HetR-Dependent and -Independent Expression of Heterocyst-Related Genes in an *Anabaena* Strain Overproducing the NtcA Transcription Factor

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Heterocyst development in the cyanobacterium *Anabaena* sp. strain PCC 7120 depends on both the global nitrogen control transcription factor NtcA and the cell differentiation regulatory protein HetR, with expression of *ntcA* and *hetR* being dependent on each other. In this study we constructed strains that constitutively express the *ntcA* gene leading to high levels of NtcA protein irrespective of the nitrogen source, and we analyzed the effects of such NtcA levels on heterocyst differentiation. In the NtcA-overproducing strain, heterocyst differentiation, induction of NtcA-dependent heterocyst development genes or operons such as *devBCA* or the *cox2* operon, and NtcA-dependent excision of the 11-kb *nifD*-intervening element only took place under nitrogen deficiency. Although functional heterocysts were produced in response to nitrogen step-down, the NtcA over-producing strain could not grow diazotrophically. Overexpression of *ntcA* in a *hetR* background promoted expression of *devBCA* in response to ammonium withdrawal and excision of the 11-kb element even in the presence of combined nitrogen. Our results show that some NtcA-dependent heterocyst-related genes can be expressed independently of HetR.

Cyanobacteria are a group of widely distributed phototrophic prokaryotes that carry out oxygenic, plant-type photosynthesis. Cyanobacteria are able to use different nitrogen sources including nitrate and ammonium, and many strains are also able to fix atmospheric nitrogen. Ammonium is assimilated in preference over nitrate, which is used in preference over dinitrogen (14, 22). Assimilation of different nitrogen sources is globally regulated in these organisms by NtcA, a transcription factor belonging to the CAP (or CRP) family that, in the absence of ammonium, activates the expression of genes required for the assimilation of nitrogen sources, including atmospheric nitrogen (22, 26, 42). NtcA binds to specific sites in the promoter regions of the regulated genes and activates their expression in response to ammonium withdrawal (26). The structure of consensus NtcA-activated promoters has been defined (26), and several NtcA-activated promoters that differ to some extent from that consensus have also been described (22). NtcA-binding sites with a repressor, rather than activating, role have been identified in a few cases (22).

The ability to fix atmospheric nitrogen depends on the oxygen-sensitive enzyme nitrogenase. Because oxygenic photosynthesis and nitrogen fixation are incompatible processes, many cyanobacteria separate them either in specific cells or at specific time periods. Some filamentous cyanobacteria, including *Anabaena* spp., are able to differentiate, in response to nitrogen deficiency, cells specialized in nitrogen fixation called heterocysts. In the *Nostocales* group, heterocysts develop from vegetative cells located at semiregular intervals in the filament and provide the microaerobic environment required for nitrogenase activity (20, 45). The differentiation process begins shortly after nitrogen deprivation and, under our experimental conditions, requires about 20 to 24 h to complete. The development of functional mature heterocysts involves the sequential activation of a large number of genes specifically related to regulatory, structural, or enzymatic aspects of heterocyst differentiation and function (20, 45). In numerous heterocyst formers, including *Anabaena* sp. strain PCC 7120, the *nifHDK* operon includes an 11-kb DNA element that interrupts the *nifD* gene encoding one of the subunits of nitrogenase. Excision of this element takes place during heterocyst differentiation and is required for the expression of nitrogenase (17, 18).

Heterocyst development requires the product of the regulatory gene *hetR*, whose expression is induced shortly after nitrogen deprivation (3, 5). Induction of *hetR* does not take place in an *ntcA* mutant (16, 32). The NtcA protein is not only required for heterocyst development (16, 44), linking nitrogen deficiency and heterocyst differentiation in the context of a hierarchy of the use of different nitrogen sources, but also for the function of mature heterocysts (22, 23). During heterocyst differentiation, expression of *ntcA* is transiently induced in a HetR-dependent manner (32). Thus, in the context of heterocyst differentiation, expression of *hetR* and *ntcA* exhibits a mutual dependency (32). We hypothesize that the double dependence on NtcA and HetR exhibited by some heterocyst development genes or operons such as *devBCA* (13), *devH* (21), or the *cox2* operon (39) could, at least in part, be operated via NtcA, with HetR being required for the transient increase of NtcA levels (23).

In an attempt to further define the roles of NtcA and HetR in heterocyst differentiation, we have constructed strains that exhibit high levels of NtcA protein irrespective of the nitrogen source and have analyzed some effects of such high levels of NtcA in both wild-type and *hetR* backgrounds.

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MATERIALS AND METHODS

Strains and growth conditions. The present study was carried out with the heterocyst-forming cyanobacterium *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 and its Het⁻ derivative strain 216, which bears a point mutation in the *hetR* gene (5). Strains CSEL1 and CSEL2 were generated in the present study (see below). They were grown photoautotrophically at 30°C in BG11₀C medium (i.e., BG11 medium [36] without NaNO₃ and supplemented with 0.84 g of NaHCO₃ liter⁻¹) 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% [vol/vol]), and supplemented with 2 μg of streptomycin and 2 μg of spectinomycin ml⁻¹ in the case of strains CSEL1 and CSEL2.

