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Expression and Mutational Analysis of the *glnB* Genomic Region in the Heterocyst-Forming Cyanobacterium *Anabaena* sp. Strain PCC 7120[▽]

Javier Paz-Yepes, Enrique Flores, and Antonia Herrero*

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, E-41092 Seville, Spain

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In the filamentous, heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, the *glnB* gene is expressed at considerable levels both in the presence and in the absence of combined nitrogen, although induction, influenced by NtcA, takes place upon combined-nitrogen deprivation likely localized to vegetative cells. In spite of extensive efforts, a derivative of PCC 7120 lacking a functional *glnB* gene could be obtained only with constructs that lead to overexpression of a downstream open reading frames (ORF), particularly all2318. Strain CSP10 [*glnB* all2318(Con)] exhibited growth rates similar to those of the wild type when it was using nitrate or ammonium, but its diazotrophic growth was impaired. However, it differentiated heterocysts with a time course and distribution pattern similar to those of the wild type, although with no cyanophycin-containing polar granules, and exhibited impaired nitrogenase activity under oxic conditions, but not under microoxic conditions. In the mutant, NtcA-dependent inducion of the *hetC* and *nifH* genes was unaltered, but induction of the *urtA* gene and urea transport activity were increased. Active uptake of nitrite was also increased and insensitive to the ammonium-promoted inhibition observed for the wild type. Thus, regulation of the nitrite transport activity requires the *glnB* gene product. In the presence of a wild-type *glnB* gene, neither inactivation nor overexpression of all2318 produced an apparent phenotype. Thus, in an otherwise wild-type background, the *glnB* gene appears to be essential for growth of strain PCC 7120. For growth with combined nitrogen but not for diazotrophic growth, the requirement for *glnB* can be overridden by increasing the expression of all2318 (and/or ORFs downstream of it).

Cyanobacteria are phototrophic prokaryotes that are responsible for a major fraction of the primary productivity in the Earth's oceans and thus play a very relevant role in the C and N cycles at the global scale (20). Many filamentous cyanobacteria have complex genomes and are capable of adaptive processes in response to changing environments, including cellular differentiation processes. In *Anabaena* sp., heterocyst differentiation takes place in response to combined-nitrogen deprivation, producing specialized cells that harbor the nitrogen fixation machinery. This leads to establishment of a filament with two interdependent cell types that exchange nutrients and regulators (11, 47). At the molecular level, the transcription factor NtcA plays a key role in the regulation of gene expression during adaptation to different nitrogen regimens in all cyanobacteria tested to date (18), including the regulation of heterocyst differentiation (19). NtcA directly binds to the promoters of multiple regulated genes to affect transcription initiation modulated by 2-oxoglutarate, which is a molecule indicative of the C-N balance of the cells (28, 40, 41, 45).

The *glnB* gene, encoding the P_{II} protein, has also been found in all the cyanobacteria tested to date. P_{II} is a trimeric protein that in the unicellular cyanobacterium *Synechococcus elongatus* has been shown to bind 2-oxoglutarate and ATP in a mutually stimulating manner (12). In *S. elongatus* and *Synechocystis* sp. strain PCC 6803, P_{II} is modified by phosphorylation at Ser49 in one, two, or three subunits, which is controlled by 2-oxoglutarate according to the C-N balance of the cells (13, 21). P_{II} exerts its regulatory effect by protein-protein interactions. In unicellular cyanobacteria, three different cellular processes have been recognized as processes that are regulated by P_{II}: the active transport of nitrate-nitrite (25), NtcA-dependent gene activation (10, 35), and arginine biosynthesis (17). P_{II} has been demonstrated to be required for the ammonium-promoted inhibition of the active uptake of nitrate and nitrite in *S. elongatus* (25), to bind and increase the catalytic activity of *N*-acetyl-L-glutamate kinase in both *S. elongatus* and *Synechocystis* sp. strain PCC 6803 (17, 27, 31), and to bind PipX, a small protein that could play a role in some NtcA-controlled processes (7, 8). Finally, P_{II} has been shown to interact in vitro with an integral membrane protein of *Synechocystis* sp. strain PCC 6803, termed PamA, which might play a role in regulating the action of some σ factors (34; for a review, see reference 29).

In heterocyst-forming cyanobacteria, the role of P_{II} is not understood. In *Nostoc punctiforme* strain ATCC 29133 (PCC 73102), no modification of the P_{II} protein could be detected in extracts from cells incubated under a variety of conditions (16). In contrast to the situation in *S. elongatus* (14), the *N. punctiforme glnB* gene could not be disrupted by targeted mutagenesis, suggesting that it has an essential role at least under the laboratory conditions tested (16). In *Anabaena* sp. strain PCC 7120, P_{II} has been described to be modified in vegetative cells,

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* Corresponding author. Mailing address: Instituto de Bioquímica Vegetal y Fotosíntesis, Centro de Investigaciones Científicas Isla de la Cartuja, Américo Vespusio 49, E-41092 Seville, Spain. Phone: 34954489522. Fax: 34954460065. E-mail: herrero@ibvf.csic.es.

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but not in heterocysts, after incubation in the absence of combined nitrogen (24). The modification in *Anabaena* sp. has been reported to consist of nitration of Tyr51 instead of the phosphorylation of Ser49 found in unicellular cyanobacteria (48).

Like the results reported for *N. punctiforme*, Laurent et al. (24) reported that attempts to isolate a P_{II}-null mutant of *Anabaena* sp. strain PCC 7120 were not successful. In contrast, generation of a derivative of PCC 7120 carrying a *glnB* gene inactivated with a cre-loxP system has been reported by Zhang et al. (48). This mutant expressed no detectable P_{II} protein, grew relatively well (approximately one-half as fast as the wild type) with ammonium, nitrate, or N_2 as the nitrogen source, and developed normal heterocysts in the absence of combined nitrogen. Here we describe a study of the expression and mutational analysis of the *Anabaena glnB* gene and the downstream open reading frame (ORF) all2318, which was aimed at gaining insight into the role of the P_{II} protein in heterocyst-forming cyanobacteria.

