0.2 μm filtered sea water. The diluted samples were kept at 4 °C until analysis. After passage over a Cd-column, nitrate was determined as nitrite on a flow injection nutrient analyzer (QuickChem, LACHAT Instruments, Loveland, CO, USA). The detection limit was 10 nM of nitrite.

Ammonium uptake
To 100 ml mid-log phase cultures grown with 0, 1, 10, 100 and 1000 μM of NO_3^-, NH_4Cl was added to a final concentration of 10 μM. Ammonium was determined on 2 ml samples taken at discrete intervals over a 6 h timespan. Samples were filtered through a 0.2 μm membrane filter (Poretics Inc.), followed by the addition of orthophtaldehyde (Holmes *et al.*, 1999). Sample fluorescence was determined on a Hoeffer Dynaquant fluorimeter (Holliston, MA, USA) 3 h after reagent addition and ammonium concentrations (with a 10 nM detection limit) were calculated using a NH_4Cl standard curve.

DNA analyses

Genomic DNA of *Trichodesmium* sp. strain IMS101 was extracted following the protocol published in West and Scanlan (1999). Genomic DNA was used as a template in PCR amplification with degenerate primers 1f and 4r used for amplification of the N-regulatory gene *ntcA* (Lindell *et al.*, 1998). The

ntcA amplicon was ^{32}P labeled using a KinaseMax oligolabeling kit (Invitrogen) and ^{32}P-α ATP, and hybridized to *Eco*RI–*Spe*I digests of *Trichodesmium* genomic DNA. A positive ~9.0 kb genomic fragment was identified by Southern blotting. A partial library was constructed by ligation of the 8.0–10.0 kb fragments into pBluescript KS+ plasmid following *Eco*RI–*Spe*I digestion of genomic DNA, and subsequent transformation into *Escherichia coli* host strain DH5α. A positive clone that contained a 9.0 kb *ntcA* fragment was identified by screening the library and was submitted to sequence analysis with primer hopping using the Dye Terminator Cycle Sequencing procedure and a model ABI 377 automated sequencer (PE-Biosystems Inc., Carlsbad, CA, USA).

NtcA expression and purification
E. coli Rosetta cells were grown on LB medium up to optical density at 600 nm of 0.6 and then induced with IPTG (0.4 mM) for 2 h. Cells were harvested by centrifugation and resuspended with 1/10 of the original volume in lysis buffer (10 mM Hepes pH 7.5, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween-20, 1 mM PMSF, 25 μg ml^{-1} DNAse, 0.2 mg ml^{-1} lysozyme, 10 mM $MgCl_2$ and 1:200 dilution of a protease inhibitor cocktail (Sigma, St Louis, MO, USA; P8465). The cells were disrupted using a French Press at 900 psi. The extract was centrifuged for 15 min at 10 000 *g*. The supernatant containing the MBP-NtcA fusion protein was then mixed with amylase beads, and incubated for 2 h at 4 °C with gentle agitation. The mixture was centrifuged (3500 r.p.m. for 3 min at 4 °C). Pellets containing the beads were passed through eight cycles of washes with column buffer (10 mM Hepes pH 7.5, 150 mM NaCl, 3.4 mM EDTA and 0.005% Tween-20), followed by centrifugation. Finally, the MBP-NtcA protein was eluted to exhaustion using 10 mM maltose in the column buffer. Purity of eluted fraction was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

PCR amplification of promoter regions
Promoter regions of cyanobacterial *ntcA* and *glnA* (glutamine synthetase) were inspected for putative NtcA-binding sites. Each of the promoter regions was PCR amplified using specific primers to yield DNA fragments of 100–200 bp in size. PCR amplicons were purified with a Promega kit (Wizard, SV gel and PCR clean-up system) and submitted to DNA sequence analysis of both strands. PCR products of 780 bp were cut out from the 1% agarose gels, purified with Promega (Madison, WI, USA) 'Wizard SV Gel and PCR clean-up system' kit and submitted to DNA sequence analysis of both strands.

