

# Purification and binding analysis of the nitrogen fixation regulatory NifA protein from *Azospirillum brasilense*

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## Abstract

NifA protein activates transcription of nitrogen fixation operons by the alternative  $\sigma^{54}$  holoenzyme form of RNA polymerase. This protein binds to a well-defined upstream activator sequence (UAS) located at the -200/-100 position of *nif* promoters with the consensus motif TGT-N<sub>10</sub>-ACA. NifA of *Azospirillum brasilense* was purified in the form of a glutathione-S-transferase (GST)-NifA fusion protein and proteolytic release of GST yielded inactive and partially soluble NifA. However, the purified NifA was able to induce the production of specific anti-*A. brasilense* NifA-antiserum that recognized NifA from *A. brasilense* but not from *K. pneumoniae*. Both GST-NifA and NifA expressed from the *E. coli tac* promoter are able to activate transcription from the *nifHDK* promoter but only in an *A. brasilense* background. In order to investigate the mechanism that regulates NifA binding capacity we have used *E. coli* total protein extracts expressing *A. brasilense nifA* in mobility shift assays. DNA fragments carrying the two overlapping, wild-type or mutated UAS motifs present in the *nifH* promoter region revealed a retarded band of related size. These data show that the binding activity present in the C-terminal domain of *A. brasilense* NifA protein is still functional even in the presence of oxygen.

## Key words

- NifA protein
- NifA antibodies
- *Azospirillum brasilense*
- Gel shift assay
- NifA activity
- Upstream activator sequence

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## Introduction

In many diazotroph organisms, the nitrogen fixation genes (*nif*) are transcribed by an alternative holoenzyme form of RNA polymerase and require an activator protein, NifA. Both activity and synthesis of NifA are regulated in response to environmental effectors. Two major signals regulate nitrogen fixation, oxygen and ammonia, and this regulation occurs mainly at the level of *nif* gene transcription mediated by NifA. NifA acts as a DNA-binding protein which recognizes

typical upstream promoter sequences (TGT-N<sub>10</sub>-ACA) showing many enhancer element properties located upstream of promoters of the -24/-12 type (1). The integration host factor protein (IHF) stimulates *K. pneumoniae* NifA-mediated activation of *nif* promoters by facilitating DNA loop formation (2).

NifA activates *nif* transcription under oxygen and nitrogen-limiting conditions but becomes inactive when levels of fixed nitrogen or oxygen increase (3). This inactivation has been attributed to the NifL protein, which

senses changes in the oxygen and/or nitrogen status of the cell in *Klebsiella pneumoniae* and *Azotobacter vinelandii* (4-7). In *A. brasilense* and members of the Rhizobiaceae family, where NifL has not been found, the NifA protein itself is inactivated by oxygen. The NifA protein contains a conserved motif of cysteine residues, which is absent in *K. pneumoniae* and *A. vinelandii*, involved in sensitivity to oxygen in *Bradyrhizobium japonicum* and possibly in *A. brasilense* (8,9).

NifA proteins are structurally similar to each other, and three independent domains, separated by two inter-linker-domains (IDL), were defined (8,10,11). The C-terminal domain contains a helix-turn-helix DNA binding motif (12,13); the central domain, involved in catalysis, contains the nucleotide binding site (10,14) and the N-terminal domain is not essential for *K. pneumoniae* NifA activity. In *K. pneumoniae* this latter domain, that has no homologue, appears to decrease the sensitivity to NifL (10,12,15). In *A. brasilense*, NifA is synthesized in an inactive form under conditions not compatible with nitrogen fixation (16). Moreover, its regulation differs considerably from that found in other free living diazotrophs. It has been proposed that, in *A. brasilense*, the N-terminal domain plays an inhibitory role which leads to the inactivation of NifA in the absence of P<sub>II</sub> protein (17,18). P<sub>II</sub> protein plays an essential role in the regulation of nitrogen metabolism in *E. coli* by controlling the activity of GS (19,20) and it appears that *glnB* is essential for nitrogen fixation in *A. brasilense* (18). Another important feature of *A. brasilense* is the regulation of nitrogenase activity at the post-translational level by a "switch-off" mechanism in response to environmental changes in ammonia concentration (21). Therefore, the plant growth-promoting *A. brasilense*, which associates with roots of economically important grasses, has some characteristic features at the level of nitrogen fixation regulation.

In this paper, we report the production of

NifA from *A. brasilense* in *E. coli* cells, its purification, the production of specific antiserum and the analysis of the activity of the recombinant NifA protein. We also show that the recombinant NifA proteins were active only in *A. brasilense* cells and were able to induce transcription from the *nifH* promoter. The ability of *A. brasilense* NifA to bind to upstream activator sequences present on *nif* genes even in its inactive form in response to oxygen is also demonstrated.

## Material and Methods

### Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in the present study are listed in Tables 1 and 2. LB medium (22) was used for growing *E. coli* strains at 37°C. YT medium (22) was used to induce the glutathione-S-transferase (GST)-NifA fusion protein on *E. coli* BL21 strains at 30°C. *A. brasilense* strains were grown at 30°C in either LB medium or MMAB minimal medium (23) using 0.5% malate as carbon source. MMAB without ammonium chloride was used when nitrogen-free medium was required. The medium was supplemented with the antibiotics tetracycline (Tc), ampicillin (Ap) or kanamycin (Km) when necessary, at concentrations of 10, 100 or 30 µg/ml, respectively.

Conjugation experiments were performed on D-plates (8 g/l Bacto nutrient broth, 0.25 g/l magnesium sulfate, 1 g/l potassium chloride, 0.01 g/l manganese chloride). MMAB medium supplemented with Tc and Km was used to select *A. brasilense* transconjugants.

### DNA manipulations, nitrogenase assays, β-galactosidase assays and conjugation

Plasmid DNA preparation, restriction enzyme analysis, transformation and hybridization were performed as described in reference 22. Restriction endonucleases and other

enzymes were purchased from Pharmacia (Uppsala, Sweden), Gibco/BRL (Gaithersburg, MD) or Promega (Madison, WI) and used according to the manufacturer's instructions.

