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Transcription of *hupSL* in *Anabaena variabilis* ATCC 29413 Is Regulated by NtcA and Not by Hydrogen▽

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Nitrogen-fixing cyanobacteria such as *Anabaena variabilis* ATCC 29413 use an uptake hydrogenase, encoded by *hupSL*, to recycle hydrogen gas that is produced as an obligate by-product of nitrogen fixation. The regulation of *hupSL* in *A. variabilis* is likely to differ from that of the closely related *Anabaena* sp. strain PCC 7120 because *A. variabilis* lacks the excision element-mediated regulation that characterizes *hupSL* regulation in strain PCC 7120. An analysis of the *hupSL* transcript in a nitrogenase mutant of *A. variabilis* that does not produce any detectable hydrogen indicated that neither nitrogen fixation nor hydrogen gas was required for the induction of *hupSL*. Furthermore, exogenous addition of hydrogen gas did not stimulate *hupSL* transcription. Transcriptional reporter constructs indicated that the accumulation of *hupSL* transcript after nitrogen step-down was restricted primarily to the microaerobic heterocysts. Anoxic conditions were not sufficient to induce *hupSL* transcription. The induction of *hupSL* after nitrogen step-down was reduced in a mutant in the global nitrogen regulator NtcA, but was not reduced in a mutant unable to form heterocysts. A consensus NtcA-binding site was identified upstream of *hupSL*, and NtcA was found to bind to this region. Thus, while neither hydrogen gas nor anoxia controlled the expression of *hupSL*, its expression was controlled by NtcA. Heterocyst differentiation was not required for *hupSL* induction in response to nitrogen step-down, but heterocyst-localized cues may add an additional level of regulation to *hupSL*.

Cyanobacteria may possess up to three different enzymes with hydrogenase activity: NiFe uptake hydrogenase, NiFe bidirectional hydrogenase, and nitrogenase (32). Nitrogenases produce hydrogen gas as an obligate by-product of the reduction of nitrogen to ammonia (32). To recapture the electrons used to make this hydrogen, most (but not all [19]) nitrogen-fixing cyanobacteria have an uptake hydrogenase encoded by the *hup* genes (30, 31). This uptake hydrogenase comprises a small and a large subunit (*hupS* and *hupL*, respectively) and contains an NiFe cofactor. The uptake hydrogenase captures much of the hydrogen that is produced by nitrogenase, and eliminating this enzyme results in a threefold increase in hydrogen production in heterocyst-forming cyanobacteria (11, 30, 42).

Because hydrogen is produced under N_2-fixing conditions in cyanobacteria, *hupSL* transcription is coordinately regulated with nitrogenase. In *Anabaena variabilis* ATCC 29413 (11), *Anabaena* sp. strain PCC 7120, *Nostoc punctiforme* ATCC 73102 (17), and *Gloeothece* sp. strain ATCC 27152 (23), the transcription of *hupSL* increases in response to nitrogen step-down. In a medium lacking a source of fixed nitrogen, approximately 10% of the cells in some filamentous cyanobacteria differentiate into microaerobic cells called heterocysts, where the oxygen-labile nitrogenase functions under aerobic growth conditions. The transcription of *hupSL* in these cyanobacteria has been reported to occur concomitantly with the differentiation of heterocysts (11). The expression of *hupSL* in *Anabaena* sp. strain PCC 7120 is restricted to heterocysts because there is an excision element in *hupL* that is removed during heterocyst development (4). However, other species, such as *A. variabilis*, lack this excision element, so the role of heterocyst differentiation in the regulation of *hupSL* is unknown.

Factors that may control *hupSL* in *A. variabilis* include hydrogen, nitrogen status or other heterocyst development signals, anoxia (15), and Ni availability (1). In several cyanobacterial species, uptake hydrogenase activity increases in the presence of hydrogen. In *A. variabilis*, exposure to hydrogen resulted in a slight transient increase in uptake hydrogenase activity in whole cells (39). Uptake hydrogenase activity in *Nostoc muscorum* and *Anabaena cylindrica* was up-regulated in response to hydrogen, but only after exposure for several days to the elevated hydrogen concentrations (34). In *Anabaena* sp. strain PCC 7120 and *N. punctiforme* ATCC 73102, the addition of hydrogen to aerobically grown cells caused an increase in uptake hydrogenase activity (14, 24). The addition of hydrogen had no effect on the reversible hydrogenase activity of *Anabaena* sp. strain PCC 7120 (14).

The induction of *hupSL* transcription by hydrogen in some bacteria is controlled by a hydrogen-sensing and the transcriptional regulation system, HupUV, HupT, and HupR (7). While the sequenced genomes of cyanobacteria lack genes similar to this sensory hydrogenase, transcriptional up-regulation of *hupSL* by hydrogen has been reported for *Nostoc muscorum* and *Nostoc punctiforme* (1).

In addition to regulation by hydrogen, nitrogen status may control the transcription of hydrogenase genes in nitrogen-fixing species. The promoters of several cyanobacterial uptake hydrogenase genes contain the consensus sequence for binding the global regulator of nitrogen metabolism, NtcA, suggesting potential regulation by nitrogen status cues (16, 17, 23).