Electrophoretic mobility shift assays
PCR products (20–60 ng) were incubated at 30 °C for 20 min with different concentrations of MBP-NtcA purified protein (0–3 μg protein), in binding buffer (25 mM Tris-HCl pH 8.0, 12% glycerol, 60 mM KCl and 4 mM spermidine), in 25 μl final volume. At the end of the incubation, 12.5 μl of 60% sucrose was added and the samples were separated on non-denaturing 6% polyacrylamide gels at 4 °C and 15 mA with TAE (40 mM Tris-acetate, 1 mM EDTA) containing 1 mM $MgCl_2$ as running buffer. The gels were stained with Vista-Green (Amersham, Piscataway, NJ, USA), and diluted 10 000 times in TAE buffer for 20 min. The gels were washed with TAE buffer and scanned using a phosphorimager (Fuji FLA300) at 473 nm emission.

RNA extraction
Samples (250 ml) of *Trichodesmium* culture were harvested halfway through the light period and filtered onto 5 μm pore size, polycarbonate membrane filters (Poretics). Filters were immediately transferred into 1 ml of ice-cold lysis buffer (100 mM LiCl, 30 mM Na-EDTA, 1% w/v SDS, 50 mM Tris-HCl pH = 8.0) and stored at −70 °C. Samples were thawed at room temperature and heated at 65 °C for 5 min, extracted once with an equal volume of acid phenol (pH = 4.5, pre-warmed at 65 °C), followed by phenol/chloroform and chloroform extraction. After isopropanol precipitation and ethanol washes, samples were dried at room temperature for 30 min and redissolved in sterile distilled water. Precipitates were then treated with RQ1 RNase-Free DNase (Promega) at 37 °C for 1 h, followed by phenol/chloroform extraction, 100% ethanol precipitation and 70% ethanol washes. The DNAse-treated RNA pellet was dissolved in 100 μl of RNAse-free water, and submitted to serial dilution. RNA samples were aliquoted to avoid contamination and RNA degradation during repeated thawing and freezing of samples.

RT-PCR
Semiquantitative reverse transcriptase-PCR (RT-PCR) protocols that compare relative transcript levels of target genes with those of housekeeping genes (including 16S or 18S rRNA) also use the internal control to titrate the exact amount of total RNA added to each reaction (Nicoletti and Sassy-Prigent, 1996; Ogretmen *et al*., 1998; Wang *et al*., 2002). The expression of N-regulated genes, *ntcA*, *napA* and *nifH*, was studied using this semiquantitative RT-PCR method with minor modifications. Sequences of forward and reverse primers and the expected size of PCR products are listed in Table 1. RT-PCR was performed using the Access RT-PCR System (Promega). A typical RT-PCR reaction of 25 μl contained 1 μl $MgSO_4$ (25 mM), 0.5 μl dNTPs (10 mM), 2.5 μl Buffer (10×), 1 μl of forward and reverse primers (25 μM), 0.5 μl of AMV (Avian Myeloblastosis Virus) reverse transcriptase (Promega), 0.5 μl of *Tfl* DNA polymerase, 1–10 μl of

Table 1 Sequence of primers and expected product sizes used in the reverse transcriptase-PCR (RT-PCR) expression studies

Gene	*Primer*	*Sequence*	*Size of product*
16S[a]	359F	5′-GGGGAATYTTCCGCAATGGG-3′	
	1313R	5′-CTTCACGTAGGCGAGTTGCAGC-3′	1.0 kb
nifH	nif-1F	5′-CTTAAACGCTAAAGCTCAAACTAC-3′	
	nif-1R	5′-CACGGATAGGCATTGCAAATCC-3′	275 bp
napA	nir-9F	5′-GCTGCACTCTATGGTGTG-3′	
	nir-10R	5′-GCAGGCAGCATAGAAACTACTGG-3′	405 bp
ntcA	ntc-1F	5′-CCAGGAGATCCTGCCGAGCGTGTC-3′	
	ntc-4R	5′-CCAATTGCTTCTGCAATTGCTTGGTG-3′	445 bp

[a]The primers for cyanobacterial 16S rRNA were reported in West and Scanlan (1999).