TABLE 1. Oligodeoxynucleotide primers used in this work

Oligodeoxynucleotide	Sequence
NIF1	5′-GTACTGCAAGGGGCGTGTGGG-3′
NIF2	5′-CACGCCTGTTTGAGCTATGGC-3′
HC5	5′-AGAGTTGAGCCAAAACTGG-3′
HC6	5′-GTAAGGGTAACTGCAACG-3′
glnB-7120-1	5′-AAATCGCTTTAGTCAACGC-3′
glnB-7120-2	5′-AATCCTTACTTACACGGC-3′
glnB-7120-3	5′-CAAGGAGTCAAGAGGTAG-3′
glnB-7120-5	5′-CTCGAGACCAAGCAACCG-3′
glnB-7120-6	5′-TTCATCTAGATTAAATGGGC-3′
glnB-7120-8	5′-TCTAGAGAAGAATACAGAAGCCGTG-3′
glnB-7120-9	5′-AAAGTCGTTAGTGCAAGGAGTCA-3′
all2318-2	5′-GCCAGTGTAAGTCATACCTG-3′
all2318-4	5′-CTCGAGATCATATTGCTTGCAA-3′
all2318-5	5′-CCCAAATTCAATTAGCAGCAGGTG-3′
all2318-6	5′-CGCACCGGCAATATAATTCAAG-3′
gfp3	5′-GATCTAGATATGAGTAAAGGAGAAG-3′
gfp4	5′-GTCTAGACATTTATTTGTATAGTTC-3′
sacB1	5′-CTTGAGGTACAGCGAAGTG-3′
sacB2	5′-TCTGCAAAAGGCCTGGAGG-3′
URT11	5′-CACAAGCAAATCCCTTAGTTT-3′
URT18	5′-CGGCTCTGCAACTATTGGTAGC-3′

MATERIALS AND METHODS

Strains and growth conditions. *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 was grown axenically at 30°C in the light (85 microeinsteins $\cdot$ m^{-2} $\cdot$ s^{-1}) in a shaker. BG11 (nitrate-containing) medium (38), BG11$_0$ medium (BG11 medium lacking $NaNO_3$), or BG11$_0$ medium supplemented with 4 mM NH_4Cl and 8 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH buffer (pH 7.5) was used. For mutants, the medium was supplemented with 5 to 10 μg neomycin (Nm) $\cdot$ ml^{-1} (for strains carrying gene cassette C.K1 or C.K3) or 2 to 5 μg spectinomycin (Sp) $\cdot$ ml^{-1} and 2 to 5 μg streptomycin (Sm) $\cdot$ ml^{-1} (for strains carrying gene cassette C.S3). For plates, the medium was solidified with 1% separately sterilized agar (Difco). Cultures used for RNA isolation were grown in medium BG11$_0$C (BG11$_0$ medium supplemented with 10 mM $NaHCO_3$), in medium BG11C (BG11 medium supplemented with 10 mM $NaHCO_3$), or in medium BG11$_0$C supplemented with 8 mM NH_4Cl and 16 mM TES-NaOH buffer (pH 7.5) and bubbled with a mixture of CO_2 (1%, vol/vol) and air. RNA was also isolated from cells grown in the last medium, and when cultures reached a density of 4 to 5 μg of chlorophyll *a* [Chl] $\cdot$ ml^{-1}, they were harvested at room temperature, washed twice with medium BG11$_0$C, resuspended in medium BG11$_0$C, and incubated for the indicated times under culture conditions with CO_2-enriched air in the medium indicated. Cyanobacterial cell mass was estimated by measuring the concentrations of Chl in the cultures, which were determined using methanolic extracts of the cells (30).

To test growth of the mutants in liquid medium, cells of the strains that had been grown in BG11 medium (with antibiotics for the mutants) were harvested, washed with BG11$_0$ medium, and resuspended in BG11$_0$ medium, BG11 medium, or BG11$_0$ medium supplemented with 4 mM NH_4Cl and 8 mM TES-NaOH buffer (pH 7.5) at a concentration of 0.2 μg Chl $\cdot$ ml^{-1}. After incubation for the indicated times, 0.2-ml samples were taken, and their protein contents were determined (32). The growth rate constant ($\mu = \ln_2/t_d$, where μ is the growth rate constant and t_d is the doubling time) was calculated from the increase in the protein content. To test growth of the mutants on solid medium, drops (10 μl) of cell suspensions of the different strains at a concentration of 1 μg Chl $\cdot$ ml^{-1} were spotted on plates of BG11$_0$ medium, BG11 medium, or BG11$_0$ medium supplemented with 4 mM NH_4Cl and 8 mM TES-NaOH buffer (pH 7.5), and the plates were incubated under culture conditions.

Escherichia coli strain DH5α was grown in LB medium to which antibiotics were added, when necessary, at the following concentrations: ampicillin, 50 μg $\cdot$ ml^{-1}; Sp, 25 μg $\cdot$ ml^{-1}; Sm, 25 μg $\cdot$ ml^{-1}; and chloramphenicol, 30 μg $\cdot$ ml^{-1}.

DNA and RNA isolation, manipulation, and analysis. Isolation of genomic DNA from *Anabaena* sp. was carried out as described previously (3). Isolation of total RNA from *Anabaena* sp. was performed as described previously for gram-negative bacteria (1), and isolation of total RNA from isolated heterocysts was performed as described by Valladares et al. (42).

For Northern blots, 20 μg of RNA was loaded per lane and electrophoresed in denaturing 1% agarose formaldehyde gels. DNA probes were generated by PCR using *Anabaena* DNA and oligonucleotide primers (Table 1), as follows: for *glnB*, the probe was a 299-bp fragment amplified with oligonucleotides glnB-7120-1 and glnB-7120-2; for all2318, the probe was a 446-bp fragment amplified with oligonucleotides all2318-5 and all2318-6; for *hetC*, the probe was a 1,598-bp fragment amplified with oligonucleotides HC5 and HC6; for *nifH*, the probe was a 1,231-bp fragment amplified with oligonucleotides NIF1 and NIF2; and for *urtA*, the probe was a 1,222-bp fragment amplified with oligonucleotides URT11 and URT18. Hybridization was performed as previously described (36). As a control for RNA loading and transfer efficiency, the filters were hybridized with a probe for the RNase P RNA gene (*rnpB*) from strain PCC 7120 amplified by PCR with universal and reverse primers using plasmid pT7-7120 as the template (46). Probes were labeled with a DNA labeling kit (Ready to Go; Amersham Pharmacia Biotech) and [α-^{32}P]dCTP. Radioactive areas in Northern hybridization blots were visualized and quantified with a Cyclone storage phosphor system (Packard).