In an attempt to further define the signals controlling *hupSL*

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expression in *A. variabilis*, we tested the effect of hydrogen, anoxia, heterocyst differentiation, and NtcA on transcription of *hupSL*. We demonstrated that neither hydrogen nor anoxic conditions affected the expression of *hupSL*. However, NtcA, but not heterocyst differentiation, per se, was required for the induction of *hupSL* after nitrogen step-down.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. variabilis* strain FD was derived from *A. variabilis* ATCC 29413 by selecting for increased growth at 40°C (6). Strain Avm13 was kindly provided by the laboratory of Thomas Happe (11). All cyanobacterial strains were grown photoautotrophically on agar-solidified Allen and Arnon medium (AA) or in a liquid medium composed of an eightfold dilution of AA (AA/8) (35) either with or without 5.0 mM NH_4Cl and 10 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; pH 7.2), unless otherwise noted. Cultures were routinely grown at 30°C with shaking (200 rpm) and illuminated with cool white fluorescent bulbs at 50 to 80 $\mu E\ m^{-2}\ s^{-1}$. The following antibiotics were added to cyanobacterial strains at the indicated concentrations: neomycin (5 μg/ml for liquid, 40 μg/ml for agar plates), spectinomycin (0.3 μg/ml for liquid, 3 μg/ml for agar plates), or streptomycin (0.3 μg/ml for liquid, 3 μg/ml for agar plates).

In experiments requiring cells to be shifted from AA/8 supplemented with NH_4/TES to AA/8 lacking fixed nitrogen, cells were washed three times in AA/8. The anaerobic induction was performed on cells grown for several transfers in medium containing 5 mM fructose. Cells were washed as described above to remove fixed nitrogen from the medium and resuspended in AA/8 containing 5 mM fructose and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Cultures were grown in the light and were bubbled vigorously with nitrogen or argon gas to remove trace amounts of oxygen. Cells exposed to hydrogen were grown in 250 ml AA/8 in a 500-ml flask bubbled with air alone or air augmented with 10% hydrogen gas. Samples were withdrawn at the indicated time points.

Cultures of *Escherichia coli* were grown overnight in Luria-Bertani (LB) liquid medium or LB agar medium supplemented, when appropriate, with ampicillin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), or spectinomycin (50 μg/ml). Cloning techniques were performed according to protocols described previously in reference 28.

Construction of plasmid pBP283. To make a *hupL*::*lacZ* fusion, a 5.2-kb SalI-to-EcoRI fragment containing *hupSL* was removed from pAAWY14832, a clone used for sequencing the *A. variabilis* genome by the Joint Genome Institute. This fragment was ligated into pBR322, digested with the same enzymes (replacing part of Tet^r), and renamed pBP273. After being digested with EcoRV, pBP273 was ligated with a 1.9-kb SmaI-digested fragment containing the *aadA* gene from pRL5801 and named pBP275. To make pBP283, a 5-kb fragment, containing the promoterless *lacZ* gene from *Escherichia coli* and the neomycin/kanamycin resistance gene C.K3 (Nm^r), was removed from pPE20 by using SmaI (9). This 5-kb fragment was ligated to HpaI-digested pBP275 and was named pBP283. This plasmid was conjugated into *A. variabilis* strains FD and NF76, selecting for single recombination, to make strains BP283/FD and BP283/NF76, respectively.

Hydrogen and acetylene reduction assays. For the detection of hydrogen, 1 ml of culture was injected into a 10-ml serum vial, sealed with an air-tight rubber stopper that had been sparged with argon gas, shaken at 30°C with illumination as described above, and sampled after 1 h. Samples from the headspace (1 ml) were injected into a 5890 series II (HP) gas chromatograph fitted with a Carboxen 1010 Plot fused-silica capillary column (30 m by 0.53 mm; Sigma), and hydrogen was detected by thermal conductivity using argon as the carrier gas. Acetylene reduction assays were performed as described previously in reference 21.

RNA extractions. Cells were grown as described above and centrifuged, and all liquid medium was removed. Cell pellets were flash frozen with liquid nitrogen and stored at −80°C until extraction. Extractions were performed using TRIzol (Sigma) as described previously (25). DNA was removed using the Turbo DNA-Free kit (Ambion) following the manufacturer's instructions. RNA was assayed for traces of DNA by performing PCR as described below for reverse transcription-PCR (RT-PCR) but using *Taq* as the sole polymerase with RnpB-L and RnpB-R primers (Table 1).

Semiquantitative and QRT-PCR. Semiquantitative RT-PCR was performed as described previously in reference 25. Primers were as follows: for *hupSL*, HupSRT-L2 and HupSRT-R2; for *nifH1*, NifH1-L and NifH1234-R; for *nifH2*, NifH2-L and NifH1234R; and for *rnpB*, RnpB-L and RnpB-R. Quantitative RT-PCR (QRT-PCR) was performed using the Superscript III Platinum one-step QRT-PCR kit with ROX (Invitrogen) in 20-μl reaction mixtures according to the manufacturer's recommended protocol. Primers (qNifH-2L and qNifH-2R, qHupSL-2L and qHupSL-2R, and qRnpB-2L and qRnpB-2R) were used at a final concentration of 150 nM each. Assays were performed on a Prism 7700 (ABI) using the following thermal cycle: 55°C for 10 min, 95°C for 5 min, 95°C for 10 s, 58°C for 30 s, 72°C for 30 s, and 40 cycles of steps 3 to 5. The QRT-PCR cycle was immediately followed by a melting curve: 95°C for 15 s, 60°C for 20 s, ramp to 95°C over 19:59 min with data collection, and a final 95°C hold for 15 s. Triplicate reactions were performed for all samples subjected to QRT-PCR. Reaction efficiencies were approximately 1.8, as measured by regression (26). Thus, approximate differences can be calculated by raising the efficiency, 1.8, to the difference in threshold cycle (C_T) value.

