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

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22

23 **Abstract**

24

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

36

37 **Introduction**

38

39 Cyanobacteria are a group of widely distributed phototrophic prokaryotes that
40 carry out oxygenic, plant-type photosynthesis. Cyanobacteria are able to use
41 different nitrogen sources including nitrate and ammonium and many strains
42 can also fix atmospheric nitrogen. Ammonium is assimilated in preference over
43 nitrate, which is used in preference over dinitrogen (Flores & Herrero, 1994;
44 Herrero *et al.,* 2001). Some filamentous cyanobacteria, including *Anabaena*
45 spp., are able to differentiate, in response to nitrogen deficiency, cells
46 specialized in nitrogen fixation called heterocysts. Assimilation of different

47 nitrogen sources is globally regulated in these organisms by NtcA, a
48 transcriptional regulator belonging to the CAP (or CRP) family that, in the
49 absence of ammonium, activates the expression of genes required for the
50 assimilation of alternative nitrogen sources including atmospheric nitrogen
51 (Vega-Palas *et al.,* 1992; Frías *et al.,* 1994; Luque *et al.,* 1994; Wei *et al.,* 1994;
52 Herrero *et al.,* 2001). NtcA binds to specific sites in the promoter regions of the
53 regulated genes and activates their expression in response to ammonium
54 withdrawal (Luque *et al.,* 1994). The structure of consensus NtcA-binding sites
55 has been defined (Luque *et al.,* 1994) and several NtcA-activated promoters
56 have been shown to carry an NtcA-binding sequence in the form $GTAN_8TAC$,
57 which is located about 22 nucleotides upstream from the promoter –10 hexamer
58 (Herrero *et al.,* 2001). NtcA-binding sites with a repressor, rather than
59 activating, role have been identified in a few cases (Herrero *et al.,* 2001). The
60 NtcA protein appears to have as a positive effector 2-oxoglutarate (Vázquez-
61 Bermúdez *et al.,* 2002; Vázquez-Bermúdez *et al.,* 2003; Luque *et al.,* 2004),
62 which is an indicator of the C to N balance in cyanobacterial cells (Muro-Pastor
63 *et al.,* 2001). For some promoters, the P_{II} protein is also needed for full
64 activation by NtcA under N deficiency (Aldeni *et al*., 2003; Paz-Yepes *et al*.,
65 2003).
66 A number of cases have been described in which NtcA-mediated
67 nitrogen regulation is not directly operated by NtcA (Herrero *et al.,* 2001;
68 Herrero *et al.,* 2004). In those cases, one would expect that NtcA activates the
69 expression of regulatory proteins that would then be responsible for direct
70 regulation, but no such effector is yet known. Here we describe a protein with

71 homology to two-component response regulators whose expression is directly
72 operated by NtcA in *Anabaena* sp. strain PCC 7120.
73
74 Materials and methods
75 Strains and growth conditions
76 This study was carried out with the heterocyst-forming cyanobacterium
77 *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 and derivative
78 strains CSE2, an insertional mutant of the *ntcA* gene (Frías *et al.,* 1994), and
79 216, which bears a point mutation in the *hetR* gene (Buikema & Haselkorn,
80 1991). They were grown photoautotrophically at 30°C in $BG11_0C$ medium
81 (BG11 medium [Rippka *et al.,* 1979] without $NaNO_3$ and supplemented with 10
82 mM of $NaHCO_3$) supplemented with 6 mM NH_4Cl plus 12 mM *N*-tris
83 (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES)-NaOH buffer (pH
84 7.5), bubbled with a mixture of CO_2 and air (1% vol/vol), and supplemented with
85 2 $\mu g \cdot ml^{-1}$ of streptomycin and 2 $\mu g \cdot ml^{-1}$ of spectinomycin in the case of strain
86 CSE2 and those strains bearing fusions between the region upstream from
87 *all4312* and the *gfp* gene.
88 For RNA isolation, cells growing exponentially in $BG11_0C$ medium
89 supplemented with NH_4Cl were harvested at room temperature and either used
90 directly (time 0) or washed with $BG11_0C$ medium, resuspended in BG11C
91 (nitrate-containing) or in $BG11_0C$ (nitrogen-free) medium and further incubated
92 under culture conditions for the number of hours indicated in each experiment.
93 For the analysis of *gfp* expression, cells growing exponentially in
94 $BG11_0C$ medium supplemented with NH_4Cl were harvested at room

95 temperature and either used directly (time 0) or washed with $BG11_0C$ medium,
96 resuspended in $BG11_0C$ (nitrogen-free) medium and further incubated under
97 culture conditions for the number of hours indicated.

