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Posttranscriptional Regulation of Glutamine Synthetase in the Filamentous Cyanobacterium *Anabaena* sp. PCC 7120: Differential Expression between Vegetative Cells and Heterocysts[▽]

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Genes homologous to those implicated in glutamine synthetase (GS) regulation by protein-protein interaction in the cyanobacterium *Synechocystis* sp. strain PCC 6803 are conserved in several cyanobacterial sequenced genomes. We investigated this GS regulatory mechanism in *Anabaena* sp. strain PCC 7120. In this strain the system operates with only one GS inactivation factor (inactivation factor 7A [IF7A]), encoded by open reading frame (ORF) *asl2329* (*gifA*). Following addition of ammonium, expression of *gifA* is derepressed, leading to the synthesis of IF7A, and consequently, GS is inactivated. Upon ammonium removal, the GS activity returns to the initial level and IF7A becomes undetectable. The global nitrogen control protein NtcA binds to the *gifA* promoter. Constitutive high expression levels of *gifA* were found in an *Anabaena ntcA* mutant (CSE2), indicating a repressor role for NtcA. *In vitro* studies demonstrate that *Anabaena* GS is not inactivated by *Synechocystis* IFs (IF7 and IF17), indicating the specificity of the system. We constructed an *Anabaena* strain expressing a second inactivating factor, containing the amino-terminal part of IF17 from *Synechocystis* fused to IF7A. GS inactivation in this strain is more effective than that in the wild type (WT) and resembles that observed in *Synechocystis*. Finally we found differential expression of the IF system between heterocysts and vegetative cells of *Anabaena*.

Glutamine synthetase (GS) catalyzes the ATP-dependent amidation of glutamate to yield glutamine. This enzyme operates sequentially with the enzyme glutamate synthase (GOGAT), which catalyzes the transfer of the amide group from glutamine to 2-oxoglutarate to yield two molecules of glutamate. This pathway (commonly known as the GS-GOGAT cycle) represents the connecting step between carbon and nitrogen metabolism. In most of the systems studied, control of GS activity responds to carbon and nitrogen signals. In the presence of abundant carbon sources, nitrogen deficiency results in a high level of GS activity. In contrast, when the nitrogen source is abundant, GS activity is downregulated (12, 14).

In cyanobacteria, GS type I (here referred to as GS) is modulated at the transcriptional and posttranscriptional levels, depending on the carbon and nitrogen supply (19). The posttranslational modification by adenylylation that occurs in enterobacterial glutamine synthetase does not exist in cyanobacteria. However, in these organisms an ammonium-dependent GS posttranslational regulation mechanism involving protein-protein interaction has been reported (8, 28). This system has been studied in detail in the cyanobacterium *Synechocystis* sp. strain PCC 6803 and consists of a reversible interaction of GS with two small proteins, inactivation factor 7 (IF7) and IF17, encoded by the *gifA* and *gifB* genes, respectively (8). The analysis of mutant strains devoid of IF7, IF17, or both revealed that each of these proteins contributes to GS inactivation *in vivo*, and a maximal level of inactivation was observed when both proteins were present (8). The expression of *gifA* and *gifB* genes, encoding IF7 and IF17, respectively, is repressed by NtcA, the main factor responsible for nitrogen control in cyanobacteria (9, 10). Thus, when ammonium is added to the medium, IF7 and IF17 are expressed and GS is inactivated. Ammonium removal provokes repression of *gif* genes and also determines the rapid degradation of IF7 and IF17 previously accumulated upon ammonium addition (6).

In filamentous cyanobacteria, early studies by Orr and Haselkorn demonstrated that GS from *Anabaena* sp. strain PCC 7120 is controlled neither by adenylylation nor by feedback inhibition by glutamine; however, levels of glutamine synthetase are lower in ammonium-grown cells than in cells grown using nitrate or dinitrogen as the nitrogen source (25, 26). As in other cyanobacteria, expression of the structural gene for glutamine synthetase (*glnA*) is regulated at the transcriptional level in *Anabaena* and this control is mediated by NtcA (5). The promoter region of the *glnA* gene has a complex structure in this strain and has been well characterized under different nitrogen regimens in vegetative cells and heterocysts (31).

Genes homologous to *gifA* or *gifB* have been found in many cyanobacterial genomes, although they seem to be absent in *Prochlorococcus*. However, ammonium-promoted downregulation of glutamine synthetase activity has been well documented only in *Synechocystis* sp. PCC 6803. Therefore, we found it interesting to explore the operation of the GS regulatory mechanism mediated by inactivating factors (IF7 or IF17 homologs) in other cyanobacterial groups. *Anabaena* sp. PCC 7120 possesses a single ferredoxin-dependent GOGAT (Fd-GOGAT) enzyme, whereas *Synechocystis* harbors both NADH-dependent and ferredoxin-dependent GOGAT enzymes (16, 22). In addition, Fd-GOGAT is absent in heterocysts from *Anabaena*, indicating

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the lack of a complete GS-GOGAT pathway in these cells (16). Hence, we decided to further investigate GS regulation and ammonium sensing in both cell types from this model cyanobacterium. Here we demonstrate that GS from a filamentous cyanobacterium is also regulated posttranscriptionally by the IF-mediated system, which is NtcA dependent. We also analyze the specificity of the interaction between IFs and GSs from different strains. Furthermore, using *gfp* as a reporter gene, we show a differential ammonium sensing between heterocysts and vegetative cells.

MATERIALS AND METHODS

Strains and culture conditions. *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 wild-type (WT) strains and the *Anabaena* strains generated in this work, the Δ*gifA*, ACHI, and AGFP strains, were grown photoautotrophically at 30°C in BG11 medium (29) supplemented with 1 g liter^{-1} $NaHCO_3$ (BG11C) and bubbled with a continuous stream of 1% (vol/vol) CO_2 in air under continuous fluorescent illumination (50 μmol photons · m^{-2} · s^{-1} white light). The CSE2 strain was cultivated in BG11C medium supplemented with 5 mM NH_4Cl and 10 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) buffer, pH 7.5. *Thermosynechococcus elongatus* BP-1 was grown under the same conditions described above for *Anabaena* and *Synechocystis* but at 45°C. For plate cultures, BG11C liquid medium was supplemented with 1% (wt/vol) agar. Ammonium treatment of cultures was performed by addition of 10 mM NH_4Cl and 20 mM TES buffer, pH 7.5. Ammonium removal was carried out by harvesting the cells by filtration, washing them, and resuspending them with BG11C. To place cultures under dinitrogen growth conditions, cells from BG11C medium or from BG11C supplemented with NH_4Cl were harvested by filtration at room temperature, washed, and resuspended in $BG11_0C$ medium (BG11C medium without $NaNO_3$).

Insertional mutagenesis of the *gifA* gene in *Anabaena*. Two fragments of 575 and 400 bp, encompassing part of the *asl2329* locus and the 5′ region and part of the *asl2329* locus and the 3′ region, respectively, were amplified by PCR using *Anabaena* genomic DNA. These fragments were cloned into pGEM-T plasmid (Promega), generating the plasmid pAN3. This plasmid contains a deletion of the *asl2329* locus and also a BamHI restriction site. This site was used to clone a Sm^r Sp^r C.S3 cassette (27) from pRL463 (pUC18/19 containing L.HEH1 and C.S3; nomenclature of Elhai and Wolk [4]) in both orientations, generating plasmids pANSP(+) and pANSP(−), respectively. XhoI-digested fragments from pANSP(+) or pANSP(−) were ligated to XhoI-digested pRL278 vector (1), generating the targeting plasmids pRLANSP(+) and pRLANSP(−), respectively. To generate Δ*gifA*(+) and Δ*gifA*(−) strains, plasmids pRLANSP(+) and pRLANSP(−), respectively, were introduced into the *Anabaena* wild-type strain by conjugation (3). Substitution of wild-type *gifA* by C.S3-interrupted versions was confirmed by Southern blot analysis.

Sequencing of *gifA* locus. The *gifA* locus was PCR amplified from genomic DNA of wild-type *Anabaena* and the CSE2 mutant strain by using primers 5′CTCTTGCAGTGTTCTGTTGCTGG3′ and 5′GAGTTACTTCCTCTAATA ACAACC3′. Direct sequencing of PCR products was carried out by the Eurofins MWG operon sequencing service.

