All4312, an NtcA-regulated two-component response regulator in *Anabaena* sp. strain PCC 7120

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**Abstract**

All4312, encoded by open reading frame *all4312* in the genome of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, exhibits a CheY-like receiver domain and an output domain similar to that of OmpR, characteristic of two-component response regulators. Expression of *all4312* was directly regulated by NtcA, the global transcriptional regulator of nitrogen assimilation in cyanobacteria. Features characteristic of NtcA-activated promoters were also found upstream from genes encoding All4312 homologues in several other cyanobacterial genomes. Expression of *all4312* was however unaffected in a mutant of *hetR*, which encodes a regulator triggering heterocyst development. The function of All4312 may be related to the cellular response to nitrogen deprivation.

**Introduction**

Cyanobacteria are a group of widely distributed photo-trophic prokaryotes that carry out oxygenic, plant-type photosynthesis. Cyanobacteria are able to use different nitrogen sources including nitrate and ammonium and many strains can also fix atmospheric nitrogen. Ammonium is assimilated in preference over nitrate, which is used in preference over dinitrogen (Flores & Herrero, 1994; Herrero et al., 2001). Some filamentous cyanobacteria, including *Anabaena* spp., are able to differentiate, in response to nitrogen deficiency, cells specialized in nitrogen fixation called heterocysts. Assimilation of different nitrogen sources is globally regulated in these organisms by NtcA, a transcriptional regulator belonging to the CAP (or CRP) family that, in the absence of ammonium, activates the expression of genes required for the assimilation of alternative nitrogen sources including atmospheric nitrogen (Vega-Palas et al., 1992; Frias et al., 1994; Luque et al., 1994; Wei et al., 1994; Herrero et al., 2001). NtcA binds to specific sites in the promoter regions of the regulated genes and activates their expression in response to ammonium withdrawal (Luque et al., 1994). The structure of consensus NtcA-binding sites has been defined (Luque et al., 1994) and several NtcA-activated promoters have been shown to carry an NtcA-binding sequence in the form GTAN₈TAC, which is located about 22 nucleotides upstream from the promoter – 10 hexamer (Herrero et al., 2001). NtcA-binding sites with a repressor, rather than activating, role have been identified in a few cases (Herrero et al., 2001). The NtcA protein appears to have as a positive effector 2-oxoglutarate (Vázquez-Bermúdez et al., 2002, 2003; Luque et al., 2004), which is an indicator of the C to N balance in cyanobacterial cells (Muro-Pastor et al., 2001). For some promoters, the PII protein is also needed for full activation by NtcA under N deficiency (Aldehni et al., 2003; Paz-Yepes et al., 2003).

A number of cases have been described in which NtcA-mediated nitrogen regulation is not directly operated by NtcA (Herrero et al., 2001, 2004). In those cases, one would expect that NtcA activates the expression of regulatory proteins that would then be responsible for direct regulation, but no such effector is yet known. Here we describe a protein with homology to two-component response regulators whose expression is directly operated by NtcA in *Anabaena* sp. strain PCC 7120.

**Materials and methods**

**Strains and growth conditions**

This study was carried out with the heterocyst-forming cyanobacterium *Anabaena* sp. (also known as *Nostoc* sp.)
strain PCC 7120 and derivative strains CSE2, an insertional mutant of the ntcA gene (Frias et al., 1994), and 216, which bears a point mutation in the hetR gene (Buikema & Haselkorn, 1991). They were grown photoautotrophically at 30 °C in BG11C medium [BG11 medium (Rippka et al., 1979) without NaN3O and supplemented with 10 mM of NaHCO₃] supplemented with 6 mM NH₄Cl plus 12 mM N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES)–NaOH buffer (pH 7.5), bubbled with a mixture of CO₂ and air [1% volume in volume (v/v)], and supplemented with 2 µg mL⁻¹ of streptomycin and 2 µg mL⁻¹ of spectinomycin in the case of strain CSE2 and those strains bearing fusions between the region upstream from \( \text{all4312} \) and the \( \text{gfp} \) gene.