98 *E. coli* strains were grown in Luria broth (LB) supplemented, when
99 necessary, with antibiotics added at standard concentrations (Ausubel *et al.,*
100 2005).

101

102 **DNA and RNA isolation and manipulation**

103 Total RNA from *Anabaena* sp. strain PCC 7120 and its derivatives was isolated
104 as previously described (Muro-Pastor *et al.,* 2002). Primer extension analysis of
105 the *all4312* transcripts was carried out as described previously (Muro-Pastor *et*
106 *al.,* 1999). The oligonucleotide used as primer was all4312-1 (complementary to
107 positions +104 to +84 relative to the translation start of *all4312*). Plasmid
108 pCSAM113 (see below) was used to generate dideoxy-sequencing ladders
109 using the same primer. Sequencing was carried out by the dideoxy chain-
110 termination method, using a T^7Sequencing^{TM} kit (Amersham Biosciences) and
111 [α-^{35}S]-thio dATP. Northern analysis was carried out as described (Muro-Pastor
112 *et al.,* 1999). The *all4312* probe used was a 714-bp *Nco*I-*Hinc*II internal
113 fragment that covers almost the whole open reading frame, isolated from
114 pCSAM115.

115 Plasmid isolation from *E. coli*, transformation of *E. coli*, digestion of DNA
116 with restriction endonucleases, ligation with T4 ligase, and PCR were performed
117 by standard procedures (Ausubel *et al.,* 2005).

118

119 **Plasmids**

120 Plasmid pCSAM113 contains a 602-bp DNA fragment PCR-amplified using
121 oligonucleotides all4312-2 (corresponding to positions -488 to -467 with respect
122 to the translational start of *all4312*) and all4312-1 (see above) and
123 chromosomal DNA from *Anabaena* sp. strain PCC 7120 as template, cloned
124 into the pGEM-T vector (Promega). In pCSAM113a the orientation of the insert
125 is such that sequences corresponding to the all4312-1 oligonucleotide are close
126 to the *Spe*I site in the polylinker of pGEM-T. In pCSAM113b the insert is cloned
127 in the opposite orientation. Plasmid pCSAM115 contains a 1,102-bp DNA
128 fragment PCR-amplified with oligonucleotides all4312-*Nco* (corresponding to
129 positions -12 to +10 with respect to the translational start of *all4312*, introducing
130 a *Nco*I site at the start codon) and all4312-3 (complementary to positions +331
131 to +309 with respect to the translational stop of *all4312*) cloned into the pGEM-T
132 vector. This fragment contains the complete *all4312* open reading frame plus
133 331 bp downstream of *all4312*.

134 **Disruption of *all4312***

135 *all4312* in plasmid pCSAM115 was disrupted by introducing *Acc*I-ended SmSp-
136 resistance cassette C.S3, excised from pRL463 (pRL138/LHEH1[*Bam*HI]/C.S3,
137 nomenclature as in [Elhai & Wolk, 1988a]), into the *Cla*I site internal to *all4312*
138 rendering pCSAM116. The ca. 3.5-kbp *Pvu*II fragment from pCSAM116,
139 containing the disrupted *all4312* plus some downstream sequences
140 (*all4312*::C.S3), was cloned into the Klenow-filled *Bgl*II site of *sacB* vector
141 pRL278 (Black *et al.,* 1993). The resulting plasmid, pCSAM118 was transferred
142 to *Anabaena* sp. strain PCC 7120 by conjugation as described (Elhai & Wolk,
143 1988b), using the helper plasmid pRL623 (Elhai *et al.,* 1997), and SmSp

144 resistant colonies were selected. Isolation of double recombinants was

145 attempted but, under our culture conditions, no sucrose-resistant, Nm-sensitive

146 colony could be obtained in several rounds of selection.

