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Modulation of NifA Activity by P_{II} in *Azospirillum brasilense*: Evidence for a Regulatory Role of the NifA N-Terminal Domain

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***Azospirillum brasilense* NifA, which is synthesized under all physiological conditions, exists in an active or inactive form depending on the availability of ammonia. The activity also depends on the presence of P_{II}, as NifA is inactive in a *glnB* mutant. To investigate further the mechanism that regulates NifA activity, several deletions of the *nifA* coding sequence covering the amino-terminal domain of NifA were constructed. The ability of these truncated NifA proteins to activate the *nifH* promoter in the absence or presence of ammonia was assayed in *A. brasilense* wild-type and mutant strains. Our results suggest that the N-terminal domain is not essential for NifA activity. This domain plays an inhibitory role which prevents NifA activity in the presence of ammonia. The truncated proteins were also able to restore *nif* gene expression to a *glnB* mutant, suggesting that P_{II} is required to activate NifA by preventing the inhibitory effect of its N-terminal domain under conditions of nitrogen fixation. Low levels of nitrogenase activity in the presence of ammonia were also observed when the truncated gene was introduced into a strain devoid of the ADP-ribosylation control of nitrogenase. We propose a model for the regulation of NifA activity in *A. brasilense*.**

The two major signals that regulate nitrogen fixation in most diazotrophs are oxygen and ammonia. This regulation occurs mainly at the level of *nif* gene transcription, which requires a *nif*-specific activator, NifA. NifA acts as an enhancer binding protein (EBP) that recognizes sequences (typical TGT-N10-A CA, designated UAS), located upstream of promoters of the −24/−12 type controlled by RNA polymerase containing the alternative σ^{54} factor (3, 35, 36).

The mode of activation of *nif* gene transcription by NifA appears to be common to a large number of diazotrophs including *Azospirillum brasilense*, whereas the regulation of *nifA* transcription differs from one organism to another (13, 33). Regulation of NifA activity has also been reported in several diazotrophs. This regulation is mediated by NifL in response to oxygen and/or ammonia in *Klebsiella pneumoniae* and *Azotobacter vinelandii* (references 11 and 39 and references therein). In members of the family *Rhizobiaceae* and *A. brasilense*, in which NifL has not been detected, the NifA protein itself is inactivated by oxygen. The NifA protein contains a conserved motif of cysteine residues (C-X11-C-X19-C-X5-C), which is absent in *K. pneumoniae* and *A. vinelandii* and which is probably involved in the sensitivity to oxygen (14, 15).

The deduced amino acid sequences of several NifA proteins from diverse diazotrophs have been compared. NifA proteins are structurally similar to each other and to other EBPs, such as NtrC, for which the activity is modulated by phosphorylation (20, 38). On the basis of these similarities, three independent domains separated by two interdomain linkers of variable length have been defined (9, 10, 14, 38) (see Fig. 1). The C-terminal domain contains the helix-turn-helix motif responsible for DNA binding. The central domain interacts with the σ^{54} factor-RNA polymerase. It contains a nucleotide binding site. Catalysis of the formation of the RNA polymerase-σ^{54} open complex of transcription requires NifA NTPase activity (4, 36, 38, 45). The central and C-terminal domains are separated by an interdomain linker present in rhizobia but absent in *K. pneumoniae*, which contains the cysteine residues mentioned above (14, 15, 38, 45). A short and very hydrophilic region, designated the Q-linker, separates the N-terminal domain from the central domain (46). There is little conservation between the N-terminal domains of the different NifA proteins. This domain is not essential for NifA activity in *K. pneumoniae*, *Rhizobium meliloti*, and *Bradyrhizobium japonicum*, and it is absent in a strain of *Rhizobium leguminosarum* bv. trifolii (2, 10, 14, 21, 24, 30, 33, 38).