Inactivation of *glnB*. Two DNA fragments were amplified by PCR using primers glnB-7120-5 and glnB-7120-6 and primers glnB-7120-8 and alr2318-4 (Table 1). The PCR products were cloned in vector pGEM-T (Promega), and the plasmids generated were designated pCSP25 (bearing the 5′ end terminal part of *glnB* and sequences upstream of it) and pCSP26 (bearing the 3′ end terminal part of the gene and downstream sequences, including all2318), respectively. Both plasmids were digested with XbaI plus ScaI and religated, producing plasmid pCSP29, in which the terminal parts of the *glnB* gene are joined at the XbaI site. The 2-kb Sm^r Sp^r gene cassette C.S3 from pRL463 [pRL138/LHEH1 (BamHI)/C.S3; nomenclature of Elhai and Wolk (6)], the 1.4-kb Nm^r gene cassette C.K1 from pRL161 [S.A1/LHEH1 (BamHI)/C.K1; nomenclature of Elhai and Wolk (6)], or the 1.4-kb Nm^r gene cassette C.K3 from pCSE48 [pRL500/LEHE2 (SalI)/C.K3 (SalI/XhoI) from pRL278 (2); nomenclature of Elhai and Wolk (6)] excised with XbaI was inserted into the unique XbaI site that was present in the pCSP29 insert, producing plasmids pCSP34, pCSP35, and pCSP32, respectively. In both pCSP35 and pCSP32, the *npt* gene was in the same orientation as *glnB*. The insert in plasmids pCS34, pCS35, and pCS32 was excised with XhoI and cloned into XhoI-linearized plasmid pRL278 carrying an Nm^r determinant and the *sacB* gene (2) (the insert from pCSP34) or into pRL277 carrying an Sm^r Sp^r determinant and *sacB* (2) (the inserts from pCSP35 and pCSP32), producing plasmids pCSP39, pCSP36, and pCSP37, respectively. These plasmids were transferred by triparental mating to *Anabaena* sp. strain PCC 7120 using pRL623 as a helper plasmid (5). To test segregation of the mutant chromosomes, PCR analyses were performed for exconjugants using primers glnB-7120-3 and glnB-7120-2 (Table 1).

To complement the *glnB* mutation of strain CSP10, a 0.75-kb DNA fragment of the *glnB* region from position −403 to position 348 with respect to the translational start site of the gene, including the full *glnB* gene, was amplified by PCR using primers glnB-7120-9 and glnB-7120-2 (Table 1). The PCR product was cloned in the pMBL-T vector (Dominion), and the plasmid generated was designated pCSP78. Once this plasmid was checked by sequencing, the insert was excised with PstI and EcoRI and cloned between the PstI and EcoRI sites of plasmid pCSEL24, which carries a fragment of the α megaplasmid from strain PCC 7120 and gene cassette C.S3 encoding an Sm^r Sp^r determinant inserted into the *nucA* gene (33), producing plasmid pCSP82. This plasmid was transferred to strain CSP10 by conjugation (see above). The presence of a wild-type *glnB* gene in strain CSP10 was tested by PCR using primers glnB-7120-1 and glnB-7120-2 (Table 1).

Inactivation of all2318. An internal fragment of all2318 encompassing nucleotides 155 to 600 with respect to the translation start site of all2318 was amplified by PCR using primers all2318-5 and all2318-6 (Table 1) and whole DNA from strain PCC 7120 as the template. The PCR product was cloned in the vector pGEM-T Easy (Promega), and the plasmid generated was designated pCSP54. The insert in this plasmid was excised with SphI and SpeI and cloned between the SphI and SpeI sites of plasmid pRL271 (2), from which it was extracted with PstI and SpeI and cloned into the PstI and SpeI I sites of plasmid pRL277 (see above), producing plasmid pCSP68. Plasmid pCSP68 was transferred by conjugation to *Anabaena* sp. strain PCC 7120 (see above). To test segregation of the mutant chromosomes, PCR analysis was performed with DNA from exconjugants using primers glnB-7120-8 and all2318-2 (Table 1).

Construction of a GlnB-GFP translational fusion. A 736-bp DNA fragment encompassing the *gfp* gene, without its promoter, was amplified from plasmid pAM1819 (4, 9) with primers gfp3 and gfp4 (Table 1) and cloned in the vector pGEM-T, producing plasmid pCSP46. The *gfp* gene was excised from pCSP46 with XbaI ends and ligated into XbaI-linearized plasmid pCSP25, which contains the promoter of the *glnB* gene (see above), producing plasmid pCSP75, which bears P_{glnB} directing expression of the fused first 35 bp of *glnB* and the *gfp* gene. Finally, the insert of pCSP75, excised with XhoI and SpeI, was cloned into the XhoI and SpeI sites of conjugative plasmid pRL278, which carries an Nm^r determinant (2), producing plasmid pCSP76. This plasmid was transferred to strain PCC 7120 by conjugation, and selection for resistance to Nm was performed. The genomic structure of exconjugants was analyzed by PCR with the oligonucleotide pairs gfp3/gfp4 and sacB1/sacB2 (Table 1). Accumulation of the green fluorescent protein (GFP) reporter was analyzed by laser confocal microscopy using a Leica HCX PLAN-APO 63× 1.4 NA objective and a Leica TCS SP2 microscope (Leica, Wetzlar, Germany).

Nitrogenase and nitrite and urea transport activities. Nitrogenase activity was measured in cultures grown in BG11$_0$C medium supplemented with 8 mM NH$_4$Cl and 16 mM TES-NaOH buffer (pH 7.5) (in the presence of Nm for mutant strain CSP10) and bubbled with a mixture of CO$_2$ (1%, vol/vol) and air. The filaments were harvested, washed, and incubated for 24 h in BG11$_0$C medium bubbled with a mixture of CO$_2$ (1%, vol/vol) and air, and the acetylene reduction assay was carried out under oxic and microoxic conditions as described previously (43). Nitrite uptake was measured in cells grown in medium BG11C (supplemented with 5 μg Nm · ml^{-1} for the CSP10 mutant) bubbled with a mixture of CO$_2$ (1%, vol/vol) and air, using 100 μM NaNO$_2$ in 25 mM glycine buffer (pH 9.6) in the presence or absence of 500 μM NH$_4$Cl (25). Urea transport activity was measured in cells grown in medium BG11$_0$C supplemented with NH$_4$Cl (in the presence of antibiotics for the mutants), bubbled with a mixture of CO$_2$ (1%, vol/vol) and air, and incubated for 3 h in the same medium or in medium BG11$_0$C without antibiotics bubbled with a mixture of CO$_2$ (1%, vol/vol) and air, using 0.1 μM [^{14}C]CO(NH$_2$)$_2$ as described previously (44).