Conjugation of wild-type and mutated *gifA* to the CSE2 strain. The *gifA* wild-type version was amplified by PCR using primers 5′GATCAGATCTCTC TTGCAGTGTTCTGTTGCTGG3′ and 5′GATCAGATCTGGAAGTAACTT CAACAATGAG3′ and *Anabaena* DNA as template. The *gifA* mutated version was generated by a two-step PCR process using primers 5′GATGCGCTAATA TTAGCAAGTGAAGAATCG3′ and 5′CGATTCTTCACTTGCTAATATTA GCGCATC3′ to introduce mutations, and the same primers were used to amplify the wild-type version in the second step. Fragments containing both *gifA* versions were BglII digested and ligated to BglII-digested pCSAV81 (31), generating plasmids pWTgifA and pMTgifA. These plasmids were transferred by conjugation (3) to strain CSE2. The correct integration of these constructs in the *nucA-nuiA* region of the *Anabaena* α megaplasmid was checked by PCR.

GS assay. GS activity was determined *in situ* by using the Mn^{2+}-dependent γ-glutamyltransferase assay in cells permeabilized with mixed alkyltrimethylammonium bromide (MTA) (17). For the analysis of the *in vitro* GS-IF interaction, binding reactions were carried out in a final volume of 20 μl containing purified *Anabaena* or *Synechocystis* GS and increasing amounts of IF7, IF17, IF7A, or IF17N/IF7A in HEPES-NaOH buffer, pH 7.0, 50 mM KCl. After the GS-IF complex formation, the same GS assay described above but without MTA addition was performed. One unit of GS activity corresponds to the amount of enzyme that catalyzes the synthesis of 1 μmol min^{-1} of γ-glutamylhydroxamate.

RNA isolation and Northern blot analysis. Total RNA was isolated from 25-ml samples of *Anabaena* cultures at the mid-exponential phase (3 to 5 μg/ml chlorophyll). Extractions were performed by vortexing cells in the presence of phenol-chloroform and acid-washed baked glass beads (0.25- to 0.3-mm diameter; Braun, Melsungen, Germany) as previously described (7). For Northern blotting, 15 μg of total RNA was loaded per lane and electrophoresed on denaturing formaldehyde-containing 1.2% agarose gels. Transfer to nylon membranes (Hybond N-plus; Amersham Pharmacia Biotech), prehybridization, hybridization, and washes were performed as recommended by the manufacturer. PCR-synthesized fragments encompassing the entire *gifA*, *gifB*, or *glnA* genes were used as probes. As a control, the filters were reprobed with a 640-bp DNA fragment containing the constitutively expressed RNase P RNA gene (*rnpB*) from *Anabaena* (33). Hybridization signals were quantified with a Cyclone Phosphor system (Packard).

Protein expression and purification. For IF7A-His_6, IF7-His_6, IF17-His_6, and IF17N/IF7A-His_6 expression, NdeI-XhoI fragments containing the corresponding *gif* gene were synthesized by PCR and cloned into the pET24a(+) plasmid (Novagen, La Jolla, CA) to generate pASET24, pSET24, pLET24, and pALSET24 plasmids, respectively. Construction of the *gifB*/*gifA* chimeric gene for IF17N/IF7A protein expression is described below. Exponentially growing *Escherichia coli* BL21 cells transformed with each of these plasmids were treated with 0.5 mM isopropyl-β-D-thiogalactoside for 4 h. IF17-His_6 was purified from *E. coli* as previously described (6). IF7A-His_6, IF7-His_6, and IF17N/IF7A-His_6 were purified by Ni-affinity chromatography using His-Bind matrix (Novagen) following the manufacturer's instructions. Fractions that showed GS inactivation activity were pooled and subjected to gel filtration chromatography using a HiLoad 16/60 Superdex 75 gel filtration column (GE Healthcare) running on an Akta fast protein liquid chromatography (FPLC) system.

For *Anabaena* GS expression, a NdeI-BamHI fragment including a histidine-tagged modified version of the *glnA* gene was synthesized by PCR and cloned into the pET-3a plasmid (Novagen, La Jolla, CA) to generate pAGS. Exponentially growing *E. coli* BL21 cells transformed with the pAHGS plasmid were treated with 0.5 mM isopropyl-β-D-thiogalactoside for 4 h. *Anabaena* His-GS was purified by Ni-affinity chromatography using His-Bind matrix (Novagen) following the manufacturer's instructions. Fractions that showed GS activity were pooled and subjected to gel filtration using a HiLoad 16/60 Superdex 200 gel filtration column (GE Healthcare) running on an Akta FPLC system.

For *Synechocystis* GS expression, a previously described histidine-tagged modified version of the *glnA* gene (8) was cloned into pBluescript SK(+) in the same orientation as the *plac* promoter to generate the pSHGS plasmid. *Synechocystis* His-GS was purified from *E. coli* DH5α cells transformed with pSHGS using the same methods described above for *Anabaena* His-GS.

For *Anabaena* NtcA expression, a NdeI-XhoI fragment encompassing the entire *ntcA* gene was synthesized by PCR and cloned into the pET24a(+) plasmid (Novagen, La Jolla, CA) to generate pANtcA. Exponentially growing *E. coli* BL21 cells transformed with pANtcA plasmid were treated with 0.5 mM isopropyl-β-D-thiogalactoside for 4 h. *Anabaena* NtcA was purified by Ni-affinity chromatography using His-Bind matrix (Novagen) following the manufacturer's instructions. For further purification, the sample was subjected to gel filtration chromatography using a HiLoad 16/60 Superdex 75 gel filtration column (GE Healthcare) running on an Akta FPLC system.

Anti-IF7A antibody production and Western blotting. Anti-IF7A antiserum was obtained according to standard immunization protocols by injecting purified IF7A-His_6 into rabbits. Purified polyclonal antibodies obtained against *Synechococcus* sp. strain PCC 6301 glutamine synthetase (15) were used to detect *Anabaena* glutamine synthetase. Antibodies obtained against *Synechocystis* sp. PCC 6803 thioredoxin A (TrxA) were used to detect *Anabaena* TrxA. For Western blot analysis, proteins were fractionated by 15% SDS-PAGE according to the method of Laemmli (11) and immunoblotted with anti-IF7A (1:2,000), anti-TrxA (1:3,000), or anti-GS (1:15,000). The ECL Plus immunoblotting system (Amersham) was used to detect the different antigens with anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:12,000). Enhanced chemiluminescence (ECL) signals were quantified using a ChemiDocXRS apparatus (Bio-Rad, Hercules, CA) and the QuantityOne program.

Primer extension analysis. Oligonucleotide PEIF1 (5′GATATTGGCGCAT CATAATGG3′; from nucleotide 47 to 23 of the coding region), end labeled with T4 polynucleotide kinase and [γ-^{32}P]dATP (3,000 Ci mmol^{-1}), was used for primer extension analysis of *gifA*. Annealing and extension reactions using total RNA from *Anabaena* were performed as previously described (9). Extension products were analyzed on a polyacrylamide sequencing gel together with a sequencing reaction mixture of the *gifA* 5′ region using PEIF1 oligonucleotide.

Gel retardation assays. NtcA-His$_6$, expressed and purified as described above, was used in gel retardation assays. DNA fragments were end labeled with [α-^{32}P]dCTP using Sequenase version 2.0 enzyme. The binding reactions and electrophoresis were carried out as previously described (21). The P*gifA1* promoter probe was obtained by BglII digestion of a PCR-amplified fragment using oligonucleotides NtcA1 (5′CTAGCGGCCGCAGTGTTCTGTTGC3′) and NtcA2 (5′GCGCAGATCTCCTATG3′). The P*gifA2* promoter probe was obtained by NotI digestion of a PCR-amplified fragment using oligonucleotides NtcA2 and NtcA3 (5′CTAGCGGCCGCAATTACATAAGTATTACA3′).

Generation of the ACHI *Anabaena* strain. To generate a chimeric gene between *gifB* from *Synechocystis* and *gifA* from *Anabaena*, under the control of the *gifB* promoter, two overlapping DNA fragments were amplified by PCR. A fragment containing the *gifB* promoter and part of the coding region was amplified from *Synechocystis* genomic DNA using oligonucleotides P17EcoRI (5′C ATCCAGCCCGAATTCCATCTCCCTCG3′) and A17NH (5′ATAGACATTT GGCTGGGAGCCGCAGCGAC3′). Another fragment, containing the *gifA* coding region and part of the 3′ region, was amplified from *Anabaena* genomic DNA using oligonucleotides AIFNH (5′CCAGCCAAATGTCTATTCAAGAA AAATCTCG3′) and AIFPstI (5′GATCCTGCAGGGAAGTAACTTCAACAA TGAG3′). The chimeric gene was PCR synthesized from these two fragments and cloned after EcoRI/PstI digestion into pCSEL24 plasmid (23), digested with the same enzymes, rendering pACHI. This plasmid was introduced into *Anabaena* by conjugation (3). The correct integration of this construct in the *nucA-nuiA* region of the *Anabaena* α megaplasmid was checked by PCR.