total RNA in proper dilution and RNase free water to 25 µl. To minimize tube-to-tube variation, Reaction buffer, $MgSO_4$, dNTP and water were assembled in advance as a master mix and aliquoted, immediately before each RT-PCR reaction, followed by the addition of enzymes and primers, and RNA template. RT-PCR reactions were run on a PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Waltham, MA, USA) with the following conditions: incubation at 48 °C for 1 h, denaturation at 94 °C for 2 min, followed by Taq polymerase addition and 26–30 cycles of 94 °C for 1 min, 45–55 °C for 1 min and 68 °C for 1 min 20 s, and then an extra step of elongation at 68 °C for 5 min. Annealing temperature was 45 °C for *napA* amplification, 50 °C for 16S rRNA and 55 °C for *nifH* and *ntcA* amplifications. For the purpose of quantification, template amounts ranging from 3 pg to 300 ng of total RNA were tested over 28 cycles to find suitable template concentration that permit exponential amplification for each of the target genes. The amount of total RNA was titrated based on the 16S rRNA gene as internal standard, and transcript levels for each gene were normalized to the transcript level of 16S rRNA in the same sample. A parallel RT-PCR negative control without AMV reverse transcriptase was carried out for each sample to ascertain absence of DNA contamination. Products of RT-PCR were run on 1.2% agarose/EtBr gels, viewed under ultraviolet light and analyzed by densitometry with Quantitiy One (Bio-Rad, Hercules, CA, USA). All experiments were replicated independently for 2–4 times.

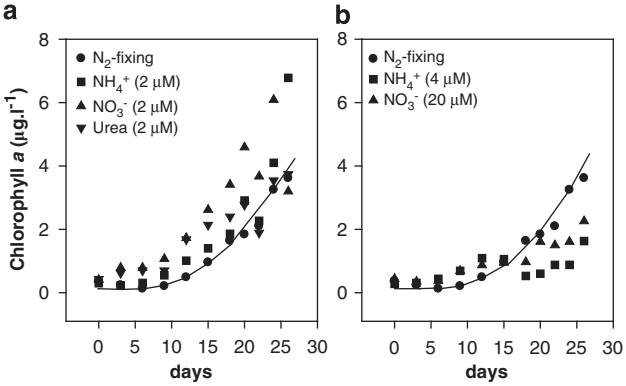

Figure 1 Diazotrophic growth (solid line) of *Trichodesmium* sp. IMS101 as compared with growth in the presence of low (2 µM) concentrations of nitrate and ammonium (**a**) and at elevated (4–20 µM) concentrations of these N sources (**b**).

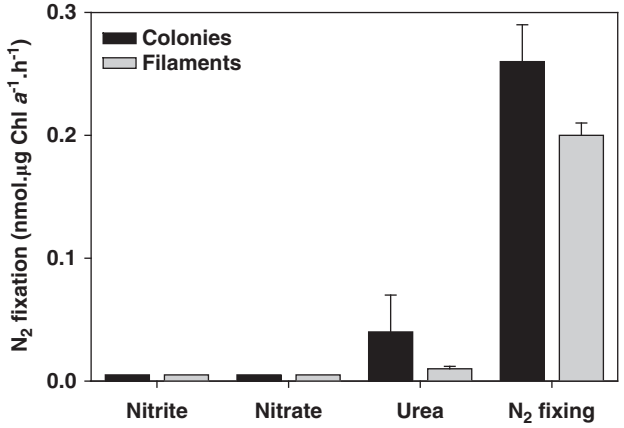

Figure 2 Ethylene reduction activities of *Trichodesmium* sp. IMS101 cultures grown on different N sources: nitrate (10 µM), nitrite (10 µM) and urea (5 µM) and in the absence of combined N (N_2 fixing).

Results

Nitrogen physiology

Although marine *Trichodesmium* spp. are known as abundant diazotrophs, they do have a capability to utilize inorganic N sources as well as organic N compounds like urea (Goering *et al*., 1966; Saino and Hattori, 1978). *Trichodesmium* IMS101 grew at a faster rate in TMV medium amended with 2 µM ammonium, nitrate or urea than in TMV medium lacking a combined N source (Figure 1a). However, growth was retarded or even fully inhibited at 4 µM ammonium and 20 µM nitrate, respectively (Figure 1b), and 40 µM urea (data not shown). N_2 fixation by *Trichodesmium* IMS101 was also affected by N source. N_2 fixation was much slower in *Trichodesmium* cultures with urea than in N-deplete controls (Figure 2). N_2 fixation was fully inhibited in the presence of 10 µM nitrate or 10 µM nitrite.