RESULTS AND DISCUSSION

Expression of the *Anabaena glnB* gene. A search of the complete genomic sequence of *Anabaena* sp. strain PCC 7120 (22) for ORFs encoding homologues of GlnB from the unicellular cyanobacterium *S. elongatus* strain PCC 7942 identified the all2319 ORF, which should encode a protein exhibiting 89% identity to the *Synechococcus* GlnB protein and 99% identity to GlnB of the heterocyst former *N. punctiforme* strain PCC 73102.

The expression of the *Anabaena glnB* gene was analyzed using whole filaments of the wild-type strain incubated under different nitrogen regimens, as well as the two different cell types, vegetative cells and heterocysts, of diazotrophically grown filaments. When expression was tested by Northern analysis, substantial levels of a ca. 0.55-kb transcript (the size of all2319 is 339 bp) were detected using RNA from whole filaments grown with ammonium. The level of this transcript, however, increased rapidly upon transfer of the cells to media lacking combined nitrogen, reaching a maximum of ca. 1.7-fold greater than the levels found in the presence of ammonium about 2 h after the transfer (Fig. 1). A smeared signal that indicated the presence of longer transcripts was also observed in the absence, but not in the presence, of ammonium (Fig. 1).

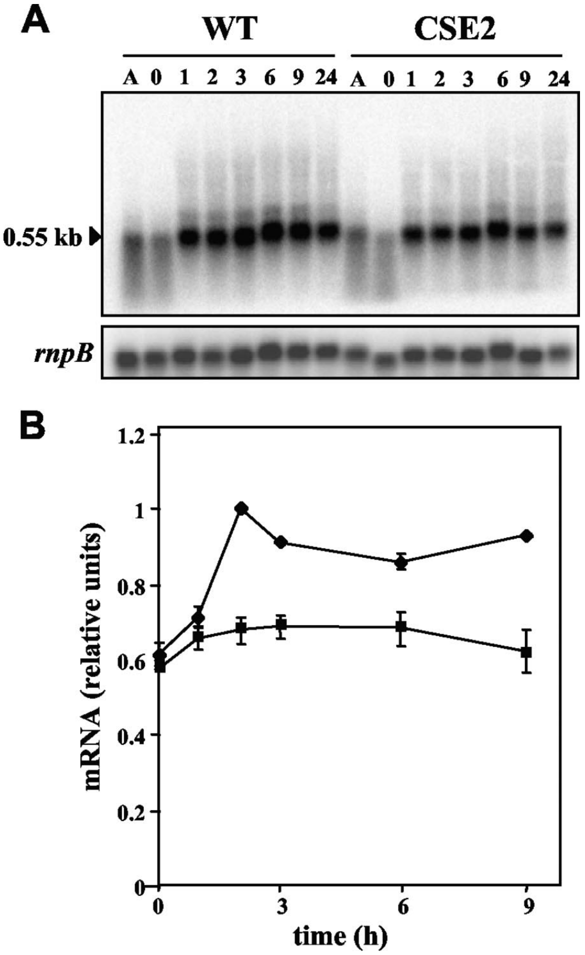


FIG. 1. Expression of the *glnB* gene in *Anabaena* sp. strains PCC 7120 and CSE2. (A) Expression of *glnB* analyzed by Northern blotting with RNA isolated from whole filaments grown with ammonium (lanes A) and incubated without combined nitrogen for the times indicated above the lanes (in hours). The filter was subsequently hybridized with a probe for the strain PCC 7120 *rnpB* gene (see Materials and Methods for details). The size of the predominant band hybridizing with the *glnB* probe is indicated on the left. WT, wild type. (B) Quantification of the results of Northern blot assays. The data are the means and standard deviations of the values, normalized to the *rnpB* levels, for five independent experiments.

Because these results indicated that there was modulation of *glnB* expression by nitrogen, expression was also analyzed in strain CSE2, a mutant of strain PCC 7120 containing an inactivated version of the *ntcA* gene (15). Figure 1 shows that the increase in *glnB* transcript levels that occurred in the wild-type strain upon removal of ammonium was impaired in the mutant, since the maximum level reached was ca. 1.25-fold greater than the level in the presence of ammonium.

Northern analysis was also performed with RNA isolated from whole filaments grown with ammonium, nitrate, or dinitrogen as the nitrogen source, as well as from heterocysts isolated from diazotrophically grown filaments (Fig. 2). In all cases, the 0.55-kb transcript was detected, and its level was

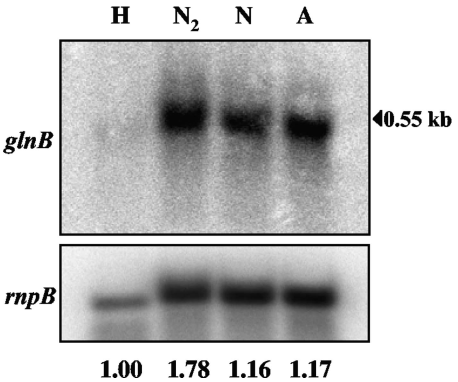


FIG. 2. Expression of the *glnB* gene in filaments of strain PCC 7120 grown with different N sources. Expression of *glnB* was analyzed by Northern blotting with RNA isolated from whole filaments grown with ammonium (lane A), with nitrate (lane N), or without combined nitrogen (lane N_2) or from isolated heterocysts (lane H). The filter was subsequently hybridized with a probe for strain PCC 7120 *rnpB* (see Materials and Methods for details). The size of the predominant band hybridizing with the *glnB* probe is indicated on the right. The numbers under the images indicate the relative amounts, normalized to the *rnpB* amounts, of the *glnB* transcript.

somewhat higher (ca. 1.6-fold) in whole filaments from the diazotrophic cultures than in whole filaments grown with combined nitrogen (either ammonium or nitrate). Moreover, in diazotrophic cultures the transcript levels were higher (ca. 1.8-fold) in whole filaments than in isolated heterocysts, suggesting that the expression levels of *glnB* in vegetative cells are about twice those in heterocysts.