A. brasilense Sp7 is able to fix nitrogen under microaerobic conditions in media devoid of ammonia. This bacterium also regulates nitrogenase activity by a switch-off mechanism described for photosynthetic bacteria. This mechanism corresponds to ADP ribosylation of the Fe protein in response to environmental changes in the ammonia concentration (32). Another important feature unique to *A. brasilense* is the regulation of the synthesis and activity of NifA. Significant transcription of *nifA* was observed even under conditions incompatible with nitrogen fixation, i.e., in the presence of air and/or ammonia (31). This response suggested the existence of a regulatory mechanism controlling NifA activity that could be mediated by P_{II}, the product of the *glnB* gene (8, 29). In a *glnB* mutant, *nifA* is transcribed whereas *nifH* is not. *K. pneumoniae* NifA can restore nitrogen fixation to this mutant whereas *A. brasilense* NifA cannot. Consistently, in a *glnB*-mutated background, *Azospirillum* NifA was in an inactive form, whereas *Klebsiella* NifA remained active (8, 29). We can then postulate that a particular domain of *Azospirillum* NifA may be involved in the regulation of its activity and that P_{II} may be required to maintain NifA in an active form. To document this hypothesis we now report the properties of different NifA proteins with a deletion of the N-terminal part. We show here that the N-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype(s) or phenotype(s)	Source or reference
Strains		
E. coli		
TG1	*Δlac-pro supE thi hsdD5* F′ *traD36 lacI*^{q} *lacZΔM15*	19
S17.1	*pro thi hsdR recA*, chromosomal integration of RP4-2-Tc::Mu-Km::Tn*7*, Sm^{r} Tp^{r} Tra^{+}	42
A. brasilense		
Sp7	Wild type	44
7606	Nif^{-}, *glnB-km* mutant of Sp7	8
7067	Nif^{-}, *nifA*-Tn*5* mutant of Sp7	31
76067	Nif^{-}, *nifA-gm glnB-km* double mutant of Sp7	This work
UB3	Nif^{+}, *draTG-km* mutant of Sp7	48
7567	Nif^{-}, derivative from UB3, *draTG-km nifA-gm* double mutant of Sp7	This work
7358	Nif^{+}, derivative of Sp7, contains the *nifH-lacZ* fusion recombined into the chromosome, Km^{r} Spe^{r}	1
70671	Nif^{-}, derivative of 7067, contains the *nifH-lacZ* fusion recombined into the chromosome, Km^{r} Spe^{r}	1
76061	Nif^{-}, derivative of 7606, contains the *nifH-lacZ* fusion recombined into the chromosome, Km^{r} Spe^{r}	This work
760671	Nif^{-}, derivative of 76067, contains the *nifH-lacZ* fusion recombined into the chromosome, Km^{r} Spe^{r}	This work
Plasmids		
pTZ18R	Cloning and sequencing vector, Amp^{r}	Pharmacia
pBCSK^{-}	Cloning vector, Cm^{r}	Stratagene
pUC1318	Cloning vector, Amp^{r}	26
pUC1318Gm	Amp^{r}, source of the gm cartridge	This laboratory
pPHU281	Tc^{r}	23
pSUP202	Amp^{r} Tc^{r} Cm^{r}	42
pAB5	5.6-kb *Eco*RI fragment contains a part of *nifA*	31
pAB51	1.9-kb *Eco*RI-*Sal*I fragment contains the end of *nifA*	31
pAB57	pVK100[a] derivative carrying a 2.5-kb *Sal*I fragment containing the *nifA* gene inserted in the opposite orientation of the *km* gene	31
pAB53	Same construction as pAB57, in the same orientation as the *km* gene	31
pAB530	pTZ18R derivative carrying a 2.5-kb *Sal*I fragment containing the *nifA* gene inserted in frame with the *lacZ* gene	This work (Fig. 1)
pAB530b	Same construction as pAB530, in the opposite orientation of the *lacZ* gene	This work
pAB534	pAB530 derivatives carrying *Sph*I deletion	This work (Fig. 1)
pAB531, pAB532, pAB533	pAB530 derivatives carrying *nifA* deletions constructed by PCR	This work (Fig. 1)
pAB5340	pVK100 derivative carrying the *Sal*I fragment of pAB534	This work (Fig. 1)
pAB5310, pAB5320, pAB5330	pVK100 derivatives carrying the *Sal*I fragment containing *nifA* deletions	This work (Fig. 1)
pAB5701	pVK100 derivative carrying the *Sal*I fragment containing a disrupted *nifA* gene	This work
pAB6	3.6-kb *Eco*RI fragment contains beginning of *nifA*	31
pAB61	*Kpn*I-*Eco*RI fragment of pAB6 inserted in pBCSK^{-}	This work (Fig. 1)
pAB62	*Eco*RI-*Bam*HI fragment of pAB530 inserted in pAB61	This work (Fig. 1)
pAB63	Derivative of pPHU281 containing the *Bgl*II-*Bam*HI fragment of pAB62 with gm cartridge in *nifA* at the *Eco*RI site	This work (Fig. 1)
pAB359	*nifH-lacZ* transcriptional fusion Amp^{r} Spe^{r} Km^{r} pSUP202 derivative	1

[a] pVK100 is a low-copy-number broad-host-range vector derived from RK2 and can be transferred by conjugation from *E. coli* to *A. brasilense.*

terminal domain is not required for NifA activity. However, this domain plays an inhibitory role which leads to the inactivation of NifA in the absence of P_{II} or in the presence of ammonia in a P_{II} normal background.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used are listed in Table 1, and the plasmid constructions are schematically represented in Fig. 1. The rich culture media used were Luria-Bertani broth for *Escherichia coli* and nutrient broth (Difco) for *A. brasilense*. Minimal lactate medium was prepared as previously described (18). Generally, *E. coli* TG1 was used as the recipient of transformation with the plasmids constructed. Transfer of plasmids by conjugation from *E. coli* to *A. brasilense* was performed with *E. coli* S17.1 as the donor, as previously described (18). The following antibiotics were used at the indicated concentrations (expressed in micrograms per milliliter): tetracycline, 5; kanamycin, 20; spectinomycin, 100; gentamicin, 40; carbenicillin, 100; and chloramphenicol, 50.

Cartridge and gene fusion recombination into the host genome. Molecular biology techniques were performed according to conventional methods (40). pAB530 carries *A. brasilense nifA* on a 2.4-kb *Sal*I fragment (31) inserted into pTZ18R in the same orientation as the *lacZ* gene (Fig. 1). Plasmid pAB63 was obtained as follows: (i) pAB61 was first constructed by inserting a *Kpn*I-*Eco*RI fragment into pBCSK^{-}; (ii) the *Eco*RI-*Sal*I fragment of pAB530 was then inserted into pAB61 by digestion with *Eco*RI and *Bam*HI (from the polylinker), yielding pAB62; (iii) an *Eco*RI fragment carrying a *gm* cartridge purified from pUC1318Gm was inserted into the *Eco*RI site of the *nifA* gene of pAB62 (data not shown); and (iv) a *Bgl*II-*Bam*HI fragment containing *nifA-gm*, purified from the resulting plasmid, was subsequently inserted into the *Bam*HI site of pPHU281, yielding pAB63 (Fig. 1). Plasmid pAB63 has been used to construct *nifA* mutants by gene disruption after introduction of the *gm* cartridge into the chromosome of different *A. brasilense* recipients by marker exchange (Table 1), as previously described (18). As expected, recombination of the cartridge into the Nif^{+} strains led to a Nif^{-} derivative. The *nifH-lacZ* fusion from pAB359 was introduced into the chromosomes of different recipients after a single recombination event so that the wild-type *nifH* gene remained intact (Table 1), according to a previous report (1). As expected, introduction of the *nifH-lacZ* fusion did not modify the Nif phenotype of the recipients. In all the constructed strains, correct recombination was verified by DNA-DNA hybridization with appropriate probes (data not shown).

