BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH
www.bjournal.com.br

ISSN 1414-431X
Volume 45 (12) 1102-1340 December 2012

BIOMEDICAL SCIENCES
AND
CLINICAL INVESTIGATION

Braz J Med Biol Res, December 2012, Volume 45(12) 1135-1140

doi: 10.1590/S0100-879X2012007500146

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Brazilian Journal of Medical and Biological Research (2012) 45: 1135-1140
ISSN 1414-431X Short Communication

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Abstract

Azospirillum brasilense is a diazotroph that associates with important agricultural crops and thus has potential to be a nitrogen biofertilizer. The *A. brasilense* transcription regulator NifA, which seems to be constitutively expressed, activates the transcription of nitrogen fixation genes. It has been suggested that the nitrogen status-signaling protein GlnB regulates NifA activity by direct interaction with the NifA N-terminal GAF domain, preventing the inhibitory effect of this domain under conditions of nitrogen fixation. In the present study, we show that an N-terminal truncated form of NifA no longer required GlnB for activity and lost regulation by ammonium. On the other hand, *in trans* co-expression of the N-terminal GAF domain inhibited the N-truncated protein in response to fixed nitrogen levels. We also used pull-down assays to show *in vitro* interaction between the purified N-terminal GAF domain of NifA and the GlnB protein. The results showed that *A. brasilense* GlnB interacts directly with the NifA N-terminal domain and this interaction is dependent on the presence of ATP and 2-oxoglutarate.

Key words: *Azospirillum brasilense*; NifA protein; GlnB protein; GAF domain; Nitrogen fixation

Introduction

Azospirillum brasilense is a microaerophilic diazotrophic α-proteobacterium associated with several plants of agricultural interest, such as maize, rice, sorghum, wheat, and sugar cane (1). This bacterium reduces N_2 to NH_3 by the nitrogenase enzyme complex, which is encoded by the *nifHDK* genes. Nitrogen fixation requires large amounts of metabolic energy, which suggests why this process is highly regulated at the level of both *nif* gene expression and nitrogenase activity.

The two major environmental factors that regulate nitrogen fixation in most diazotrophs are oxygen and ammonium (2). In Proteobacteria, control of the activity of the *nif* gene transcription activator, the NifA protein, by these factors is the key step in the regulation of nitrogenase expression. The NifA protein consists of three typical structural domains (2). The N-terminal GAF domain is the most variable among NifA proteins and has a regulatory function. The N-terminal and central domains are separated by a glutamine-rich linker named QL. The central AAA+ domain is the catalytic domain and contains ATPase and σ^{54}-binding sites. The C-terminal domain contains a conserved helix-turn-helix motif, which is involved in DNA binding. In *A. brasilense* and other diazotrophs, such as *Bradyrhizobium japonicum* and *Herbaspirillum seropedicae*, the two latter domains are connected by an interdomain linker region (2,3). Two cysteines present in this region, together with two cysteines from the central domain, may be involved in oxygen sensitivity of the NifA proteins of these organisms (2,4).

In *A. brasilense* and *H. seropedicae* the N-terminal GAF domain is not essential for NifA activity (4,5), although it is required for inhibition of NifA activity in the presence of fixed nitrogen. It has been proposed that this control involves direct interaction between the NifA GAF domain and the GlnB protein in *A. brasilense* (6,7). This model suggests that GlnB binds to NifA under low levels of fixed nitrogen and relieves the inhibition by the GAF domain

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Received May 21, 2012. Accepted August 27, 2012. Available online September 21, 2012. Published December 17, 2012.

of the central AAA+ domain (5-8). Recent studies have shown that amino acid residues 66-88 and 165-176 in the NifA GAF domain are responsible for the interaction with GlnB (8). GlnB is a homotrimeric protein of the P_{II} family that can sense the cellular nitrogen, carbon and energy levels and relay these signals to a variety of target proteins by means of protein-protein interactions. These protein interactions are modulated by the uridylylation status of GlnB, which, in turn, reflects the availability of the nitrogen-signaling molecule glutamine. Furthermore, GlnB-targeting interactions are influenced by the allosteric binding of 2-oxoglutarate, ATP and ADP to GlnB. The levels of 2-oxoglutarate reflect the nitrogen/carbon availability while the competitive binding of ATP and ADP to GlnB reflects the energy level (reviewed in Ref. 9). Two different P_{II} proteins, namely GlnB and GlnZ, have been identified in *A. brasilense* (10); however, only GlnB is able to activate NifA under nitrogen limitation (6).