Differential expression of *glnB* in the two cell types of diazotrophic filaments of strain PCC 7120 was also examined by using GFP as a reporter. The *gfp* gene was inserted, maintaining the translation frame, at nucleotide 35 after the translation start site of *glnB* in the *Anabaena* genome (see Materials and Methods). The selected clone (strain CSP15) had incorporated the P_{glnB}-*gfp* construct by single crossover, thus keeping an intact copy of the wild-type P_{glnB}-*glnB* region (not shown). Fluorescence from GFP was analyzed for diazotrophically grown filaments of strain CSP15. It was observed to be consistently higher in vegetative cells than in heterocysts (Fig. 3), in agreement with the results obtained by Northern analysis described above.

Inactivation of the *glnB* gene. In order to study the function of the GlnB protein in strain PCC 7120, we aimed at generation of a mutant strain lacking a functional *glnB* gene. Different constructs were generated in vitro (see Materials and Methods for details) and consisted of a DNA fragment from the strain PCC 7120 *glnB* region, extending from 1,049 bp upstream of the gene to 1,036 bp downstream of all2318, carrying gene cassette C.K1 (including a kanamycin resistance [Km^r] determinant expressed from a promoter that is weak in *Anabaena* and lacking transcription terminators), gene cassette C.K3 (including a Km^r determinant expressed from a strong promoter in *Anabaena* and lacking transcription terminators), or gene cassette C.S3 (including an Sm^r Sp^r determinant and transcription terminators) (6) substituting for most of the *glnB* gene. These constructs were inserted into conjugative plasmids bearing the *sacB* gene that confers sensitivity to sucrose (3) and were transferred to strain PCC 7120 by conjugation with selection for resistance to the determinant of the gene cassette inserted into *glnB*. Antibiotic-resistant clones were then selected on the basis of resistance to 5% sucrose, and the selected clones were checked for sensitivity to the antibiotic for which a resistance determinant was present in the vector portion of the conjugative plasmid transferred to *Anabaena* sp. Clones exhibiting the antibiotic resistance of the inserted gene cassette, resistance to sucrose, and sensitivity to the antibiotic of the vector-encoded resistance were expected to have incorporated the mutagenic construct into the *Anabaena* genome by double crossover replacing the wild-type region with the mutant region. The genomic structure of the *glnB* region was analyzed by PCR for a number of clones for each different construct. Clones with only mutant chromosomes could be obtained with gene cassette C.K3, and these segregated clones were obtained with a high frequency; ca. 40% of the clones showed Km resistance, resistance to sucrose, and sensitivity to Sm and Sp. One of these clones was selected and designated strain CSP10 (Fig. 4). As expected, strain CSP10 did not express the *glnB* gene (Fig. 5). Despite exhaustive trials, including numerous rounds of filament fragmentation and growth under different conditions (liquid or solid medium and different N sources), no segregation of the mutant chromosomes could be obtained with either of the other two gene cassettes used. Also, despite numerous trials, we were unable to isolate a fully segregated clone bearing only mutant versions of the *glnB* gene generated either by markerless deletion of nucleotides 36 to 314 of the gene or by removal of the C.K3 gene cassette present in the genome of strain CSP10.

Given that in strain CSP10 the C.K3 gene cassette inserted into the *glnB* gene expresses the Km^r determinant from a strong promoter and that it does not bear transcription terminators (6), we checked whether expression of ORF all2318, which is located downstream from *glnB* (Fig. 4), was altered. The expression of all2318 in strain CSP10 was compared to the expression in the wild type by performing a Northern analysis with RNA isolated from cultures grown with ammonium or grown with ammonium and incubated in the absence of combined nitrogen. The hybridization levels (including signals corresponding to transcript sizes similar to those found in the wild

FIG. 3. Expression of the *glnB* gene along diazotrophic filaments of *Anabaena:* transmitted light (A), autofluorescence (B), and GFP fluorescence (C) images of a filament of strain CSP15 grown in the absence of combined nitrogen (see Materials and Methods for details). The arrow in panel C indicates a heterocyst.

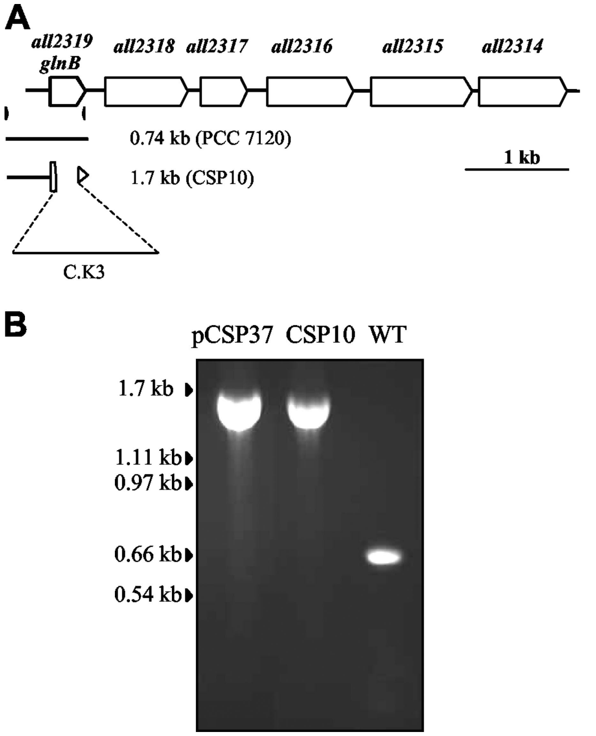


FIG. 4. Genomic structure of strain CSP10 in the *glnB* region. (A) Diagram of the genomic region including the *glnB* gene, ORFs downstream of it, and the mutation introduced to generate strain CSP10 (see Materials and Methods for details). (B) PCR analysis performed with DNA from strain PCC 7120 (WT) or CSP10 or plasmid pCSP37 as the template and oligonucleotides glnB-7120-3 and glnB-7120-2 as the primers (positions indicated in panel A). The positions of bands of ClaI-digested phage λ DNA, used as size standards, are indicated on the left.