Generation of AGFP *Anabaena* strain and visualization of green fluorescent protein (GFP). A promoterless *gfp* gene was PCR amplified from pCSEL19 (18) with primers 5′CTAGGACTGTATGTCTAAAGGAGAAGAAC3′ and 5′CTA GGACTGTACGTCTTATTTGTATAGTTCATCCATGC3′. This fragment was AhdI digested and ligated to pANSP(−) plasmid (described above), containing the *Anabaena gifA* genomic region with a deletion of the *asl2329* open reading frame (ORF), digested with the same enzyme. The resulting plasmid, pAGFP1, contains a translational fusion between the *gifA* promoter region and promoterless *gfp*. A XhoI-digested fragment from pAGFP1 was ligated to XhoI-digested pRL278 vector (1), generating the targeting plasmid pAGFP2. To generate the AGFP strain, pAGFP2 was introduced into the *Anabaena* wild-type strain by conjugation (3). These plasmids can be integrated upon homologous recombination in the *gifA* locus of *Anabaena*. The correct integration was confirmed by Southern blot analysis.

The accumulation of the GFP reporter was analyzed by laser confocal microscopy as described previously (18).

RESULTS

Comparative GS inactivation/reactivation processes in different cyanobacteria. Taking into account that ORFs homologous to the *Synechocystis gifA* and *gifB* genes are present in several cyanobacterium sequenced genomes, we decided to examine if the addition of ammonium causes inactivation of GS in other members of the phylum, as described for *Synechocystis* (8). Figure 1 shows comparative kinetics of this process in the heterocyst-forming, model cyanobacterium *Anabaena* sp. PCC 7120, the thermophilic strain *Thermosynechococcus elongatus* BP-1, and *Synechocystis* sp. PCC 6803. Addition of ammonium provokes a quick drop in GS activity in the *Thermosynechococcus* and *Synechocystis* strains; however, in *Anabaena* the process is slower, and by 6 h following ammonium addition, GS activity reaches only about 60% of the initial level. Ammonium removal leads to a rapid recovery of GS activity in all the strains analyzed (Fig. 1).

Deletion mutants of *asl2329* ORF from *Anabaena* sp. PCC 7120 are impaired in GS inactivation. The *Anabaena asl2329* ORF shares homology with both GS inactivation factors from *Synechocystis* (IF7 and IF17) (8). To test whether this ORF is involved in *Anabaena* GS inactivation, we constructed deletion mutants of this gene (Fig. 2A) and investigated the ammonium-dependent GS inactivation process in wild-type (WT) *Anabaena* and mutant strain cultures. GS activities were similar in the two strains generated [Δ*gifA*(+) and Δ*gifA*(−) strains]. Moreover, as shown in Fig. 2B, GS activity shows only minor changes (less than 15%) after ammonium addition in *asl2329* ORF deletion mutants compared to the wild-type strain. These results demonstrate that this gene is the *gifA* ortholog in *Anabaena* (8) and that the product of this ORF is a GS inactivation factor (here called IF7A).

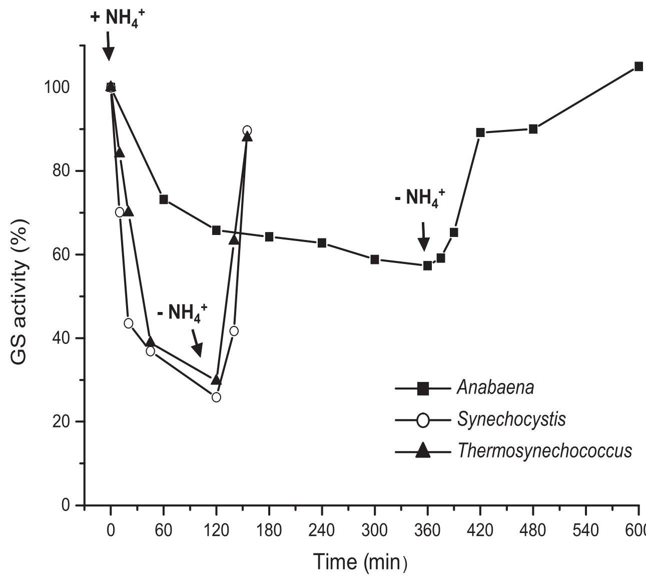

FIG. 1. Time course of the GS inactivation and reactivation processes in three different cyanobacteria. Cells of *Anabaena* 7120, *Synechocystis* 6803, and *Thermosynechococcus elongatus* were grown in BG11C medium using nitrate as nitrogen source. At the time indicated by an arrow, 10 mM NH_4Cl was added and GS transferase activity was determined *in situ*. An arrow also indicates the time at which cells were washed with ammonium-free medium and GS reactivation took place. One hundred percent GS activities correspond to 926, 1,540, and 1,138 mU mg of protein^{-1} for *Anabaena*, *Synechocystis*, and *Thermosynechococcus*, respectively.

***Anabaena gifA* gene expression depends on nitrogen status.** As a first step in the characterization of *gifA* gene expression, we analyzed the *gifA* mRNA level in parallel with IF7A accumulation in the ammonium-mediated GS inactivation/reactivation processes. To monitor the cellular level of IF7A, we produced specific antibodies against this protein. Figure 3A shows that ammonium addition to nitrate-grown cells provokes an increase of *gifA* mRNA and that this level remains elevated during the ammonium treatment. Ammonium removal results in a drop of *gifA* transcript, reaching the steady-state level observed in nitrate-grown cells within 1 h. With respect to the protein IF7A, it was undetectable in nitrate-grown cells and accumulated after ammonium addition. However, ammonium removal led to a rapid decrease in IF7A abundance, and the protein was not detectable 1 h after ammonium elimination (Fig. 3B). In parallel with Northern and Western blotting experiments, we measured GS activity over the same time course (Fig. 3C). A precise inverse correlation between IF abundance and GS activity was observed. As a control for protein loading, membranes of Western blotting experiments were incubated also with anti-TrxA antibodies. Thioredoxin A (TrxA) is constitutively expressed, independently of the nitrogen source (2).

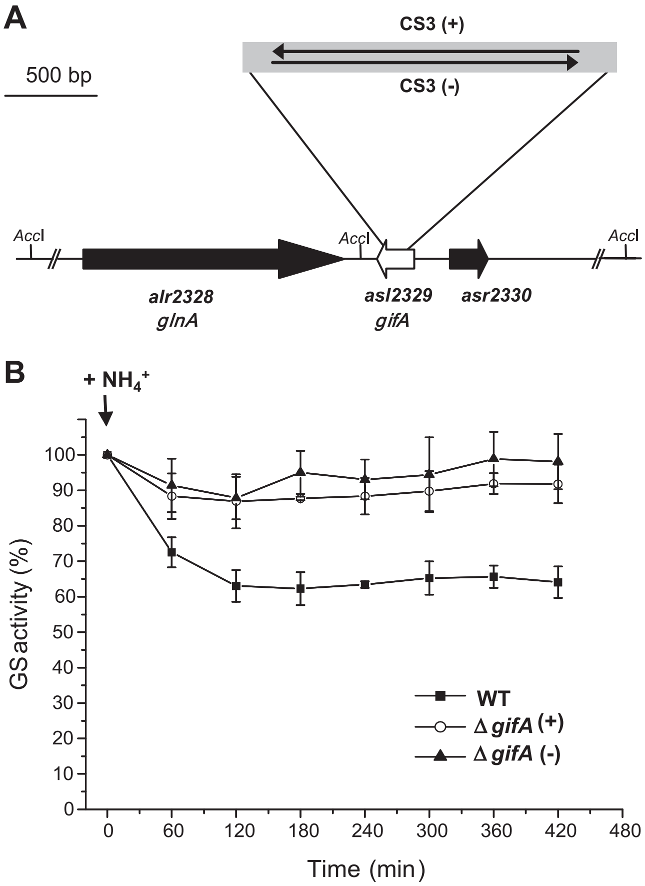

FIG. 2. Analysis of the *Anabaena asl2329* mutant strain. (A) Schematic representation of the *asl2329* genomic region in the wild-type strain and site of insertion of the CS3 cassette, containing the *aadA* gene in both orientations, to generate Δ*gifA*(+) and Δ*gifA*(−) strains, respectively. (B) Ammonium-dependent GS inactivation in *Anabaena* WT and Δ*gifA* mutants. Cells were grown in BG11C medium using nitrate as nitrogen source. A 10 mM concentration of NH_4Cl was added (arrow), and GS transferase activity was determined *in situ* at the indicated times. The curves represent arithmetic means from three independent experiments and their standard deviation values.