β-Galactosidase-mediated in situ localization. In situ localization was performed essentially as described previously (38). Briefly, cells were fixed in 0.01% glutaraldehyde, washed, and exposed to C-12-fluorescein-β-D-galactoside (C_{12}-FDG, a substrate that fluoresces after cleavage by β-galactosidase). Filaments were viewed with an epifluorescence microscope (Zeiss) fitted with a fluorescein filter set and a short-pass filter to block biliprotein fluorescence (excitation, 450 to 490 nm; dichroic mirror, 510 nm; barrier filter, 520 nm). Images were acquired using a Retiga EXi (QImaging) cooled, charge-coupled device camera with IP Labs 4.0 software (BD Biosciences). Exposure time was 1.0 s for fluorescence pictures and about 0.05 s for bright-field images.

RNA ligase-mediated RT-PCR. The transcription start site of *hupSL* was determined essentially as described previously by reference 2. Briefly, total RNA was extracted from cultures of *A. variabilis* strain FD grown in AA/8 in the absence of fixed nitrogen, and DNA was removed as described above. RNA (5 to 10 μg) was treated with 25 U tobacco acid phosphatase (Epicentre Biotechnologies) according to manufacturer's instructions or was left untreated as a control. RNA was extracted with 1 volume of phenol-chloroform and precipitated with ethanol. The RNA oligonucleotide PE (Table 1) was ligated to the free 5′ ends of RNA with T4 RNA ligase (120 U; New England Biolabs). The ligated RNA was extracted and precipitated again as described above, and the RNA was reverse transcribed by using 1 pmol of the primer hupSL-RRT and Superscript III (100 U; Invitrogen) at 50°C for 1 h according to the manufacturer's instructions. DNA from the reverse transcribed (2-μl) reaction mixture was amplified by PCR using the nested primers P1 and hupSL/R (Table 1) in the cycle of 95°C for 5 min, 95°C for 30 s, 56°C for 30 s, and 72°C for 60 s and 30 cycles of steps 3 to 5. A small band (ca. 200 bp) observed only in samples treated with tobacco acid phosphatase was excised from the gel, cleaned (PCR cleanup kit; Qiagen), and cloned using the pCR2.1 TA cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmid from five colonies was purified and sequenced using the M13 forward and reverse primers to determine the 5′ transcription start site.

NtcA-binding assay. NtcA-binding reactions were carried out essentially as described previously (20). PCR products of the 431-bp and 301-bp *hupSL* upstream regions of *A. variabilis* (created using the primer hupSntcAtest-R with hupSupntcA-L and hupSdownntcA-L, respectively), a 282-bp PCR product of the *hetC* promoter region of *Anabaena* sp. strain PCC 7120 (using primers HC1 and HC2 [described previously in reference 22]) or a ca. 500-bp PCR product of the *rnpB* gene from *A. variabilis* (obtained with the rnpB-L and rnpB-R primers [Table 1]) was end labeled with [γ-^{32}P]ATP and incubated for 30 min at room temperature with native purified NtcA (1.25 to 3.75 ng/μl final concentration) in 15 μl binding buffer (HEPES-NaOH, 12 mM; Tris-HCl, 4 mM; KCl, 60 mM; EDTA, 1 mM; dithiothreitol, 1 mM; pH 8.0) that contained salmon sperm DNA (0.05 mg/ml), 8% glycerol, 2-oxoglutarate (0.6 mM), and bovine serum albumin (0.05 mg/ml). The DNA fragments were separated on a 6% polyacrylamide gel. Some experiments included the addition of 20 ng (25-fold excess) unlableled PCR product.

RESULTS

Analysis of *hupSL* transcription. In the filamentous cyanobacterium *A. variabilis* ATCC 29413, *hupSL* expression is up-regulated during the differentiation of heterocysts after nitrogen step-down (11). The expression of *hupSL* began to increase at 12 and 14 h after nitrogen step-down (Fig. 1). The timing of the increase in mRNA agrees with the induction of uptake hydrogenase activity reported elsewhere (11, 39). The expression of *nifH1* followed a pattern similar to that of *hupSL*, with transcript increasing between 12 and 14 h after nitrogen step-down (Fig. 1).