Nitrogenase activity was measured by the acetylene reduction method described by Burris (24). Ethylene production was quantified on a Plot fused silica column (50 m x 0.32 mm, 5 µm Al<sub>2</sub>O<sub>3</sub>/KCl, Chrompack) installed in a Hewlett Packard 5890A gas chromatograph. Propane was used as internal standard.

Determination of β-galactosidase activity of the *nif-lacZ* promoter fusions was carried out according to the standard procedure of Miller (as described in reference 22) at 25°C.

Plasmid mobilization from *E. coli* S17.1 to *A. brasilense* Sp7 by biparental conjugation was performed as previously described (25).

### Plasmid constructions

The strategy used to obtain the pKKAbA plasmid is shown in Figure 1A. DNA from plasmid p10 was digested to generate the 2.2-kb *SmaI/SalI* DNA fragment carrying the coding region for NifA from *A. brasilense*. The *SmaI* site is located at 91 bp from the ATG start codon. In the pKKAbA (pKK233-3 vector) construct the *A. brasilense nifA* gene is under the control of the *tac* promoter.

The *nifA* gene from *A. brasilense* was also cloned into three other plasmid vectors. Plasmid pKKAbA was digested with *SalI* and the entire construction, *ptac-nifA*, was cloned into the *SalI* site of the pACYC184 vector (pAAbA plasmid). The pGEX 4T-1 vector was used to generate a GST-NifA fusion protein. The *SmaI/SalI* 2.2-kb DNA fragment from plasmid p10 was also cloned into the pGEX 4T-1 *SmaI/SalI* digested vector. In this way the *nifA* gene was cloned in frame with GST and the recombinant plasmid, pGEXAbA, was able to produce a GST-NifA fusion protein (Figure 2A).

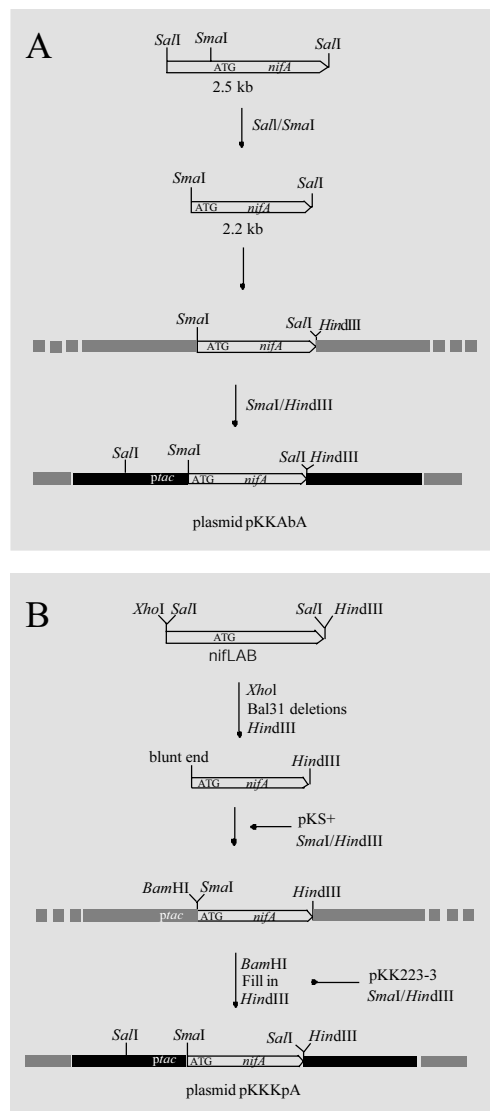
Table 1 - Bacterial strains used in the present study.

Bacteria	Strain	Relevant characteristics	Reference
A. brasilense	Sp7	wild-type strain, ATCC 29145	37
	Sp7067	Sp7 mutant Nif <sup>-</sup> ; nifA::Tn5	31
	Sp7077	Sp7 mutant Nif <sup>-</sup> ; nifA::Tn5	31
E. coli	MC1061	hsdR mcrB araD 139Δ(araABC-leu) 7679 ΔlacX74 galU galK rpsL thi	38
	BL21	hsdS gal (λclts857 ind1 Sam7 nin5 lacUV5-T7 gene1)	39
	DH5α	hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44 ΔlacU169 (φ80lacZΔM15)	22
	S17.1	thi endA recA hsdR RP4-2-Tc::Mu-Kan::Tn7	40

Table 2 - Plasmids used in the present study.

Plasmid	Relevant characteristics	Reference
pBluescript	Ap <sup>R</sup>	Stratagene
pUC18	Ap <sup>R</sup>	22
pKK223-3	Ap <sup>R</sup>	Pharmacia
pGEX 4T	GST gene fusion vector, Ap <sup>R</sup>	Pharmacia
pLAFR3	pRK290 derivative having M13mp18 polylinker and λ cos sites, Tc <sup>R</sup>	41
pCK3	pRK290 derivative having K. pneumoniae nifA gene	42
pACYC184	Tc <sup>R</sup> , Cm <sup>R</sup>	43
p10	pSUP202 derivative having 2.5-kb <i>SalI</i> nifA from <i>A. brasilense</i> , Ap <sup>R</sup>	Dr. Fabio Pedrosa, UFPR, Brazil
pMCH	pnifH-lacZ, promoter from <i>A. brasilense</i> nifH, Ap <sup>R</sup>	27
pKKAbA	pKK223-3 derivative, <i>ptac-nifA</i> ( <i>A. brasilense</i> nifA gene), Ap <sup>R</sup>	this paper
pAAbA	pACYC184 derivative, <i>ptac-nifA</i> ( <i>A. brasilense</i> nifA gene), Cm <sup>R</sup>	this paper
pGEXAbA	pGEX 4T-1 derivative, GST-NifA fusion ( <i>A. brasilense</i> nifA gene), Ap <sup>R</sup>	this paper
pLKSAbA	pLAFR3 derivative, <i>ptac-nifA</i> ( <i>A. brasilense</i> nifA gene), Tc <sup>R</sup>	this paper
pLpucNifA	pLAFR3 derivative, GST-NifA fusion ( <i>A. brasilense</i> nifA gene), Tc <sup>R</sup>	this paper
pKKKpA	pKK223-3 derivative, <i>ptac-nifA</i> ( <i>K. pneumoniae</i> nifA gene), Ap <sup>R</sup>	this paper
pKSKpLAB	pBluescript KS+ derivative, carrying a 2.5-kb <i>SalI</i> DNA fragment from <i>K. pneumoniae</i> nifLAB operon, Ap <sup>R</sup>	this laboratory
pAKpA	pACYC184 derivative, <i>ptac-nifA</i> ( <i>K. pneumoniae</i> nifA gene), Cm <sup>R</sup>	this paper
pKSwT	pBluescript derivative containing the <i>A. brasilense</i> nifH promoter	27
pKSUAS1	pBluescript derivative containing the <i>A. brasilense</i> nifH promoter UAS1 mutant	27
pKSUAS2	pBluescript derivative containing the <i>A. brasilense</i> nifH promoter UAS2 mutant	27
pGEXKpA	pGEX 4T-3 derivative, GST-NifA fusion ( <i>K. pneumoniae</i> nifA gene), Ap <sup>R</sup>	this paper