Nitrate utilization in turn was affected by the presence of ammonium (Figure 3). Nitrate acquisition was progressively inhibited with ammonium additions of increasing concentration (Figure 3a). Conversely, ammonium assimilation was not affected by nitrate and identical acquisition rates of ammonium were determined in the concentration range of 0 to 1 mM of this oxidized N source (Figure 3b). Thus, *Trichodesmium* preferentially assimilated ammonium over nitrate, and nitrate assimilation over N_2 fixation. Ammonium addition immediately downregulated nitrate assimilation and N_2 fixation. In the non-N_2-fixing *Synechococcus* WH7803, *ntcA* transcription is below detection when ammonium concentrations exceed 1 μM, and transcript levels are upregulated in nitrate-grown cells and become maximal under N-deplete conditions (Lindell and Post 2001; Lindell *et al*., 1998, 2005). It has been reported that ambient ammonium accumulates to ~2 μM in N_2-fixing (=N-deplete) cultures of *Trichodesmium* IMS101 (Mulholland *et al*., 2001), thus raising questions about the mechanisms of ammonium control over N assimilation and N_2 fixation in this organism. This led us to study aspects of transcription patterns for the N-regulatory gene, *ntcA,* in *Trichodesmium*.

Identification of ntcA

We PCR amplified a 449 bp product of the *Trichodesmium* IMS101 *ntcA* gene and used it to screen a clone library of *Eco*RI–*Spe*I-digested genomic DNA fragments. A positive clone of 9.5 kb molecular size was identified and sequenced (Genbank accession AF382392). Figure 4 shows the open reading frames (ORFs) deduced from the primary DNA sequence.

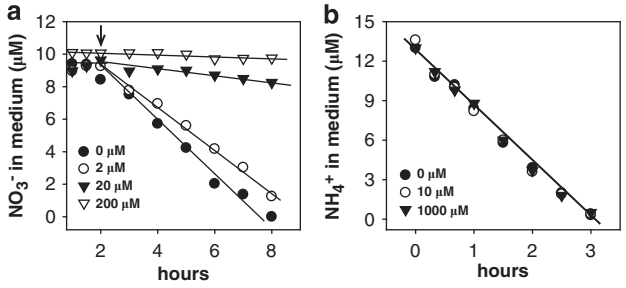

Figure 3 Effect of different ammonium additions (arrow) on nitrate uptake in nitrate-grown cultures of *Trichodesmium* IMS101 (**a**) and the effect of different nitrate concentrations on ammonium uptake by such cultures (**b**).

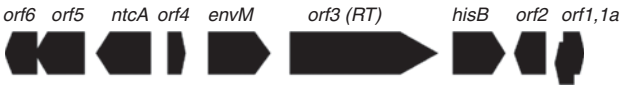

Figure 4 Gene arrangement of a 9 kb genomic DNA fragment of *Trichodesmium* IMS101 carrying the N-regulatory gene *ntcA* and its flanking regions. Block arrows denote the individual ORFs with their direction of transcription.