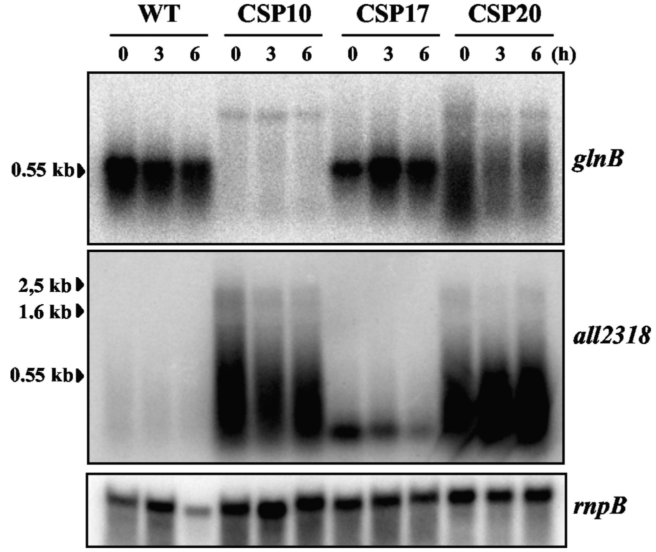
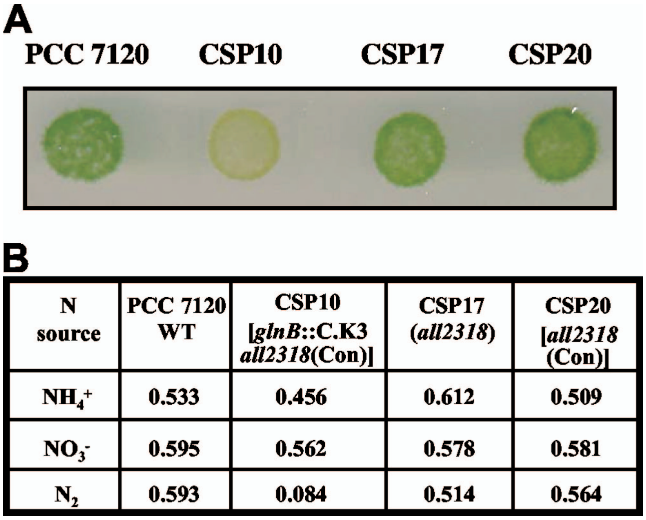


FIG. 5. Expression of the *glnB* gene and ORF all2318 in strains PCC 7120 (WT), CSP10, CSP17, and CSP20. Expression of *glnB* and all2318 was analyzed by Northern blotting with RNA isolated from whole filaments grown with ammonium and incubated without combined nitrogen for the numbers of hours indicated above the lanes. The filter was successively hybridized with probes for *glnB*, all2318, and *rnpB* (see Materials and Methods for details). The sizes of some apparent bands are indicated on the left.

type plus signals from longer transcripts that should have originated inside the gene cassette inserted into *glnB*) were similar in the presence and absence of ammonium, and they were much higher (ca. 20 times higher from ca. 2.4 kb down) in CSP10 than in the wild-type strain. Thus, in strain CSP10 all2318 is expressed from the strong constitutive promoter introduced with gene cassette C.K3.

These results can be explained by assuming that in *Anabaena* sp. strain PCC 7120 the *glnB* gene is essential, at least under the standard laboratory conditions used, and that the lack of a functional *glnB* gene can, at least under certain conditions, be overridden by overexpression of a downstream ORF(s). Consistent with these results are the previously reported unsuccessful attempts to isolate a *glnB* insertional mutant of this strain (24) or of another heterocyst-forming cyanobacterium, *N. punctiforme*, by insertion of cassette Ω that bears transcription terminators (16). On the other hand, as mentioned above, isolation of a *glnB* null mutant of strain PCC 7120 with a cre-loxP system has been reported by Zhang et al. (48). Because this strain is able to grow with ammonium, nitrate, or N_2 as the nitrogen source and to form apparently normal heterocysts, it has been concluded that P_{II} is not required for heterocyst differentiation (48). Because in this mutant a long foreign DNA fragment, including an Sm^r determinant and a plasmid vector, is inserted between ORF all2320 (located upstream from *glnB* in the wild-type chromosome) and all2318, overexpression of all2318 could explain the apparent inconsistency between the results obtained by Zhang et al. (48) and our results. Alternatively, the *glnB* mutant strain used (48) could carry some kind of suppressor mutation.

Phenotype of strain CSP10. We analyzed the phenotype of strain CSP10 [*glnB* all2318(Con)] with respect to growth with different nitrogen sources and expression of NtcA-regulated genes. Whereas strain CSP10 could grow on solid medium with either ammonium or nitrate to an extent similar to that of the wild type, growth was clearly impaired in the absence of combined nitrogen (Fig. 6A). In liquid media, growth rate constants were calculated; the values were similar for CSP10 and PCC 7120 in medium supplemented with nitrate and slightly lower for CSP10 with ammonium, and the values for CSP10 were only about 14% those for the wild type in medium lacking combined nitrogen (Fig. 6B). In spite of the very poor growth of strain CSP10 under diazotrophic conditions, this organism was able to differentiate heterocysts with a time course and distribution along the filament similar to those observed for the wild-type strain (Fig. 7). However, heterocysts of strain CSP10 lacked cyanophycin granules (a polymer of aspartate and arginine constituting a reservoir of N [39]) at the heterocyst poles (Fig. 7). A lack of heterocyst cyanophycin granules has also been described for a mutant of strain PCC 7120 with an impaired *prpS* gene encoding a putative P_{II} phosphatase (24). In strain CSP10, the lack of cyanophycin granules in the heterocysts could result from deficient N_2 fixation activity or from a lack of activation by P_{II} of the arginine biosynthesis enzyme *N*-acetylglutamate kinase, which has been described for unicellular cyanobacteria (17, 31) (see above). However, the last possibility would not explain the impaired diazotrophic



N source	PCC 7120 WT	CSP10 [*glnB*::C.K3 *all2318*(Con)]	CSP17 (*all2318*)	CSP20 [*all2318* (Con)]
NH_4^+	0.533	0.456	0.612	0.509
NO_3^-	0.595	0.562	0.578	0.581
N_2	0.593	0.084	0.514	0.564

FIG. 6. Growth of mutant strains CSP10, CSP17, and CSP20 with different N sources. (A) Cell suspensions of the strains grown with nitrate (and supplemented with the appropriate antibiotics in the case of mutants) and washed with combined-nitrogen-free medium were used to inoculate (10 ng Chl per spot) plates of $BG11_0$ medium that were incubated for 15 days under culture conditions (see Materials and Methods for details). (B) Cell suspensions of strains PCC 7120, CSP10, CSP17, and CSP20 grown with nitrate (and supplemented with the appropriate antibiotics in the case of mutants) and washed with combined-nitrogen-free medium were used to inoculate (0.2 μg Chl ml^{-1}) liquid cultures in media with the indicated N sources that were incubated under culture conditions (see Materials and Methods for details). Aliquots were withdrawn from the cultures at different times up to 80 h to determine the protein content. The numbers are specific growth rate constants (in day^{-1}). WT, wild type.