For DNA and RNA isolation, cells growing exponentially in BG11₀C medium supplemented with NH₄Cl were harvested at room temperature and either used directly or washed with BG11₀C medium and resuspended in either BG11₀C (nitrogen-free) medium or nitrate-containing BG11C medium and further incubated under culture conditions for the number of hours indicated in each experiment.

To determine the ability of *ntcA*-overexpressing strains to grow with ammonium, nitrate, or dinitrogen as nitrogen sources, cells that had been grown in BG11 or in BG11₀ medium supplemented with NH₄Cl were harvested at room temperature, washed with BG11₀, and used to inoculate flasks with 25 ml of BG11₀ (nitrogen free), BG11 (nitrate-containing medium), or BG11₀ medium supplemented with NH₄Cl. Cells were inoculated at 0.2 μg of chlorophyll *a* (Chl) per ml. Chl was determined in methanol extracts (28). The cultures were incubated at 30°C in the light (75 microeinsteins m⁻² s⁻¹) with shaking (80 to 90 rpm), and samples of 0.2 ml were withdrawn and frozen at different times. The amount of protein in these samples was determined by a modified Lowry procedure (29), with bovine serum albumin as a standard. The amount of cells containing 1 μg of Chl contains about 25 to 30 μg of protein.

Escherichia coli strains were grown in Luria broth (LB). *E. coli* strains containing pCSM1 (see below) were grown in LB medium supplemented with 2% glucose in order to reduce basal expression of the *ntcA* gene.

Plasmids. Plasmid pCSAM60 contains an ~350-bp DNA fragment from the *ntcA* gene of *Anabaena* sp. strain PCC 7120 cloned into NcoI/EcoRI-digested expression vector pTrc99A (1). This fragment extends from the start codon of the *ntcA* gene (cloned in frame with the site of initiation of translation located after the *trc* promoter in pTrc99A) to the ClaI site located inside the *ntcA* reading frame. In order to prevent regulation of the *trc* promoter by LacI in *Anabaena* and allow selection of integration of pCSAM60 into the *Anabaena* chromosome (see below), the Sm/Sp resistance cassette C.S3 (11) was introduced (as an EcoRV-HincII fragment excised from pCSE67, a plasmid that contains HindIII-ended cassette C.S3 cloned into the HindIII site of vector pIC20R [30]) into the EcoRV site located in the *lacI*�q gene in the vector portion of pCSAM60 rendering pCSM1. Because constitutive expression of *ntcA* from the *trc* promoter in pCSM1 is deleterious for *E. coli*, pCSM1 was maintained in *E. coli* BL21(DE3) (37) in the presence of pREP4 (Qiagen), which expresses high levels of the LacI�q repressor.

Construction of *ntcA*-overexpressing strains. Plasmid pCSM1 was transferred to *Anabaena* sp. strain PCC 7120 and *hetR* strain 216 (5) by conjugation (9) using the helper plasmid pRL623 (8). Integration of pCSM1 into the *Anabaena* chromosome was selected in BG11 medium supplemented with 5 μg of streptomycin and 5 μg of spectinomycin ml⁻¹.

DNA and RNA isolation and manipulation. Total DNA (6) and RNA (32) from *Anabaena* sp. strain PCC 7120 and its derivatives was isolated as previously described. Sequencing was carried out by the dideoxy chain-termination method with a T7Sequencing kit (Amersham Biosciences) and [α-³⁵S]thio-dATP.

Plasmid isolation from *E. coli*, transformation of *E. coli*, digestion of DNA with restriction endonucleases, ligation with T4 ligase, and PCR were performed by standard procedures (2).

Southern and Northern blotting and hybridization. Southern analysis was carried out according to standard procedures (2). Northern analysis was carried out as described previously (31). The *ntcA* probe (see Fig. 1) was a 338-bp ClaI-EcoRI fragment corresponding to the second half of *ntcA*. The *nir* probe was a 757-bp fragment amplified by PCR with the oligonucleotides *nir*-15 (corresponding to positions +130 to +149 with respect to the translational start of the *nir* gene) and *nir*-16 (complementary to positions +884 to +866 with respect to the translational start of the *nir* gene) and pCSE26 (15) as a template. The *rbcL* probe was a 1,139-bp fragment PCR amplified by using oligonucleotides RL3 (corresponding to positions +188 to +206 with respect to the translational start of the *rbcL* gene) and RL4 (complementary to positions +1324 to +1304 with respect to the translational start of the *rbcL* gene) and pCSAV61 as a template. (pCSAV61 contains this *rbcL* fragment cloned in pGEM-T [Promega].) The *coxB2* (*alr2514*) probe was a 966-bp fragment PCR amplified by