In the present study, we analyzed the *in trans* regulatory function of the N-terminal GAF domain of *A. brasilense* NifA using *Escherichia coli* strains with a chromosomal *nifH'-lacZ* fusion and expressing an N-truncated variant of NifA. We also demonstrate that the GlnB protein binds the N-terminal domain of NifA *in vitro* in the presence of ATP and 2-oxoglutarate, and discuss the biological relevance of this result.

Material and Methods

Reagents

All chemicals were of analytical or molecular biology grade and were purchased from Merck (Germany), Sigma (USA), J.T. Baker (The Netherlands), or Invitrogen (USA). Restriction enzymes were from Fermentas (Lithuania) or Invitrogen. Oligonucleotides were from Invitrogen. HiTrap Chelating and HiTrap Heparin chromatographic columns were products of GE Healthcare (Sweden). MagneHis™ Ni-particles were from Promega (USA). SYPRO® Ruby was from Invitrogen.

Bacterial strains and plasmids

The bacterial strains and plasmids used are listed in Table 1.

Plasmid construction

The DNA fragment encoding the N-truncated NifA protein was excised from the plasmid pCNpETCCT (11) as an *Xba*I/*Hin*dIII fragment and cloned into pDK5 (12), yielding the plasmid pCNK5CCT. The sequence encoding the N-terminal GAF domain of the NiFA protein was amplified using the primers NifA5'NT (5'-GGTGTCGCATATGCCGGTG-3') and NifA3'Cent (5'-CATGAAGCTTTACTCCTCGGCC-3'), which introduced *Nde*I and *Hin*dIII restriction sites, respectively

Table 1. Bacterial strains and plasmids used.

	Genotype/characteristics	Reference/source
Escherichia coli		
BL21(λDE3)*pLysS*	*hsdS gal* (λcI*ts* 857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene *1*)	Novagen
DH10B	SmR, F'[*proAB* + *lacZ*ΔM15]	Invitrogen
YMC10(*nifH'-lacZ)*	*ΔlacU169 endA1 thi-1 hsdR17 supE44 hutCK* [Km^R-*Φ*(*nifH'-lacZ*)]	15
RB9060(*nifH'-lacZ)*	*ΔlacU169 endA1 thi-1 hsdR17 supE44 hutCKΔglnB2306* [Km^R-*Φ*(*nifH'-lacZ*)]	15
WCH30(*nifH'-lacZ)*	*ΔlacU169 endA1 thi-1 hsdR17 supE44 hutCKΩGmrΔglnK1* [Km^R-*Φ*(*nifH'-lacZ*)]	15
UNF3435(*nifH'-lacZ)*	*ΔlacU169 endA1 thi-1 hsdR17 supE44 hutCKΔglnB2306 ΩGmrΔglnK1* [Km^R-*Φ*(*nifH'-lacZ*)]	15
Plasmids		
pET28a	Expression vector/T7 promoter, Km^R	Novagen
pT7-7	Expression vector/T7 promoter, Amp^R	New England Biolabs
pDK5	Expression vector/*tac* promoter, Amp^R	12
pDK7	Expression vector/*tac* promoter, Cm^R	12
pCR2.1	Cloning vector, Km^R, Amp^R	Invitrogen
pLMA4	*A. brasilense glnB* in pT7-7, Amp^R	6
pLANTpET	His-tagged *A. brasilense nifA* N-terminal region cloned as *Nde*I/*Hin*dIII fragment in pET28a	Present study
pCNK5CCT	His-tagged *A. brasilense* N-truncated *nifA* cloned as *Hin*dIII/*Xba*I fragment in pDK5, Amp^R	Present study
pCNK7NT	His-tagged *A. brasilense nifA* N-terminal region cloned as *Hin*dIII/*Xba*I fragment in pDK7, Cm^R	Present study

(underlined), and cloned into the pCR2.1 cloning vector (Invitrogen), yielding the plasmid pLANTTA. The amplified fragment was sequenced to confirm gene integrity. The *Nde*I/*Hin*dIII fragment obtained from pLANTTA was then cloned into pET28a to yield the plasmid pLANTpET. To obtain the plasmid pCNK7NT, the *Xba*I/*Hin*dIII fragment excised from pLANTpET was cloned into pDK7 (12).