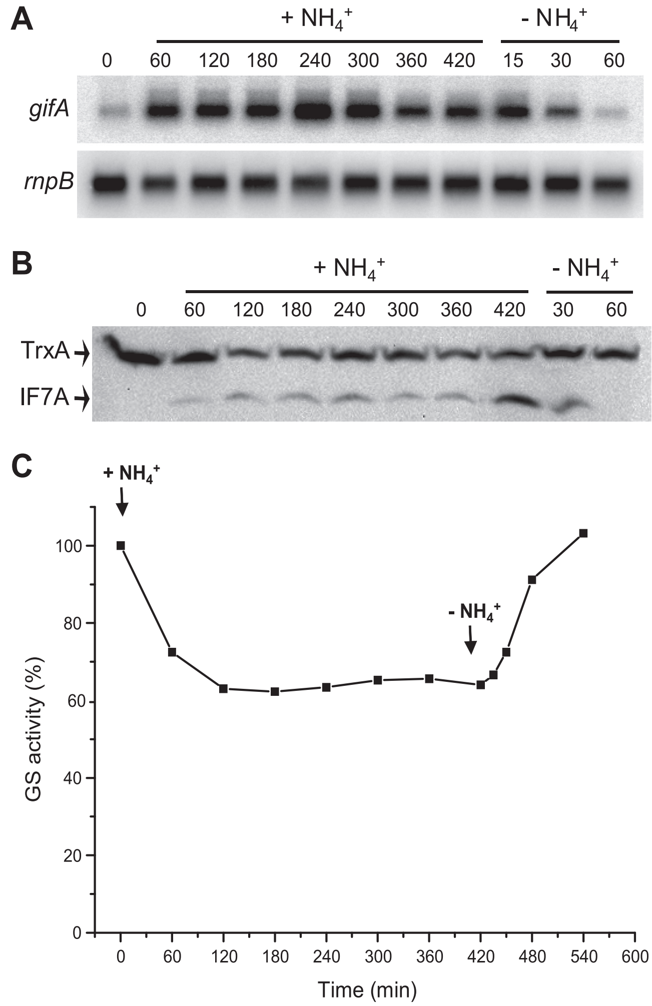

FIG. 3. IF7A expression during the GS inactivation/reactivation processes. At the time indicated by an arrow, 10 mM NH_4Cl was added to *Anabaena* cells cultivated with nitrate as nitrogen source. An arrow also indicates the time at which cells were washed with ammonium-free medium and GS reactivation took place. (A) Northern blot assay of the *gifA* gene under different nitrogen conditions. Total RNA was isolated from cells grown with nitrate (0) and after ammonium addition ($+NH_4^+$) or removal ($-NH_4^+$) at the indicated times (min). Gels were blotted and hybridized with the *gifA* probe. The filters were stripped and rehybridized with an *rnpB* probe as a loading control. (B) Western blot assay of IFA during GS inactivation/reactivation processes. From the same culture as that used for Northern blot analysis, samples were taken from nitrate-grown cells (0) and after ammonium addition ($+NH_4^+$) or removal ($-NH_4^+$) at the indicated times (min). Total proteins were isolated and resolved by SDS-PAGE, blotted, and incubated with anti-IF7A and anti-TrxA antibodies. (C) Time course of the GS activity. Samples were taken, during the inactivation and reactivation processes, for determination of GS activity.

***Anabaena* GS is not inactivated by the *Synechocystis* IFs.** To characterize *in vitro* the GS-IF7A interaction, we purified *Anabaena* GS and IF7A expressed in *E. coli*. In order to study the specificity of the GS-IF interaction, we analyzed also the components of the previously characterized GS inactivation system from *Synechocystis* (GS, IF7, and IF17) (8). The three purified inactivation factors (IF7, IF17, and IF7A) inhibited *Synechocystis* GS, but only IF7A was able to inhibit *Anabaena* GS (Fig. 4). In addition, IF7A-*Synechocystis* GS interaction seems to be stronger than IF7A-*Anabaena* GS interaction, because equal amounts of this inactivating factor provoke stronger inhibition of *Synechocystis* GS than of *Anabaena* GS (Fig. 4C).

NtcA regulates the *Anabaena gifA* promoter. To identify the promoter region of the *gifA* gene (P*gifA*), the transcription start point (TSP) of the gene was determined by primer extension analysis. The *gifA* TSP was mapped to nucleotide −43 with respect to the translation start codon (Fig. 5A). Four nucleotides upstream of the TSP, a putative −10 box in the form TATATT was found. No obvious −35 box was detected. As observed in RNA blotting experiments, the primer extension product from the *gifA* promoter was more abundant in samples from cells grown in the presence of ammonium than in samples from those grown in the presence of nitrate or using RNA from nitrogen-fixing cells. Taking into account the influence of nitrogen status on the expression of *gifA* and the

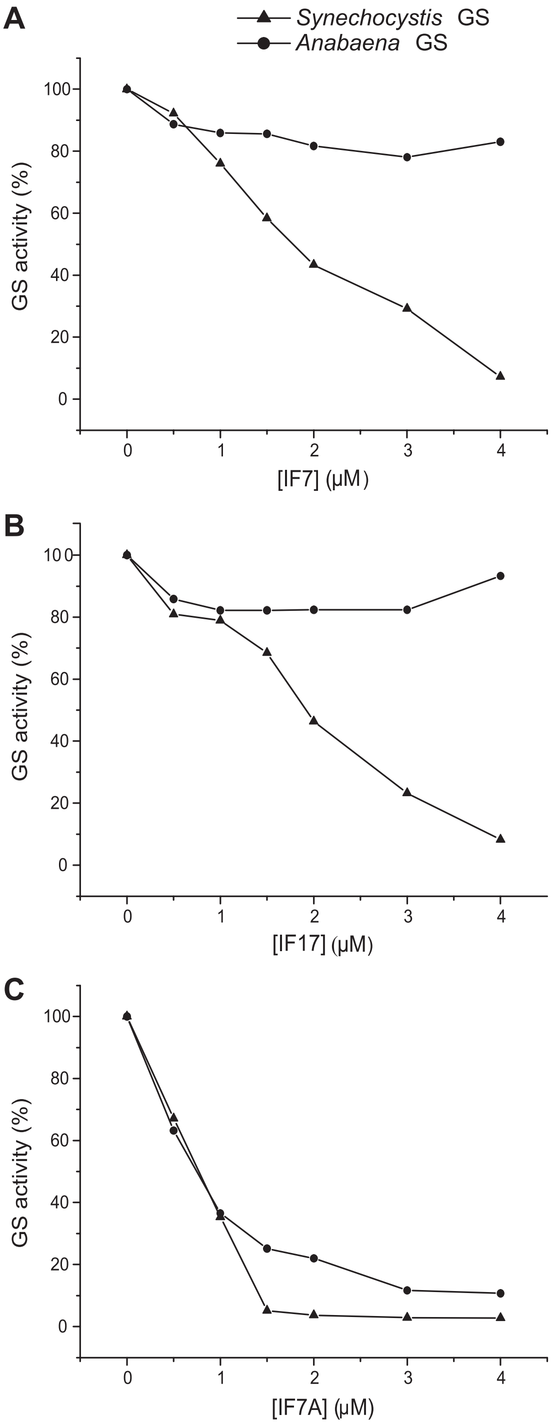

FIG. 4. *In vitro* reconstitution of *Synechocystis* and *Anabaena* GS inactivation. *Synechocystis* GS (1.7 μg) and *Anabaena* GS (2.2 μg) were incubated with increasing quantities of IF7 (A), IF17 (B), and IF7A (C) in a final volume of 20 μl. Inactive GS-IF complexes were allowed to form during 2 min, and GS transferase activity was determined. One hundred percent activity corresponds to 0.4 unit of GS.

previously described NtcA-dependent repression of *gif* genes from *Synechocystis* sp. PCC 6803 (9), the presence of NtcA binding sites in the *gifA* promoter region was analyzed. Two consensus NtcA binding sites (13) centered at positions −28.5 and −77.5, respectively, upstream of the *gifA* TSP were found (Fig. 5B). It is worth noting that the NtcA consensus site centered at position −28.5 with respect to the TSP is located at exactly the same distance from the −10 box as is the repressing NtcA binding site described for the *gifA* promoter from *Synechocystis* (Fig. 5B).