Upstream of *ntcA* we identified *fabI* (=*envH*, enoyl carrier reductase) and *hisB* (imidazole-glycerol phosphate dehydrogenase). Interestingly, an ORF located between these genes showed >60% amino-acid identity with reverse transcriptase of group II introns. Finally, the 3′ end of the 9.5 kb fragment consisted largely of non-coding DNA with the exception of an ORF with some similarity to transposons/transposases. The *ntcA* gene was found near the 5′ end of the fragment and was similar to *ntcA* of *Nostoc azollae* (E-value 3e–120), *Nostoc punctiforme* (E-value 1e–119) and *Nostoc* PCC 7120 (E-value 8e–109). Two ORFs (ORF5 and ORF6) immediately downstream of *ntcA* were in the same orientation as *ntcA* (Figure 4). ORF5 was first identified in *Anabaena* PCC 7120 (Ramasubramanian *et al*., 1994, 1996) and *ntcA*-ORF5 form a conserved gene pair in nearly all cyanobacteria (AF Post, unpublished data). As no clear promoter elements were identifiable upstream of ORF5, we studied the transcription of *ntcA* and ORF5 by RT-PCR. Attempts to amplify an RT product that linked the 3′ end of *ntcA* with the 5′ end of ORF5 were unsuccessful. However, Figure 5 shows that both genes were co-transcribed in N_2-fixing filaments of *Trichodesmium* IMS101. We identified a putative NtcA-binding motif CGTG..N_8..AACT starting at 96 nt upstream of the *ntcA* initiation codon in *Trichodesmium*, thus setting the stage for autoregulation of *ntcA* transcription. A second putative, atypical binding site (AGTA..N_9..TACA) was identified starting 307 nt upstream of the initiation codon. Overexpressed *Prochlorococcus* MED4 NtcA retarded DNA fragments with the *ntcA* promoter regions of *Prochlorococcus* MED4, but also that of *Synechococcus* WH7803 (Figure 6, lanes 5 and 6). Additions of NtcA to DNA with the *ntcA* and *glnA* promoters of the diazotrophs *Trichodesmium* IMS101 and *Anabaena* PCC 7102 (lanes 1–4) showed significant retardation of these fragments. This retardation was least pronounced for P*ntcA* of *Trichodesmium* where DNA binding caused a smear with only a faint product at higher molecular weight (Figure 6).

Transcriptional regulation

Semiquantitative RT-PCR was employed to assess *ntcA* transcript levels in *Trichodesmium* IMS101 under different N treatments. Figure 7 shows that

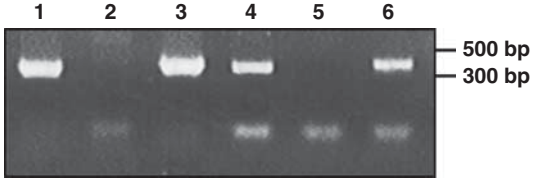

Figure 5 Co-transcription of *ntcA* (lanes 1–3) and ORF5 (lanes 4–6) in N_2-fixing filaments of *Trichodesmium* IMS101. PCR amplicons were derived from genomic DNA (lanes 1 and 4) and total RNA before (lanes 2 and 5) and after (lanes 3 and 6) reverse transcriptase treatment.

ntcA RNA was readily amplified from total RNA of N_2-fixing filaments after RT. NH_4^+ additions of 2–2000 μM enhanced *ntcA* transcript levels in *Trichodesmium* after 1 h of incubation (Figure 7a). Similarly, NO_3^- additions of 0.5–1000 μM caused an approximate 40% increase in *ntcA* transcript level (Figure 7b). The effect of N source on *ntcA* transcription was not of a transient nature and transcript levels stayed high for at least 24 h (Figure 7c).

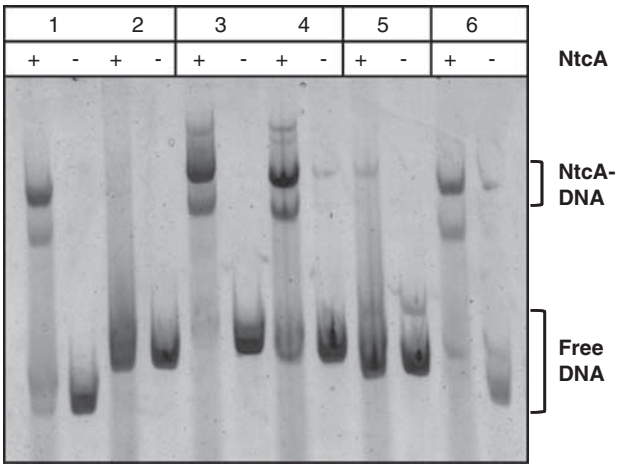

Figure 6 NtcA binding to N-regulated promoters in electrophoretic mobility shift assay (EMSA) experiments with over-expressed, maltose-column purified NtcA of *Prochlorococcus* strain MED4. (Lanes 1 and 2) P*glnA* and P*ntcA* of *Trichodesmium* IMS101, (lanes 3 and 4) P*glnA* and P*ntcA* of *Anabaena PCC 7120*, (lane 5) P*ntcA* of *Synechococcus* WH7803, and (lane 6) P*ntcA* of *Prochlorococcus* MED4.