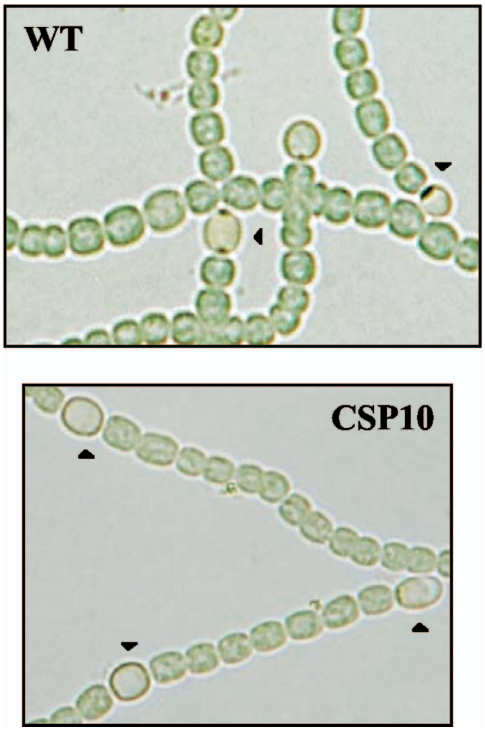


FIG. 7. Microscopic examination of filaments of strain CSP10 incubated without combined N. Cell suspensions of strains PCC 7120 (WT) and CSP10 grown with ammonium and incubated without combined N for 24 h were observed by optical microscopy. The arrowheads indicate heterocysts. Note the lack of polar granules in the heterocysts of strain CSP10.

growth of strain CSP10, as cyanophycin synthesis has been shown to be dispensable in *Anabaena* sp. strain PCC 7120 (37).

The nitrogenase activity in strain CSP10 was measured under both oxic and microoxic conditions and compared to that in PCC 7120 after incubation in combined-nitrogen-free medium. Whereas under microoxic conditions the activity levels were comparable for the two strains (47 and 35 nmol ethylene formed μg $chlorophyll^{-1}$ min^{-1} for strains PCC 7120 and CSP10, respectively [averages of two independent experiments]), the levels in strain CSP10 were only ca. 15% those in the wild type under oxic conditions (30 and 4 nmol μg $chlorophyll^{-1}$ min^{-1} for the wild type and the mutant, respectively). These results show that P_{II}, acting either directly or indirectly, has a role in protection of the nitrogenase enzyme against oxygen in the heterocyst. The observed impairment of the nitrogenase activity of strain CSP10 can explain the impairment of its diazotrophic growth.

Expression of several genes that in the wild-type strain are activated by the transcriptional regulator NtcA upon withdrawal of ammonium (see reference 19) was studied in strain CSP10 by performing a Northern analysis with RNA isolated from cells grown with ammonium or grown with ammonium and incubated in the absence of combined nitrogen. For both conditions, the *nifH* (encoding nitrogenase reductase) and *hetC* (encoding an ABC-type transporter involved in heterocyst differentiation [23]) expression levels in strain CSP10 were similar to those in the wild type (not shown). On the other hand, the expression levels of *urtA* (encoding the substrate-binding protein of the Urt urea transporter [44]) were ca. 1.7 higher in strain CSP10 than in PCC 7120 3 h after withdrawal of ammonium, a time point at which the maximum level of expression of the gene was observed (Fig. 8A). These results are consistent with the higher urea transport activity exhibited by strain CSP10 than by the wild type (Fig. 8B). Taken together, the results for characterization of strain CSP10 show that this strain is not impaired in the general response to nitrogen step-down.

Because in *S. elongatus* the P_{II} protein is required for the inhibition by ammonium of the active transport of nitrate and nitrite mediated by the Nrt transporter (25), the uptake of nitrite carried out at pH 9.6 to minimize diffusion of nitrous acid (25) and the effect of ammonium in strain CSP10 were tested and compared with those in strain PCC 7120. Figure 9 shows that the transport activity was somewhat higher (ca. 1.3-fold) in CSP10 than in the wild type. Moreover, whereas 98% inhibition of the uptake activity by ammonium was observed for strain PCC 7120, a considerably smaller effect (14% inhibition) was observed for strain CSP10. Thus, nitrite transport activity is regulated by ammonium with a requirement for the *glnB* gene product in *Anabaena* sp. strain PCC 7120.

Generation of mutants with an alteration in ORF all2318. To test whether the impairment of diazotrophic growth of strain CSP10 resulted from inactivation of *glnB* or from overexpression of all2318, we generated two different strains, one