Figure 1 - Cloning strategy for the insertion of *nifA*, from *A. brasilense* (A) and from *K. pneumoniae* (B), into the pKK223-3 vector. The figure also shows the restriction map of plasmid p10 containing the *nifA* gene from *A. brasilense* (A) and plasmid pCRA37 containing the *nifLAB* genes from *K. pneumoniae* (B). The black boxes represent the pKK223-3 vector and the gray boxes represent the pUC18 vector in A and the pBluescript vector in B.



For the complementation experiments, the *nifA* gene was cloned into the pLAFR3 cosmid. Figure 3A depicts the steps used to generate plasmid pLKSAbA carrying the *ptac-nifA* construction and Figure 3B shows the pLpucNifA derivative containing the GST-NifA fusion construction.

The *nifA* gene of *K. pneumoniae* was also cloned into the transcriptional vector pKK223-3 and into the translational fusion vector pGEX 4T-3. Plasmid pCRA37 (26) was digested with *SalI*, yielding a 2.9-kb DNA fragment carrying the *K. pneumoniae nifLAB* region. This fragment was cloned

into the pBluescript KS+ vector generating the pKSKpLAB plasmid. To remove the *nifL* gene, pKSKpLAB was digested with *XhoI* and Bal 31 deletions were carried out. Two different deleted DNA fragments have been selected and confirmed by DNA sequencing. The first one, shown in Figure 1B, has only 91 bp upstream from the *nifA* ATG start codon and was cloned into the pKK223-3 vector under the control of the *tac* promoter to produce the recombinant plasmid pKKKpA. To construct plasmid pAKpA, the *ptac-nifA* region from pKKKpA was removed by *SalI* digestion and cloned into the pACYC184 *SalI* digested vector. The second Bal 31 deleted fragment was cloned into the pBluescript vector, named pKS3.1(5.1), and completely sequenced. Figure 2B shows the strategy used to clone the NifA as a fusion protein with GST into the pGEX 4T-3 vector to produce the plasmid pGEXKpA.

#### Cell extracts and detection of NifA by Western blot

Coupled transcription/translation in S30 extracts (Promega), using either pKKAbA or pKKKpA plasmids, were prepared following the instructions provided by the manufacturers.

*E. coli* BL 21 extracts, carrying pGEXAbA or pGEXKpA plasmids, and DH5 $\alpha$ , carrying plasmids pKKAbA or pAAAbA, were prepared after growing the cells for 2 h at 30°C in YT medium supplemented with ampicillin. The fusion protein was induced in aerobically grown cultures by the addition of 0.1 mM IPTG and the cells were harvested after growing for another 3 h at the same temperature. Proteins (1/4 of the culture) were separated on 10% SDS/polyacrylamide gels and visualized by Coomassie blue staining. Western blotting was performed as described in reference 22. Detection was carried out using monoclonal antibodies against GST or *A. brasilense* NifA antiserum. The reaction was incubated with

anti-mouse antibody labeled with alkaline phosphatase and developed with a goat anti-mouse (IgG) secondary antibody using the substrate B-CIP/NBT detection system (Sigma, St. Louis, MO).

**Purification of NifA protein and immunological procedures**

*A. brasilense* NifA protein was purified from *E. coli* harboring the pGEXAbA plasmid as described in the GST Gene Fusion System (Pharmacia 2nd edn.). The purified NifA protein was used as immunogen for the generation of mouse antiserum as follows. Five female BALB/c white mice were immunized by intradermal injection with NifA. The purified protein (10 mg) was mixed with an equal volume of Freund's complete adjuvant for the first injection, or with Freund's incomplete adjuvant for the second injection 12 days later. One week after the second injection, NifA antiserum was collected, diluted 1:100 and used for Western immunoblot experiments.

**Gel shift motility assays**

DNA-binding assays were performed as described before (27).

The oligonucleotides used in the PCR reactions were 5'TGCATCCCCCGTGCCAGTTCGCG3' and 5'CCTGACGCTGGCTCTGACGCTGG3'. The first oligonucleotide was labeled with digoxigenin (DIG) and the detection system was that described in the DIG gel shift kit (Boehringer, Mannheim, Germany).

**Results and Discussion**

**Detection of NifA activity on transcriptional fusions**

To demonstrate the activity of *A. brasilense* NifA protein *in vitro* (binding assays), we directed the synthesis of this protein from an expression vector (pKK223-3) in a