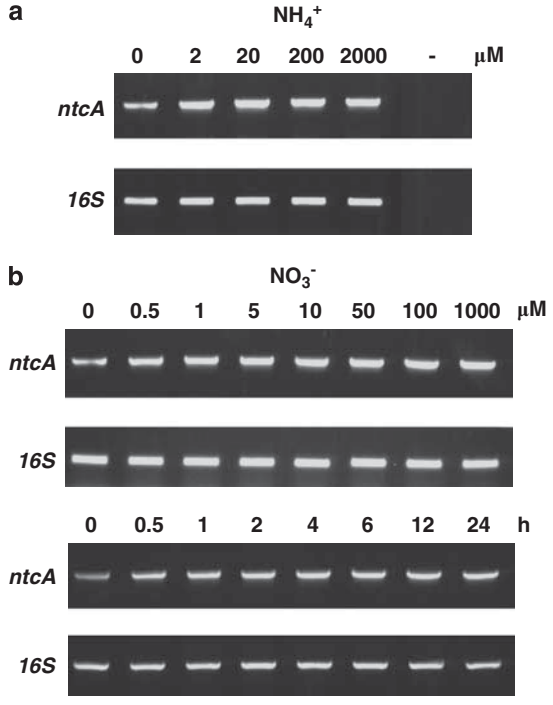

Figure 7 Levels of *ntcA* transcripts in *Trichodesmium* IMS101 filaments after different ammonium treatments for 1 h (**a**), at different concentrations of nitrate for 1 h and for different times with 100 μM nitrate (**b**). Total RNA was titrated to equal amounts using 16S rRNA as internal control.

N_2-fixing filaments of *Trichodesmium* IMS101 accumulated high levels of *nifH* transcript as expected (Figure 8). However, similar transcript levels of *nifH* were found in the presence of nitrate (100 μM) or ammonium (2–20 μM). Such filaments have significantly lower nitrogen-fixation rates (Figure 2) and utilize the combined N source. At ammonium concentrations in excess of 20 μM, *nifH* transcript levels declined and no *nifH* transcripts were detected at NH_4^+ concentrations of $\geqslant$200 μM (Figure 8).

Nitrate utilization requires the expression of the nitrate transporter encoded by *napA*, which forms part of the *nirA* operon in *Trichodesmium* IMS101 (Wang *et al.*, 2000). Both N_2-fixing and ammonium-utilizing filaments contained low levels of *napA* transcripts (Figure 9a), as compared with the levels found under nitrate nutrition (Figure 9b). However, the nitrate effect on *napA* expression was counteracted by addition of ammonium. Transcript levels of *napA* declined sharply when ammonium was

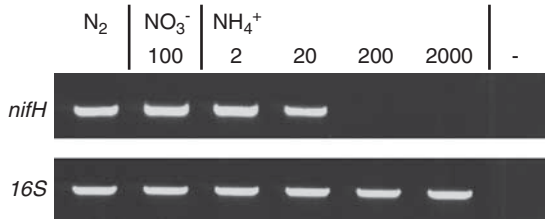

Figure 8 *nifH* transcript levels in *Trichodesmium* IMS101 filaments cultured in N_2-fixing condition, in 100 μM NO_3^- or in NH_4^+ of different concentrations. The amount of total RNA of different samples was titrated to the same level using house-keeping 16S rRNA gene as internal control as shown in the down row of each panel. The results shown are representative of at least two independent experiments.