147

148 **Fusions to the green fluorescent protein**

149 Fusions of the promoter region of *all4312* to the *gfp* gene encoding green

150 fluorescent protein were prepared as follows. SmSp-resistance cassette C.S3,

151 excised from pRL463 (see above) as an *Xba*I fragment, was inserted into the

152 *Spe*I site located in the polylinker of pCSAM113b upstream from the *all4312*

153 promoter, rendering pCSAM114. A *Sal*I-*Nco*I (Klenow-filled) fragment from

154 pCSAM114, containing C.S3 followed by the promoter region of *all4312*, was

155 placed upstream from the *gfp* gene in *Sal*I, *Eco*RV-digested pCSEL19,

156 rendering pCSAM117. (pCSEL19 contains a promoterless *gfp* gene PCR-

157 amplified using oligonucleotides gfp-1 [5'GGAGATATCCATATGAGTAAAGG3',

158 introducing a *Eco*RV site upstream from the start codon of the *gfp* gene] and

159 gfp-2 [5'AACAGAAGCTTGCATGCCTG] and plasmid pKEN2-GFPmut2 [(Ezaz-

160 Nikpay *et al.,* 1994; Cormack *et al.,* 1996)] as a template, cloned into the

161 pGEM-T vector in the same orientation of the beta-lactamase gene). A *Pst*I

162 fragment from pCSAM117, containing C.S3 followed by a transcriptional fusion

163 between the region upstream from *all4312* and the *gfp* gene, was cloned in both

164 orientations into the *Pst*I site of pCSAV80 (a derivative of pCSAM28 [Muro-

165 Pastor *et al.,* 1992] in which the *nucA* gene has been inactivated by digestion

166 with *Hind*III followed by Klenow treatment and religation), designed for

167 integration of constructs into the *nucA* region located in the α megaplasmid of

168 *Anabaena* sp. strain PCC 7120. The resulting plasmids, pCSAM119a and

169 pCSAM119b, were transferred to *Anabaena* by conjugation as described above
170 and SmSp resistant colonies were selected. The C.S3 cassette bears
171 transcriptional terminators that are effective in *Anabaena* sp. strain PCC 7120
172 (Frías *et al.,* 1997), ensuring that the P*all4312*::*gfp* fusion is not transcribed from
173 an external promoter other than P*all4312*.
174 The accumulation of GFP reporter was analysed by laser confocal
175 microscopy. Samples were observed using a Leica HCX PLAN-APO 63X 1.4
176 NA oil immersion objective attached to a Leica TCS SP2 confocal laser-
177 scanning microscope. GFP was imaged using the 488 nm line supplied by an
178 argon ion laser. Fluorescent emission was monitored by collection across
179 windows of 500-570 nm (GFP imaging) and 630-700 nm (cyanobacterial
180 autofluorescence). All confocal images were collected using the same settings,
181 so that the intensities can be compared.
182
183 **Band-shift assays**
184 A 330-bp *Dra*I-*Spe*I fragment from pCSAM113a was used in band shift assays
185 with purified NtcA. This fragment includes sequences -220 to +104 with respect
186 to the translation start of *all4312*. DNA fragments were end-labeled with T4
187 polynucleotide kinase and [γ-^{32}P]dATP. Assays were carried out as described
188 previously (Luque *et al.,* 1994) in the presence or absence of 0.6 mM 2-
189 oxoglutarate (Vázquez-Bermúdez *et al.,* 2002), and they contained about 0.5
190 fmol of labeled fragment and 2.5 to 15 pmol of purified His-tagged NtcA (Muro-
191 Pastor *et al.,* 1999).
192