Protein purification procedure

To purify the NifA N-terminal GAF domain, the *E. coli* strain BL21(λDE3)*pLysS* carrying plasmid pLANTpET was induced with 0.5 mM IPTG at 18°C overnight. After harvesting, cells were resuspended in buffer S (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM DTT, 10% glycerol) and lysed by sonication. The crude extract was clarified by centrifugation at 12,000 *g* for 30 min. The soluble fraction containing the N-terminal domain was loaded onto a Hi-trap-chelating-Ni^{2+} column pre-equilibrated in buffer S. Two washing steps were performed using 15 column volumes each of buffer W1 (50 mM Tris-HCl, pH 6.3, 500 mM NaCl, 10 mM imidazole) and buffer W2 (50 mM Tris-HCl, pH 6.3, 500 mM NaCl, 40 mM imidazole). The proteins were eluted by a stepwise increase of imidazole (50, 100, 300, 500, and 1000 mM) in buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10% glycerol), using five column volumes of each concentration. The *A. brasilense* GlnB and GlnZ proteins were purified by the method of Araújo et al. (13), except that a heat treatment of the crude extract at 80°C for 10 min was included (14).

Protein analysis

Protein concentration was determined by the Bradford method using bovine serum albumin as a standard. Protein expression and purification was analyzed by 12.5% SDS-PAGE and proteins were stained with Coomassie blue R-250 or SyPro Ruby.

Pull-down assays

A MagneHis™ Ni^{2+}-particle suspension was equilibrated in binding buffer containing 50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.05% Tween-20, and 20 mM imidazole, pH 8.0. The NifA N-terminal domain, at a concentration of 0.25 µM, was immobilized by pre-incubation in a total volume of 500 µL containing 25 µL of the magnetic bead suspension. After 5 min, the beads were washed in the above buffer and *A. brasilense* GlnB was added to a final concentration of 0.8 µM in a volume of 500 µL, in the presence or absence of the ATP or ADP effectors (3.5 mM) and 2-oxoglutarate (2 mM). After an additional 15 min of incubation, the beads were washed with binding buffer in the presence or absence of the effectors, the buffer was removed, and elution performed with elution buffer (binding buffer with 1 M imidazole). Aliquots of each sample were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis. The gels were stained with the fluorescent dye SyPro Ruby, visualized using a 302 UV transilluminator and recorded with a cooled CCD camera (Biochemi, UVP, USA). The LabWorks Image Acquisition and Analysis software (UVP) version 4.0.0.8 was used for analysis of the gel images.

Transcription activation of a *nifH'-lacZ* fusion

E. coli strains containing a *Klebsiella pneumoniae nifH'-lacZ* chromosomal fusion (15) and carrying plasmids expressing *A. brasilense* N-truncated NifA and NifA N-terminal GAF domain were grown overnight at 37°C in nitrogen-free Davis and Mingioli (NFDM) medium (16) containing 5% LB medium, 5 mg/L $CaCl_2$, 0.1 g/L NaCl, 5 µg/mL thiamine, 100 µg/L glutamine and antibiotics as required. The cultures were diluted to an absorbance at 600 nm of 0.1 in NFDM medium plus 5 µg/L thiamine, 5 mg/L $CaCl_2$, 0.1 g/L NaCl, 100 µg/L glutamine, 100 µg/L serine, 0.5 mM IPTG and antibiotics. The cell suspensions were incubated for 16 h at 30°C in the presence or absence of 20 mM NH_4Cl, under air or N_2, and assayed for β-galactosidase activity. The relative levels of N-truncated NifA and NifA N-terminal domain proteins were determined by immunoblots.

β-galactosidase activity was determined using *o*-nitrophenyl-β-galactoside as described by Miller (17) and is reported in Miller units.

