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

Received 2 October 2008/Accepted 13 January 2009

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 underoxic conditions, but not under microoxic conditions. In the mutant, NtcA-dependent induction of the hetC and nifH genes was unaltered, but induction of the uralA 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 PII protein, has also been found in all the cyanobacteria tested to date. PII 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, PII is modified by phosphorylation at Ser49 in one, two, or three subunits, which is controlled by 2-oxoglutarate and according to the C-N balance of the cells (13, 21). PII exerts its regulatory effect by protein-protein interactions. In unicellular cyanobacteria, three different cellular processes have been recognized as processes that are regulated by PII: the active transport of nitrate-nitrite (25), NtcA-dependent gene activation (10, 35), and arginine biosynthesis (17). PII 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, PII 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 PII is not understood. In Nostoc punctiforme strain ATCC 29133 (PCC 73102), no modification of the PII 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, PII has been described to be modified in vegetative cells,
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 PII-null mutant of Anabaena sp. strain PCC 7120 were not successful. In contrast, generation of a derivative of PCC 7120 carrying a glnBII gene inactivated with a cre-loxP system has been reported by Zhang et al. (48). This mutant expressed no detectable PII protein, grew relatively well (approximately one-half to as fast as the wild type) with ammonium, nitrate, or N2 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 glnBII gene and the downstream open reading frame (ORF) all2318, which was aimed at gaining insight into the role of the PII protein in heterocyst-forming cyanobacteria.

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 microinocultures - m² - 1 s⁻¹) in a shaker. BG11 (nitrate-containing) medium (38), BG110 medium (BG11 medium lacking NaNO₃), or BG110 medium supplemented with 4 mM NH₄Cl 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) · ml⁻¹ (for strains carrying gene cassette CK1 or CK3) or 2 to 5 μg spectinomycin (Sp) · ml⁻¹ and 2 to 5 μg streptomycin (Sm) · ml⁻¹ (for strains carrying gene cassette CS3). For plates, the medium was solidified with 1% separately sterilized agar (Difco). Cultures used for RNA isolation were grown in medium BG110C (BG110 medium supplemented with 10 mM NaHCO₃, in medium BG11C (BG11 medium supplemented with 10 mM NaHCO₃), or in medium BG11C supplemented with 8 mM NH₄Cl and 16 mM TES-NaOH buffer (pH 7.5) and bubbled with a mixture of CO₂ (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) · ml⁻¹, they were harvested at room temperature, washed twice with medium BG11C, resuspended in medium BG11C, and incubated for the indicated times under culture conditions with CO₂-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 BG111 medium, and resuspended in BG111 medium, BG111 medium, or BG111 medium supplemented with 4 mM NH₄Cl and 8 mM TES-NaOH buffer (pH 7.5) at a concentration of 0.2 μg Chl · 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 (μ = ln₂t₀, where μ is the growth rate constant and t₀ 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 · ml⁻¹ were spotted on plates of BG11 medium, BG11 medium, or BG11 medium supplemented with 4 mM NH₄Cl 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 · ml⁻¹; Sp, 25 μg · ml⁻¹; Sm, 25 μg · ml⁻¹; and chloramphenicol, 30 μg · 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 glnBII, 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, and for ort4, 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 (mpRP) by strain PCC 7120 amplified by PCR with universal and reverse primers using plasmid pT7-7120 as the template (26). Probes were labeled with a DNA labeling kit (Ready to Go; Amersham Pharmacia Biotech) and [α-32P]dCTP. Radioactive hybridization blots were visualized and quantified with a Cyclone storage phosphor system (Packard).

Inactivation of glnBII. 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 of the gene and the downstream fragment of the gene and the 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 glnBII gene are joined at the XbaI site. The 2-kb Smr Spr gene cassette C.S3 from pRL463 [pRL138/LHEH1 (BamHI) gene, and the down-
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 1 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 Xbal ends and ligated into XbaI-linearized plasmid pCSPT5, which contains the promoter of the glnB gene (see above), producing plasmid pCSPT5, which bears PnrtB directing expression of the fused first 35 bp of glnB and the gfp gene. Finally, the insert of pCSP75, excised with Xhol and SpeI, was cloned into the Xhol and SpeI sites of conjugal plasmid pRL278, which carries an Nm 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 BG11C medium supplemented with 8 mM NH4Cl and 16 mM TEA-NaOH buffer (pH 7.5) (in the presence of Nm for mutant strain CSP10) and bubbled with a mixture of CO2 (1%, vol/vol) and air. The filaments were harvested, washed, and incubated for 24 h in BG11C medium bubbled with a mixture of CO2 (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 μM Nm and 1 for the CSP10 mutant) bubbled with a mixture of CO2 (1%, vol/vol) and air, using 100 μM NaNO2 in 25 mM glycine buffer (pH 9.6) in the presence or absence of 500 μM NH4Cl (25). Urea transport activity was measured in cells grown in medium BG11C supplemented with NH4Cl (in the presence of antibiotics for the mutants), bubbled with a mixture of CO2 (1%, vol/vol) and air, and incubated for 3 h in the same medium or in medium BG11C without antibiotics bubbled with a mixture of CO2 (1%, vol/vol) and air, using 0.1 μM [14C]CO(NH2)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 Synechococcus PCC 7942 identified the all2318 ORF, which should encode a protein exhibiting 89% identity to the Synechococcus Glb protein and 99% identity to Glb 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).

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
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 grown with different N sources, no segregation of the mutant chromosomes could be obtained with either of the other two gene cassettes used. Also, despite 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' 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. 

Expression of ORF all2318 in strain CSP10 was compared to the expression in the wild type by performing a Northern analysis using 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. 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 N₂) 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.

**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 presence of combined nitrogen (see Materials and Methods for details). The arrow in panel C indicates a heterocyst.
type plus signals from longer transcripts that should have originated inside the gene cassette inserted into glnB (see Materials and Methods for details). The positions of bands of ClaI-digested phage λ DNA, used as size standards, are indicated on the left.

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 /H9024 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 N₂ as the nitrogen source and to form apparently normal heterocysts (Fig. 7). However, heterocysts of strain CSP10 lacked cyanophycin granules (a polymer of aspartate and arginine constituting a reservoir of N) 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 PII phosphatase (24). In strain CSP10, the lack of cyanophycin granules in the heterocysts could result from deficient N₂ fixation activity or from a lack of activation by PII 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
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/g chlorophyll/min 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/g chlorophyll/min 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
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 Smr Spr. 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 N₂ (Fig. 6) and levels of expression of the nifH, devB (not shown), and urtA genes (Fig. 8A) similar to those of strain PCC 7120.

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 1/11002 to position 348 with respect to the translational start of the gene and flanked by sequences of the strain PCC 7120 megaplasmid and an Sm r- and Sp r-encoding determinant. A clone bearing a wild-type glnB gene inserted into the megaplasmid was selected and designated strain CSP20. (It should be pointed out that in strain PCC 7120 the copy number of the 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 N₂ (Fig. 6) and levels of expression of nifH, devB (not shown), and urtA genes (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 in-
crease 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 O2 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 over-expressed.

Acknowledgments

We thank Victoria Merino-Puerto for the nitrogenase activity measurements and Rosio Lpez-Igual for nitrite uptake measurements. J.R. was the recipient of an FPI fellowship from the Ministerio de Educaci n y Ciencia (Spain). This work was supported by grants BFU2004-00872 and BFU2007-60457 from the Ministerio de Educaci n y Ciencia (Spain).

References