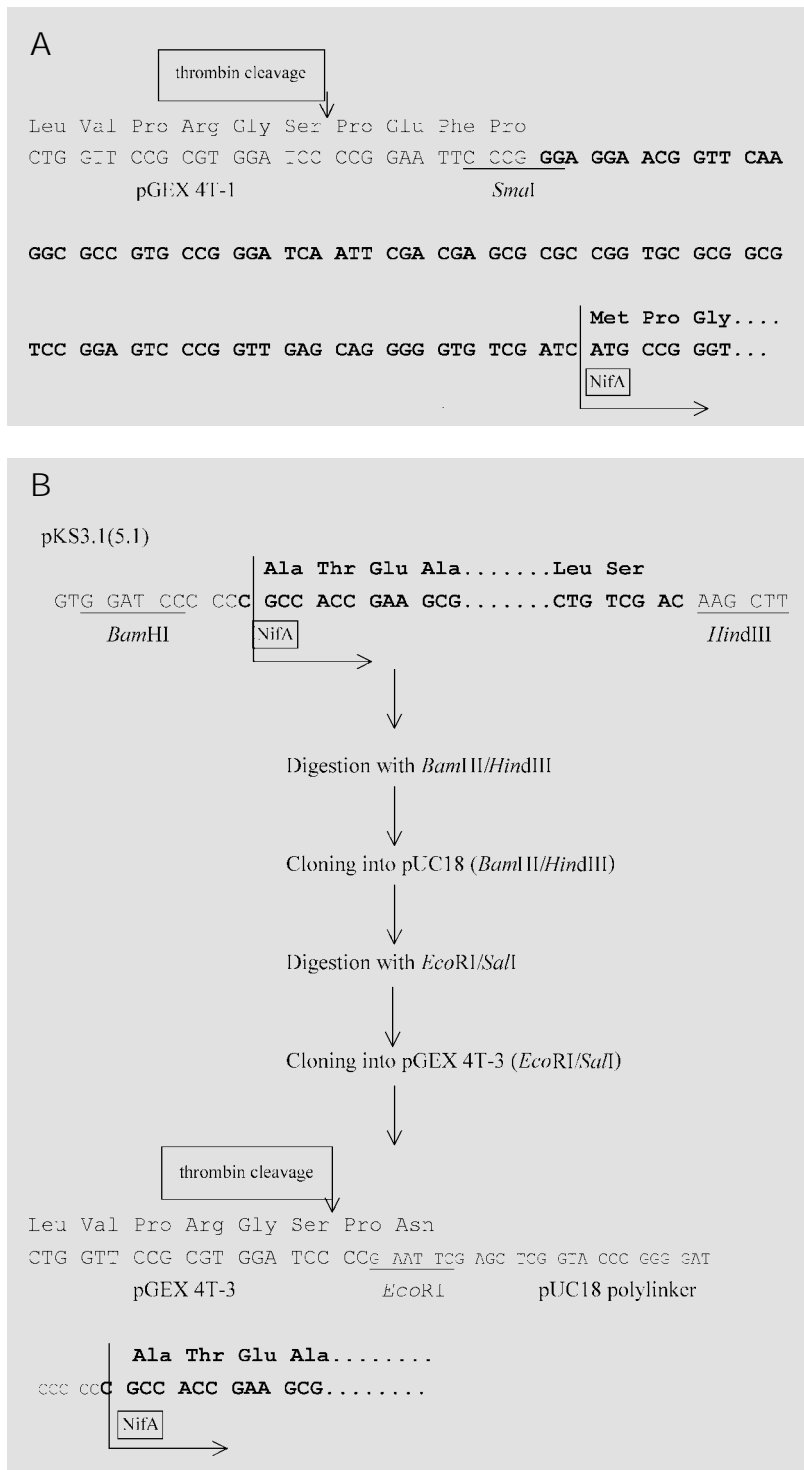


Figure 2 - Cloning strategy for the insertion of *nifA* from *A. brasilense* (A) and from *K. pneumoniae* (B) into the pGEX 4T vector. The nucleotide sequence from the *nifA* gene is indicated in bold type. The first amino acids for the coding region of NifA are indicated in bold above the nucleotide sequence. Small capital letters represent the pUC18 polylinker. The region that represents the *nifA* gene/NifA protein is marked by an arrow.