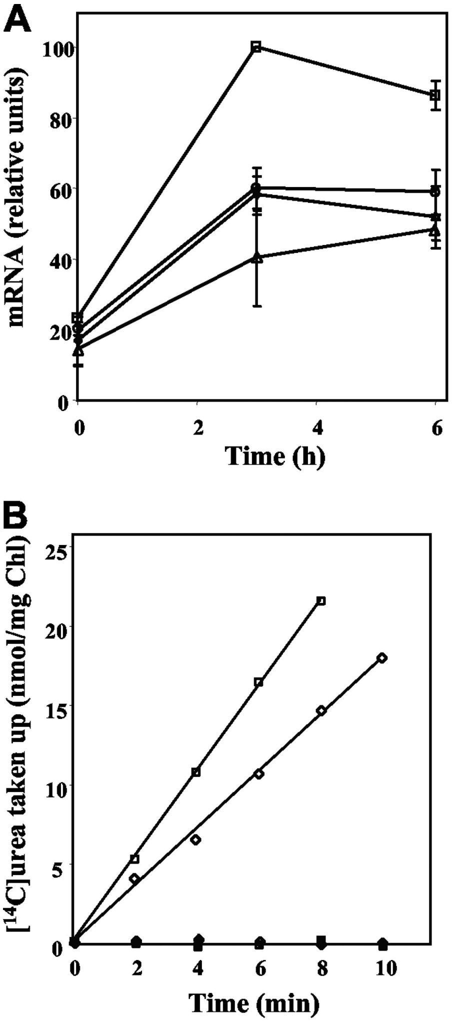


FIG. 8. Transcript levels of the *urtA* gene and urea transport activities of strain PCC 7120 and mutants. (A) Expression of *urtA* was analyzed by Northern blotting with RNA isolated from whole filaments of strain PCC 7120 (diamonds), CSP10 (squares), CSP17 (circles), and CSP20 (triangles) grown with ammonium and incubated without combined nitrogen for the indicated numbers of hours. The filter was subsequently hybridized with a probe for the strain PCC 7120 *rnpB* gene (see Materials and Methods for details). The data are the means and standard deviations of the values resulting from quantification of the Northern blot assays, normalized to the *rnpB* values, from four independent experiments. (B) Filaments of strain PCC 7120 (diamonds) or CSP10 (squares) grown with ammonium and incubated for 3 h without combined nitrogen (open symbols) or with ammonium (filled symbols) were used for urea transport activity assays (see Materials and Methods). At the indicated times, the radioactivity incorporated by the cells was measured using aliquots withdrawn from the assay mixtures. Data from a representative experiment are shown.

with all2318 inactivated and the other overexpressing this ORF, both of which had a wild-type *glnB* gene. To inactivate all2318, strain PCC 7120 was used as the recipient for conjugal transfer of plasmid pCSP68, which carries an internal fragment of all2318 extending from position 155 to position 600 cloned in vector pRL277 encoding $\mathrm{Sm^r}$ $\mathrm{Sp^r}$. This plasmid integrated by single recombination into the all2318 locus, and a clone exhibiting resistance to Sm and Sp and lacking wild-type copies of all2318 (not shown) was selected and designated strain CSP17. As expected, no transcript covering all2318 could be detected in strain CSP17 (Fig. 5), which, however, exhibited levels of the ca. 0.55-kb *glnB* transcript similar to those in the wild-type strain (Fig. 5). Strain CSP17 exhibited growth rates using ammonium, nitrate, or N_2 (Fig. 6) and levels of expression of the *nifH*, *devB* (not shown), and *urtA* genes (Fig. 8A) similar to those of strain PCC 7120.

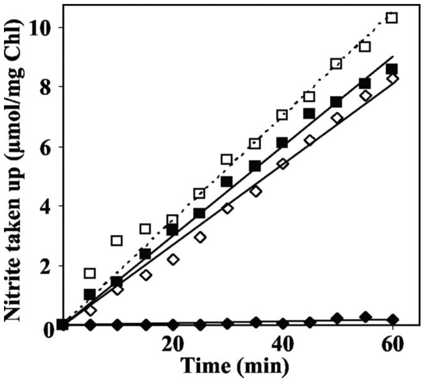


FIG. 9. Nitrite uptake activities of strains PCC 7120 and CSP10. Filaments of strain PCC 7120 (diamonds) or CSP10 (squares) grown with nitrate were used for nitrite uptake activity assays in the absence (open symbols) or presence (filled symbols) of ammonium (see Materials and Methods). At the indicated times, nitrite was measured using aliquots withdrawn from the assay mixtures. Data from a representative experiment are shown.

To generate a strain overexpressing all2318 in the presence of a wild-type *glnB* gene, strain CSP10 was complemented for its *glnB* mutation. Strain CSP10 was used as the recipient for conjugal transfer of plasmid pCSP82, which carries a fragment of the *glnB* genomic region extending from position -403 to position 348 with respect to the translational start of the gene and flanked by sequences of the strain PCC 7120 α megaplasmid and an $\mathrm{Sm^r}$- and $\mathrm{Sp^r}$-encoding determinant. A clone bearing a wild-type *glnB* gene inserted into the α megaplasmid was selected and designated strain CSP20. (It should be pointed out that in strain PCC 7120 the copy number of the α megaplasmid is similar to that of the chromosome [26].) As expected, in strain CSP20 the hybridization signals corresponding to all2318 were similar to those in strain CSP10, whereas *glnB* was expressed at levels substantially higher than those in strain CSP10 (Fig. 5). Strain CSP20 exhibited growth rates using ammonium, nitrate, or N_2 (Fig. 6) and levels of expression of *nifH*, *devB* (not shown), and *urtA* (Fig. 8A) similar to those of strain PCC 7120.

A comparison of the results obtained with strains CSP10 [*glnB* all2318(Con)], CSP17 (all2318), and CSP20 [all2318(Con)] indicates that the impairment of diazotrophic growth (as well as the increase in *urtA* expression and urea transport activity) exhibited by strain CSP10 results from inactivation of the *glnB* gene rather than from overexpression of a downstream ORF(s).

Concluding remarks. In *Anabaena* sp. strain PCC 7120, the *glnB* gene is expressed at high levels under all N conditions tested and in both vegetative cells and heterocysts when it is growing diazotrophically. However, expression appears to increase in vegetative cells when there is combined-nitrogen deprivation in a process influenced by NtcA. The permanent expression of this gene, together with the difficulty of inactivating it in a wild-type background, suggests that its product has an essential role in cellular functions under all conditions tested.

The requirement for a functional *glnB* gene can be overridden by overexpression of an ORF(s) downstream of this gene during growth with combined nitrogen, but not during diazotrophic growth. Although the levels of expression of *glnB* are lower in heterocysts than in vegetative cells, the *glnB* product appears to have a role, which cannot be counteracted by overexpression of a downstream ORF(s), in protection of the nitrogenase system against O_2 involving an as-yet-unidentified target(s). In vegetative cells, the *glnB* gene product appears to be required for the ammonium-promoted regulation of nitrite transport activity. However, the *glnB* gene is not needed for a general response of *Anabaena* sp. strain PCC 7120 to nitrogen deprivation, including NtcA-dependent activation of gene expression, at least when an ORF(s) downstream of *glnB* is overexpressed.

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