TABLE 1. Bacterial strains, plasmids, and primers used in this work

Strain, plasmid, or primer	Description	Reference or source
Bacterial strains		
A. variabilis ATCC 29413	Wild type	C. P. Wolk
NF76	Point mutant unable to form heterocysts	5
NF76/BP283	*lacZ*-Nm^r inserted into *hupL* by single crossover	This work
A. variabilis ATCC 29413 strain FD	Wild-type strain selected for growth at 40°C	6
Avm13	Nm^r gene replacing *hupS-hupL* genes	11
MM3	Nm^r gene interrupting the *ntcA* gene	38
JE21	Nm^r gene replacing the *nifUII-nifDII* region of the nifII genes	37
JE9	Nm^r gene replacing the *xisA-nifEI* region of the nifI genes	37
BP283/FD	*lacZ*-Nm^r inserted into *hupL* by single crossover	This work
Plasmid		
pBP283	pBR322-derived vector with *lacZ*-Nm^r inserted into *hupL*	This work
Primers		
qNifH-2L	5′-TTAGCCAAGAAAATCATCAACAAC	
qNifH-2R	5′-CTGCGTGCTTAGAATCATCATC	
qHupSL-2L	5′-CCCCCGCTACTTCTATGGATTGTT	
qHupSL-2R	5′-GTCACGCCGAGTTCATAGGATTTG	
qRnpB-2L	5′-AGCAAGGCCGAAGGAACTATGGT	
qRnpB-2R	5′-ATTGCTTTACACGAGGGCGATTAT	
HupSRT-L2	5′-TTAATGCCCCCAACGGTACA	
HupSRT-R2	5′-GCGGTTGCAGGAAGAATGAG	
NifH1-L	5′-CGGCATGACCTATTGGTAGC	
NifH1234-R	5′-GGTGARATGATGGCGATGTAYGC	
NifH2-L	5′-TAGACTACTACTGCTTTGCC	
RnpB-L	5′-AGAGTAGGCGTTGGCGGTTGC	
RnpB-R	5′-ATTGCTTTACACGAGGGCGATTAT	
HupSupntcA-L	5′-ATATAAGCGTGGCTACAACTAACCAA	
HupSdownntca-L	5′-CTGGCAGGATCAAAAAAGCCTCA	
HupSntcAtest-R	5′-GACGCTTGTAGAGGTTCACCTGT	
HC1	5′-TAGTACATCGGTGAGGGGTG	
HC2	5′-TGTGAGCAACATCGACATCTG	
HupSL-RRT	5′-TCGCATACTGTCGGTTCTTC	
HupSL/R	5′-ATATACCCGGGTAGTTTTAGTGGCATTGGCTATTAGTTTGTATATTG	
PE	5′-CTAGTACTCCGCTATTGCGGTACCCTTGTACGCCTGTTTTATA	
P1	5′-GGTATTGCGGTACCCTTGTAC	

Effect of hydrogen on *hupSL* transcription. To understand whether the induction of *hupSL* transcript is controlled by heterocyst-specific developmental cues or is regulated by the hydrogen made by nitrogenase, we examined *hupSL* expression by real-time QRT-PCR in the strain JE9. This mutant, in which part of the *nifD1* excision element and the 3′ end of *nifD1* were replaced by an Nm^r gene, lacks the Mo-nitrogenase and thus produces no hydrogen (Fig. 2B and C) (36). Hydrogen produced from other nitrogenases (the anaerobically induced Nif2 nitrogenase and the Mo-repressed V nitrogenase) was not observed, because these alternative nitrogenases are not expressed under aerobic conditions in the presence of Mo (35, 37). While strain JE9 lacks nitrogenase activity and dies after 2 or 3 days due to nitrogen starvation (Fig. 2A), it still differentiates heterocysts (36). Despite the lack of nitrogenase activity and its concomitant hydrogen production, *hupSL* transcript increased in abundance in strain JE9 (Fig. 2D). This finding suggests that hydrogen was not required for the induction of *hupSL* transcription and that in *A. variabilis*, other cues are involved in *hupSL* regulation.

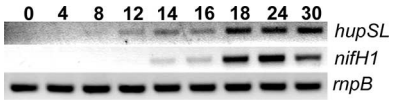

FIG. 1. Induction of *hupSL* and *nifH1* after nitrogen step-down. Time course for *hupSL*, *nifH1*, and *rnpB* transcription measured by RT-PCR for 30 h after nitrogen step-down. Hours after nitrogen step-down are indicated above each lane.

While hydrogen is not required to activate *hupSL* expression in *A. variabilis*, *hupSL* expression might still be modified by hydrogen. To test whether *hupSL* expression is affected by hydrogen, we exposed *A. variabilis* grown in AA/8 to air augmented with 10% hydrogen and measured the abundance of *hupSL* transcript by QRT-PCR. At 8 h, the average change in C_T value relative to a *t* value of 0 for *hupSL* was 0.22 (±0.19) or 0.38 (±0.10) for cells exposed to air alone or to air plus 10% hydrogen, respectively. At 24 h, the average change in C_T value relative to a *t* value of 0 for *hupSL* was 1.44 (±0.63) or 1.22 (±0.85) for cells exposed to air alone or to air plus 10% hydrogen, respectively. Thus, after 8 h or 24 h of exposure to H_2, no increase in *hupSL* transcript was observed that could be attributed to the presence of hydrogen.

Effect of anoxia on *hupSL* transcription. The microaerobic conditions in heterocysts are not established until late in development; hence, the timing of the induction of *hupSL* and