193 ## Results

194

195 **Expression of *all4312***

196 As a result of a search for NtcA boxes upstream from *Anabaena* sp. strain PCC
197 7120 open reading frames and their corresponding homologs in *Nostoc*
198 *punctiforme*, *all4312* was identified as a gene exhibiting a putative regulatory
199 NtcA box in similar positions in both organisms (Jeff Elhai and Alicia M. Muro-
200 Pastor, unpublished results). Genes putatively encoding homologs of All4312
201 were identified in 10 cyanobacterial genomes. The regions upstream from the
202 corresponding open reading frames contained NtcA boxes with the consensus
203 sequence GTAN$_8$TAC centered at positions ranging from 66 to 130 nucleotides
204 upstream of the predicted translational start (Fig. 1). Furthermore, in four cases,
205 the NtcA box contains the nucleotides CA in the second and third positions after
206 the GTA triplet, a feature conserved in many consensus-type NtcA-binding sites
207 (Herrero *et al.,* 2001). The observation that NtcA boxes are located in such a
208 position in all ten genomes suggests that NtcA might be involved in expression
209 of the corresponding genes.

210 Because NtcA is known to regulate expression of genes in response to
211 the nitrogen status of the cells, expression of *all4312* in *Anabaena* sp. strain
212 PCC 7120 was analysed in ammonium-grown filaments incubated for 4 or 24 h
213 in the presence of nitrate or in the absence of combined nitrogen. Northern blot
214 hybridization (Fig. 2A) showed that expression in the wild-type strain was very
215 low in the presence of ammonium, was slightly induced in the presence of
216 nitrate as sole nitrogen source and was strongly induced after 4 h of nitrogen
217 deficiency. A time-course of induction of *all4312* in response to nitrogen

218 deprivation was also carried out (Fig. 2B). Induction of *all4312* took place, in
219 wild-type cells, after less than 3 h of nitrogen deficiency, and expression was
220 highest between 6 and, at least, 12 h. Induction of expression did not take place
221 in the *ntcA* mutant strain CSE2. Transcript 5´ ends for the *all4312* gene in
222 *Anabaena* sp. strain PCC 7120 were analyzed by primer extension (Fig. 3). A
223 unique transcription start point was identified 27 nucleotides upstream from the
224 translation start of *all4312*. Consistent with Northern hybridization results shown
225 in Fig. 2, expression from this start point was very low in ammonium-grown cells
226 and increased after 3 h of nitrogen deficiency. Also, the 5' end of this mRNA
227 could not be detected in the *ntcA* mutant CSE2 (Fig. 3).
228 Expression of *all4312* in the *hetR* mutant strain 216 was also analysed by
229 Northern hybridization (Fig. 2) and primer extension (Fig. 3). Expression of
230 *all4312* was not altered in the *hetR* mutant, and utilization of the transcription
231 start point located 27 nucleotides upstream from the translational start of
232 *all4312* in the *hetR* mutant was similar to that observed in the wild type.
233
234 **Analysis of *gfp* fusions**
235 Expression of transcriptional fusions of the *all4312* promoter region to a
236 promoterless *gfp* gene was analysed in ammonium-grown filaments incubated
237 in nitrogen-free medium for 9 or 24 h. Ammonium-grown filaments were also
238 analysed for comparison. Several *Anabaena* clones carrying fusions to *gfp* were
239 analysed, and the results for two clones, bearing the promoter-*gfp* fusion in both
240 orientations with respect to vector sequences, are shown in Fig.4. GFP
241 fluorescence of strains carrying the P*all4312*::*gfp* fusions was low in
242 ammonium-grown filaments but was higher after nitrogen deprivation.