For RNA isolation, cells growing exponentially in BG11C medium supplemented with NH₄Cl were harvested at room temperature and either used directly (time 0) or washed with BG11C medium, resuspended in 11C (nitrate-containing) or in BG11C (nitrogen-free) medium and further incubated under culture conditions for the number of hours indicated in each experiment. For the analysis of \( \text{gfp} \) expression, cells growing exponentially in BG11C medium supplemented with NH₄Cl were harvested at room temperature and either used directly (time 0) or washed with BG11C medium, resuspended in BG11C (nitrate-containing) or in BG11C (nitrogen-free) medium and further incubated under culture conditions for the number of hours indicated.

Escherichia coli strains were grown in Luria broth (LB) supplemented, when necessary, with antibiotics added at standard concentrations (Ausubel et al., 2005).

DNA and RNA isolation and manipulation

Total RNA from \textit{Anabaena}\ sp. strain PCC 7120 and its derivatives was isolated as previously described (Muro-Pastor et al., 2002). Primer extension analysis of the \textit{all4312} transcripts was carried out as described previously (Muro-Pastor et al., 1999). The oligonucleotide used as primer was \( \text{all4312} \) (complementary to positions +104 to +84 relative to the translation start of \( \text{all4312} \)). Plasmid pCSAM113 (see below) was used to generate dideoxy-sequencing ladders using the same primer. Sequencing was carried out by the dideoxy chain-termination method, using a T7-\text{SequenaseTM} kit (Amersham Biosciences, Uppsala, Sweden) and \([\alpha\text{-}35\text{S}]\)-thio dATP. Northern analysis was carried out as described (Muro-Pastor et al., 1999). The \textit{all4312} probe used was a 714-bp \text{NcoI–HincII} internal fragment that covers almost the whole open reading frame, isolated from pCSAM115.

Plasmid isolation from \textit{E. coli}, transformation of \textit{E. coli}, digestion of DNA with restriction endonucleases, ligation with \text{T4} ligase, and PCR were performed by standard procedures (Ausubel et al., 2005).

Plasmids

Plasmid pCSAM113 contains a 602 bp DNA fragment PCR-amplified using oligonucleotides \text{all4312-2} (corresponding to positions –488 to –467 with respect to the translational start of \( \text{all4312} \)) and \text{all4312-1} (see above) and chromosomal DNA from \textit{Anabaena}\ sp. strain PCC 7120 as template, cloned into the pGEM-T vector (Promega, Madison, WI). In pCSAM113a the orientation of the insert is such that sequences corresponding to the \text{all4312-1} oligonucleotide are close to the SpeI site in the polynucleotide pGEM-T. In pCSAM113b the insert is cloned in the opposite orientation. Plasmid pCSAM115 contains a 1102 bp DNA fragment PCR-amplified with oligonucleotides \text{all4312-Nco} (corresponding to positions –12 to +10 with respect to the translational start of \( \text{all4312} \), introducing a \text{NcoI} site at the start codon) and \text{all4312-3} (complementary to positions +331 to +309 with respect to the translational stop of \( \text{all4312} \)) cloned into the pGEM-T vector. This fragment contains the complete \( \text{all4312} \) ORF plus 331 bp downstream of \( \text{all4312} \).

Disruption of \textit{all4312}

\( \text{all4312} \) in plasmid pCSAM115 was disrupted by introducing AccI-ended SmSp-resistance cassette C.S3, excised from pRL463 [pRL138/LHEH1(BamHI)/C.S3, nomenclature as in Elhai & Wolk, 1988a], into the \text{ClaI} site internal to \( \text{all4312} \) rendering pCSAM116. The ca. 3.5-kbp \text{PvuII} fragment from pCSAM116, containing the disrupted \( \text{all4312} \) plus some downstream sequences (\text{all4312-}:C.S3), was cloned into the Klenow-filled \text{BglII} site of \text{SacB} vector pRL278 (Black et al., 1993). The resulting plasmid, pCSAM118 was transferred to \textit{Anabaena}\ sp. strain PCC 7120 by conjugation as described (Elhai & Wolk, 1988b), using the helper plasmid pRL623 (Elhai et al., 1997), and SmSp-resistant colonies were selected. Isolation of double recombinants was attempted but, under our culture conditions, no sucrose-resistant, Nm-sensitive colony could be obtained in several rounds of selection.