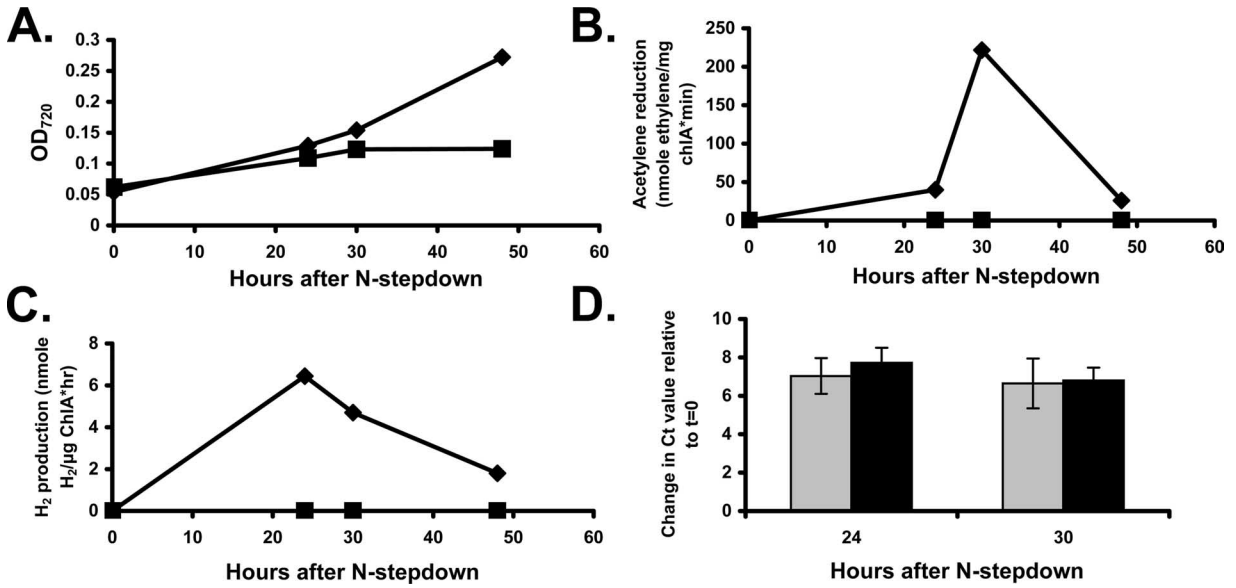

FIG. 2. Hydrogen production by *A. variabilis*. Growth curves (A), acetylene reduction (B), and hydrogen production (C) of strains FD (diamonds) and JE9 (squares) during 48 h after nitrogen step-down. (D) Expression of *hupSL* measured by QRT-PCR in two strains (FD [gray bars] and JE9 [black bars]) expressed as change in C_T value from 0 to 24 h or 0 to 30 h after nitrogen step-down. Thus, each unit represents an approximately twofold difference in the amount of RNA (see Materials and Methods). Error bars indicate standard deviations from the mean for at least three independent experiments. OD_{720}, optical density at 720 nm.

nifH1 transcription might reflect a response to the microaerobic conditions in the heterocyst. Thus, growth under anaerobic conditions in the absence of fixed nitrogen might induce *hupSL*. Cells were washed to remove ammonium from the growth medium and then exposed to either anaerobic or aerobic conditions. Anaerobic conditions were confirmed by acetylene reduction assay at 6 h, showing that the anaerobically induced Nif2 system was active (data not shown) (36). Aerobic cultures had no acetylene reduction activity at 6 h. The transcription of *hupSL* in anaerobic cultures did not increase compared to that in aerobic cultures at either 8 or 24 h after nitrogen step-down (Fig. 3). As a control for anaerobic induction, *nifH2* was found to be expressed strongly at 8 h, with slightly less transcript present at 24 h after nitrogen step-down. Thus, oxygen status does not appear to be the heterocyst-linked cue regulating the transcription of *hupSL*.

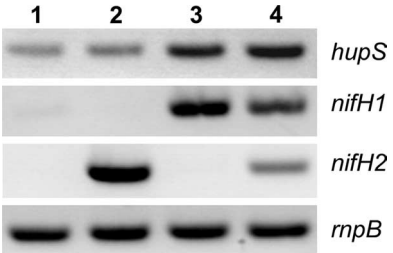

FIG. 3. Effect of anoxia on expression of *hupSL*. Expression of *hupSL*, *nifH1*, and *nifH2* under aerobic and anaerobic conditions 8 and 24 h after nitrogen step-down. Cells were grown in the presence of NH_4-TES (5 mM) and fructose (5 mM), washed, and exposed to aerobic or anaerobic conditions. Samples were harvested at the indicated times for RT-PCR analysis. Lane 1, aerobic conditions at 8 h after nitrogen step-down; lane 2, anaerobic conditions at 8 h after nitrogen step-down; lane 3, aerobic conditions at 24 h after nitrogen step-down; lane 4; anaerobic conditions at 24 h after nitrogen step-down 4. Experiments were repeated at least three times, and representative figures are shown.

Effect of heterocysts and NtcA on *hupSL* transcription. To test whether *hupSL* expression is controlled by heterocyst developmental cues, two mutant strains of *A. variabilis* that are unable to form heterocysts were tested for *hupSL* expression. The first strain, MM3, bears a mutation in *ntcA*, a gene involved in the global regulation of nitrogen status in cyanobacteria (38). Strain MM3 does not differentiate heterocysts and has the delayed phycobilisome degradation following the removal of fixed nitrogen from the growth medium that is typical of *ntcA* mutants (29). Transcripts of *hupSL* and *nifH1* did not accumulate in strain MM3 24 h after nitrogen step-down (Fig. 4A). The low-level transcription of *hupSL* observed when cells are grown in the presence of ammonium was not affected in the *ntcA* mutant (data not shown). Thus, it appears that either NtcA or the downstream signals initiated by this protein during heterocyst differentiation are required for *hupSL* induction in response to nitrogen step-down.