Construction of *nifA* deletions. Plasmid pAB530 carries a 2.5-kb *Sal*I fragment. This fragment contains the entire *nifA* coding sequence and 440 nucleotides of the upstream region including the *nifA* promoter region (31). The deletions for

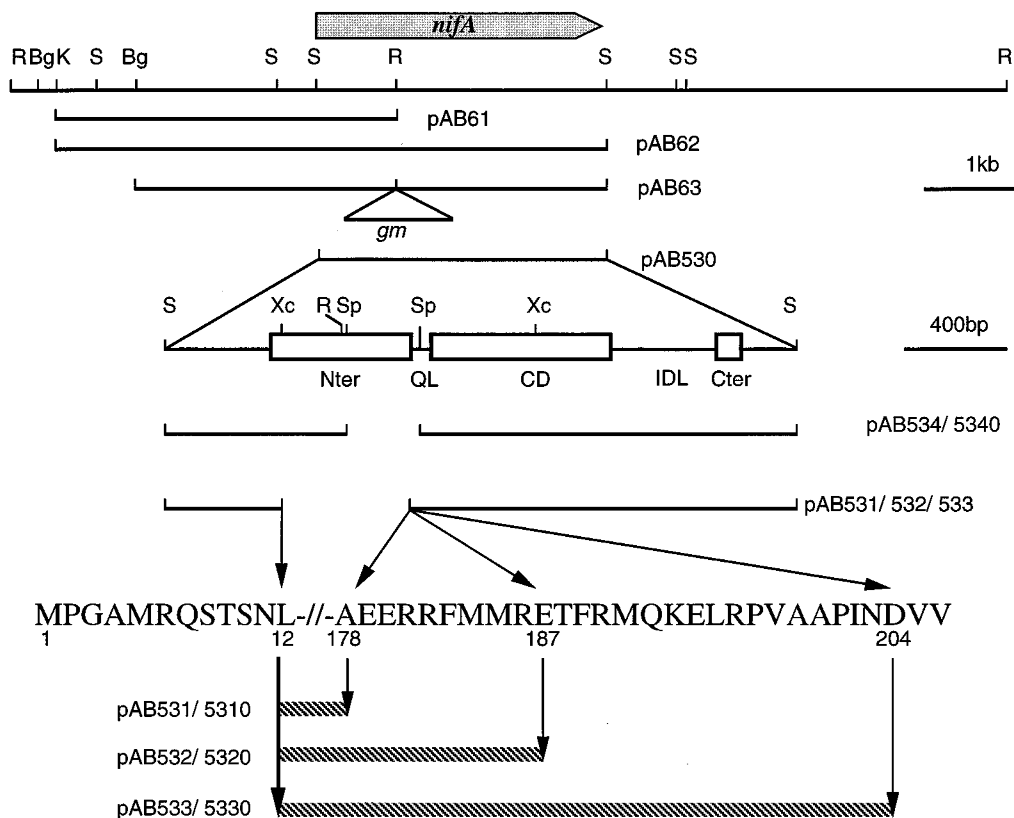
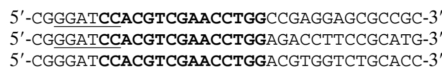


FIG. 1. Physical map of *nifA* region. Vector maps are not shown. Construction of the plasmids is described in Materials and Methods. The sizes of the deletions are indicated at the bottom. Nter, N-terminal domain; QL, Q-linker; CD, central domain; IDL, interdomain linker; Cter, C-terminal domain. Restriction sites: Bg, *Bgl*II; K, *Kpn*I; R, *Eco*RI; S, *Sal*I; Sp, *Sph*I; Xc, *Xcm*I.

this work have been designed so that only part of the coding sequence is deleted and the promoter region remains intact. Digestion of pAB530 with *Sph*I yielded pAB534, with an in-frame deletion of 294 nucleotides (98 amino acids [aa]) of the N-terminal domain coding sequence of *nifA*. The *Sal*I fragment of pAB534 with the deletion was then inserted into pVK100 to yield pAB5340 (Fig. 1). Other in-frame deletions were constructed according to a strategy based on PCR amplification. Two *Xcm*I sites are present in *nifA*: one located in the N-terminal coding sequence (7th to 12th codons) and another located in the central domain (Fig. 1). This enabled us to design three pairs of oligonucleotide primers overlapping these sites such that the amplified fragments carried the desired deletions.

Each pair of primers used in PCR amplification contained a 25-mer primer complementary to the coding strand (5′-CGGT**GGTTGGACTTGGA**CCTAGG GC-3′) synthesized to overlap the *Xcm*I site of the central domain.

The three other oligonucleotides were 35-mers complementary to the noncoding strand. All carried the sequence corresponding to the N-terminal *Xcm*I sites at the 5′ end and sequences corresponding to nucleotides around codons 178, 187, and 204 (oligonucleotides 1 to 3, respectively) as follows:


5′-CGGGATC**CACGTCGAACCTGG**CCGAGGAGCGCCGC-3′
5′-CGGGATC**CACGTCGAACCTGG**AGACCTTCCGCATG-3′
5′-CGGGATC**CACGTCGAACCTGG**ACGTGGTCTGCACC-3′

The *Xcm*I site CCA-N9-TGG is indicated in boldface type, and all four contained a *Bam*HI site at the 5′ end (underlined).

The DNA template used to perform PCR amplification was pAB51 (Table 1), since this plasmid carries only a part of *nifA* from the *Eco*RI to the *Sal*I site and thus is devoid of the beginning of the gene. Amplifications were performed with 20 ng of plasmid pAB51 and 100 ng of each oligonucleotide. The products of amplification, with lengths of 541, 514, and 463 bp, respectively, were digested with *Bam*HI and inserted into pTZ18R for verification by nucleotide sequencing that they carried the correct deletion and that no other mutation was introduced. Each resulting plasmid was digested by *Xcm*I and the fragments with the appropriate deletions were inserted into pAB530 by substitution of the wild-type 1-kb *Xcm*I fragment of the *nifA* gene. This yielded three different deletions of 495, 522, and 573 nucleotides carried by pAB531, pAB532, and pAB533, respectively (Fig. 1). Then, each *nifA* gene with a deletion was introduced as a *Sal*I fragment at the *Xho*I site of pVK100 to yield pAB5310, pAB5320, and pAB5330 (Fig. 1). *nifA* with the deletion is in the same orientation as the *km* gene in pAB5310 and in the opposite orientation from that in pAB5320 and pAB5330.

Nitrogenase and β-galactosidase assays. Determination of β-galactosidase activity in *A. brasilense* strains carrying *lacZ* fusions was done with cells incubated in minimal medium with and without ammonia under microaerobic conditions (99.5% Ar–0.5% O_2) or in air, as previously described (31). β-Galactosidase activity was assayed as described by Miller (34). Nitrogen fixation was determined by the acetylene reduction test according to the derepression protocol described by Galimand et al. (18).