To test if NtcA binds to P*gifA*, electrophoretic mobility shift assays using purified *Anabaena* NtcA protein were performed. *Anabaena* NtcA was expressed in *E. coli* and purified as a histidine-tagged version. Binding assays were performed with two DNA fragments, P*gifA1*, which spans positions −134 to +36 with respect to the TSP, and a shorter fragment, P*gifA2*, which spans positions −77 to +36 and lacks the GTA triplet of the consensus NtcA binding site centered at position −77.5. When NtcA was incubated with P*gifA1*, two NtcA-DNA complexes were detected (Fig. 6A); however, when the P*gifA2* probe was used, only one NtcA-DNA complex could be detected (Fig. 6B). These results indicate that NtcA binds *in vitro* to both consensus recognition sites found in the *gifA* promoter region. On the other hand, 2-oxoglutarate has been reported to increase the binding affinity of NtcA for several nitrogen-regulated promoters (32). As shown in Fig. 6A, the presence of this metabolite in the binding assay has a positive effect on NtcA recognition of P*gifA*.

To demonstrate that the transcriptional regulator NtcA controls the synthesis of the *gifA* mRNA, we determined the level of *gifA* transcript in the NtcA mutant strain CSE2 (5). As a control, we checked the level of expression of *glnA*, the transcription of which is positively controlled by NtcA (5, 31). We analyzed the steady-state mRNA levels of these genes in the wild type and in the CSE2 strain under three different conditions: nitrate utilization, ammonium utilization, and nitrogen deprivation. Ammonium-grown CSE2 or wild-type cells were transferred for 6 h to nitrate- or ammonium-containing medium or to nitrogen-free medium, and samples were taken for RNA isolation. As previously reported, the amount of *glnA* mRNA in the wild-type strain increased upon incubation in medium containing nitrate or no combined nitrogen. However, induction was severely impaired in CSE2 mutant cells (Fig. 7A). *gifA* transcript levels were high in wild-type ammonium-grown cells and were downregulated upon incubation in medium containing nitrate or no combined nitrogen. In contrast, *gifA* transcript levels in CSE2 cells remained high under all conditions tested. These results demonstrate that NtcA represses the *gifA* promoter.

In addition we studied the accumulation of the IF7A protein in the wild type and the CSE2 mutant strain under the three nitrogen regimens analyzed. For this purpose, we performed Western blot analysis and found that IF7A accumulated in the wild-type cells cultivated with ammonium, but this protein was undetectable under the other nitrogen conditions. On the other hand, IF7A was also undetectable in CSE2 cells under all conditions tested. We also analyzed the level of GS in both strains under the same conditions, using anti-GS antibodies. As shown in Fig. 7B, the amount of GS is lower in CSE2 cells than in the WT, under the different nitrogen regimens. If the GS-IF interaction is critical for IF stability as described for the *Synechocystis* system (6), the low level of the IF7A target protein (GS) found in CSE2 cells may contribute to the lack of IF7A accumulation in this strain. However, the difference in

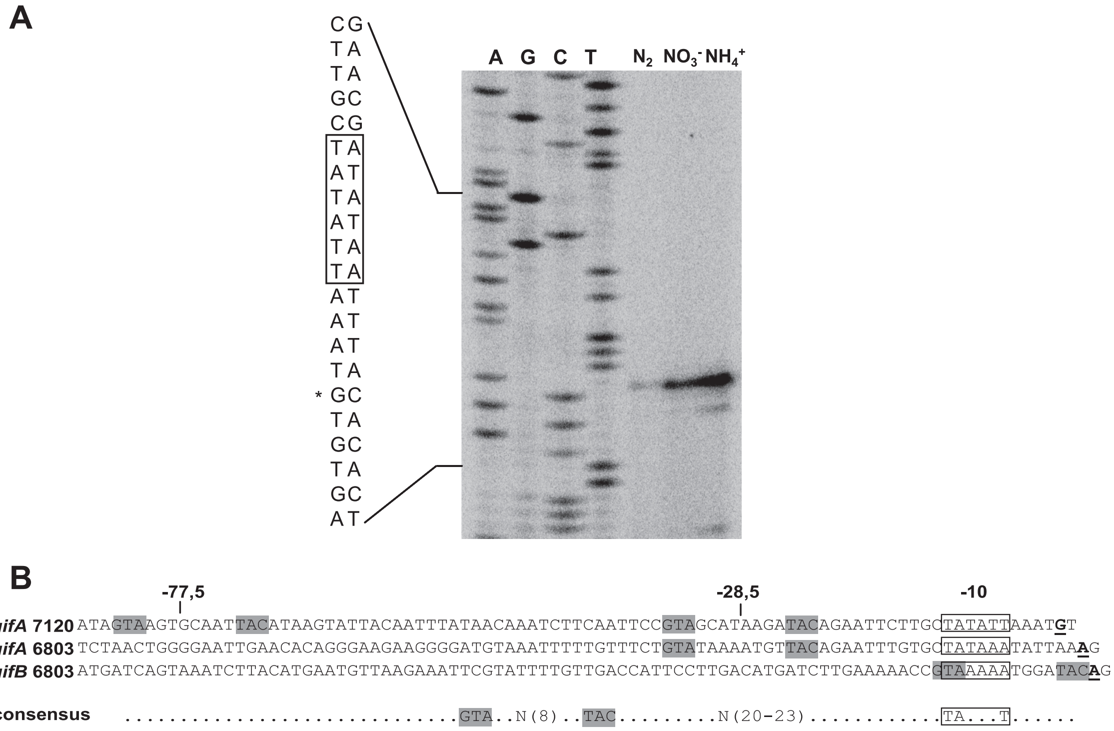

FIG. 5. Primer extension analysis of the *gifA* transcript. (A) Total RNA (20 μg) from mid-log *Anabaena* cells grown with ammonium (NH_4^+) or nitrate (NO_3^-) or grown with nitrate and incubated for 7 h in the absence of any nitrogen source (N_2) was used for primer extension analysis, as described in Materials and Methods. A sequencing ladder carried out with the same primer as that used for primer extension is also shown. The transcriptional start site is marked with an asterisk on the sequence, and a putative −10 sequence is boxed. (B) Alignment of *Anabaena* and *Synechocystis gif* promoters. NtcA binding sites are shaded in gray, and −10 regions are boxed. The consensus sequence of the NtcA-activated promoter is also shown.

amounts of GS between the two strains, WT and CSE2, is not that much. To analyze the possibility that the absence of IF7A in CSE2 cells was due to another reason, we decided to sequence the PCR-amplified *gifA* gene from genomic DNA of both strains. A point mutation, *gifA49C>T*, encoding the substitution Q17STOP, was found in four independent PCR amplifications of the *gifA* gene from the CSE2 strain. The mutation position is indicated as the nucleotide distance from the first nucleotide of the start codon to the mutation site. No mutation was found in the *gifA* gene amplified from the WT *Anabaena* strain. The early termination of IF7A protein in the CSE2 strain must be the reason why we could not observe

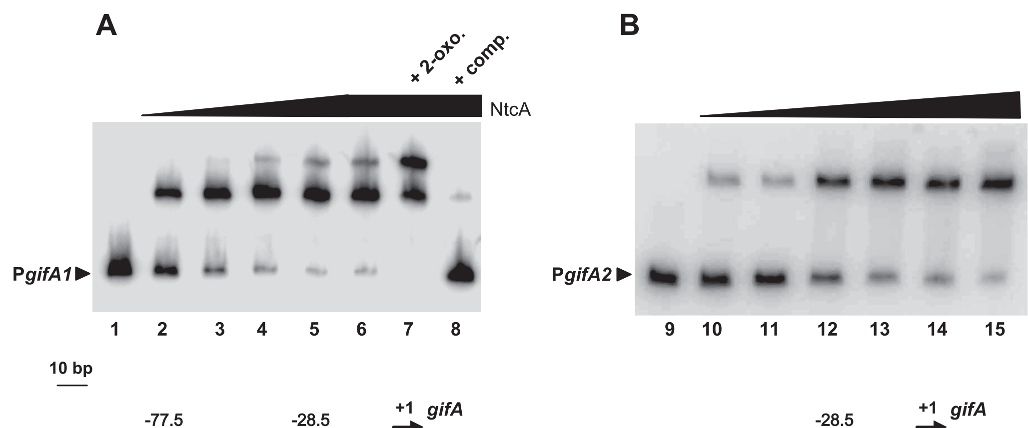

FIG. 6. Gel retardation analysis of the binding of NtcA to the *gifA* promoter region. Two DNA fragments were used: P*gifA1*, encompassing the wild-type *gifA* promoter (A), containing two putative NtcA binding sites, and P*gifA2*, depleted of the first triplet of one of the NtcA binding sites (B). Each triplet of putative NtcA binding sites is represented by a small black box in P*gifA1* and P*gifA2* schemes. Both probes were end labeled and incubated in the presence of purified NtcA protein, 5 to 250 nM (lanes 1 to 7 and 9 to 15, respectively). P*gifA1* was also incubated with 250 nM NtcA in the presence of 0.6 mM 2-oxoglutarate (lane 7) or a 200-fold excess of the same unlabeled fragment (lane 8).