Results and Discussion

The N-terminal GAF domain of NifA regulates the activity of the catalytic domains

Previous results have shown that the NifA protein from *A. brasilense* requires co-expression of the cognate GlnB protein to activate *nif* gene expression in an *E. coli* background (8). In its native background, the NifA activity is regulated by the prevailing ammonium levels and the N-terminal GAF domain of NifA has been implicated in ammonium-dependent regulation through interaction with the GlnB protein (5,7). To test the ability of the *A. brasilense* N-truncated NifA protein to activate the transcription of *nif* genes in a heterologous background, we used the *E. coli* strains YMC10 (wild-type), RB9060 (*glnB*$^-$), WCH30 (*glnK*$^-$) and UNF3435 (*glnB*$^-$ and *glnK*$^-$) carrying a *K. pneumoniae nifH'-lacZ* chromosomal fusion (15) and plasmid pCNK5CCT, which expresses an N-truncated form of *A. brasilense* NifA. The *E. coli* strains expressing the N-truncated NifA protein showed 5-8 times higher β-galactosidase activity in cells assayed in the absence of oxygen, confirming that this variant of the NifA protein is active and reinforcing the evidence that the oxygen sensitivity of NifA is not related to the N-terminal domain (Figure 1). Also, the activity was similar in cells assayed in the presence or absence of ammonium, confirming that removal of the N-terminus of NifA leads to loss of ammonium regulation. Nishikawa et al. (11) have reported similar results. In addition, we observed that the N-truncated NifA was active and non-regulated regardless of the presence of genes encoding endogenous P_{II} proteins

(*glnK* or *glnB*) in the *E. coli* backgrounds, although the β-galactosidase activity was approximately 2-fold higher in strains RB9060 (*glnB*) and UNF3435 (*glnB*/*glnK*) (Figure 1). We also determined the effect of *in trans* expression of the *A. brasilense* NifA N-terminal domain on the activity of the N-truncated NifA protein in the same *E. coli* strains (Figure 1). The expression of these NifA variants under all conditions reported in Figure 1 was confirmed by immunoblot analysis (data not shown). The co-expression of a His-tagged variant of the N-terminal GAF domain (NifANT-His) and the N-truncated NifA caused a substantial decrease in β-galactosidase activity in all strains under low ammonium and anaerobiosis conditions, confirming the inhibitory role of the NifA N-terminal GAF domain. The expression of the GAF domain led to partial recovery of ammonium-dependent regulation of the N-truncated NifA activity in the wild-type and *glnK* strains (YMC10 and WCH30, respectively) (Figure 1). In contrast, no regulation was observed in the strains lacking *glnB* (RB9060 and UNF3435, respectively). These results indicate that the NifA GAF domain can inhibit *in trans* the N-truncated NifA protein and that regulation by ammonium requires the *E. coli* GlnB but not GlnK (Figure 1). The effects observed were not due to lack of expression of the NifA protein variants in the *E. coli* strains, since immunoblot assays with antibodies raised against the N-truncated NifA and the N-terminal domain revealed expression of both proteins under the β-galactosidase assay conditions (data not shown). It has been reported that the *E. coli* GlnB but not GlnK is able to activate the native *A. brasilense* NifA in an *E. coli* background, supporting evidence that GlnB, but not GlnK, plays a role in NifA regulation (6).

The N-terminal GAF domain of NifA interacts with the GlnB protein

To determine if the NifA GAF domain interacts with the P_{II} proteins *in vitro*, pull-down assays were performed us-