Cell extracts, SDS-PAGE, and immunoblotting. *E. coli* TG1 carrying pAB530 or its derivatives was incubated at 37°C in 2YT medium supplemented with carbenicillin and isopropyl-β-D-thiogalactopyranoside (IPTG) when indicated. After 3 h, when the culture reached an optical density at 600 nm of 0.6, 0.4-ml samples were centrifuged for extraction. *A. brasilense* strains were incubated at an optical density at 600 nm of 0.1 under derepression conditions in nitrogen-free minimal lactate medium alone or supplemented with 20 mM ammonia under microaerobic conditions. After 4 h at 33°C, 20-ml suspensions were centrifuged for extraction. In both cases, the pellets were resuspended in Laemmli buffer, and each sample was boiled for 5 min. *A. brasilense* extracts were sonicated for 6 min to break the DNA. The extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10 or 7.5% polyacrylamide gel. Proteins were transferred to nitrocellulose by semidry electroblotting for 1 h at 200 mA or classical methods with the use of Tris-glycine buffer overnight at 150 mA. Detection was carried out as described by Sambrook et al. (40), using antibodies against *B. japonicum* NifA (37). The cross-reactivity was revealed by using secondary anti-rabbit antibodies and by following the procedure for enhanced chemiluminescence (Amersham).

RESULTS

Immunodetection of NifA in crude extracts of *A. brasilense* strains incubated in the presence or absence of ammonia. A plasmid-borne *nifA-lacZ* fusion was previously shown to be expressed in the presence of ammonia (31). Immunodetection with antibodies against *B. japonicum* NifA (37) was carried out

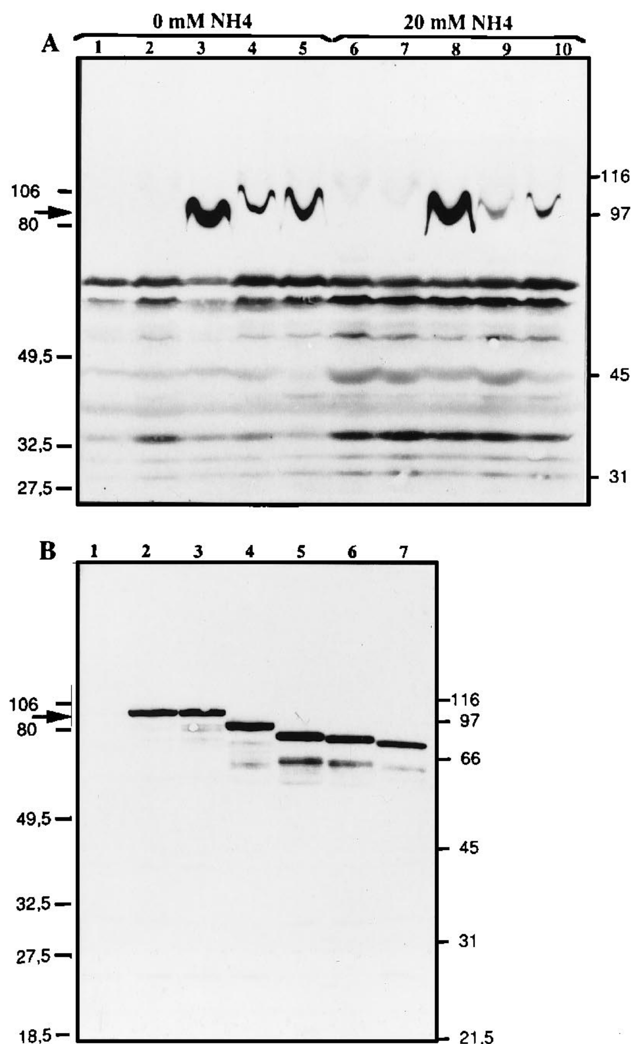


FIG. 2. (A) Immunodetection of NifA from *A. brasilense* cells incubated in the absence (lanes 1 to 5) or presence (lanes 6 to 10) of 20 mM ammonia. Lanes: 1 and 6, *nifA*-Tn*5* mutant strain carrying no plasmid; (2, 3, 7, and 8) derivatives pVK100 with a disrupted *nifA* gene (pAB5701) (lanes 2 and 7) or with an intact *nifA* gene (pAB53) (lanes 3 and 8); 4 and 9, wild-type strain (Sp7); 5 and 10, *glnB*-km mutant strain (7606). (B) Immunodetection of the *A. brasilense* NifA from *E. coli* TG1 strains containing pTZ18R derivatives grown in the absence of IPTG (lane 1) or in the presence of 12.5 μM (lane 2) or 25 μM (lane 3 to 7) IPTG. The TG1 strains contain the following pTZ18R derivatives: pAB530 (WT NifA) (lanes 1 to 3), pAB534 (NifA deleted from 98 aa) (lane 4), pAB531 (NifA deleted from Leu-12 to Asn-178 [165 aa]) (lane 5), pAB532 (NifA deleted from Leu-12 to Glu-187 [174 aa]) (lane 6), and pAB533 (NifA deleted from Leu-12 to Asp-204 [191 aa]) (lane 7). The arrows indicate the position of the wild-type NifA. Molecular mass markers (in kilodaltons) are indicated.

to verify that expression of the fusion was correlated with NifA synthesis. The antibodies used were prepared against an 18-amino-acid peptide, located in the central domain, conserved in most NifA proteins. A certain degree of conservation is also observed for other EBPs (38). In its central domain, *A. brasilense* NifA possesses 16 residues identical to this 18-amino-acid peptide and NtrC possesses 14 of the conserved residues (28). Thus, we expected to detect NifA as well as other proteins.