243 Expression of transcriptional fusions was similar in all cells of the filament.
244 Some highly fluorescent cells appeared occasionally, but no correlation could
245 be established with proheterocysts or mature heterocysts.
246

247 **Analysis of the *all4312* promoter**

248 The analysis of the region upstream from *all4312* indicated that the transcription
249 start point determined by primer extension was located at the standard distance
250 from the NtcA boxes found in consensus Class II NtcA-activated promoters.
251 Thus, the NtcA box is centered at about -41.5 nucleotides with respect to the
252 tsp, which is preceded by a -10 box in the form TAN_3T (see figure 1). Band-shift
253 assays were carried out with purified NtcA protein and a fragment from the
254 *all4312* upstream region containing the NtcA box (Fig. 5). Retardation of the
255 labelled DNA fragment in response to the addition of increasing amounts of
256 NtcA was observed, indicating binding of NtcA. Affinity of NtcA for this promoter
257 fragment was higher in the presence of 2-oxoglutarate.
258

259 Discussion

260 The observations described in this work indicate that expression of *all4312* is
261 directly regulated by NtcA, the global transcriptional regulator for N control in
262 cyanobacteria. In response to nitrogen deficiency, an increase in the expression
263 of the *ntcA* gene (Muro-Pastor *et al.,* 2002) and accumulation of the NtcA
264 protein (Olmedo-Verd *et al.,* 2005) take place in *Anabaena* sp. strain PCC 7120.
265 Such increased expression of NtcA requires HetR, a positive-acting factor for
266 heterocyst differentiation (Buikema & Haselkorn, 1991). Because HetR is
267 required for the transient increase of NtcA levels that takes place during

268 nitrogen deficiency (Muro-Pastor *et al.,* 2002), the observation that HetR is not
269 involved in the expression of *all4312* suggests that those increased levels of
270 NtcA are not required for *all4312* expression. Expression of *all4312* would thus
271 result from the activation of NtcA in response to nitrogen deficiency. Combined
272 nitrogen deprivation would provoke an increase in the cellular levels of 2-
273 oxoglutarate (Laurent *et al.,* 2005), which has the effect of increasing NtcA
274 affinity for binding to the *all4312* promoter (Fig. 5). Thus, one would predict that
275 this promoter is activated early, and probably in all cells of the filament, upon
276 combined nitrogen deprivation (Herrero *et al.,* 2004). This appears to be
277 actually the case, as shown by the P*all4312-gfp* transcriptional fusions analysed
278 in this work (Fig. 4). A number of promoters are activated by NtcA upon
279 ammonium withdrawal (Herrero *et al.,* 2001). One of them, the *glnA* P_1 promoter
280 (Valladares *et al.,* 2004), bears an NtcA binding site that is identical in sequence
281 to that of the *all4312* promoter characterized in this work.
282 Genomic-wide analysis of genes encoding multi-domain proteins in
283 *Anabaena* sp. strain PCC 7120 have identified a remarkably large number of
284 genes for two-component systems (Ohmori *et al.,* 2001; Wang *et al.,* 2002).
285 Such abundance of regulatory elements might reflect the complexity of
286 *Anabaena* regulatory networks and physiology. All4312 would be the first known
287 response regulator of a two-component regulatory system that might be
288 involved in NtcA-mediated regulation. The nature of the corresponding sensor
289 component, if any, is currently unknown. Genomic analysis of all two-
290 component systems and signaling proteins that can be identified in *Anabaena*
291 sp. strain PCC 7120 reveals no clustering of *all4312* with any signaling protein
292 (Wang *et al.,* 2002). It is conceivable that All4312 integrates signals from NtcA,

293 the global nitrogen regulator activating the expression of the response regulator,
294 and from whatever sensor component that might modify the activity of this
295 protein.

296 Because we have failed in the isolation of a fully segregated *all4312*
297 insertional mutant, we do not know which physiological traits may be regulated
298 by All4312. However, the fact that increased expression of *all4312* in response
299 to N step-down takes place in all vegetative cells of the filament, rather than
300 being localized to pro-heterocysts or heterocysts, suggests a function for
301 All4312 related to a response to N stress rather than specifically to heterocyst
302 differentiation. Additionally, the expression of *all4312*, which is increased in
303 response to N step-down, decreases in the wild type by 24 h post-induction,
304 when N_2 fixation has started (Fig. 2). A function related to N stress would also
305 be consistent with the presence of All4312 homologs exhibiting similar
306 regulatory features (i.e., NtcA boxes in their promoter regions) in non-nitrogen-
307 fixing cyanobacteria.
308