Fusions to the green fluorescent protein

Fusions of the promoter region of \( \text{all4312} \) to the \( \text{gfp} \) gene encoding green fluorescent protein were prepared as follows. SmSp-resistance cassette C.S3, excised from pRL463 (see above) as an \text{XbaI} fragment, was inserted into the \text{SpeI} site located in the polynucleotide of pCSAM113b upstream from the \( \text{all4312} \) promoter, rendering pCSAM114. A \text{SalI–NcoI} (Klenow-filled) fragment from pCSAM114, containing C.S3 followed by the promoter region of \( \text{all4312} \), was placed upstream from the \( \text{gfp} \) gene in \text{SalI}, EcoRV-digested pSEL19, rendering pCSAM117 [pSEL19 contains a promoterless \( \text{gfp} \) gene PCR-amplified using oligonucleotides...
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gfp-1 (5′GGAGATATCCATATGATGAAGG3′, introducing an EcoRV site upstream from the start codon of the gfp gene) and gfp-2 (5′AAGCAGGCTTGGCAATGGCGT3′) and plasmid pKEN2-GFPmut2 (Ezzaz-Nikpay et al., 1994; Cormack et al., 1996) as a template, cloned into the pGEM-T vector in the same orientation of the β-lactamase gene. A PsI fragment from pCSAM117, containing C.S3 followed by a transcriptional fusion between the region upstream from all4312 and the gfp gene, was cloned in both orientations into the PsI site of pCSAV80 [a derivative of pCSAM28 (Muro-Pastor et al., 1992) in which the nucA gene has been inactivated by digestion with HindIII followed by Klenow treatment and religation], designed for integration of constructs into the nucA region located in the α-megaplasmid of Anabaena sp. strain PCC 7120. The resulting plasmids, pCSAM119a and pCSAM119b, were transferred to Anabaena by conjugation as described above and SmSp resistant colonies were selected. The C.S3 cassette bears transcriptional terminators that are effective in Anabaena sp. strain PCC 7120 (Frias et al., 1997), ensuring that the Pall4312:gfp fusion is not transcribed from an external promoter other than Pall4312.

The accumulation of GFP reporter was analysed by laser confocal microscopy. Samples were observed using a Leica HCX PLAN-APO 63X 1.4 NA oil immersion objective confocal microscopy. Samples were observed using a Leica TCS SP2 confocal laser-scanning microscope (Leica, Wetzlar, Germany). GFP was imaged using the attached to a Leica TCS SP2 confocal laser-scanning microscope (Leica, Wetzlar, Germany). GFP was imaged using the

Results

Expression of all4312

As a result of a search for NtcA boxes upstream from Anabaena sp. strain PCC 7120 ORFs and their corresponding homologues in Nostoc punctiforme, all4312 was identified as a gene exhibiting a putative regulatory NtcA box in similar positions in both organisms (J. Elhai and A. M. Muro-Pastor, unpubl. results). Genes putatively encoding homologues of All4312 were identified in 10 cyanobacterial genomes. The regions upstream from the corresponding open reading frames contained NtcA boxes with the consensus sequence GTAN\textsubscript{8}TAC centered at positions ranging from 66 to 130 nucleotides upstream of the predicted translational start (Fig. 1). Furthermore, in four cases, the NtcA box contains the nucleotides CA in the second and third positions after the GTA triplet, a feature conserved in many consensus-type NtcA-binding sites (Herrero et al., 2001). The observation that NtcA boxes are located in such a position in all ten genomes suggests that NtcA might be involved in expression of the corresponding genes.

Because NtcA is known to regulate expression of genes in response to the nitrogen status of the cells, expression of all4312 in Anabaena sp. strain PCC 7120 was analysed in ammonium-grown filaments incubated for 4 or 24 h in the presence of nitrate or in the absence of combined nitrogen.

Band-shift assays

A 330 bp DraI–SpeI fragment from pCSAM113a was used in band shift assays with purified NtcA. This fragment includes sequences −220 to +104 with respect to the translation start of all4312. DNA fragments were end-labelled with T4 polynucleotide kinase and [γ-32P]dATP. Assays were carried out as described previously (Luque et al., 1994) in the presence or absence of 0.6 mM 2-oxoglutarate (Vázquez-Bermúdez et al., 2002), and they contained about 0.5 fmol of labelled fragment and 2.5–15 pmol of purified His-tagged NtcA (Muro-Pastor et al., 1999).