Figure 2A shows a typical immunoblot of protein extracts from cells incubated in the absence of ammonia (lanes 1 to 5) or in the presence of 20 mM ammonia (lanes 6 to 10). Although a certain number of bands were detected in all cases, clearly a band of about 80 kDa was missing in extracts of the *nifA*-Tn*5* mutant strain 7067 (lanes 1 and 6), whereas the band was revealed in extracts of the same strain complemented by a plasmid (pAB53) carrying the wild-type *nifA* gene (lanes 3 and 8). Similar data were obtained with strain 7067 complemented by pAB57, which carries *nifA* expressed from its own promoter (data not shown). The 80-kDa polypeptide was not produced when the *nifA*-Tn*5* mutant carried a noncomplementing plasmid, composed of the same vector containing the same insert but with a disrupted *nifA* gene (lanes 2 and 7). This polypeptide had an apparent molecular mass of about 80 kDa, whereas the predicted NifA molecular mass deduced from the nucleotide sequence was 67 kDa (31). This discrepancy could be due to the high percentage of proline residues in the interdomain linker of NifA; a high proline content is known to cause anomalous migration in SDS-PAGE (17). In addition, detection of the NifA polypeptide requires that large amounts of protein extracts be loaded onto the gels, a requirement which probably accounts for the wave-type migration observed.

A polypeptide with a size of 80 kDa was also detected in extracts from wild-type Sp7 (lanes 4 and 9) and the *glnB* mutant 7606 (lanes 5 and 10) both in the absence (lanes 4 and 5) and the presence (lanes 9 and 10) of ammonia, in agreement with previous observations obtained with a *nifA-lacZ* fusion and primer extension experiments. The lower signal intensity observed in the presence of ammonia can be correlated with a lower level of transcription (31).

Construction of plasmids encoding N-terminal truncated NifA polypeptides. Comparison of the amino acid sequences of *K. pneumoniae* and *A. brasilense* NifA enabled the N-terminal domain of *A. brasilense* to be localized from the first residue to Ala-178. The region corresponding to the so-called Q-linker could not be precisely defined. However, on the basis of the alignment of other NifA sequences, it should be located between residues 179 and 204. Thus, we constructed a set of deletions encoding the N-terminal domain and the Q-linker. The following four deletions were constructed: pAB531/5310 encodes a deletion of 165 aa, from Leu-12 to Asp-178; pAB532/5320 encodes a deletion of 174 aa, from Leu-12 to Glu-187; pAB533/5330 encodes a deletion of 191 aa, from Leu-12 to Asp-204; and pAB534/5340 encodes a deletion of 97 aa, from Met-94 to Gln-192 (see Materials and Methods and Fig. 1).

To verify the constructions, immunodetection with NifA antibodies was done with extracts of *E. coli* TG1 carrying the pTZ derivatives (pAB530 to pAB534) (Fig. 2B). In these plasmids *nifA* was expected to be expressed either from its own promoter or from the promoter of *lacZ*, which is in the same orientation as *nifA*. In the absence of IPTG (Fig. 2B, lane 1), no band was detected, indicating that the *nifA* promoter was not functional in *E. coli*. In the presence of IPTG (Fig. 2B, lanes 2 to 7), a polypeptide was detected in each case, indicating that the different *nifA* genes were expressed from the *lacZ* promoter. The relative decrease in the size of the NifA polypeptide was in agreement with the size of the deleted regions (Fig. 2B, lanes 3 to 7). The sizes of the polypeptides detected were about 15 kDa larger than expected if translation proceeded from the *nifA* initiation codon. It appears that for each construction, the *nifA* gene was inserted in frame with the gene encoding the α peptide of β-galactosidase. Thus, the polypeptides detected in *E. coli* correspond to a fusion between NifA and the α peptide, including the translation product of the *nifA* promoter region. By contrast, in the constructions used for *A. brasilense*, which are derivatives of pVK100, *nifA* expression is driven by the *nifA* promoter (see Materials and Methods).