using oligonucleotides CB2-4 (corresponding to positions +20 to +40 with respect to the translational start of the *coxB2* gene) and CB2-5 (complementary to positions +974 to +955 with respect to the translational start of the *coxB2* gene) and pCSAV128 (39) as a template. The *nifH* probe was a 1,231-bp fragment PCR amplified by using oligonucleotides NH1 (corresponding to positions −333 to −310 with respect to the translational start of the *nifH* gene) and NH4 (complementary to positions +888 to +868 with respect to the translational start of the *nifH* gene) and pCSAV60 as a template. (pCSAV60 contains this *nifH* fragment cloned in pGEM-T.) The *nifD* probe used to test excision of the 11-kb element was a 541-bp fragment PCR amplified by using oligonucleotides ND1 (corresponding to positions +329 to +348 with respect to the translational start of the *nifD* gene) and ND2 (complementary to positions +860 to +841 with respect to the translational start of the *nifD* gene) and pCSEL7 as a template. (pCSEL7 contains this *nifD* fragment cloned in pGEM-T [Promega].) The *devB* probe was a 1,423-bp fragment PCR amplified by using oligonucleotides O34 (corresponding to positions +1 to +19 with respect to the translational start of the *devB* gene) and O18 (complementary to positions +1423 to +1400 with respect to the translational start of the *devB* gene) and plasmid pIM11 (12) as a template. Fragments used as probes were ³²P-labeled with a Ready-to-Go DNA labeling kit (Amersham Biosciences) using [α-³²P]dCTP. Images of radioactive filters were obtained and quantified by using a Cyclone storage phosphor system and OptiQuant image analysis software (Packard).

Primer extension analysis. Primer extension analysis of the *ntcA* transcripts was carried out as described previously (31). The oligonucleotide used as primer was NA1 (complementary to positions +59 to +40 relative to the translation start of *ntcA*). Plasmid pCSM1 was used to generate dideoxy-sequencing ladders using the same primer.

Enzymatic activities. Nitrogenase activity was determined in exponentially growing cells (1 to 5 μg of Chl ml⁻¹) subjected to nitrogen deficiency for the number of hours indicated in each case. A total of 2 ml of culture (containing 2 to 10 μg of Chl) was incubated under an atmosphere of 13.3% acetylene in air, with shaking in the light at 30°C, and the production of ethylene was determined by gas chromatography in samples taken after 30 and 60 min of incubation.
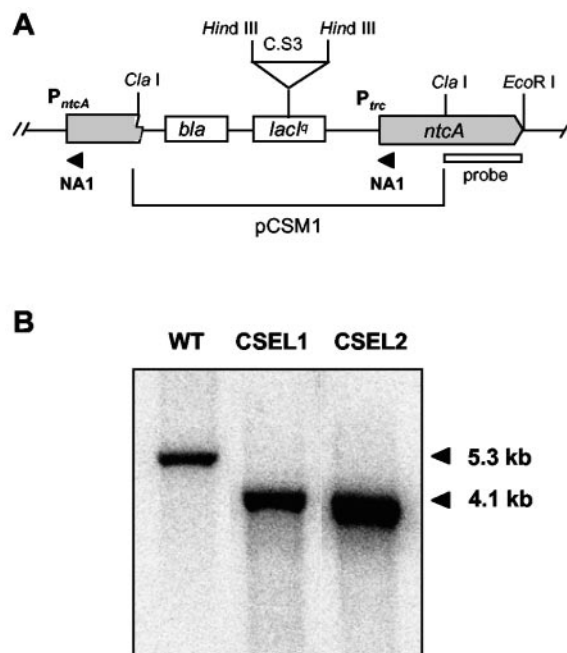

FIG. 1. Structure of the *ntcA* region in *ntcA*-overexpressing strains CSEL1 and CSEL2. (A) Sequences from the *ntcA* gene are shown in gray. DNA regions present in plasmid pCSM1 (see Materials and Methods for details), the *ntcA* fragment used as probe for Northern hybridization, and the position of oligonucleotide NA1 used for primer extension experiments are indicated. P*ntcA*, wild-type *ntcA* promoter; P*trc*, *trc* promoter. (B) Southern blot analysis of the structure of the *ntcA* region in strains CSEL1 and CSEL2. Total DNA from wild-type strain PCC 7120 and *ntcA*-overexpressing strains CSEL1 and CSEL2 was digested with HindIII and hybridized to the probe shown in panel A. Sizes (in kilobases) are indicated on the right. WT, wild-type strain PCC 7120.

Western blotting. Proteins in crude extracts were separated by standard electrophoresis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes (Bio-Rad). Immunodetection of NtcA was carried out with polyclonal antibodies generated in rabbits against His-tagged NtcA protein purified from *E. coli* cells bearing pCSAM70 (31). An enhanced chemiluminescence detection system (Amersham Biosciences) was used according to the instructions of the manufacturer.

Alcian blue staining. Alcian blue was used to stain heterocyst-specific envelopes. A 0.5% Alcian blue (Sigma) solution in 50% ethanol was mixed with an equal volume of culture before microscopic examination.

RESULTS

Construction of *Anabaena* *ntcA*-overexpressing strains. Derivatives of *Anabaena* sp. strain PCC 7120 with high levels of transcription of the *ntcA* gene from a *trc* promoter were generated both in the wild-type and *hetR* backgrounds. Plasmid pCSM1, which contains the first half of the *ntcA* gene located downstream from the *trc* promoter, was introduced into *Anabaena* sp. strain PCC 7120 and the *hetR* strain 216 (5) by conjugation. Integration of pCSM1 through a single recombination event into the *Anabaena* chromosome led to a partial duplication of the *ntcA* region (Fig. 1A). The strains obtained, CSEL1 and CSEL2 (wild-type and *hetR* backgrounds, respectively), contained a wild-type *ntcA* gene whose expression was under the control of the *trc* promoter, whereas the native promoter of the *ntcA* gene directed expression of a truncated version of the gene. Vector sequences located between the duplicated regions include the *bla* gene and the *lacI*^{q} gene interrupted by the Sm/Sp resistance cassette C.S3 (11). Because *lacI*^{q} is disrupted, there is no LacI-mediated regulation of the *trc* promoter in these strains. The chromosomal structure of strains CSEL1 and CSEL2, which were homozygous for the mutant chromosomes, was assessed by Southern hybridization (Fig. 1B).