![Fig. 1](https://academic.oup.com/femsle/article/256/1/171/498209)

Identification of putative NtcA boxes in the regions upstream from genes encoding All4312 and its homologues in several cyanobacterial genomes. Sequences are aligned at the predicted NtcA boxes and distances to the putative translation start (GTG or ATG codon) of the corresponding genes are indicated. The transcription start point determined for all4312 is underlined, and the corresponding putative promoter – 10 hexamer is indicated. Sequences aligned correspond to those upstream from all4312 (first line) and genes encoding All4312 homologues from Anabaena variabilis strain ATCC 29413 (gi75907486), Nostoc punctiforme strain PCC 73102 (gi53687472), Trichodesmium erythraeum strain IMS101 (gi71676548), Synechocystis sp. strain PCC 6803 (gi16332107), Crocosphaera watsonii strain WH 8501 (gi67924898), Synechococcus elongatus strain PCC 7942 (gi45513869), Synechococcus elongatus strain PCC 6301 (gi56751647), Gloeobacter violaceus strain PCC 7421 (gi35212842), and Thermosynechococcus elongatus strain BP-1 (gi22295054).

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Northern blot hybridization (Fig. 2a) showed that expression in the wild-type strain was very low in the presence of ammonium, was slightly induced in the presence of nitrate as sole nitrogen source and was strongly induced after 4 h of nitrogen deficiency. A time-course of induction of all4312 in response to nitrogen deprivation was also carried out (Fig. 2b). Induction of all4312 took place in wild-type cells, after less than 3 h of nitrogen deficiency, and expression was highest between 6 and, at least, 12 h. Induction of expression did not take place in the ntcA mutant strain CSE2. Transcript 5' ends for the all4312 gene in Anabaena sp. strain PCC 7120 were analysed by primer extension (Fig. 3). A unique transcription start point was identified 27 nucleotides upstream from the translation start of all4312. Consistent with northern hybridization results shown in Fig. 2, expression from this start point was very low in ammonium-grown cells and increased after 3 h of nitrogen deficiency. Also, the 5' end of this mRNA could not be detected in the ntcA mutant CSE2 (Fig. 3).

Expression of all4312 in the hetR mutant strain 216 was also analysed by northern hybridization (Fig. 2b) and primer extension (Fig. 3). Expression of all4312 was not altered in the hetR mutant, and utilization of the transcription start point located 27 nucleotides upstream from the translational start of all4312 in the hetR mutant was similar to that observed in the wild type.

### Analysis of gfp fusions

Expression of transcriptional fusions of the all4312 promoter region to a promoterless gfp gene was analysed in ammonium-grown filaments incubated in nitrogen-free medium for 9 or 24 h. Ammonium-grown filaments were also analysed for comparison. Several Anabaena clones carrying fusions to gfp were analysed, and the results for two clones, bearing the promoter-gfp fusion in both orientations with respect to vector sequences, are shown in Fig. 4. GFP fluorescence of strains carrying the Pall4312::gfp fusions was low in ammonium-grown filaments but was higher after nitrogen deprivation. Expression of transcriptional fusions was similar in all cells of the filament. Some highly fluorescent cells appeared occasionally, but no correlation could be established with proheterocysts or mature heterocysts.

### Analysis of the all4312 promoter

The analysis of the region upstream from all4312 indicated that the transcription start point determined by primer extension was located at the standard distance from the NtcA boxes found in consensus Class II NtcA-activated promoters. Thus, the NtcA box is centered at about −41.5 nucleotides with respect to the tsp, which is preceded by a −10 box in the form TAN₇T (see Fig. 1). Band-shift assays were carried out with purified NtcA protein and a fragment from the all4312 upstream region containing the NtcA box (Fig. 5). Retardation of the labelled DNA fragment in response to the addition of increasing amounts of NtcA was observed, indicating binding of NtcA. Affinity of NtcA for this promoter fragment was higher in the presence of 2-oxoglutarate.