Activity of truncated NifA proteins. The activity of the trun-

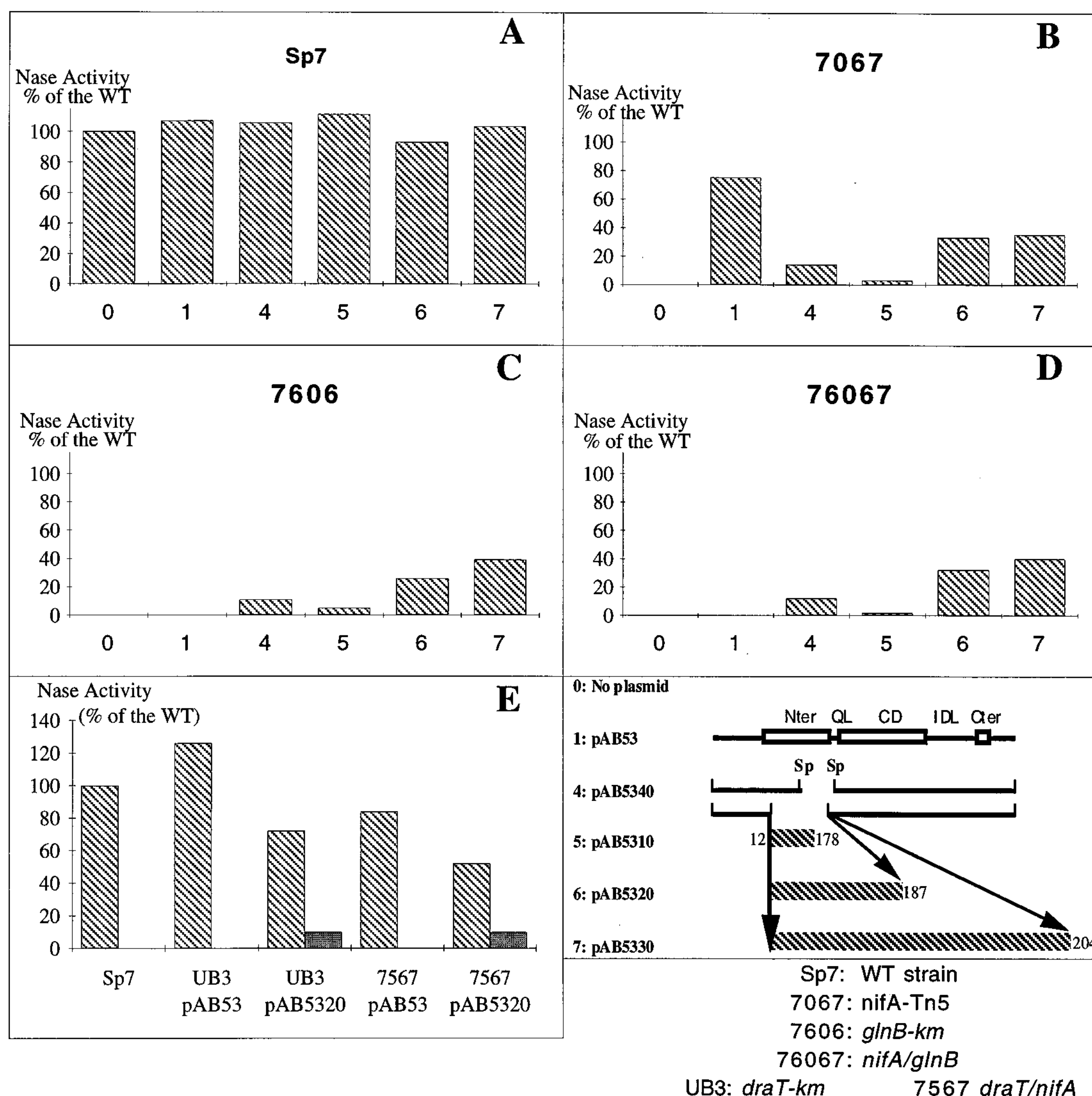


FIG. 3. Nitrogenase activity of *A. brasilense* strains containing plasmids carrying the *nifA* genes. The relative activity is expressed as a percentage of activity in strain Sp7. Cells were incubated for 3 h under nitrogen fixation conditions (striped column) or in the presence of 20 mM ammonia (closed column). Data are the averages for at least three experiments. The standard deviation was less than 20% of the values. Absence of activity means no activity was WT, wild type. Abbreviations are defined in the legend to Fig. 1.

cated NifA proteins was determined by their ability to restore nitrogen fixation to the *nifA*-Tn*5* (strain 7067), *glnB-km* (strain 7606), and *nifA-gm/glnB-km* (strain 76067) mutant strains under nitrogen fixation conditions. In addition, it was of interest to verify whether the activity of truncated NifA was still regulated in response to ammonia. The nitrogenase activity of the transconjugants could not be assayed in the presence of ammonia, since the nitrogenase of *A. brasilense* is inactivated by ADP ribosylation. For this reason, we recombined a *nifH-lacZ* fusion into the wild-type and mutant strains (see Materials and Methods).

Figure 3A to D shows the percentages of nitrogenase activity of the wild-type and mutant strains. The presence of the different plasmids did not significantly modify the nitrogenase activity in the wild type (Fig. 3A). The four NifA deletions (pAB5310, pAB5320, pAB5330, and pAB5340) were able to restore a Nif^+ phenotype to a *nifA*-Tn*5* (7067) strain (Fig. 3B), although the specific activity was reduced compared with the complementation observed with pAB53 (or pAB57 [data not shown]). This finding suggests that neither the N-terminal domain nor the Q-linker is essential for NifA activity. Similar results were obtained with the *glnB-km* (7606) strain carrying these deletions (Fig. 3C) and with the *nifA-glnB* mutant (Fig. 3D). Thus, the truncated NifA proteins are active independently of P_{II} in the absence of ammonia. In particular, the presence of wild-type NifA in an inactive form in the *glnB-km* strain did not interfere with the activity of the deletion form.

With regard to the expression of *nifH-lacZ* in the absence of any plasmid, the results obtained with the chromosomally integrated fusion (Fig. 4) were similar to those with the plasmid-borne fusions (31); i.e., *nifH* was expressed only in the wild type under nitrogen fixation conditions (Fig. 4A to C, 0). In addition, in the double *nifA-glnB* mutant, the fusion was expressed at the basal level (Fig. 4D, 0), in agreement with the phenotype of the single-mutant strains. The use of the chromosomal *nifH-lacZ* fusion enabled us to compare the activities of *K. pneumoniae* NifA (pCK3) and *A. brasilense* NifA (pAB53 and pAB57) which are expressed in the presence of ammonia. The