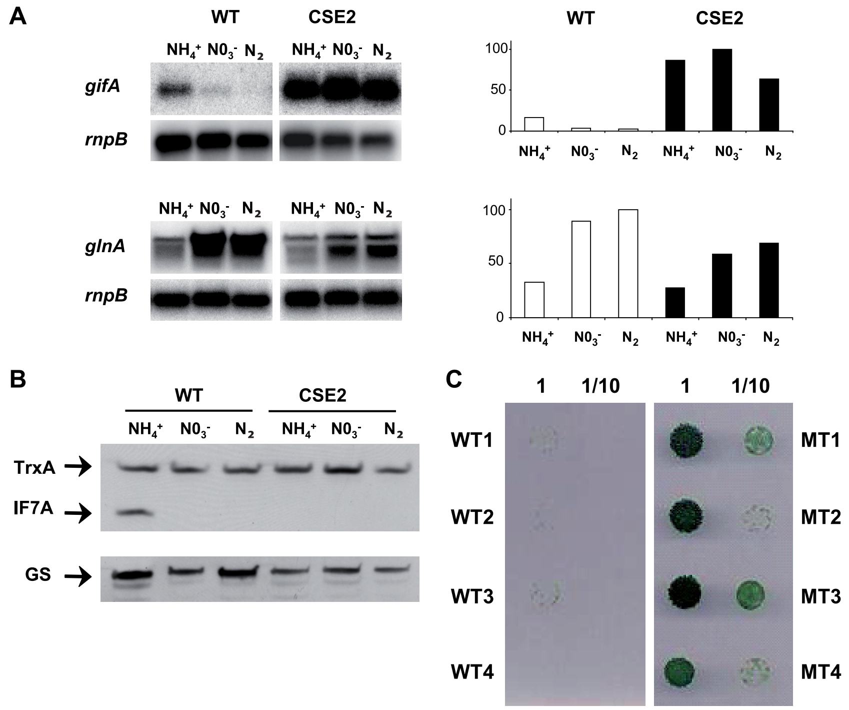

FIG. 7. Expression levels of NtcA-controlled genes in the CSE2 mutant. Ammonium-grown wild-type and CSE2 *Anabaena* cultures were divided into three aliquots. Aliquots were transferred for 6 h to nitrate-containing medium (NO_3^-) or to nitrogen-free medium (N_2) or were maintained in ammonium-containing medium (NH_4^+). (A) Samples were taken for total RNA isolation. Fifteen micrograms of total RNA was denatured, separated by electrophoresis, blotted, and hybridized with *gifA* and *glnA* probes. Hybridization signals were quantified with a Cyclone Storage Phosphor system autoradiography apparatus. *gifA* and *glnA* levels were normalized to those of *rnpB*. It should be noted that 100% corresponds to the maximal signal of hybridization for each probe and, thus, signals from different probes cannot be compared. (B) From the same cultures as those used for Northern blot analysis, samples were taken for Western blotting. Total proteins were isolated and resolved by SDS-PAGE, blotted, and incubated with anti-IF7A and anti-TrxA antibodies. The filter was stripped and incubated with anti-GS antibodies. (C) Growth of CSE2 exconjugants harboring wild-type (WT1 to WT4) or mutant (MT1 to MT4) versions of the *gifA* gene. Five microliters of cellular suspensions from the different strains and a 1/10 dilution were spotted on ammonium-containing BG11 plates. Photos were obtained after 2 weeks of incubation.

accumulation of that protein in this strain. To further investigate if this finding is meaningful, we decided to introduce two different constructs in the CSE2 strain. One of them contained the wild-type *gifA* gene and its promoter region, and the other contained the same DNA fragment but with two point mutations in the *gifA* coding region: *gifA40C*>*T*, encoding the substitution Q14STOP, and *gifA46C*>*T*, encoding the substitution Q16STOP. After conjugation, colonies were obtained using CSE2 cells as recipient and either plasmid pWTgifA (wild-type *gifA* gene) or plasmid pMTgifA (mutant *gifA* gene). However, further growth of exconjugants bearing the wild-type version of *gifA* integrated in the *nucA-nuiA* region of the α megaplasmid was very poor. Two amounts from cellular suspensions of exconjugants were spotted on plates (Fig. 7C). Whereas all exconjugants bearing the mutant version of *gifA* grew well, the growth of strains containing the wild-type version of *gifA* was negligible.

Analysis of an *Anabaena* strain expressing two inactivation factors. *Synechocystis* and *Thermosynechococcus elongatus* both display rapid inactivation of GS following addition of ammonium compared to *Anabaena* (6) (Fig. 1). Since both *Synechocystis* and *Thermosynechococcus elongatus* harbor two inactivating factors, IF7 and IF17, and two IF17 homologous proteins, respectively, it might be inquired whether the slow response in *Anabaena* is related to the lack of any IF17 homologous protein. We also know from previous studies that IF7 and IF17 display different stabilities and that the 82-residue-long amino-terminal part of IF17 may be responsible for the different stabilities observed (6; unpublished results). Thus, we proceeded to generate an *Anabaena* strain expressing an IF17-like GS inactivation factor. For this purpose we constructed a chimeric gene between *gifB* from *Synechocystis* and *gifA* from *Anabaena*, in order to express a modified version of IF7A with the 82-residue-long amino-terminal part of IF17 fused to its amino terminus, under the control of the *gifB* promoter. This construct was introduced in *Anabaena* by conjugation (3) and integrated, through homologous recombination, in the *nucA-nuiA* region of the α megaplasmid. The resulting *Anabaena* strain, ACHI, contains the unaltered *gifA* gene in the chromosome and the chimeric gene *gifB*/*gifA* in the α megaplasmid. Before analyzing GS inactivation/reactivation processes in the ACHI strain, we wanted to test *in vitro* inactivation of *Anabaena* GS by the chimeric protein IF17N/IF7A. For this purpose we purified this protein expressed in *E. coli* and studied its capacity to inactivate *Anabaena* GS comparatively with IF7A. Figure 8A shows that the chimeric protein is much less effective than IF7A on GS inactivation. We proceeded then to the *in vivo* analysis of WT and ACHI strains; we tested in both