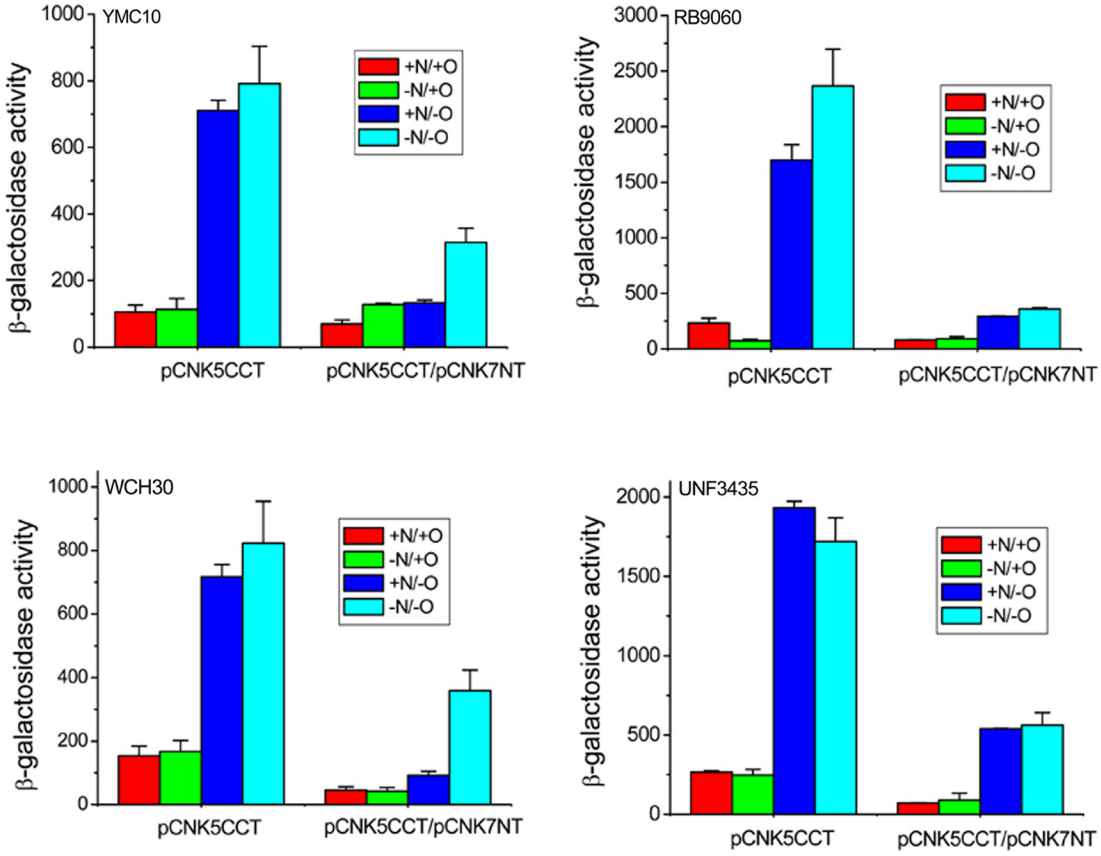

Figure 1. Transcriptional activation of *Klebsiella pneumoniae nifH'-lacZ* by *Azospirillum brasilense* N-truncated NifA in the presence or absence of the NifA N-terminal GAF domain. Assays were carried out in *Escherichia coli* strains YMC10 (wild type), RB9060 (*glnB*$^-$), WCH30 *(glnK*$^-$), and UNF3435 (*glnB*$^-$/*glnK*$^-$) transformed by pCNK5CCT (N-truncated NifA domain) and/or pCNK7NT (NifA N-terminal GAF domain). Samples were incubated under air (+O) or under N_2 (-O), and in the presence (+N) or absence (-N) of 20 mM NH_4Cl as described in Material and Methods. β-galactosidase activity is indicated in Miller units. Cells carrying no plasmid or only the pCNK7NT plasmid showed background activity of less than 150 Miller units. Data are the average of at least three independent experiments.

ing NifANT-His as bait and the *A. brasilense* GlnB or GlnZ proteins. The NifANT-His protein bound to MagneHis-Ni^{2+} beads was incubated with *A. brasilense* GlnB or GlnZ proteins. After extensive washing with binding buffer, the proteins bound to the MagneHis-Ni^{2+} were eluted with imidazole and analyzed by SDS-PAGE (Figure 2). GlnB co-precipitated with NifANT-His only in the presence of MgATP and 2-oxoglutarate (Figure 2A), while GlnZ did not interact with NifANT-His under any tested conditions (Figure 2B), confirming our previous hypothesis that GlnB but not GlnZ is involved in NifA regulation (6). Bovine serum albumin, used as a negative control, failed to bind to the MagneHis-Ni^{2+} beads charged with NifANT-His (data not shown).

Chen et al. (7) have previously shown that the *A. brasilense* NifA GAF domain could interact with GlnB using yeast two-hybrid studies. Our *in vitro* analysis confirmed this observation and extended it by showing that this interaction requires the GlnB effectors MgATP and 2-oxoglutarate (Figure 2). The interaction between the NifA N-terminal GAF domain of *H. seropedicae* and the GlnK protein is also stabilized by MgATP and 2-oxoglutarate *in vitro* (18).