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316

317

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421

422 **FIGURE LEGENDS**

423 Figure 1. Identification of putative NtcA boxes in the regions upstream from
424 genes encoding All4312 and its homologs in several cyanobacterial genomes.
425 Sequences are aligned at the predicted NtcA boxes and distances to the
426 putative translation start (GTG or ATG codon) of the corresponding genes are
427 indicated. The transcription start point determined for *all4312* is underlined, and
428 the corresponding putative promoter –10 hexamer is indicated. Sequences
429 aligned correspond to those upstream from *all4312* (first line) and genes
430 encoding All4312 homologs from *Anabaena variabilis* strain ATCC 29413
431 (gi75907486), *Nostoc punctiforme* strain PCC 73102 (gi53687472),
432 *Trichodesmium erythraeum* strain IMS101 (gi71676548), *Synechocystis* sp.
433 strain PCC 6803 (gi16332107), *Crocosphaera watsonii* strain WH 8501
434 (gi67924898), *Synechococcus elongatus* strain PCC 7942 (gi45513869),
435 *Synechococcus elongatus* strain PCC 6301 (gi56751647), *Gloeobacter*
436 *violaceus* strain PCC 7421 (gi35212842) and *Thermosynechococcus elongatus*
437 strain BP-1 (gi22295054).
438

439 Figure 2. Expression of *all4312* in *Anabaena* sp. strain PCC 7120, the *ntcA*
440 insertional mutant CSE2, and the *hetR* strain 216. (A) RNA from wild-type
441 *Anabaena* sp. strain PCC 7120 was isolated from ammonium-grown filaments
442 (lanes labelled 0) or from ammonium-grown filaments incubated in nitrate-
443 containing or nitrogen-free medium for 4 or 24 h and hybridized to an *all4312*
444 probe. Samples contained 20 μg of RNA. Size standards are indicated on the
445 right. (B) RNA was isolated from ammonium-grown filaments (lanes labelled 0)
446 or from ammonium-grown filaments incubated in nitrogen-free medium for the

447 number of hours indicated in each case and hybridized to an *all4312* probe.
448 Samples contained 25 μg of RNA. Lower panels correspond in all cases to
449 hybridization to an *rnpB* probe (Vioque, 1997), which was used as a loading and
450 transfer control. Similar results to those shown in panel B were obtained with
451 RNA samples from an independent induction experiment (not shown). WT, wild-
452 type strain PCC 7120.
453
454 Figure 3. Primer extension analysis of expression of *all4312* in *Anabaena* sp.
455 strain PCC 7120, the *ntcA* insertional mutant CSE2, and the *hetR* strain 216.
456 Assays were carried out with RNA isolated from ammonium-grown filaments
457 (lanes labelled 0) or from ammonium-grown filaments incubated in nitrogen-free
458 medium for the number of hours indicated in each case. The oligonucleotide
459 used for extension was all4312-1 (see materials and methods). Sequence
460 ladders were generated with the same oligonucleotide and plasmid pCSAM113.
461 Arrowhead points to the putative tsp identified 27 nucleotides upstream from the
462 translation start of the gene.
463
464 Figure 4. Expression of fusions between the promoter region upstream from
465 *all4312* and the *gfp* gene. Fluorescent emission was determined in ammonium-
466 grown filaments or in ammonium-grown filaments incubated in nitrogen-free
467 medium for the number of hours indicated. GFP fluorescence (left panels) and
468 cyanobacterial autofluorescence (right panels) is shown. Cells lacking
469 autofluorescence are mature heterocysts. A and B show fluorescent emission of
470 two different clones (see materials and methods for details). Other clones

471 analyzed showed similar results. White triangles point to proheterocysts or

472 heterocysts.

473

474 Figure 5. Binding of purified NtcA to the upstream region of *all4312*. Band-shift

475 assays were carried out with a fragment of the *all4312* upstream region (see

476 materials and methods for details) in the absence (lanes 1 to 5) or in the

477 presence of 0.6 mM 2-oxoglutarate (lanes 6 to 9). The amounts of purified NtcA

478 protein used in the assays were: (1) no NtcA protein added; (2,6) 2.5 pmol; (3,7)

479 5 pmol; (4,8) 10 pmol; (5,9) 15 pmol. Solid arrowhead points to the free

480 fragment, white arrowhead points to the retarded NtcA bound fragment.