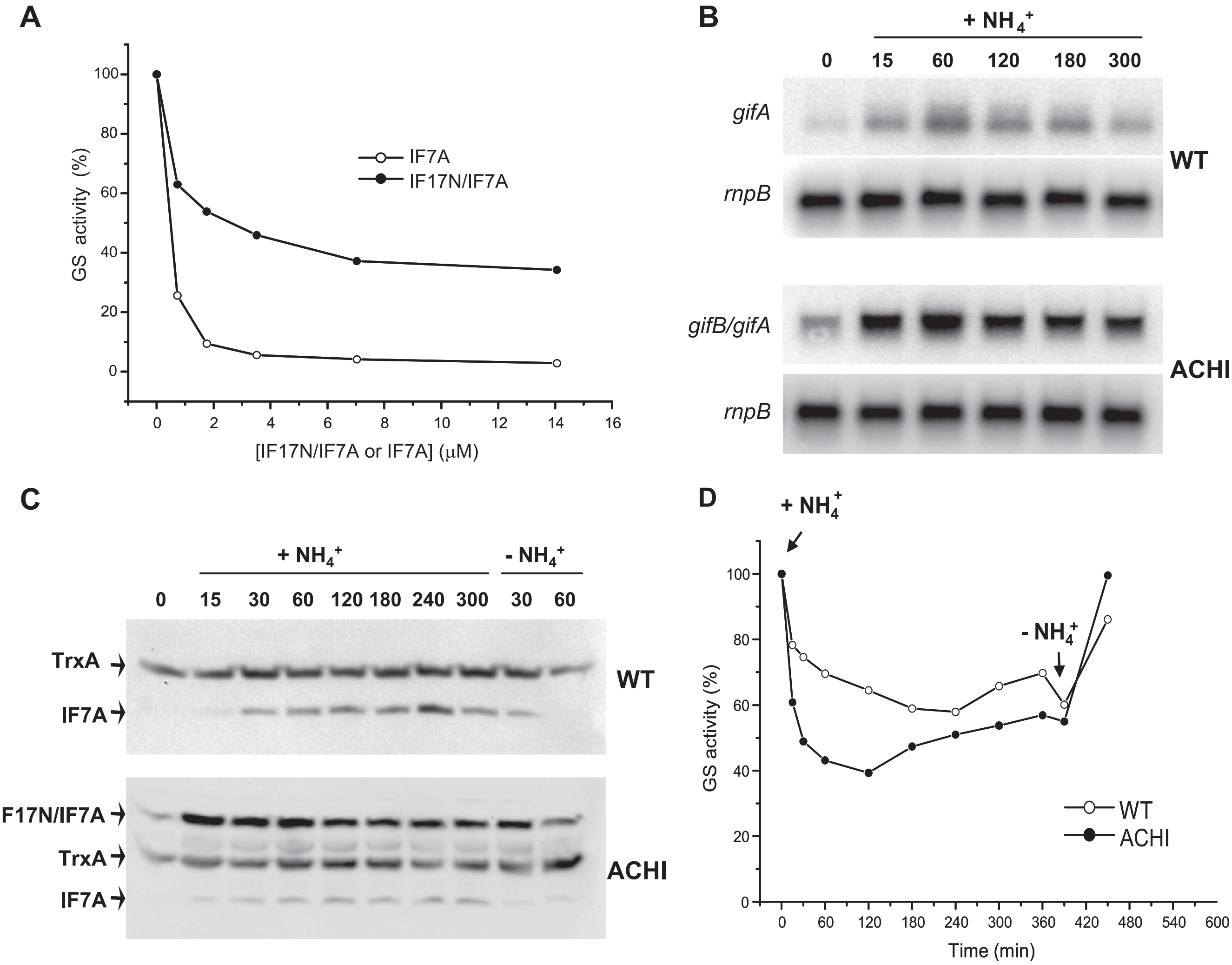

FIG. 8. GS inactivation/reactivation processes in the ACHI strain. (A) *In vitro* inactivation of *Anabaena* GS with IF7A or IF17N/IF7A. *Anabaena* GS (1.65 μg) was incubated with increasing quantities of IF7A or IF17N/IF7A in a final volume of 20 μl. Inactive GS-IF complexes were allowed to form for 2 min, and GS transferase activity was determined. One hundred percent activity corresponds to 0.3 unit of GS. Wild-type and ACHI *Anabaena* strains were grown in BG11C using nitrate as nitrogen source. At the time indicated by an arrow in panel D, 10 mM NH_4Cl was added. An arrow in panel D also indicates the time at which cells were washed with ammonium-free medium and GS reactivation took place. (B) Northern blot assay of the *gif* genes under different nitrogen conditions. Total RNA was isolated from cells grown with nitrate (0) and after ammonium addition ($+NH_4^+$) at the indicated times (min). Gels were blotted and hybridized with *gifA* (WT strain) and *gifB* (ACHI strain) probes. Filters were stripped and rehybridized with an *rnpB* gene probe as a loading control. (C) Western blot assay of IF7A and IF17N/IF7A proteins during the GS inactivation/reactivation processes. Samples were taken from nitrate-grown cells (0) and after ammonium addition ($+NH_4^+$) or removal ($-NH_4^+$) at the indicated times (min). Total proteins were isolated and resolved by SDS-PAGE, blotted, and incubated with anti-IF7A and anti-TrxA antibodies. (D) From the same cultures as those used for Northern and Western analysis, GS transferase activity was determined *in situ*.

strains the mRNA level of *gif* genes in parallel with IF7A and IF17N/IF7A accumulation in the ammonium-mediated GS inactivation/reactivation processes. Figure 8B shows that ammonium addition to nitrate-grown cells provokes an increase of *gifA* mRNA in the WT strain and of the *gifB*/*gifA* chimeric gene in the ACHI strain. With respect to the protein levels, in the ACHI strain both proteins, IF7A and IF17N/IF7A, accumulated after ammonium addition and decreased upon ammonium removal (Fig. 8C). In parallel with Northern and Western blotting experiments, we measured GS activity over the same time course in the two strains. A clear difference in GS activity could be observed after ammonium addition (Fig. 8D). Whereas the ACHI strain reached about 40% of the initial activity in the first 2 h, the WT strain showed the typical slow *Anabaena* GS inactivation.

P*gifA* is repressed in heterocysts. The GS regulatory system is strictly dependent on the global nitrogen control regulator NtcA in both *Synechocystis* (9) and *Anabaena* (see above). Notably, a differential *ntcA* gene expression between heterocysts and vegetative cells has been described in *Anabaena* (23, 24). Therefore, it would be of interest to analyze *gif* expression along the *Anabaena* filaments. For this study we analyzed *in vivo* the expression of a *gifA*-*gfp* translational fusion. This construct was introduced in *Anabaena* by conjugation (3) and

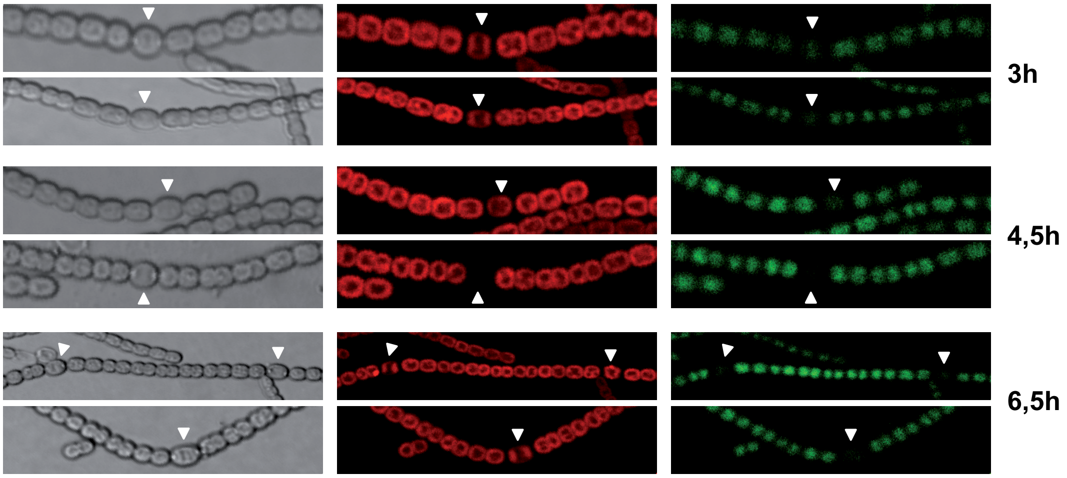

FIG. 9. GFP fluorescence of *Anabaena* strain carrying the *gifA-gfp* fusion (AGFP1). GFP fluorescence micrographs of diazotrophically grown filaments after incubation with 10 mM ammonium for 3, 4.5, or 6.5 h. Light transmission micrographs (left column), phycobiliprotein autofluorescence (middle column), and GFP fluorescence (right column) are shown for each condition. White triangles point to heterocysts.

integrated through homologous recombination in the *gifA* locus. The resulting *Anabaena* strain, AGFP1, was examined by fluorescence microscopy. A delayed ammonium-dependent induction of *gif* genes in nitrogen-starved cells has been reported for *Synechocystis* (9). This delay is likely due to the high 2-oxoglutarate levels under these conditions (20). Assuming a similar scenario in nitrogen-fixing *Anabaena* cells (16, 21, 30), we analyzed by Northern blotting *gifA* induction after ammonium addition in wild-type *Anabaena* cells from diazotrophic or nitrate-supplied cultures. A delayed *gifA* induction was observed in nitrogen-fixing cells (not shown). Taking this into account, we monitored P*gifA* induction in the AGFP1 strain. Cells of this strain were cultivated in nitrate-containing medium and then transferred to nitrogen-free medium. Once mature heterocysts were observed by light microscopy (24 h), 10 mM ammonium was added and samples were taken for fluorescence microscopy analysis at 3, 4.5, and 6.5 h. Upon ammonium addition, GFP expression in vegetative cells becomes higher than that observed in heterocysts. Such differential expression is clearly observed at 4.5 and 6.5 h (Fig. 9). This observation suggests that derepression of the *gifA* promoter is not observed in heterocysts, and thus, the GS inactivation system is repressed in this type of cell.