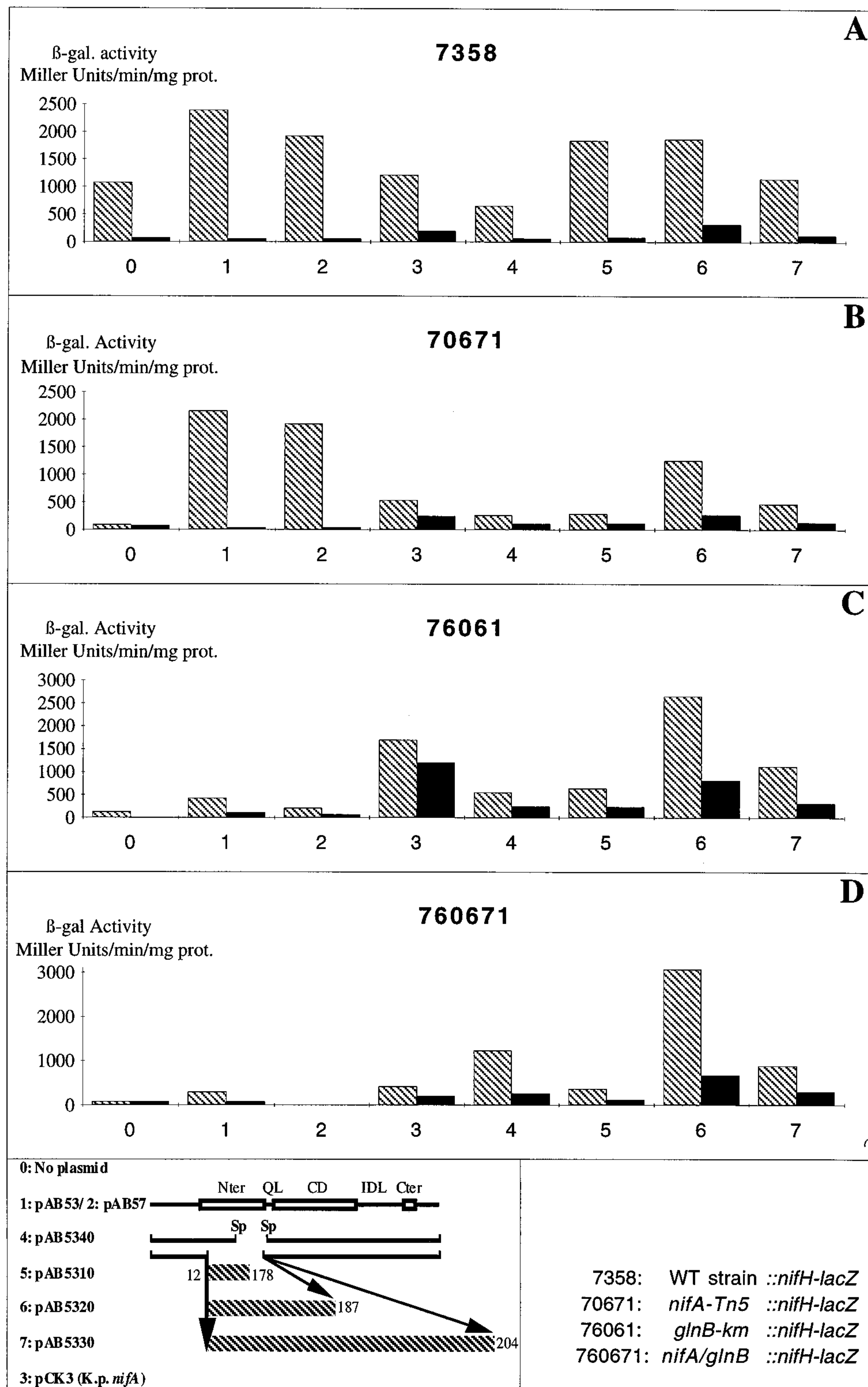


FIG. 4. β-Galactosidase activity of *A. brasilense* strains carrying a chromosomal *nifH-lacZ* fusion and containing plasmids carrying *nifA* genes as indicated. Activity is expressed in Miller units per milligram of protein (prot.). Cells were incubated for 4 h under nitrogen fixation conditions (striped column) or in the presence of 20 mM ammonia (closed column) (2D, not tested). Data are the averages for at least three experiments. The standard deviation was less than 20% of the values. (D) Abbreviations are defined in the legend to Fig. 1. WT, wild type.

data showed that *K. pneumoniae* NifA, in contrast to *A. brasilense* NifA, was active in a *glnB* mutant even in the presence of ammonia. Surprisingly, a higher level of *nifH* expression was observed when both *K. pneumoniae* NifA and *A. brasilense* NifA were present (compare Fig. 4C, 3, with Fig. 4B or 4D, 3).

As expected from the nitrogenase assays, the transfer of the four *nifA* genes carrying deletions to the *nifA*, *glnB*, and *nifA/glnB* mutants led to restoration of *nifH* expression in the absence of ammonia (Fig. 4B to D, 4 to 7). In the presence of ammonia, significant expression of *nifH* was observed, suggesting that the truncated proteins were still active under these conditions in contrast to wild-type NifA. Thus, the N-terminal domain is probably involved in inactivation of NifA in response to ammonia. No β-galactosidase activity was detected when the cultures were incubated in the presence of air, suggesting that truncated NifA retained its sensitivity to oxygen (data not shown).

The four truncated NifA proteins were not equally active for *nifH* expression. The truncated protein constructed by deletion of Leu-12 to Glu-187 (Fig. 4, 6) was the more active protein, both in the absence and in the presence of ammonia. The N-terminal domain of this protein was completely truncated, except the 12 first residues, whereas the Q-linker, or part of it, remained. Thus, only the N-terminal domain may be involved in the modulation of NifA activity. In pAB5330, the part encoding the Q-linker is removed; however, the truncated protein did not yield a higher level of expression of *nifH* or a significantly higher level of nitrogenase activity.

Complementation of *draTG* mutant with deletions of *nifA*. The *draT* mutant (UB3) contains a *km* cartridge insertion in *draT*, which is polar on *draG* (48). This is mutant lacks ADP-ribosyltransferase activity responsible for nitrogenase inactivation. The mutant strain expressed nitrogenase activity of the same order of magnitude as that of Sp7 and escapes switch off (48). However, the mutant is unable to fix nitrogen when cultivated in the presence of ammonia, most probably because NifA is inactive. Strain UB3 containing pAB53 (Fig. 3E) did not display nitrogenase activity in the presence of 2 mM ammonia. The same result was observed for a *nifA-draT* double mutant (7567) constructed for this work when complemented with pAB53 (Fig. 3E). Because an N-terminally truncated NifA could activate *nifH* expression in the presence of ammonia, it was of interest to assay the nitrogenase activities in the *draT* and the *nifA-draT* strains (UB3 and 7567) containing pAB5320. In the presence of ammonia, a low but significant level of nitrogenase activity, ca 10% of the value obtained for the wild type under conditions of nitrogen fixation, was observed for the two strains. This finding shows that truncation of the N-terminal part of NifA can lead to partial nitrogenase activity in the presence of ammonia if the ADP ribosylation process is removed. A second mechanism of posttranslational regulation of nitrogenase activity in response to ammonium has been recently described. This mechanism could account for the low levels of nitrogenase activity obtained in the UB3 and 7567 strains containing the truncated *nifA* gene (47).

DISCUSSION

Four plasmids encoding NifA-truncated proteins were constructed, and NifA activity was assayed either by monitoring expression of a *nifH-lacZ* fusion or, when possible, by assaying nitrogenase activity. We showed here that the N-terminal domain of *A. brasilense* NifA was not essential for the activity of the protein. This result is in agreement with data observed for NifA proteins from other diazotrophs, such as *K. pneumoniae*,

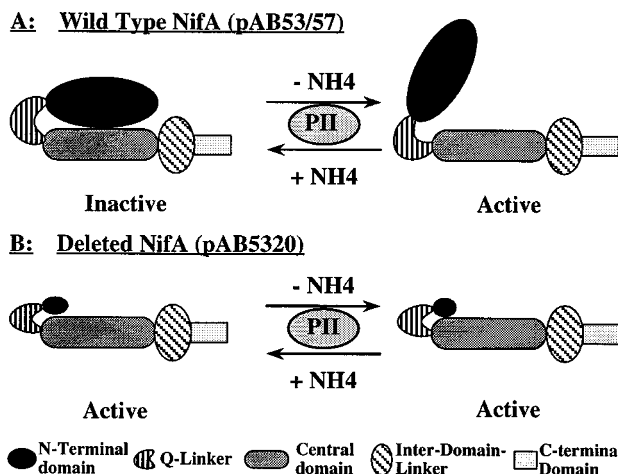


FIG. 5. Model of regulation of NifA activity in response to ammonia. It is assumed that NifA is organized into three domains separated by two interdomain linkers as indicated.