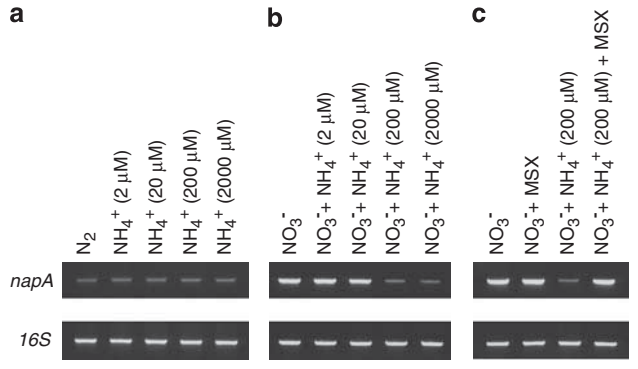

Figure 9 Levels of *napA* transcript in *Trichodesmium* IMS101 filaments 1 h following the addition of different concentrations of ammonium (**a**), with different combinations of ammonium with 100 μM NO_3^- for 1 h (**b**) and (**c**) with different combinations of nitrate (100 μM), ammonium (200 and 2000 μM) and MSX (100 μM).

present—alongside nitrate—at $\geqslant 200\,\mu M$ (Figure 9b), whereas 2–20 μM additions did not affect transcript levels. These findings indicate that ammonium repressed the transcription of *nifH* and *napA*, but did not affect *ntcA* expression. In order to establish whether nitrate-induced *napA* expression was controlled by ammonium repression or nitrate stimulation, we studied *napA* expression in the presence of 100 μM L-methionin-DL-sulfoximine (MSX), an inhibitor of glutamine synthase activity. Figure 9c shows that MSX treatment allowed for full expression of *napA* in nitrate-grown filaments of *Trichodesmium* in the presence of ammonium, whereas *napA* expression in control experiments was suppressed.

Discussion

Nitrogen utilization

The marine diazotrophic cyanobacterium *Trichodesmium* IMS101 utilized nitrate, urea and ammonium similar to previous reports (Ohki *et al.*, 1991; Mulholland and Capone, 1999; Mulholland *et al.*, 2001). Growth on combined N was sustained at low concentrations ($\leqslant 2\,\mu M$) only and N_2-fixation rates were suppressed at combined N concentrations of $\geqslant 5\,\mu M$. Ammonium repressed both N_2 fixation and nitrate assimilation at ecologically relevant concentrations. Similarly, micromolar concentrations of nitrate and nitrite inhibited N_2 fixation. These inhibitory actions establish a hierarchy in which *Trichodesmium* filaments prefer ammonium over nitrate before they resort to N_2 fixation. This hierarchy was not observed in the transcriptional regulation of N genes in *Trichodesmium* IMS101. Whereas ammonium repressed the transcription of key genes in nitrate utilization (*napA*) and N_2 fixation (*nifH*), nitrate additions failed to repress *nifH* transcript levels. Surprisingly, ammonium did not repress the transcription of the N-regulatory gene *ntcA*, and its mRNA accumulated to high levels, even in the presence of 2–2000 μM ammonium. In *Synechococcus* WH7803, *ntcA* transcript levels dropped below detection when ammonium concentrations exceed 1 μM (Lindell and Post, 2001; Lindell *et al.*, 2005).

Ammonium repression

Ammonium availability is usually considered the key factor in N-adaptive responses through its control over *ntcA* transcription (Herrero *et al.*, 2001). The use of the glutamine synthetase inhibitor MSX indicated that metabolized (as opposed to ambient) ammonium suppressed the transcription of N genes in the *Trichodesmium* filaments, similar to our findings in marine *Synechococcus* (Lindell and Post, 2001; Lindell *et al.*, 2005). This is consistent with the concept that α-oxoglutarate accumulates and acts as the NtcA activator molecule when ammonium supplies are limiting (Muro-Pastor *et al.*, 2001; Tanigawa *et al.*, 2002). NtcA-binding affinity to its DNA targets is enhanced by α-oxoglutarate (Vazquez-Bermudez *et al.*, 2002). The high *ntcA* transcript levels at $>2\,\mu M$ ammonium would be consistent with high α-oxoglutarate levels in *Trichodesmium*. Intracellular levels of α-oxoglutarate ranged between 100 and 1600 pmol per colony (equivalent to 3–200 fmol per cell) over the course of a diel cycle (Mulholland and Capone, 1999). This suggests that α-oxoglutarate might be available in sufficient amounts to continuously activate NtcA in *Trichodesmium*, especially among the high light-exposed surface populations with high rates of photosynthesis. Such populations have N_2-fixing capacity and release ammonium into the environment (Mulholland and Capone, 1999; Mulholland *et al.*, 2001). In order to sustain N_2-fixation and *nifHDK* transcription in an NtcA-dependent manner, they would have to counteract ammonium repression with high cellular levels of α-oxoglutarate.