The second mutant strain, NF76, is an uncharacterized mutant that does not form heterocysts or fix nitrogen (5). NF76 has a functional *ntcA* gene, however, since it can use nitrate as a nitrogen source (data not shown), while *ntcA* mutants cannot (41). In contrast to the results for the *ntcA* mutant, *hupSL* was induced after nitrogen step-down in strain NF76 (Fig. 4A). The induction of *nifH1* was also observed in NF76 but was reduced approximately 15-fold compared to the induction of the wild type (Fig. 4A). Despite the induction of *nifH1*, no acetylene reduction activity was measured for NF76 24 h after nitrogen step-down (data not shown). Thus, mature heterocysts are not essential for *hupSL* transcription, but either NtcA or some

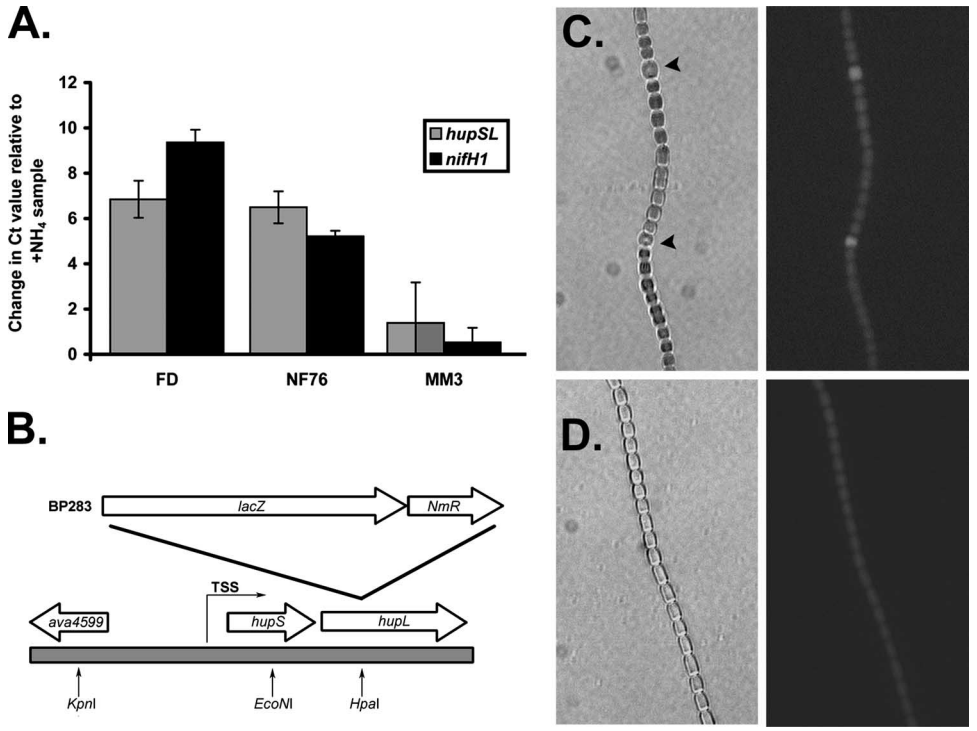

FIG. 4. (A) Induction of *hupSL* and *nifH1* in heterocyst-deficient mutants. The amount of transcript of *hupSL* (gray bars) and *nifH1* (black bars) in FD and mutants NF76 and MM3 was measured by QRT-PCR 24 h after nitrogen step-down. Error bars indicate standard deviations from the mean for at least three independent experiments. (B) Diagram showing insertion of *lacZ*:Nm^r into *hupL* in the plasmid pBP283. (C and D) In situ localization of β-galactosidase in BP283/FD (C) and BP283/NF76 (D). Cells were grown in AA/8 supplemented with NH_4-TES (5 mM), washed, and resuspended in AA/8. Cells were imaged 24 h after nitrogen step-down after incubation with C_{12}-FDG as described in Materials and Methods. Images at left and right are bright-field and epifluorescence microscopy, respectively. Arrowheads indicate heterocysts.

signal derived from NtcA activity is required for *hupSL* induction.

In situ localization of *hupSL* expression. To determine in which cells the induction of *hupSL* occurred in the NF76 mutant, reporter strains with *lacZ* inserted within *hupL* (plasmid pBP283) (Fig. 4B) were constructed. Spatial distribution of β-galactosidase activity along the filament was determined by observing the fluorescence from the intracellular cleavage of the substrate C_{12}-FDG by β-galactosidase. In strain BP283/FD, fluorescence was strongest in heterocysts (Fig. 4C). β-Galactosidase activity was also observed at a much lower level in the vegetative cells and is consistent with reports of *hupSL* expression in these cells (3, 39). Fluorescence in strain BP283/NF76 was observed throughout the vegetative cell filaments, and no localization to any spaced, cryptic proheterocyst cells was observed (Fig. 4D).