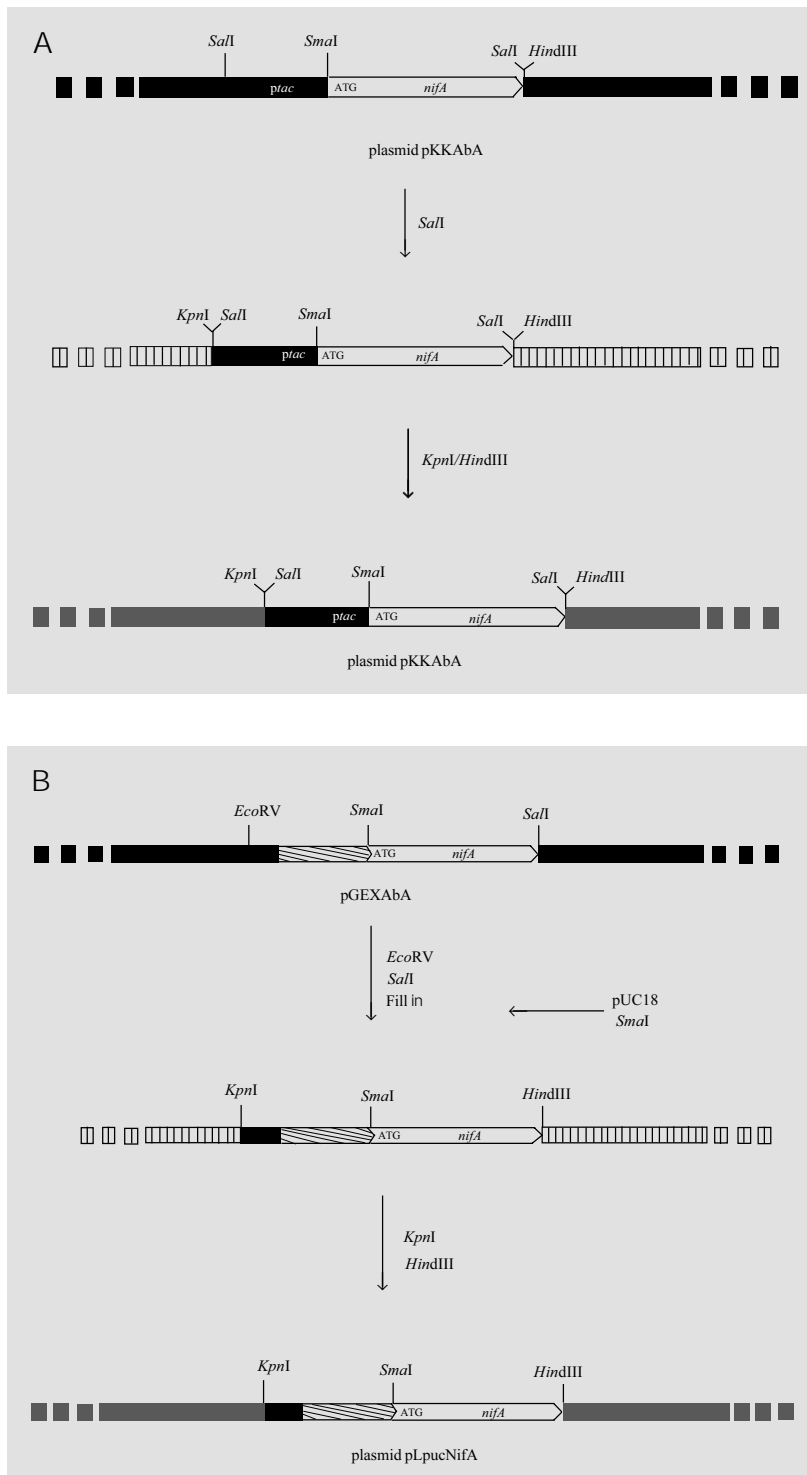


Figure 3 - Strategy for cloning the *nifA* gene from *A. brasilense* into cosmid pLAFR3. A shows the steps for cloning the transcriptional *tac-nifA* fusion construct, and B shows the steps for cloning the translational fusion GST-NifA. Black boxes indicate the pKK223-3 vector in A and the pGEX 4T-1 vector in B; striped boxes indicate the pBluescript vector in A and the pUC18 vector in B; gray boxes indicate the pLAFR3 vector. The stippled arrow indicates the sequence of the GST protein from vector pGEX 4T-1.

coupled transcription-translation system (S30 extracts). Two plasmids, pKKAbA and pKKKpA, carrying transcriptional fusions of *A. brasilense* and *K. pneumoniae nifA* genes with *tac* promoter were constructed (Figure 1) and tested. Induction of the expression from *tac* promoter in strains carrying the plasmids resulted in no detectable production of the  $^{35}\text{S}$ -labeled proteins on 10% SDS-polyacrylamide gels.

To analyze the capability of the *nifA* protein to activate the *nifH* promoter two other plasmid constructions were made. Plasmids pAAbA and pAKp6 carry the same transcriptional fusion present in pKKAbA and pKKKpA, but were cloned into a vector (pACYC184) compatible with pMCH (27), containing the *A. brasilense nifH* promoter fused to *lacZ*. Cell cultures were prepared from strain MC1061 carrying the pMCH plasmid and from the two different constructs which produced NifA either from *A. brasilense* or from *K. pneumoniae*. Transcriptional activation of the *A. brasilense nifH* promoter *in vivo* by *K. pneumoniae* NifA protein is shown in Table 3. The transcriptional fusion of *A. brasilense nifA* protein failed to activate the *nifH* promoter.

The transcription/translation coupled system was used previously to analyze the activity of NifA protein from *R. meliloti* (28) and *K. pneumoniae* (29,30). The activity present in the control construction (*tac-nifA* from *K. pneumoniae*) is in agreement with previous results which have shown that this protein is able to activate the *nifH* promoter from *A. brasilense* (27; Table 3). The failure of the *A. brasilense tac-nifA* fusion protein to activate the *nifH* promoter is probably due to its inactive form in the presence of oxygen (31).

#### Detection of NifA activity on translational fusions

In order to purify *A. brasilense* NifA and to induce the production of antibodies we

cloned the coding region as a translational fusion with GST (pGEX vector). NifA protein from *K. pneumoniae* has been previously purified as active fusion protein with MBP using the whole sequence (13), only the catalytic domain (15), only the N-terminal domain or only the C-terminal domain (32). In the present study two plasmids were constructed, pGEXAbA carrying the *A. brasilense* NifA protein fused with GST and pGEXKpA carrying the GST-NifA fused protein from *K. pneumoniae* (Figure 2). After digestion with thrombin, the *A. brasilense* NifA protein has an addition of 31 amino acids from the NifA noncoding region of *A. brasilense* in the N-terminal domain. The GST-NifA fusion protein from *K. pneumoniae* has a NifA deleted for the 32 first amino acids and an insertion of 9 extra amino acids from the pUC18 polylinker (Figure 2).

To verify the constructions,  $\beta$ -galactosidase activity was determined in *E. coli* cells carrying the pMCH plasmid and the activity of the truncated NifA from *K. pneumoniae* is shown in Table 3. The transcriptional activation shows low values due to the incompatibility between plasmids pMCH and pGEXKpA resulting in transient expression only. We demonstrated that the *K. pneumoniae* NifA fused with GST, partially deleted at the N-terminal domain, is in its active form. Similar results were reported by Berger et al. (32) using NifA fusion with MBP, showing the activity of NifA both *in vivo* and *in vitro*. However, the *A. brasilense* NifA fusion protein was unable to induce  $\beta$ -galactosidase activity from the *nifH* promoter in *E. coli* extracts.

#### Immunological detection of *A. brasilense* NifA protein

*A. brasilense* NifA protein was purified from *E. coli* extracts as a GST-NifA fusion protein (plasmid pGEXAbA). NifA was released from GST by cleavage with thrombin (Figure 4, lanes 4 and 5). Approximately

50% of the NifA protein remained in the supernatant (Figure 4, lane 5) and was purified by standard procedures (Figure 4, lane 6). NifA polypeptide has an apparent molecular mass of 51 kDa (Figure 5), whereas the predicted NifA protein size, deduced from the nucleotide sequence, was 77 kDa (31). This discrepancy was also found by Arsène et al. (33) and they suggested that it could be due to the presence of a high proline residue content. Proteins rich in proline can cause anomalous migration in SDS-PAGE.

The purified NifA protein was used to

Table 3 - Activation of *nifH* promoter by *K. pneumoniae* NifA in an *E. coli* background.

All plasmids were transformed to *E. coli* and, after growth at 37°C for 18 h,  $\beta$ -gal activity was measured. Results are reported as the means of three representative experiments. ND = Not determined.