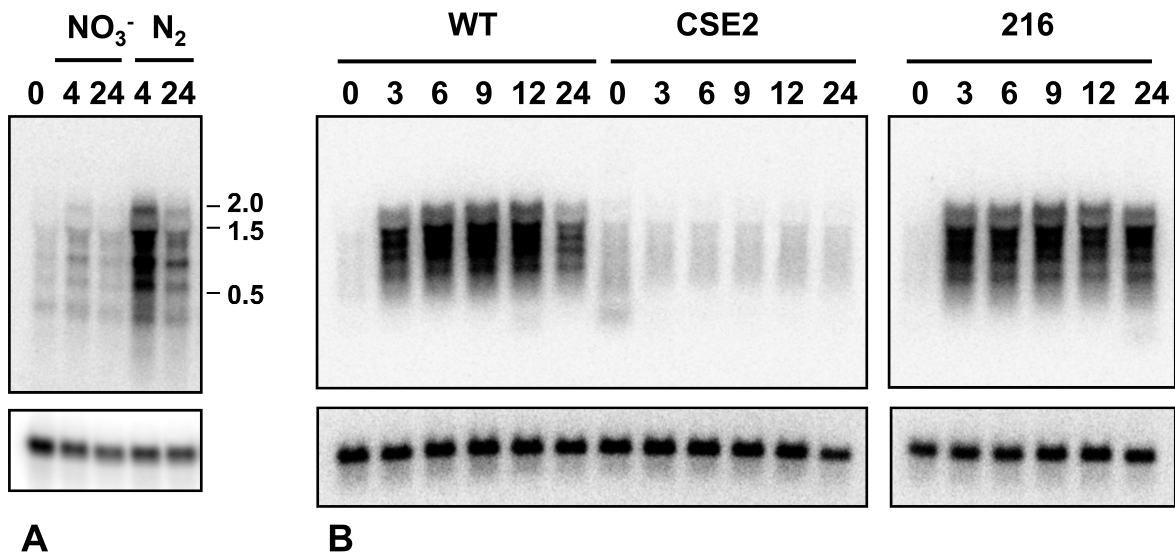
481

Muro-Pastor et al., FIG 1

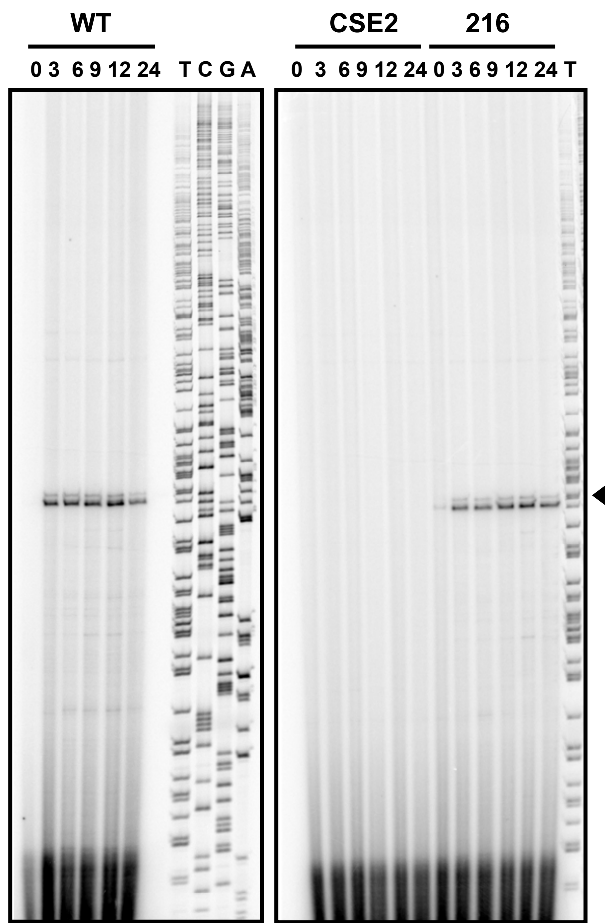
```
                  NtcA binding box                  -10 box

PCC 7120        TAGAGTAACAAAGACTACAAAACCTTGGGCATGGGCTTGTTACTTTGAAATTCATC----22nt-----GTG
A. variabilis   TAGAGTAACAAAGACTACAAAACCTTGGGCATGGGCTTGTTACTTTGAAATTCATC----22nt-----GTG
N. punctiforme  TGAAGTAACAAAGGCTACAAAACCTTAGAGATGGGCTTGTTACTTTGAAAGTCATC----23nt-----GTG
T. erythraeum   TTTAGTAGCTTCTGTTACAAAAGCGCCACAATAATTTATGTTATTTTTATACTTAG----36nt-----GTG
PCC 6803        GCAGGTAACTGTTGTTACAAAGCCTTGACATTGACTTTGTTAGATTAACAGGGAAC----22nt-----GTG