Binding of NtcA to the *hupSL* promoter. The regulation of *hupSL* by NtcA suggested that an NtcA-binding site might be present in the promoter. We identified a consensus NtcA-binding site 427 bp upstream of the transcriptional start site (TSS) reported by Happe et al. (11) (Fig. 5C). The ability of the NtcA protein to bind to this site was tested using two PCR products: a 431-bp product that spanned the putative binding site in the *hupSL* promoter and a 301-bp product that contained the *hupSL* promoter but lacked the binding site (Fig. 5C). NtcA was found to bind the fragment of the *hupSL* promoter that included the NtcA site. The presence of this site resulted in much stronger binding compared to that of the shorter fragment of the *hupSL* promoter lacking the NtcA site (Fig. 5A). The addition of the unlabeled product that included the NtcA site in 25-fold excess reduced the binding of the labeled fragment (Fig. 5B), and a negative control fragment from the *rnpB* gene did not bind to the protein (Fig. 5B). Because NtcA-binding sites are typically, but not exclusively, located approximately −40 bp before the TSS (13), we investigated whether an alternative TSS was present. By using RNA ligase-mediated RT-PCR, we identified one TSS 77 bp upstream of the TSS reported by Happe et al. (11) (Fig. 5C), but there was no evidence of a TSS near the NtcA-binding site.

DISCUSSION

Among cyanobacterial strains, the regulatory cue signaling *hupSL* induction varies considerably (31, 33). For example, *Anabaena* sp. strain PCC 7120 contains an excision element in *hupL* that is removed during heterocyst maturation (4), while *hupSL* in *Gloeothece* sp. strain ATCC 27152 is regulated by the global nitrogen regulator NtcA (23). Because *hupSL* regulatory signals vary by species and had not been characterized for *A. variabilis*, we examined several potential *hupSL* regulatory cues, such as hydrogen, anoxia, heterocyst differentiation, and the global nitrogen regulator NtcA.

The induction of *hupSL* transcription in response to nitrogen step-down, but not the basal level of expression observed

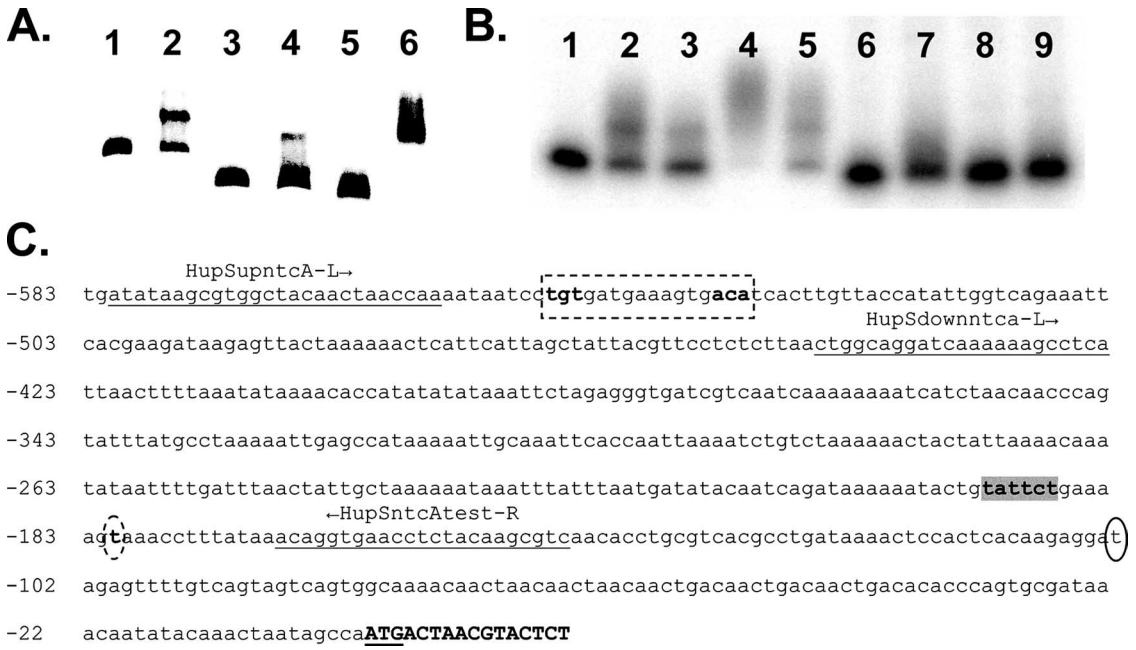

FIG. 5. NtcA binding to the *hupSL* upstream region. (A) Lanes 1 and 2, a 431-bp region upstream of *hupSL* containing a consensus NtcA-binding site incubated without (lane 1) or with (lane 2) NtcA (1.25 ng/μl). Lanes 3 and 4, a 301-bp region upstream of *hupSL* lacking a consensus NtcA-binding site incubated without (lane 3) or with (lane 4) NtcA. Lanes 5 and 6, a 282-bp region upstream of *hetC* containing a strong NtcA-binding site (22) incubated without (lane 5) or with (lane 6) NtcA. (B) Lanes 1 to 5, 431-bp region upstream of *hupSL* containing a consensus NtcA-binding site incubated with the following: no protein (lane 1), 2.5 ng/μl NtcA (lane 2), 3.125 ng/μl NtcA (lane 3), 3.75 ng/μl NtcA (lane 4), and 3.75 ng/μl NtcA with 25-fold cold competitor 431-bp PCR product (lane 5). Lanes 6 to 9, 500-bp fragment of the *rnpB* gene incubated with the following: no protein (lane 6), 3.125 ng/μl NtcA (lane 7), 3.75 ng/μl NtcA (lane 8), and 3.75 ng/μl NtcA with 25-fold cold competitor *rnpB* PCR product (lane 9). (C) Upstream region of *hupSL*. Shown are primers used to amplify the products for the NtcA-binding assay (underlined), a putative NtcA-binding site (dashed box), the TSS published previously by Happe et al. (11) (solid circle), a newly identified TSS (dashed circle), and a consensus −10 site corresponding to the new TSS (gray box). Line numbers indicate nucleotides before the beginning of the coding region. Bold uppercase letters indicate the *hupSL* open reading frame.