Plasmids		$\beta$ -Galactosidase activity (Miller's units)	
Activator	Reporter	NifA absent	NifA present
	pMCH	21.94	ND
pCK3	pMCH	ND	2383.56
pGEXKpA	pMCH	ND	708.67
pAKp6	pMCH	ND	8065.01

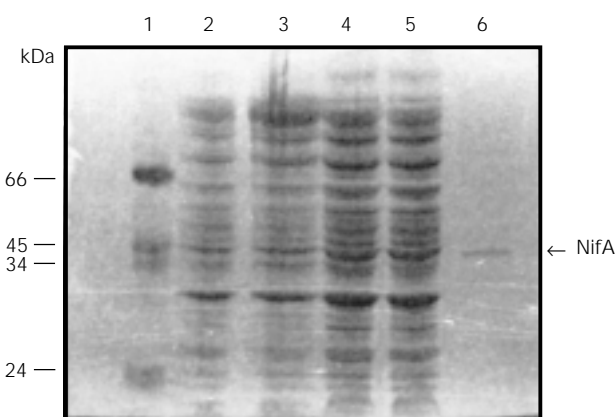


Figure 4 - Purification of NifA from *E. coli* BL21 (pGEXAbA). SDS-PAGE of denatured samples. Total *E. coli* extracts containing the GST-NifA fusion protein present in the supernatant (lane 2) and pellet (lane 3). Total *E. coli* extracts containing the *A. brasilense* NifA protein, after cleavage with thrombin, present in the supernatant (lane 4) and pellet (lane 5). Lane 6, 1  $\mu$ g of purified NifA preparation and lane 1, molecular mass standards (Pharmacia). The position of NifA is indicated by the arrow.

elicit antibodies. To detect the presence of NifA protein, Western blot analysis was performed using the anti-*A. brasilense* NifA antiserum. The antibodies reacted with the purified *A. brasilense* NifA protein (Figure 5, lane 1) and also with a related polypeptide band present in *E. coli* extracts harboring

pKKAbA or pAAbA plasmids (*ptac-nifA*) (Figure 5, lanes 3 and 4). The fusion GST-NifA protein was also detected with antiserum (Figure 5, lane 2). The extra bands present in all extracts are cross-reactive non-specific *E. coli* antibodies.

Immunodetection allowed us to verify the *A. brasilense* NifA constructs. We were able to demonstrate the expression of *A. brasilense* NifA from the *tac* promoter and also as a fusion GST-NifA protein (Figure 5). The antiserum is specific for *A. brasilense* NifA since it does not recognize NifA from *K. pneumoniae* (Figure 6, lanes 7-12). It is known that *K. pneumoniae* NifA protein shows an intrinsic tendency to aggregate and can only be purified as a fusion MBP-NifA protein (13,15,30,32). Although the NifA proteins from *A. brasilense* and *K. pneumoniae* have a high level of amino acid sequence identity (31), they differ in *nifA* expression patterns. We demonstrate here that *A. brasilense* NifA protein can be easily purified from aerobically grown *E. coli* cells, but is inactive. The present results also show that *A. brasilense* NifA can be synthesized in *E. coli* driven by the *tac* promoter and, although the protein is unable to activate the *nifH* promoter in *E. coli* cells, it is able to bind to the UAS present in this promoter (see Binding activity of the *A. brasilense* NifA protein).

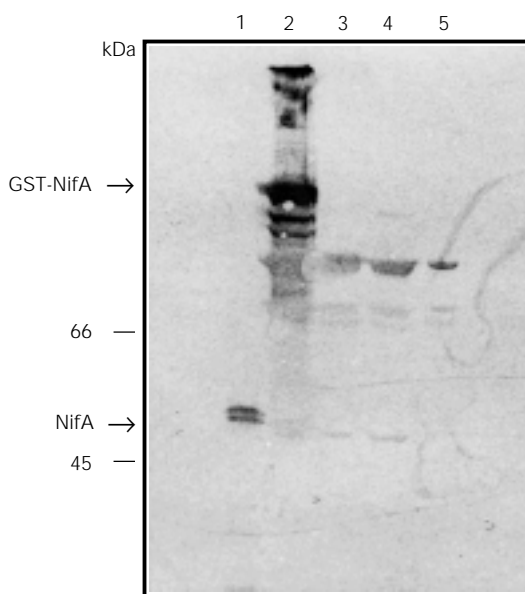


Figure 5 - Immunodetection of the *A. brasilense* NifA from *E. coli* cells using anti-*A. brasilense* NifA-antibodies. The *E. coli* strains contain the following plasmids: pGEXAbA (GST-NifA), lane 2; pKKAbA (*ptac*-NifA), lane 3; pAAbA (*ptac*-NifA), lane 4, and no plasmid, lane 5. Partially purified *A. brasilense* NifA protein (1 µg) was used as control (lane 1). The arrows indicate the position of the purified NifA or the GST-NifA fusion protein.

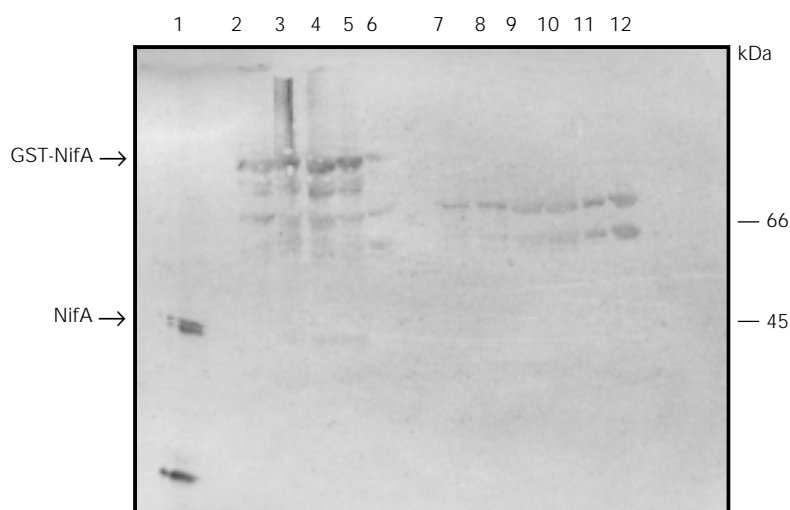


Figure 6 - Immunodetection of the *A. brasilense* NifA from *E. coli* cells harboring the pGEXAbA plasmid. Lane 1, partially purified *A. brasilense* NifA protein; lanes 2-6, *E. coli* extracts carrying the pGEXAbA plasmid, and lanes 7-12, *E. coli* extracts carrying pGEXKpA plasmid (*K. pneumoniae* GST-NifA fusion protein).