DISCUSSION

The work presented here reveals that the GS posttranscriptional regulation system described first in the *Synechocystis* sp. PCC 6803 strain is not restricted to this cyanobacterium. In fact, genes homologous to *gifA* and *gifB* from *Synechocystis* have been found in several cyanobacterial genomes but seem to be absent in strains of the genus *Prochlorococcus*. Here we show that the *gifA* gene from the filamentous, nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 is responsible for GS inactivation in this organism. It is worth noting that the genetic contexts of *gifA* genes in several genomes of filamentous cyanobacteria are similar. In these strains, *Anabaena* sp. PCC 7120, *Anabaena variabilis* ATCC 29413, *Nostoc punctiforme* sp. PCC 73102, *Nodularia* sp. strain PCC 9350, and *Anabaena azollae*, the *gifA* gene is located downstream and on the opposite strand from the GS-encoding gene, *glnA*. This fact raises the possibility of additional GS regulatory mechanisms, mediated by the *gifA* gene, affecting *glnA* at the mRNA level, which would be conserved in filamentous cyanobacteria. Furthermore, this proximal localization of the GS/IF coding genes may be related to genome reorganization phenomena or coevolutionary processes. In this sense, our observation that only IF7A is able to inactivate *Anabaena* GS *in vitro* (Fig. 4) is consistent with this possible coevolution.

The characterization of *gifA* expression in *Anabaena* reveals that ammonium-mediated upregulation of this gene is not transitory, as described for *gif* genes in *Synechocystis* (8). The slow GS inactivation observed in *Anabaena* might be the reason why *gifA* expression remains high during ammonium treatment. In *Synechocystis*, ammonium addition provokes a strong decrease in the 2-oxoglutarate pool and thus *gif* genes are derepressed. However, a quick GS inactivation leads to the increase of the 2-oxoglutarate amount and NtcA-mediated repression of *gif* genes takes place again (9, 20). In this regard, *Synechocystis* mutant strains that harbor only the *gifA* gene behave similarly to *Anabaena* with respect to GS inactivation and *gifA* expression (not shown).

Analysis of the *Anabaena gifA* promoter revealed the presence of two NtcA binding sites; one of them is centered at position −28.5 in respect to the TSP, which is a localization described for NtcA-repressed promoters, centered downstream of position −40.5 (9). The other site, centered at position −77.5, is a putative activator site because some NtcA-activated promoters have been described to bear NtcA binding sites upstream of position −41.5 not matching the structure of the canonical NtcA-activated promoter, with an NtcA binding box centered at −40.5 (9, 14). One promoter in which NtcA acts both as an activator and as a repressor has been described; this is the case of the *gltX* gene from *Synechococcus elongatus*, but its regulatory pattern is unique among the NtcA-regulated genes. In the case of the *Anabaena gifA* gene, the results obtained with the *ntcA* mutant strain (CSE2) clearly demonstrate a repressive role for NtcA in the transcription of this gene (Fig. 7). A similar role has been described for NtcA in the transcription of *gif* genes from *Synechocystis*; however, those promoters bear only one NtcA binding site, which is located at a repressive position (9). Additional studies are required to understand the *in vivo* putative role of the NtcA binding site located at an activation position in the *Anabaena gifA* promoter.

The comparative study, shown in Fig. 7, concerning *gifA* expression in the wild type and the *ntcA* mutant strain CSE2 indicates that this gene is highly expressed in CSE2 cells under all nitrogen regimens tested. It is worth noting that the highest level of *gifA* mRNA detected in wild-type cells, obtained in the presence of ammonium, is significantly lower than the one present in the CSE2 strain under any conditions. This fact clearly indicates that the *gifA* gene is partially repressed by NtcA in wild-type cells under all conditions tested. Derepression of *gif* genes in *Synechocystis*, upon ammonium addition, is dependent on the metabolism of this compound by the GS-GOGAT pathway and the subsequent decrease in the 2-oxoglutarate cellular pool (20). In *Anabaena* there is also strong evidence in support of a key regulatory role of this metabolite in transcriptional regulation mediated by NtcA (30). In addition to the capacity of using N_2, the filamentous cyanobacterium *Anabaena* differs from *Synechocystis* in others aspects of nitrogen metabolism and specifically in the GS-GOGAT pathway. *Anabaena* contains only the ferredoxin-dependent glutamate synthase (Fd-GOGAT), which is present in all cyanobacteria (16), whereas *Synechocystis* harbors, in addition, a second glutamate synthase, accepting NADH as reductant. Thus, metabolism of the nitrogen-sensing molecule 2-oxoglutarate, which is a GOGAT substrate, is also different between these two cyanobacterial strains. It is possible that the strong drop in 2-oxoglutarate amount provoked by addition of ammonium in *Synechocystis* (20) is not as pronounced in *Anabaena* and, consequently, derepression of the *gifA* gene is lower.

Despite the high *gifA* mRNA level found in CSE2 cells under any nitrogen regimen, IF7A is not detectable by Western blotting in this strain. The fact that we found a mutated allele of *gifA* in CSE2 cells, which codes for a truncated IF7A protein at residue Q16, clearly explains why the protein was not detectable by Western blotting. It is worth noting that *ntcA* mutants are selected in ammonium-containing medium, because NtcA protein is required for the utilization of any other nitrogen source (5), and also that *glnA* expression is basal under this nitrogen regimen (5) (Fig. 7A). Taking this into account, mutation of the *gifA* gene in an *ntcA*-null mutant like the CSE2 strain would be a mechanism to avoid a strong repression of GS activity. The results shown in Fig. 7C demonstrate that in an *ntcA* mutant background, expression of the *gifA* gene is deleterious and this gene might be a target of suppressor mutations in NtcA-deficient cultures.

As discussed above, 2-oxoglutarate metabolism is different between the two cyanobacteria in which GS posttranscriptional regulation has been studied, *Synechocystis* 6803 and *Anabaena* 7120. This fact, clearly related to ammonium sensing, influences the GS inactivation process. Another difference between these two cyanobacteria is the presence in *Synechocystis* of two GS inactivation factors whereas *Anabaena* has only one. The analysis shown in Fig. 8 of a modified *Anabaena* strain (ACHI) that harbors two inactivating factors indicates that the level of GS inactivation upon ammonium addition is still much lower than the one shown in *Synechocystis* (6) (Fig. 1). Taking into account that *gif* genes are correctly induced by ammonium (Fig. 8B) and that both inactivating factors, IF7A and IF17N/IF7A, accumulated in *Anabaena* cells (Fig. 8C), the reason why GS inactivation is not as efficient as expected *a priori* must be related to the affinity between *Anabaena* GS and the chimeric inactivating factor IF17N/IF7A or the inactivation capacity of this last protein. The *in vitro* study using purified proteins (Fig. 8A) clearly indicates that the fusion of the 82-residue-long amino-terminal part of IF17 to the amino terminus of IF7A affects negatively its interaction and/or the *Anabaena* GS inactivation function. In fact the *in vivo* GS inactivation observed in any strain is a combination of the level of transcription of the corresponding *gif* gene, the stability of the inactivation factor expressed, and the capacity of this inactivation factor to interact with and/or inactivate the GS enzyme. Interestingly the IF17N/IF7A chimeric protein quite effectively inactivates *Synechocystis* GS *in vitro* (not shown). These results, together with those shown in Fig. 4, tell us about the specificity of the IF-GS interaction.

Several pieces of data suggest that the 2-oxoglutarate concentration is high in heterocysts. Firstly, expression of the isocitrate dehydrogenase coding gene, *icd*, is higher in heterocysts than in vegetative cells. Secondly, the *ntcA* gene is induced in proheterocysts (23) and this gene has been described as an activator of the *icd* gene in *Synechocystis* (21). It is also well established that the GS-GOGAT pathway is not operative in the heterocysts of *Anabaena* 7120, because Fd-GOGAT is absent from these cells (16). Therefore, 2-oxoglutarate metabolism takes place mainly in vegetative cells, while in heterocysts, accumulation of this metabolite must be responsible for the differential NtcA repression of the *gifA* promoter observed using a GFP fusion (Fig. 9).

The present study reveals that the mechanism of GS regulation by protein-protein interaction, described first in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, is not restricted to this organism. In fact the system analyzed in this work in *Anabaena* sp. PCC 7120, which bears only one *gif* gene, homologous to *gifA* from *Synechocystis*, seems to be more extended than the *Synechocystis* one, based on two inactivation factors (IF7 and IF17). Most cyanobacterial strains carry genes encoding IF7-like peptides (65 to 68 amino acids) (14). The data presented here and in previous studies (9) also make evident that this GS modulation system is strictly dependent on the global nitrogen regulator NtcA and thus on the C/N balance of the cell. Actually, any metabolic characteristic affecting the GS-GOGAT pathway or other parameters related to carbon or nitrogen fluxes modulates the level of GS activity observed in each cyanobacterium.

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