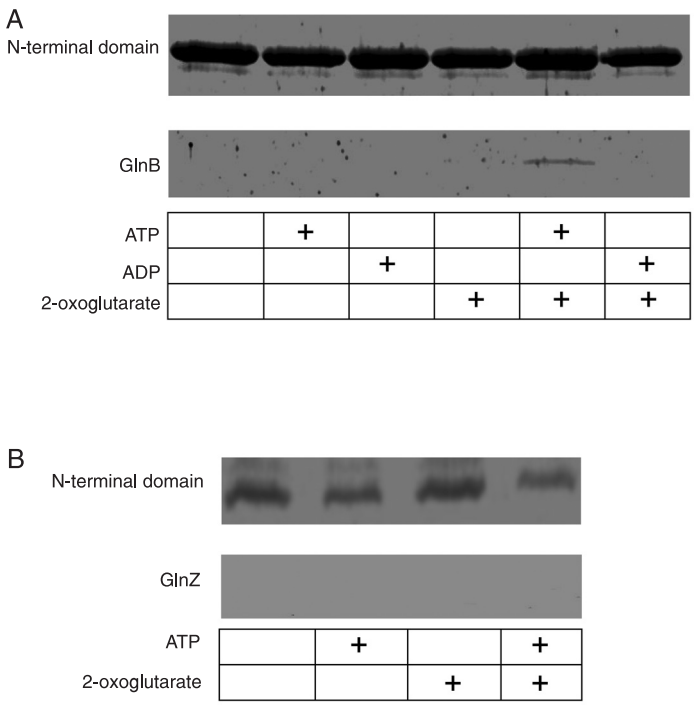

Figure 2. Pull-down assays of GlnB bound to the NifA N-terminal domain on MagneHis-Ni^{2+} particles. The assays were carried out as described in Material and Methods. The NifA N-terminal-His domain (0.25 µM) was immobilized on MagneHis-Ni^{2+} particles and incubated with 0.8 µM GlnB (Panel A) or GlnZ proteins (Panel B) in the presence of ATP or ADP (3.5 mM) and/or 2-oxoglutarate (2 mM) when indicated. Samples were run on 12.5% SDS-PAGE. The gels were stained with SYPRO Ruby.

The effectors MgATP and 2-oxoglutarate bind synergistically to nearly all P_{II} proteins studied to date. The biochemical basis for such synergy is explained by the fact that the 2-oxoglutarate binding sites are located in the vicinity of the MgATP binding sites, and the Mg^{2+} ion provides two coordination sites for 2-oxoglutarate (reviewed in Ref. 9). Based on the data reported in Figure 2, the *A. brasilense* GlnB structure would only be able to interact with NifA when the GlnB trimmer is saturated with MgATP plus 2-oxoglutarate. However, we cannot exclude the possibility that 2-oxoglutarate also binds to the *A. brasilense* NifA GAF domain since the GAF domain of NifA from *A. vinelandii* interacts with this metabolite *in vitro* (19).

Our results show for the first time a direct *in vitro* interaction between the N-terminal domain of NifA of *A. brasilense* and the signaling protein GlnB, but not with GlnZ, in the presence of MgATP and 2-oxoglutarate. The physiological significance for the requirement of MgATP and 2-oxoglutarate to promote the interaction between the NifA GAF domain and GlnB is not yet clear. The 2-oxoglutarate pool fluctuates *in vivo* in response to nitrogen levels, increasing under nitrogen limitation and decreasing when nitrogen is abundant (20). Hence, GlnB saturated with MgATP and 2-oxoglutarate should accumulate under nitrogen limitation (20). Nitrogen limitation also induces GlnB uridylylation (10,13). Although evidence suggests that GlnB uridylylation is required to relieve the inhibitory effect of the NifA GAF, the NifA-GlnB interaction might be primarily modulated by the effectors bound to GlnB rather than by the GlnB uridylylation status. We are currently investigating the interaction of uridylylated GlnB with NifA *in vitro*.

Acknowledgments

We are grateful to Roseli Prado, Valter Baura, Julieta Pie, and Marilza Lamour for technical assistance. We are also grateful to Dr. M. Geoffrey Yates (UK) for reading the manuscript. Research supported by INCT-Fixação Biológica de Nitrogênio, CNPq, CAPES, and Institutos do Milênio.

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