#### Activity of the *A. brasilense* NifA protein

The activity of the NifA protein was determined by its ability to restore nitrogen fixation to *A. brasilense nifA*-Tn5 mutant strains, under nitrogen fixation conditions. For this reason, we constructed the pLAFR3 derivative plasmids pLpucNifA and pLKSAAbA (Figure 3). In this way, we were able to analyze both NifA constructs, the GST-NifA fused protein and the *ptac*-NifA expression system. Table 4 shows the nitrogenase activity of the wild type and mutant *A. brasilense* strains. The two constructs



were able to restore a Nif<sup>+</sup> phenotype to the *nifA*-Tn5 mutant strains. The reduced activity, as compared to the wild-type strain, could be due to the structure of the NifA protein found in these constructions containing the extra amino acids or even the GST protein. Transcriptional regulation should be ruled out because no *nifA* promoter was present.

### Binding activity of the *A. brasilense* NifA protein

The *A. brasilense nifH* promoter presents two overlapping UASs and their role was examined by introducing base substitutions in both UAS sites, generating plasmids pKSUAS1 and pKSUAS2 (27). A 630-bp *Sau3A* DNA fragment from plasmids pKSwT, pKSUAS1 and pKSUAS2 (27) was used as target sequence to amplify by PCR a 200-bp fragment containing the two overlapping wild-type UASs, the UAS1 mutant and the UAS2 mutant, respectively. To assay the *A. brasilense* NifA binding capacity, total protein extracts isolated from *E. coli* MC1061 carrying plasmids p10, pAAbA or pGEXAbA were incubated with the DIG-labeled 200-bp DNA fragment (Figure 7A). Total protein extracts carrying *K. pneumoniae* NifA protein provided by plasmids pCK3, pAKpA or pGEXKpA were used as positive control. The binding reactions were carried out at 30°C for 20 min in order to maintain the NifA protein in an active form (13). A retarded band was visualized when extracts from MC1061/pCK3, MC1061/pAKpA, MC1061/pGEXKpA, MC1061/p10 and MC1061/pAAbA were incubated with the UAS-carrying fragments (Figure 7A, lanes 4-8). No shift was detected when an extract from MC1061 was used (Figure 7A, lane 2) or when an extract from MC1061/pCK3 was incubated with a 230-bp DNA fragment, with no UAS motif (Figure 7A, lane 3). Two retarded bands appeared when an extract from MC1061/pGEXAbA was used (Figure 7A, lane 9). The upper new extra band was

Table 4 - Nitrogenase activity of *A. brasilense*.

Complementation was measured in overnight cultures. The data are the average of three independent assays. ND = Not determined.

Strain	Nitrogenase activity (nmol C <sub>2</sub> H <sub>4</sub> min <sup>-1</sup> mg protein <sup>-1</sup> )		
	No.	Plasmid pLpucNifA	pLKSAbA
Sp7	39.38	ND	ND
Sp7067::Tn5	<0.1	17.87	16.85
Sp7077::Tn5	<0.1	8.75	8.66

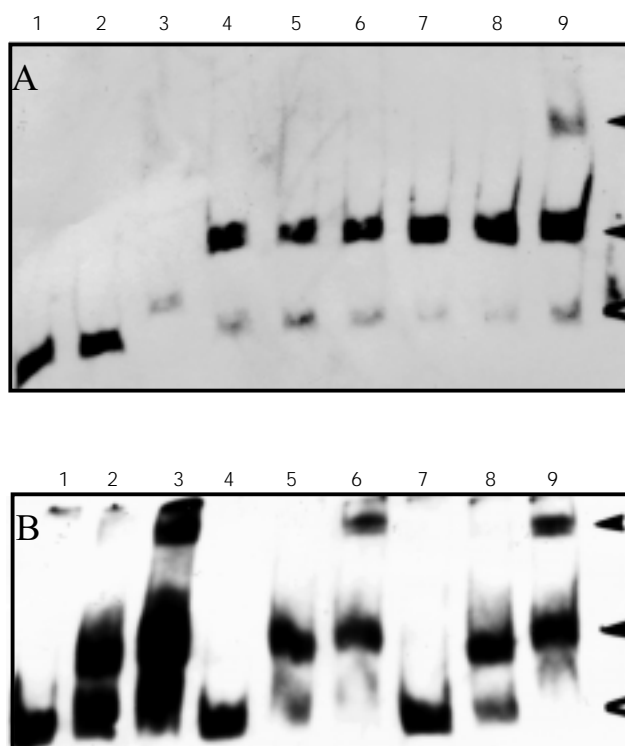


Figure 7 - Binding of NifA protein to the *nifH* UAS promoter region by gel-mobility shift assay. A, The 200-bp DIG labeled DNA fragment was incubated with crude cell supernatant from *E. coli* strains (20 µg total extract/reaction) containing the *K. pneumoniae* NifA protein synthesized by plasmids pCK3 (lane 4), pAKpA (lane 5) and pGEXKpA (lane 6) or *A. brasilense* NifA protein synthesized by plasmids p10 (lane 7), pAAbA (lane 8) and pGEXAbA (lane 9), alone or fused with GST protein. Lane 1, control of DNA fragment; lane 2, the control of the DNA fragment was incubated with MC1061 total extract, and lane 3, a 230-bp DNA fragment with no UAS motif, used as a probe, was incubated with MC1061/pCK3 total extract (negative controls). B, Lanes 1, 4 and 7 correspond to the added wild-type UAS region, UAS1 mutant and UAS2 mutant fragments, respectively. Lanes 2 and 3, wild-type fragment incubated with crude cell supernatant from *E. coli* strains (20 µg total extract/reaction): MC1061/pAAbA (lane 2) and MC1061/pGEXAbA (lane 3). Lanes 5 and 6, UAS1 mutant fragment incubated with total extracts from MC1061/pAAbA (lane 5) and from MC1061/pGEXAbA (lane 6). Lanes 8 and 9, UAS2 mutant fragment incubated with total extracts from MC1061/pAAbA (lane 8) and MC1061/pGEXAbA (lane 9). The full arrowheads represent the retarded bands and the open arrowhead represents the free-labeled fragment.

visualized only with *E. coli* harboring this plasmid and was probably due to the high molecular weight GST-NifA fusion protein.