ntcA transcripts and NtcA protein in strains CSEL1 and CSEL2. Expression of *ntcA* in strains CSEL1 and CSEL2 was analyzed by Northern blot hybridization. Parental strains *Anabaena* sp. strain PCC 7120 and *hetR* strain 216 were included for comparison (Fig. 2A). As previously shown (32), expression of *ntcA* was induced ~5-fold in the wild-type strain after 9 h of nitrogen deficiency, whereas such an increase did not take place in the *hetR* strain. The amount of *ntcA* transcript in CSEL1 and CSEL2, both under nitrogen deficiency or in ammonium-grown filaments, was much higher than the induced levels found in the wild-type strain. Quantification of the *ntcA* signals, normalized with respect to the *rnpB* signal, indicated that the level of *ntcA* transcript in CSEL1 and CSEL2 ammonium-grown cells was about 50-fold the amount of *ntcA* transcript found in ammonium-grown wild-type cells. Overexpression resulted in *ntcA* transcripts ranging in size from 0.8 to 1.4 kb, a finding similar to what was observed for the wild-type strain (35). Higher transcript levels were observed when the filaments were subjected to nitrogen deficiency, suggesting possible factor(s) other than transcription in determining *ntcA* transcript levels.

Transcript 5′ ends for the *ntcA* gene in strains CSEL1 and CSEL2 were analyzed by primer extension (Fig. 2B). Comparison of the 5′ ends identified with those observed in the wild-type strain indicated that, as expected, expression of *ntcA* in CSEL1 and CSEL2 took place mostly from the *trc* promoter irrespective of the nitrogen status of the cells. Transcription

from the wild-type promoters, which in CSEL1 and CSEL2 would direct expression of truncated versions of the *ntcA* transcript, could also be detected with the oligonucleotide used (see the −49, −136, and −180 positions indicated in Fig. 2B). Given that some of the native transcripts are NtcA-regulated, their abundance could be affected by the high levels of NtcA protein present in cells of strains CSEL1 and CSEL2.

The amount of NtcA protein in *ntcA*-overexpressing strains was determined by Western blotting with antibodies generated against *Anabaena* NtcA (Fig. 2C). Consistent with the induction of the *ntcA* transcript, the amount of NtcA protein was higher in the wild-type strain under nitrogen deficiency. Also consistent with the lack of induction of the *ntcA* transcript in the *hetR* strain (see above), there was no increase in the level of NtcA in strain 216. *ntcA*-overexpressing strains CSEL1 and CSEL2 contained high levels of NtcA both in ammonium-grown filaments and in filaments subjected to nitrogen deficiency. Western blot analysis of serial dilutions of these extracts indicated that the content of NtcA protein in whole filaments of strains CSEL1 and CSEL2 was at least 25-fold the amount of NtcA in ammonium-grown wild-type cells (not shown). These results indicate that both the expression of *ntcA* and the amount of NtcA protein were high in strains CSEL1 and CSEL2 irrespective of the nitrogen source and the presence of a functional HetR protein. No NtcA protein that could correspond to translation of the truncated *ntcA* gene was detected in these extracts (not shown).

Growth characteristics and nitrogenase activity of strains CSEL1 and CSEL2. The ability of strains CSEL1 and CSEL2 to grow using different nitrogen sources was tested. Parental strains were included for comparison. After 3 days of incubation under growth conditions, all four strains looked healthy in media containing either ammonium or nitrate and the growth rate constants were similar for the CSEL1 and CSEL2 strains with regard to their parental strains (not shown). Under diazotrophic conditions, however, only the wild-type strain grew. Microscopic examination of cultures subjected to nitrogen deficiency for 24 h showed that wild-type *Anabaena* sp. strain PCC 7120 and its *ntcA*-overexpressing derivative CSEL1 exhibited a similar frequency of heterocysts (~6% of the cells), although filaments of strain CSEL1 were highly fragmented. However, after 5 days of nitrogen deficiency, filaments of strain CSEL1 exhibited very few mature heterocysts and virtually no proheterocysts, as determined by staining of the filaments with Alcian blue (not shown). No heterocysts or proheterocysts were ever observed in *hetR* strain 216 or its derivative CSEL2. When cells incubated for 5 days in the absence of combined nitrogen were inoculated into fresh ammonium-containing, nitrate-containing, or nitrogen-free medium, normal growth was observed for the four strains (wild type, 216, CSEL1, and CSEL2) in the presence of combined nitrogen but, under diazotrophic conditions, no growth was observed for strains CSEL1, 216, and CSEL2.