Transcriptional regulation

Autoregulation of *ntcA* transcription requires NtcA-binding upstream of its own gene. We identified *ntcA* and its neighboring genes on a 9.5 kB genomic fragment in *Trichodesmium* IMS101. The DNA sequence of this fragment is identical to that found in the complete genome sequence (Genbank accession NC_008312) that was determined subsequently. The genome carries a CRP-like transcriptional activator (locus tag Tery-1557) that was predicted to control the transcription of *nifB* and *nifX-K1-W* (see http://regprecise.lbl.gov/RegPrecise). This genome further contains a single *ntcA* copy (Tery-2023) with a predicted regulon of 18 genes/operons. The NtcA regulon contains *ntcB* (Tery-4333), which encodes a transcriptional activator of the LysR family that is required for nitrate assimilation (Aichi and Omata, 1997; Frías *et al.*, 2000; Flores *et al.*, 2005). Interestingly, this NtcA regulon prediction suggests that *ntcA* transcription is not autoregulated. Two putative NtcA-binding sites upstream of the *ntcA* start codon were identified in this study. These sites are degenerate variants of the consensus-binding site and they were weakly bound by *Prochlorococcus* NtcA. Interestingly, we found that the same, heterologous NtcA bound strongly to the *Anabaena ntcA* promoter region, whereas it interacts only weakly binds with its native NtcA (Ramasubramanian *et al.*, 1994). N-induced transcription start points for *ntcA* have been identified at −49 and −180 nt in *Anabaena* PCC 7120 (Olmedo-Verd *et al.*, 2008), a configuration similar to that observed in *Trichodesmium*. NtcA is further required for development of N_2 fixation in *Anabaena* PCC 7120 (Muro-Pastor *et al.*, 1999), but it is not certain that NtcA activates *nifHDK* transcription. No correlation was observed between *ntcA* transcript levels and that of *nifHDK* in

Cyanothece BH68K (Bradley and Reddy, 1997). Moreover, *nifHDK* transcripts of *Trichodesmium* IMS101 show a clear diel pattern, whereas those of *ntcA* do not (Chen *et al.*, 1998). Transcription of *ntcA* occurs from both constitutive and induced transcription start points in *Synechococcus* spp. and thus yields two transcript populations, the latter one regulated by NtcA (Luque *et al.*, 1994; Lindell *et al.*, 1998). The *ntcA* expression pattern with high transcript levels in the presence of ammonium as presented here for *Trichodesmium* is also encountered in the N_2 fixers *Cyanothece* and *Anabaena (*Ramasubramanian *et al.*, 1996; Bradley and Reddy, 1997). The latter produces three different transcripts, two of which seem to originate from an atypical promoter that involves NtcA binding (Ramasubramanian *et al.*, 1996). Thus, *ntcA* may be involved in the development of nitrate utilization as its expression is high both in nitrate-grown *Trichodesmium* IMS101 (this study) and in *Cyanothece* BH68K (Bradley and Reddy, 1997). The upstream region of the *nir* operon (which includes the reductase genes and the nitrate transporter *napA*) in *Trichodesmium* was shown to have promoter elements (Wang *et al.*, 2000), but lacks a well-defined NtcA-binding site. However, enhanced transcription of *napA* overcame ammonium repression in the presence of the glutamine synthetase inhibitor MSX, consistent with NtcA regulation in *Synechococcus* spp. (Suzuki *et al.*, 1993; Lindell and Post, 2001; Tanigawa *et al.*, 2002; Lindell *et al.*, 2005). Not all regulatory aspects of NtcA are known in diazotrophic cyanobacteria. These may include post-transcriptional regulation, different modes of interaction with promoter regions of N genes and conformational change in NtcA protein. Whereas this remains to be addressed in future studies, our results show unequivocally that ammonium repression and *ntcA* transcription are uncoupled in the globally important marine diazotroph *Trichodesmium* sp.

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