R. meliloti, and *B. japonicum* (2, 10, 14, 21), and for other EBPs (12, 16, 22, 27). However, truncated NifA proteins are less active than wild-type NifA under nitrogen fixation conditions. Thus, even though the N-terminal domain is not required for NifA activity under these conditions, this domain is probably required for optimal activity either by maintaining other domains in their active form or by enhancing the protein stability.

An original feature of *Azospirillum* NifA is that the N-terminal domain inhibits its own activity in the presence of ammonia. The N-terminally truncated NifA proteins were still active in the presence of ammonia. However, the level of expression of a *nifH-lacZ* fusion observed for strains expressing the truncated proteins was lower in the presence of ammonia than under conditions of nitrogen fixation. This result can be explained by the expression of truncated NifA proteins from the *nifA* promoter. Liang et al. (31) have shown that even though *nifA* is expressed under all physiological conditions, the level of expression in the presence of ammonia is reduced about 50% compared with that observed under nitrogen fixation conditions. The immunological results presented here agree with those of earlier transcription studies and indicate that slightly higher levels of the NifA protein were observed in ammonia-starved cells.

An inhibitory role of the N-terminal domain has been reported for other EBPs, such as AnfA, XylR, and DctD, and also for the σ^{70}-dependent activator FixJ. For XylR, DctD, and FixJ, a model which suggests an inhibition of the central domain by the N-terminal domain has been proposed (7, 12, 22, 27, 41). For DctD and FixJ, this inhibition is suppressed after phosphorylation by DctB and FixL, respectively. In *A. vinelandii*, the Fe protein of nitrogenase is required to relieve the inhibitory effect of the N-terminal domain of AnfA (16). For *A. brasilense* NifA, the suppression of inhibition involves the P_{II} protein.

A proposed model of regulation of NifA activity, based on the similarities to the regulation of activity of other EBPs, is presented in Fig. 5. For the wild-type NifA (Fig. 5A), the protein is active only in the absence of ammonia. Under this condition, the P_{II} protein (or a component under the control of P_{II}) is required to prevent the N-terminal inhibition. In the presence of ammonia, the N-terminal domain inhibits the rest of the protein, probably because P_{II} is unable to maintain NifA in its active form. For truncated NifA (Fig. 5B), the protein is

still active in the presence of ammonia or in the absence of P_{II}, since the N-terminal domain cannot inhibit NifA activity.

The mechanism by which P_{II} prevents N-terminal inhibition under nitrogen fixation conditions is unknown. In *E. coli*, P_{II} is involved in the modulation of phosphatase activity of NtrB by the direct interaction of P_{II} with NtrB (25). For *A. vinelandii*, apparently P_{II} and the uridyltransferase-uridylyl-removing enzyme GlnD (NfrX) are involved by modulating directly or indirectly the activity of NifL (6). *A. brasilense* likely contains the equivalent of a *glnD* gene since its glutamine synthetase activity is modulated by adenylylation (5). The putative role of this gene remains to be established. Since no significant homology could be detected between NtrB and NifA and since NifL has not been identified in *A. brasilense*, it cannot be determined yet whether P_{II} interacts directly with the N-terminal domain of NifA or indirectly by modulating the activity of other proteins. It is also not known whether P_{II} is still able to modulate the activity of truncated NifA. In this context, *glnB* mutants containing *nifA* deletions display higher levels of activity than do strains which still carry an intact *glnB* gene (Fig. 4).

The most efficient deletion corresponding to Leu-12 to Glu-187 was constructed by deleting the gene encoding all the N-terminal domain and part of the Q-linker (schematized in Fig. 5B). Thus, the integrity of the Q-linker may not be important for NifA activity as it was observed for AnfA of *A. vinelandii* (16). The Q-linker is supposed to serve as an interdomain linker, and Fernandez et al. (12) have shown that this domain is important for the repressor role of the N-terminal domain of XylR.

It can be questioned whether similar mechanisms of regulation of NifA activity do exist in other species or genera. Unpublished observations from our laboratory suggest the existence of a regulation of NifA activity by ammonia in *Azorhizobium caulinodans*. A brief report also mentioned a possible involvement of the amino-terminal domain in the regulation of NifA of *Herbaspirillum seropedicae* when the *nifA* gene is expressed in a heterologous background (43). However, it is not known whether this regulation involves a P_{II}-like protein.

Regulation of NifA activity in response to oxygen is not mentioned in the model but probably acts through the cysteine residues contained in the interdomain linker. The fact that *A. brasilense* truncated NifA proteins are not active in the presence of oxygen was in agreement with the model of regulation in response to oxygen, proposed by Fischer et al. (14, 15), that involves the cysteine residues found in the interdomain linker.

Inactivation and reactivation (switch off and switch on, respectively) of *A. brasilense* nitrogenase by ADP ribosylation in response to ammonia is catalyzed by DraT and DraG, respectively (32, 48). We have shown that the double *draT-nifA* mutant, as well as the single *draT* mutant, carrying a *nifA* deletion was able to fix nitrogen in the presence of ammonia. The low level of activity obtained in the presence of ammonia can be explained as follows: (i) the deleted NifA protein is not fully active (discussed above), (ii) the level of expression is lower in the presence of ammonia (discussed above), (iii) the presence of a second mechanism of the posttranslational regulation of nitrogenase activity in response to ammonium has been recently described (47), and (iv) a modification of the metabolism in the presence of ammonia, which could lead to a limitation of energy supply for nitrogen fixation, can also cause this low level of activity.

However, a significant level of activity is obtained. Thus, even if another mechanism were involved in the nitrogen fixation regulation in the presence of ammonia, our results suggest that nitrogen fixation in *A. brasilense* is regulated mainly by two mechanisms. The first control occurs at the level of NifA inactivation through its N-terminal domain, and this control is mediated by P_{II}. The second level of regulation corresponds to the reversible nitrogenase switch off due to DraTG and/or to another, previously unknown mechanism that has been described recently (47).

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