Nitrogenase activity was determined in *ntcA*-overexpressing strains and their parental strains. Determinations were carried out in cells incubated in the absence of combined nitrogen for 20 to 26 h. Microscopic examination of the filaments indicated that, at those times, heterocyst differentiation was complete both in the wild-type and in the CSEL1 strains. Similar levels of activity, i.e., ~1 μmol of ethylene min^{-1} mg of Chl^{-1}, were
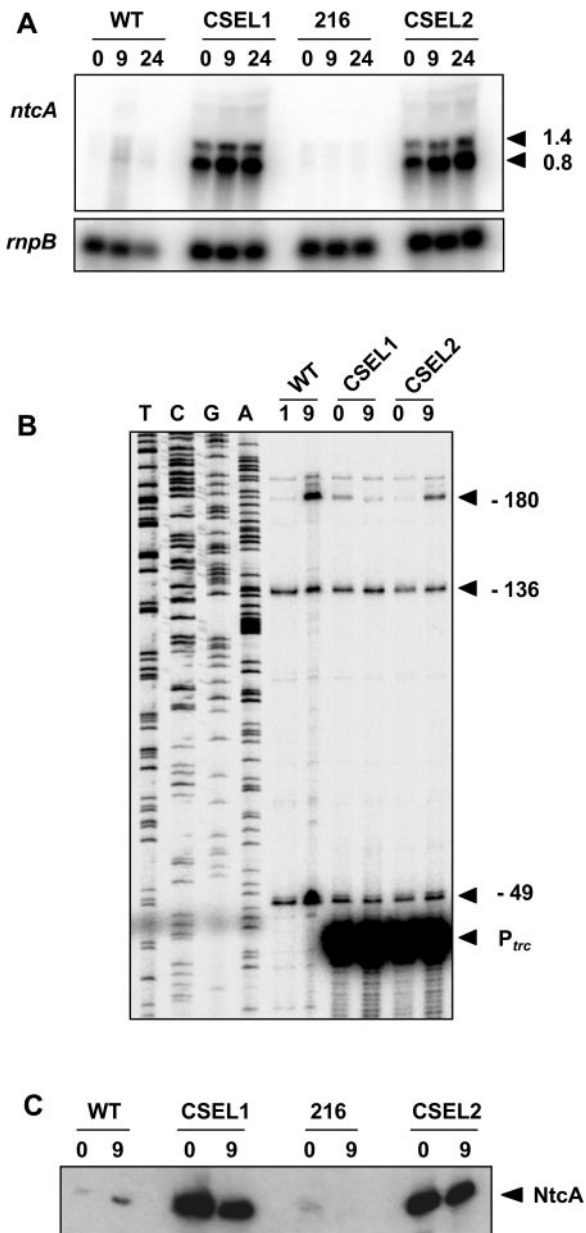
FIG. 2. Expression of *ntcA* and amount of NtcA protein in *Anabaena* sp. strain PCC 7120, *hetR* strain 216, and *ntcA*-overexpressing strains CSEL1 and CSEL2. (A) Northern blot analysis of expression of the *ntcA* gene. RNA was isolated from ammonium-grown filaments (lanes 0) or from ammonium-grown filaments incubated in the absence of combined nitrogen for 9 or 24 h. Samples contained 50 µg of RNA. Hybridizations to the *ntcA* probe (see Fig. 1) (upper panel) or to an *rnpB* probe (43) used as a loading and transfer control (lower panel) were performed. Note that the *ntcA* probe used only hybridizes to transcripts that include the second half of the *ntcA* gene. (B) Primer extension analysis of *ntcA* transcripts. RNA was isolated from ammonium-grown filaments (lanes 0) or from ammonium-grown filaments incubated in the absence of combined nitrogen for 1 or 9 h. Oligonucleotide NA1 (see position in Fig. 1) was used as a primer in assays containing 25 µg of RNA (wild type) or 5 µg of RNA (CSEL1 and CSEL2). A sequencing ladder was generated with the same oligonucleotide and plasmid pCSM1. (Note that pCSM1 does not contain the wild-type *ntcA* promoter upstream from *ntcA* but contains the *trc* promoter instead. Thus, the sequencing ladder shown corresponds to *ntcA* only for sequences downstream from the ATG codon.) The po-
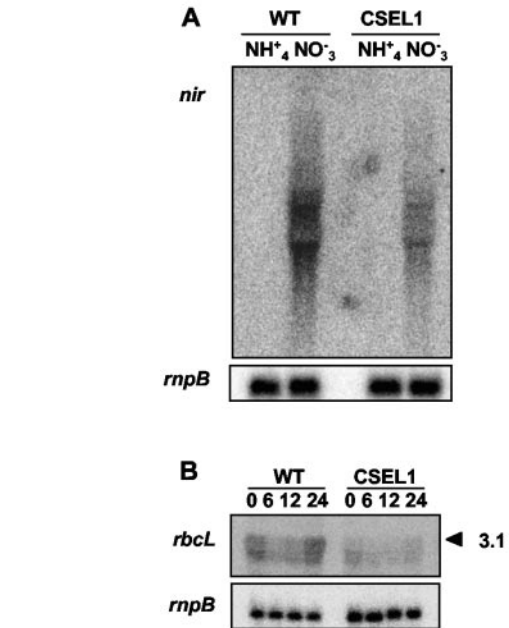
FIG. 3. Expression of the *nir* and *rbcLXS* operons in *Anabaena* sp. strain PCC 7120 and strain CSEL1. (A) RNA was isolated from ammonium-grown filaments (lanes NH_4^+) or from ammonium-grown filaments incubated in nitrate-containing medium for 4 h (lanes NO_3^-) and hybridized to a *nir* probe. Samples contained 30 µg of RNA. (B) RNA was isolated from ammonium-grown filaments (lanes 0) or from ammonium-grown filaments subjected to nitrogen deficiency for the number of hours indicated in each case and hybridized to a *rbcL* probe. The lower panels correspond to hybridization to a *rnpB* probe (43) in both cases. WT, wild-type strain PCC 7120.