### Discussion

The observations described in this work indicate that expression of all4312 is directly regulated by NtcA, the global transcriptional regulator for N control in cyanobacteria. In response to nitrogen deficiency, an increase in the expression of the ntcA gene (Muro-Pastor et al., 2002) and...
accumulation of the NtcA protein (Olmedo-Verd et al., 2005) take place in Anabaena sp. strain PCC 7120. Such increased expression of NtcA requires HetR, a positive-acting factor for heterocyst differentiation (Buikema & Haselkorn, 1991). Because HetR is required for the transient increase of NtcA levels that takes place during nitrogen deficiency (Muro-Pastor et al., 2002), the observation that HetR is not involved in the expression of all4312 suggests that those increased levels of NtcA are not required for all4312 expression. Expression of all4312 would thus result from the activation of NtcA in response to nitrogen deficiency. Combined nitrogen deprivation would provoke an increase in the cellular levels of 2-oxoglutarate (Laurent et al., 2005), which has the effect of increasing NtcA affinity for binding to the all4312 promoter (Fig. 5). Thus, one would predict that this promoter is activated early, and probably in all cells of the filament, upon combined nitrogen deprivation (Herrero et al., 2004). This appears to be actually the case, as shown by the Pall4312-gfp transcriptional fusions analysed in this work (Fig. 4). A number of promoters are activated by NtcA upon ammonium withdrawal (Herrero et al., 2001). One of them, the ghmA P₁ promoter (Valladares et al., 2004), bears an NtcA binding site that is identical in sequence to that of the all4312 promoter characterized in this work.

Fig. 3. Primer extension analysis of expression of all4312 in Anabaena sp. strain PCC 7120, the ntcA insertional mutant CSE2, and the hetR strain 216. Assays were carried out with RNA isolated from ammonium-grown filaments (lanes labelled 0) or from ammonium-grown filaments incubated in nitrogen-free medium for the number of hours indicated in each case. The oligonucleotide used for extension was all4312-1 (see Materials and methods). Sequence ladders were generated with the same oligonucleotide and plasmid pCSAM113. Arrowhead points to the putative tsp identified 27 nucleotides upstream from the translation start of the gene.

Fig. 4. Expression of fusions between the promoter region upstream from all4312 and the gfp gene. Fluorescent emission was determined in ammonium-grown filaments or in ammonium-grown filaments incubated in nitrogen-free medium for the number of hours indicated. GFP fluorescence (left panels) and cyanobacterial autofluorescence (right panels) is shown. Cells lacking autofluorescence are mature heterocysts. A and B show fluorescent emission of two different clones (see Materials and methods for details). Other clones analysed showed similar results. White triangles point to proheterocysts or heterocysts.
Genomic-wide analysis of genes encoding multi-domain proteins in *Anabaena* sp. strain PCC 7120 has identified a remarkably large number of genes for two-component systems (Ohmori *et al*., 2001; Wang *et al*., 2002). Such abundance of regulatory elements might reflect the complexity of *Anabaena* regulatory networks and physiology. All4312 would be the first known response regulator of a two-component regulatory system that might be involved in NtcA-mediated regulation. The nature of the corresponding sensor component, if any, is currently unknown. Genomic analysis of all two-component systems and signalling proteins that can be identified in *Anabaena* sp. strain PCC 7120 reveals no clustering of *all4312* with any signalling protein (Wang *et al*., 2002). It is conceivable that All4312 integrates signals from NtcA, the global nitrogen regulator activating the expression of the response regulator, and from whatever sensor component that might modify the activity of this protein.

Because we have failed in the isolation of a fully segregated *all4312* insertional mutant, we do not know which physiological traits may be regulated by All4312. However, the fact that increased expression of *all4312* in response to N step-down takes place in all vegetative cells of the filament, rather than being localized to pro-heterocysts or heterocysts, suggests a function for All4312 related to a response to N stress rather than specifically to heterocyst differentiation. Additionally, the expression of *all4312*, which is increased in response to N step-down, decreases in the wild type by 24 h post-induction, when N₂ fixation has started (Fig. 2). A function related to N stress would also be consistent with the presence of All4312 homologues exhibiting similar regulatory features (i.e. NtcA boxes in their promoter regions) in non-nitrogen-fixing cyanobacteria.

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