When the 200-bp DNA fragments carrying the mutated UAS motifs were used as a probe a gel shift band was also visualized (Figure 7B). The extra band in gel shift assays was also detected when the UAS mutant DNA fragments were mixed with *A. brasilense* NifA fused with GST (MC1061/pGEXAbA). Previously, we have demonstrated that the two overlapping UAS sequences have differential effects on *nifH* promoter activity using *K. pneumoniae* NifA protein (27). However, the binding affinity of *A. brasilense* NifA protein, fused or not with GST, revealed no specificity when we compared wild-type or mutant UAS DNA fragments.

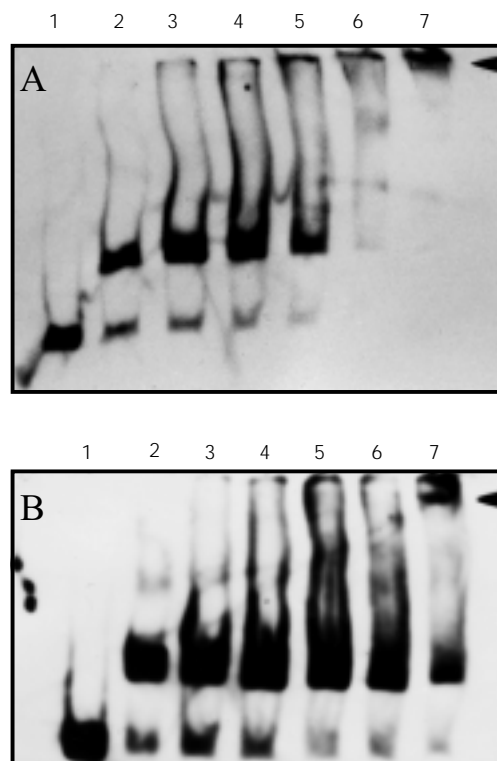
To further analyze the binding of NifA protein, mobility gel shift assays were carried out with NifA preparations (total extracts from MC1061/pAAbA and MC1061/pGEXAbA), wild-type UAS-carrying frag-

ment, and incubated with anti-*A. brasilense* NifA-antibody (Figure 8). Increasing amounts of antibody led to the formation of a high molecular weight complex which was not visible in the control reaction (Figure 8, lane 2). Since this antibody was shown to be specific for *A. brasilense* NifA protein, the supershift effect detected when both extracts were used should be due to the formation of the DNA-NifA-antiNifA complex. A similar result was also reported by Zimmer et al. (34) using extracts enriched for HoxA protein in the presence of purified HoxA antibodies.

Up to now, only NifA from *K. pneumoniae* and *A. vinelandii* have been purified. The *K. pneumoniae* NifA protein has been purified as a fusion protein with MBP, providing a large N-terminal extension (13). The *A. vinelandii* NifA protein was purified as a soluble active protein and showed *in vitro* control functions predicted from *in vivo* functions (7). In these two bacteria the activity of NifA is modulated by the negative regulatory protein NifL in response to environmental oxygen and fixed nitrogen (6,7). The *A. brasilense* NifA protein belongs to a class of proteins that are not active in the presence of oxygen, based on the model of regulation proposed by Fischer et al. (8,9) that involves the cysteine residues found in the inter-linker domain. Another feature of *A. brasilense* NifA is that the N-terminal domain inhibits its own activity in the presence of ammonia (33). This was explained by the presence of two closely related P<sub>II</sub> proteins found in *A. brasilense* (18) that are involved in different regulatory steps of nitrogen metabolism. The P<sub>II</sub> protein, a product of the *glnB* gene, is required for modulating NifA activity in response to changes in cellular nitrogen levels (18,33).

Our work demonstrates that the NifA from *A. brasilense* can be expressed in *E. coli* extracts both as a fusion protein or as NifA itself but is still inactive in aerobically grown cells. This inactivation could be due

Figure 8 - Mobility shift assay of *E. coli* extracts carrying plasmids pAAbA (A) or pGEXAbA (B) in the presence of increasing amount of purified NifA antibody. Standard binding reactions were carried out with the total extracts previously incubated overnight with different antibody concentrations. Lane 1, Wild-type fragment only; lane 2, wild-type fragment and 20  $\mu$ g of protein. Lanes 3 to 7, different amounts of antibody reacting with fragment/protein as in lane 2. Lane 3, 2  $\mu$ l of anti-NifA; lane 4, 4  $\mu$ l of anti-NifA; lane 5, 8  $\mu$ l of anti-NifA; lane 6, 16  $\mu$ l of anti-NifA and lane 7, 32  $\mu$ l of anti-NifA. The supershift band is indicated by the full arrowhead.



to the lack of *A. brasilense* P<sub>II</sub>-like protein in *E. coli* cells. However, NifA activity was obtained in *A. brasilense* *nifA* mutants. Thus, even if a different mechanism is involved in the regulation of nitrogen fixation in the presence of ammonia, it is possible that one mechanism of control occurs at the level of NifA inactivation through its N-terminal domain, mediated by P<sub>II</sub> protein (18).

The modular structure of transcriptional activators suggests that inactive NifA would be able to bind to its target sequences (35). It has been shown, in the case of *K. pneumoniae* NifA, that DNA binding activity can be separated from the transcriptional activation capacity (32). The oxygen sensitivity of the *A. brasilense* NifA is attributed to the conserved motif of cysteine residues which is present in the interdomain linker between the central and carboxy-terminal domains (8,9). Upon oxygen inactivation, it can be hypothesized that the conformation of the central domain is altered in such a way that transcriptional activation capacity is lost,

without affecting the DNA-binding capacity of the carboxy-terminal domain. In the case of NtrC-like transcriptional activators, where transcriptional activation capacity is regulated by phosphorylation, the inactive, unphosphorylated activator is still able to bind to its UAS (36). In this study we show that, although *A. brasilense* NifA protein is unable to activate *nif* promoters in aerobiosis due to its sensitivity to oxygen, the protein retains the ability to bind UAS sequences.

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