reached in the wild-type strain and CSEL1. However, the development of nitrogenase activity with time was slightly delayed in strain CSEL1 with respect to the wild type (in three independent experiments). Thus, strain CSEL1 had the unexpected phenotype of impaired diazotrophic growth while still being able to carry out at least one round of heterocyst differentiation in response to nitrogen step-down. No nitrogenase activity could be detected in strains 216 or CSEL2.

Expression of the *nir* and *rbcLXS* operons. Expression of the *nir* operon, whose transcription is activated by NtcA in vegetative cells (16), was analyzed in strain CSEL1 (Fig. 3A). The *nir* operon (almost 10 kb) produces a large transcript that is only detected as mRNA degradation products (15). As in the wild type, expression of the *nir* operon was only induced in the absence of ammonium (Fig. 3A).

sitions of the 5′ ends with respect to the ATG codon of the *ntcA* gene, as well as that corresponding to the transcripts originating from the *trc* promoter (P_{trc}), are indicated with arrowheads on the right. Note that the amount of RNA from strains CSEL1 and CSEL2 used in primer extension assays was fivefold lower than for the wild-type samples. (C) Western blot detection of the NtcA protein. Whole-cell extracts were prepared from ammonium-grown filaments (lanes 0) or from ammonium-grown filaments incubated in the absence of combined nitrogen for 9 h. Samples contained extract amounts corresponding to 1.6 µg of Chl. Arrowhead points to the NtcA protein. WT, wild-type strain PCC 7120.
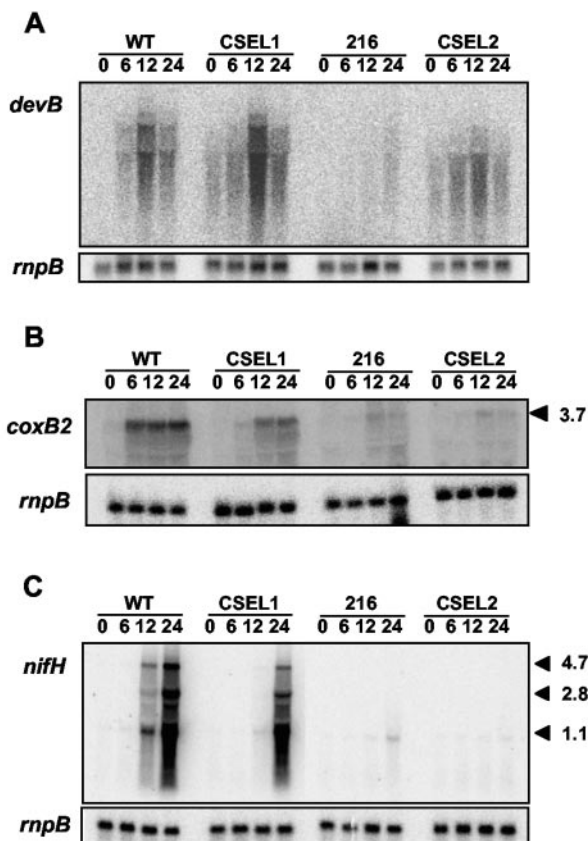
FIG. 4. Expression of *devBCA*, *cox2*, and *nifHDK* in *Anabaena* sp. strain PCC 7120, the *hetR* strain 216 and strains CSEL1 and CSEL2. RNA was isolated from ammonium-grown filaments (lanes 0) or from ammonium-grown filaments incubated in the absence of combined nitrogen for the number of hours indicated in each case. Samples were hybridized to *devB* (A), *coxB2* (B), or *nifH* (C) probes. Samples contained 25 μg of RNA (A and C) or 40 μg of RNA (B). The lower panels correspond to hybridization to a *rnpB* probe (43) in all cases. Arrowheads point to the transcripts (size in kilobases) previously described for the corresponding genes. WT, wild-type strain PCC 7120.
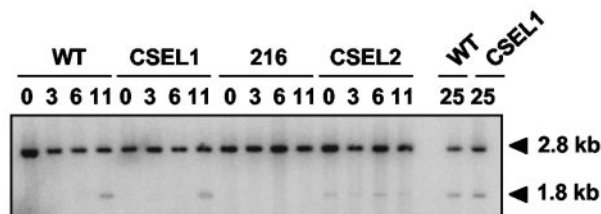

FIG. 5. Excision of the 11-kb *nifD*-intervening element in *Anabaena* sp. strain PCC 7120, the *hetR* strain 216 and strains CSEL1 and CSEL2. DNA was isolated from ammonium-grown filaments (lanes 0) or from ammonium-grown filaments subjected to nitrogen deficiency for the number of hours indicated in each case and digested with HindIII. Excision of the 11-kb element was assessed by Southern blot with a fragment of the *nifD* gene as a probe. The probe hybridizes to a 2.8-kb HindIII fragment in chromosomal DNA prior to rearrangement and to a 1.8-kb HindIII fragment after excision of the *nifD* element. WT, wild-type strain PCC 7120.

which expression of *devBCA* is hampered (13), expression was observed in its derivative strain CSEL2, indicating an NtcA-activated expression independent of HetR (Fig. 4A). Interestingly, *devBCA* transcripts were also detected in the presence of ammonium in both *ntcA*-overexpressing strains.