C. watsonii     CCAGGTAACAGATGTTACAAACTCCTGACAATACGTTTGTTAGGCTAATGACTGTC----23nt-----GTG
PCC 7942        GCTCGTAAAGGCGAATACAGAAGCCACAATGGACAGCTTGCTAGGTTAAAGTCACA----21nt-----GTG
PCC 6301        GCTCGTAAAGGCGAATACAGAAGCCACAATGGACAGCTTGCTAGGTTAAAGTCACA----21nt-----GTG
PCC 7421        GTCTGTACGCCGAGGTACTGCGCACAGAGACACAGATGGACAGCGGCCTGCGCCAG----64nt-----GTG
T. elongatus    TAAAGTATTATTCGTTACGAAATGATAGGTATTAGATTTGCTAGATTAGCATCCAA----85nt-----ATG
```

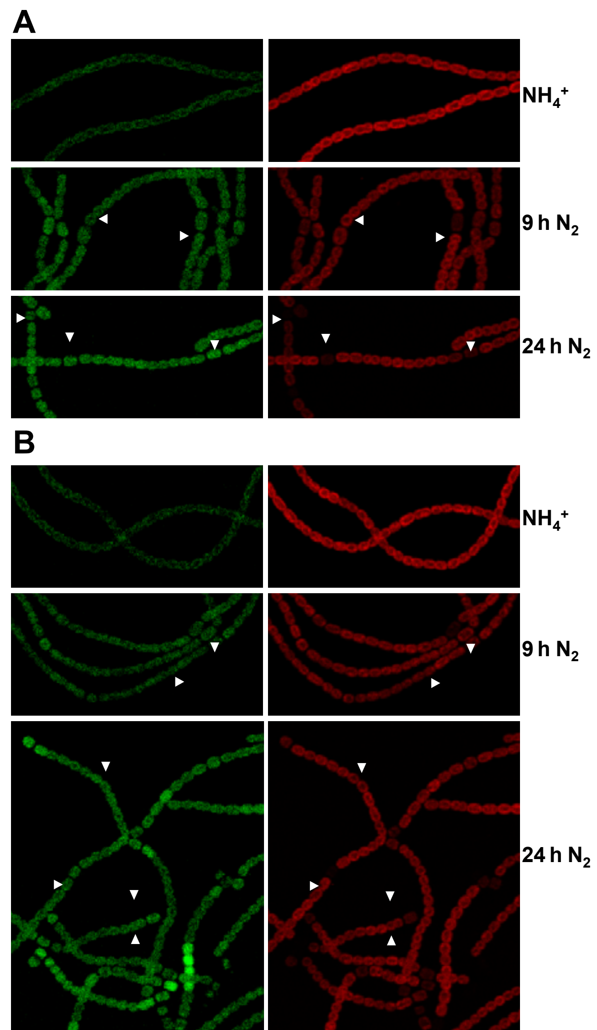
Muro-Pastor et al., FIG 2



Muro-Pastor et al., FIG. 3



Muro-Pastor et al., FIG 4



Muro-Pastor et al., FIG. 5