in cells grown with ammonium, depended on a functional *ntcA* gene, indicating that NtcA directly, or indirectly via heterocyst differentiation, controls the increased transcription of *hupSL* after nitrogen step-down. The induction of *hupSL* transcript to the same degree in both wild-type cells and the heterocyst-defective mutant NF76 suggested that heterocyst differentiation was not essential for *hupSL* induction. The mutation in NF76 is unknown; however, the strain grows with nitrate, indicating a functional NtcA. The induction of *hupSL* in NF76 might be due to an increase in spaced cells destined for heterocyst differentiation; however, the expression of β-galactosidase in all cells in the BP283/NF76 strain suggested that the increase in *hupSL* transcript occurred in all cells and not in cryptic proheterocysts. Thus, the increase in *hupSL* transcription, on a per cell basis, was much lower in NF76 than in the wild-type strain, suggesting that additional regulation from a heterocyst-related cue is necessary for the full induction observed in heterocysts. One possibility is that the mutated gene in NF76 may play a role in vegetative cell gene repression during the response to nitrogen step-down.

We identified an NtcA-binding site in the *hupSL* promoter 428 bp upstream of the *hupSL* TSS published by Happe et al. (Fig. 5C) (11). This binding site bears the consensus sequence (TGT-N_{9or10}-ACA) shown to be present in other genes controlled by NtcA, such as *xisA*, *glnA*, and *rbcL* (27). Considerable variation in NtcA-binding sequences has been observed, but the minimal consensus (GT-N_{10}-AC) fits the sequence found upstream of *A. variabilis hupSL*.

Binding sites for NtcA in genes positively regulated by NtcA are typically found −40 bp upstream of a TSS, suggesting the possibility that an alternate, NtcA-regulated TSS may exist for *hupSL* (12). We examined this possibility through RNA-ligase-mediated RT-PCR in cells grown without fixed nitrogen. Our finding of a TSS 77 bp upstream of the previously reported TSS may be a result of the different methods used to obtain the TSS. While Happe et al. employed 5′ extension, our method depended upon an intact 5′ triphosphate to which an RNA linker could be ligated and thus selected for an intact 5′ sequence (2). Despite the identification of a new TSS, the consensus NtcA site remains 350 bp upstream of the new TSS. NtcA binding sites at distances greater than the prototypical distance of −40 bp have been demonstrated for several cyanobacterial genes, such as *cox2*, *cox3*, and *furA* (13, 18).

The binding of NtcA to the *hupSL* promoter region was shown to be much stronger in a fragment of the promoter that included the NtcA consensus binding site than in a promoter fragment that lacked this site (Fig. 5C). This finding, when combined with the absence of *hupSL* induction in the *ntcA* mutant, suggests that NtcA positively regulates *hupSL*. Some binding to the smaller fragment that did not contain the consensus NtcA binding site was observed and may indicate that a second nonconsensus NtcA binding site may also be present

closer to the TSS. Because uptake hydrogenases function to recapture hydrogen produced during nitrogen fixation, our finding that NtcA controls *hupSL* expression in *A. variabilis* is consistent with the role of NtcA in nitrogen acquisition.

The transcription of *nifH* in *Nostoc* sp. strain PCC 7120 after nitrogen step-down was detected by Northern analysis between 18 and 24 h (10) or as early as 12 h using more sensitive RT-PCR (40). Consistent with this result, we observed the induction of *nifH1* transcription between 12 and 14 h after nitrogen step-down in *A. variabilis*. The transcription of *hupSL* in *A. variabilis* began at the same time and perhaps even slightly earlier than *nifH1* (Fig. 1). This further supports the idea that hydrogen produced by nitrogenase is not required for the induction of *hupSL*, because the induction occurs before nitrogenase can produce hydrogen. Thus, *hupSL* belongs in the same category of "late development" genes as does *nifH1* in the heterocyst differentiation process.

A partial fumarate nitrate-reductase regulator site was previously identified in the promoter of *hupSL*, pointing to anoxia as a potential regulator of transcription (11). However, anoxic conditions did not affect *hupSL* transcription. It has been reported that the transcription of *nifH1* could not be induced by anaerobic conditions in vegetative cells (8, 37). Thus, the anaerobic conditions found in the heterocyst are not sufficient to induce *hupSL* expression. In addition, no difference was observed when the ratio of hydrogen production to acetylene reduction was compared after anaerobic induction in wild-type FD and AVM13, a *hupSL* mutant strain (data not shown). This result suggests that the normal basal expression of *hupSL* in vegetative cells is not part of a strategy to allow the rapid production of uptake hydrogenase for the anaerobically induced Nif2 nitrogenase that is produced in vegetative cells of this strain.

The control of *hupSL* in the absence of fixed nitrogen is regulated by NtcA, a transcriptional regulator that controls cyanobacterial genes involved in nitrogen metabolism. While it is reasonable to suggest that *hupSL* transcription in *A. variabilis* might be regulated by hydrogen, our studies indicate that this is not the case. The nature of the reported hydrogen-dependent increases in hydrogenase activity is a subject for future study (1).

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