Induction of the *cox2* operon has been shown to take place upon nitrogen step-down and to depend on both NtcA and HetR (39). For the wild type, the levels of *cox2* transcripts, tested with a *coxB2* probe, were ~7-fold higher in filaments incubated in the absence of combined nitrogen for 24 h than in ammonium-grown filaments (Fig. 4B; see also reference 39). The induction of expression was lower (~3.5-fold for the 24-h time point) and retarded in strain CSEL1. Transcript levels were very low in both the *hetR* strain 216 and its derivative strain CSEL2.

nifHDK transcripts were analyzed in filaments incubated in the absence of combined nitrogen for up to 24 h (Fig. 4C). Consistent with nitrogenase activity (see above), all three transcripts described for the *nifHDK* operon, (i.e., *nifH*, 1.1 kb; *nifHD*, 2.8 kb; and *nifHDK*, 4.7 kb) (19) could be identified earlier in the wild type than in its derivative CSEL1 (Fig. 4C). Although no expression of *nifHDK* was observed in *hetR* strain 216 or its derivative CSEL2, a transcript that might correspond to *nifH* was evident in strain 216 and barely detectable in strain CSEL2 (Fig. 4C).

Excision of the 11-kb *nifD*-intervening element. The chromosomal rearrangement that leads to excision of the 11-kb element depends on NtcA (44). The results shown in Fig. 5 indicate that excision of this element also requires HetR, since there was no rearrangement in *hetR* strain 216. The same results were obtained with *hetR* insertional mutant DR884a (3) (not shown). Overexpression of NtcA did not alter excision of the 11-kb element in the wild-type background (strain CSEL1). However, in the *hetR* background overexpression of NtcA led to excision of the 11-kb element even in the presence of ammonium (Fig. 5). Excision of the 11-kb element in strain CSEL2 was also observed in nitrate-grown cells (not shown). Therefore, overexpression of NtcA forced excision of the 11-kb intervening element in the *hetR* background even in the presence of combined nitrogen.

A repressor role of NtcA has been suggested for the *rbcLXS* operon, which is expressed exclusively in vegetative cells (10), in *Anabaena* sp. strain PCC 7120 (34). In the wild-type strain, expression of this operon at the whole filament level, tested with an *rbcL* probe, was reduced at the 6- and 12-h time points after nitrogen step-down, but by 24 h of nitrogen deficiency reached levels that were similar to those found in the presence of ammonium (Fig. 3B). Consistent with a repressor role of NtcA in *rbcLXS* expression, the levels of *rbcL* transcripts in strain CSEL1 were lower than those found in the wild-type strain under all conditions tested (Fig. 3B).

Expression of *devBCA*, *cox2*, and *nifHDK*. Expression of several NtcA-dependent genes required for heterocyst differentiation and/or function was analyzed in nitrogen step-down experiments. The tested genes included *devBCA* (13), the *cox2* operon encoding a heterocyst-specific cytochrome *c* oxidase (39), and the *nifHDK* operon encoding nitrogenase.

Induction of the *devBCA* operon after nitrogen step-down, tested with a *devB* probe, was higher in strain CSEL1 than in the wild type. In contrast to results obtained with strain 216, in

DISCUSSION

Early events in heterocyst development in *Anabaena* sp. strain PCC 7120 require the induction of expression of *hetR*,

encoding a positive-acting factor that exhibits autoprotease (46) and DNA-binding (24) activities in vitro, and of *ntcA*, encoding the global transcriptional regulator of nitrogen assimilation in cyanobacteria (16, 44). The induction of expression of both genes is mutually dependent in the context of heterocyst development (32). Whereas HetR is specifically required for development and thus for diazotrophic growth, NtcA is also necessary for growth at the expense of nitrate. In the present study we have shown that induction of expression of *ntcA* upon combined nitrogen deprivation results in increased levels of NtcA protein (see Fig. 2C).

Because HetR is required for the transient increase of NtcA levels (32), it can be suggested that the double dependence on HetR and NtcA exhibited by some heterocyst-specific genes could in fact be operated at the transcriptional level via NtcA. In the present study we constructed strains in which HetR-independent high expression of *ntcA* leads to a high content of NtcA protein in the filament irrespective of the nitrogen source both in the wild-type and in the *hetR* backgrounds.

In the wild-type background, the presence of high levels of NtcA protein in the filament does not promote expression of the NtcA-dependent *nir* operon in ammonium-grown cells. This observation is consistent with recent results reported for *Synechococcus* sp. strain PCC 7942 (27) and suggests that NtcA-mediated transcriptional activation might not only involve increased levels of NtcA protein but also perception of the nitrogen status of the cells leading to some modification of NtcA and/or the presence of positive effectors. Alternatively, in the presence of combined nitrogen, NtcA could be modulated by interaction with some negative effector(s). Cyanobacteria have been suggested to perceive the nitrogen status of the cells by sensing levels of 2-oxoglutarate (33). In the presence of 2-oxoglutarate, NtcA shows increased affinity for its binding sites on DNA (40) and increased rates of transcription activation both in vitro (38) and in vivo (41). A recent report has shown that 2-oxoglutarate also has a positive effect on heterocyst development in *Anabaena* sp. strain PCC 7120 (25). In the particular case of the *nir* operon, the lack of additional regulators that are required (22) might contribute to the low level of expression observed in strain CSEL1 in the presence of ammonium. On the other hand, overexpression of NtcA results in a relative repression of the *rbcLXS* operon, whose promoter includes an NtcA binding site with a putative repressor role (34), even in the presence of ammonium. This observation shows that the repressor role of NtcA can be simply achieved by increasing the cellular NtcA levels, as previously suggested for *Synechoccocus* sp. strain PCC 7942 (27).

Strains CSEL1 and CSEL2 do not show any growth defect in the presence of combined nitrogen. However, in the absence of combined nitrogen, strain CSEL1, although capable of first-generation heterocyst differentiation and nitrogen fixation, is not able to maintain diazotrophic growth. When inoculated into fresh medium containing ammonium or nitrate, nitrogen-starved cells were able to resume growth, indicating that the effects of *ntcA* overexpression are not lethal to the cells. In addition to the repression of *rbcLXS* discussed above, strain CSEL1 shows impaired expression of *cox2* and *nifHDK* (Fig. 4B and C). A possible explanation for a deleterious effect of high levels of NtcA in the absence of combined nitrogen can be therefore related to the repressor effects of NtcA. Binding of

NtcA to DNA sequences that are not usually occupied when NtcA is present at physiological levels could lead to the repression of genes specifically required for heterocyst development or function. Alternatively, high *ntcA* expression from the P_{trc} promoter in strain CSEL1 (and CSEL2) could take place in vegetative cells but not in cells that are engaged in the differentiation process. This would result in a deficiency in NtcA that could justify an impairment in development.

Upon combined nitrogen deprivation, first-round induction of heterocyst development genes such as *nifHDK* or *cox2* is impaired in strain CSEL2 (Fig. 4B and C), suggesting that the *hetR* mutation is epistatic to *ntcA* overexpression. In contrast, expression of *devBCA* in the absence of combined nitrogen is stimulated by overexpression of *ntcA* in a *hetR* background. Furthermore, the maximum level of induction is higher in strain CSEL1 than in the wild type, and in the NtcA-overproducing strains some expression of *devBCA* is also observed in the presence of combined nitrogen, which, at least in the case of CSEL2 that does not develop heterocysts, should take place in all of the cells of the filament. These results corroborate that some heterocyst development genes can be directly activated by NtcA and that their dependence on HetR can be overcome by an increase of NtcA levels (23, 32). Interestingly, of the four promoters analyzed here, only that of *devBCA* (13) corresponds to a class II NtcA activated promoter that can be predicted to be activated early, and probably in all cells of the filament, upon combined nitrogen deprivation (23).

As an indication of the progression of heterocyst differentiation, we have also analyzed the rearrangement of the *nifHDK* region that is considered a late event in heterocyst development (18). However, since transcription of the *nifDK* genes only takes place after excision of the *nifD* element (19), the expression of all three *nifHDK* transcripts as early as 12 h (Fig. 4D) indicates that rearrangement of the *nifD* element and expression of *nifHDK* occur hours before the heterocysts are mature and appreciable levels of nitrogenase activity are detected. In the wild-type background, overexpression of *ntcA* (strain CSEL1) does not promote excision of the 11-kb *nifD*-intervening element in the presence of combined nitrogen. Surprisingly, overexpression of *ntcA* does promote excision of the 11-kb element in the *hetR* background (strain CSEL2) even in the presence of ammonium, presumably in some vegetative cells of the filament (Fig. 5). Rearrangement of the *nifHDK* region does not require heterocyst-specific factors, since expression of *xisA* (encoding the excisase of the 11-kb element) from a shuttle vector leads to excision in vegetative cells (4). Our results indicate that excision of the 11-kb element depends on HetR since it does not take place in the *hetR* mutant (Fig. 5). However, HetR also seems to exert a negative effect, perhaps preventing excision in vegetative cells (compare strains CSEL1 and CSEL2 in Fig. 5). One possibility is that the effects of NtcA and/or HetR are exerted through regulation of expression of *xisA*, which has been suggested to be positively regulated by NtcA (7, 34).

To summarize, whereas the induction of expression of some NtcA-activated genes (such as *nifHDK* or the *cox2* operon) is still dependent on HetR in the NtcA-overproducing strains, high levels of NtcA can promote expression of *devBCA* and excision of the 11-kb element in the absence of heterocyst differentiation. The observation that, in the case of *devBCA*

expression, NtcA overexpression is epistatic to the *hetR* mutation indicates that the requirement for HetR could be operated via NtcA in this case. Also, the effects of NtcA and HetR on the accumulation of transcripts could be additive.

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