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Interaction between GlnB and the N-terminal domain of NifA in *Azospirillum brasilense*

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***Azospirillum brasilense* is a diazotroph associated with many important agricultural crops and shows potential as a biofertilizer. NifA, the transcriptional activator of nitrogen fixation (*nif*) genes, and GlnB, one of P_{II} signal transduction family protein, are key proteins in the regulation of nitrogen fixation in *A. brasilense*. It was previously reported that the regulation of NifA activity in *A. brasilense* depends on GlnB. We report here that GlnB was found to interact directly with the N-terminal domain of NifA *in vivo* under nitrogen-free conditions and the N-terminal mutant of NifA in which the Tyr residues at position 18 and 53 were replaced by Phe (NifA-N-Y18/53F) strengthened the interaction with GlnB. Moreover, we also found that the amino acid residues 66—88 and 165—176 in N-terminus of NifA are responsible for the interaction with GlnB.**

Azospirillum brasilense, NifA, GlnB, protein-protein interaction

Azospirillum brasilense is a diazotroph associated with important agricultural crops, such as maize and wheat, and shows potential as a biofertilizer[1]. In this organism, as in many other diazotrophs, the nitrogen fixation process is highly regulated, and *nif* gene transcription is inhibited by ammonium and oxygen. Specifically, the regulation is involved in a specific transcriptional activator NifA, which activates σ^{54}-dependent promoters[2,3].

In addition to NifA, GlnB, one of P_{II} signal transduction family protein, is another key protein in the regulation of nitrogen fixation in *A. brasilense* [4]. P_{II} proteins constitute a family of highly conserved trimeric proteins that act as sensors of celluar carbon and nitrogen status in prokaryotes and probably in plants as well[5]. In *Escherichia coli* and many other bacteria, GlnB can be reversibly uridylylated depending on the nitrogen status of the cell. A bifunctional uridylyltransferase/uridylyl-removing enzyme GlnD (UTase/UR, gene product of *glnD*) regulates P_{II} proteins by uridylylation or deuridylylation and it is thought to be a primary sensor of intracellular nitrogen status[6,7].

The N-terminal domain of NifA contains a conserved GAF domain which is found in sensory proteins and known to be involved in the binding of small effectors[8]. Under conditions appropriate for nitrogen fixation, the GAF domain of NifA in *Azotobacter vinelandii* plays an essential role in preventing NifL inhibition by binding 2-ketoglutarate. This effector blocks the formation of the NifL-NifA complex when ammonia is absent and the 2-ketoglutarate level is elevated[9]. However, the role of GAF domain in NifA is still unknown in *A. brasilense*. The deletion of part of the N-terminal GAF domain is not essential for NifA activity, and the truncated NifA is partially active in the absence of GlnB, indicating that GlnB is required to activate NifA by antagonizing the inhibitory effect of this N-terminal domain under nitrogen-fixing conditions[4]. GlnB was found to interact with the N-terminal GAF domain of NifA, but not with its central or C-terminal domain by using yeast two-hybrid system[10]. Such transient protein-protein interactions may be involved in modifications of proteins and control and regulate a large number of cellular processes[11].

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There are 3 Tyr residues in N-terminus of NifA which can be the potential modification sites. Replacement of the Tyr residues at positions 18, 43 and 53 in *A. brasilense* NifA N-terminal domain resulted in NifA proteins that retained high nitrogenase activity in both *nifA* and *glnB* mutant backgrounds[12]. However, whether GlnB regulates NifA activity by directly interaction with N-terminal of NifA (abbreviated to NifA-N) or by covalent modification of NifA is still unknown.

The objective of this work was to further investigate the role of GlnB in the regulation of NifA activity. In order to clarify the situation, we have now used pull-down assays to investigate whether *A. brasilense* NifA-N mutants carrying substitutions in Tyr residues and/or deletion mutants of NifA-N interact with GlnB under nitrogen-free conditions in an *E. coli* background.

1 Materials and methods

1.1 Strains and plasmids

The *E. coli* strain BL21(DE3) was used in this work. Plasmids are described in Table 1.

1.2 Plasmid construction

The *glnB* gene was amplified from genomic DNA of *A. brasilense* Sp7 using primers glnB-*Sac* I (5′-AAAGAGCTCTACACGAGAGACCCATGAAG-3′) and glnB-*Xho* I (5′-AAACTCGAGAAGCACACAACGTGACAGCA-3′) based on published sequence[15]. These primers introduced *Sac* I and *Xho* I restrictions sites flanking the sequence. The amplified DNA was subcloned into the *Sac* I/*Xho* I sites of vector pET23c, yielding pETNB.

The 5 plasmids pNC11, pLN1zf, pTZ18nifAY43F, pTZ18nifAY53F and pJR4zf (Table 1), containing the wild type or mutated *nifA*, were used as templates for PCR amplification. Oligonucleotide primers AU1-*Bam*H I (5′-TTATGGATCCGTG TCGATCATGCCGGGTGC-3′) and AD1-*Sac* I (5′-TTAAGAGCTCATGAAGCGGCGCTCCTCGGC-3′) containing a *Bam*H I and a *Sac* I site respectively were designed to amplify a 560 bp fragment of 5′-terminal of *nifA*. Each fragment was digested with *Bam*H I and *Sac* I, and then cloned into the pET28a vector digested with the same enzymes, yielding pETAN, pETAN18, pETAN43, pETAN53 and pETAN18/53, respectively.

The plasmids encoding different regions of NifA-N (residues 21－176, 44－176, 66－176, 89－176, 1－120, 1－143 and 1－164) were constructed by amplifying DNA fragments corresponding to the individual coding regions and then subcloned into pET28a as an N-terminal His-tag fusion. The primers used for PCR amplification to create fusion constructs containing deleted NifA-N fragments are listed as follows: AU1-*Bam*H I (5′-TTATGGATCCGTGTCGATCATGCCGGGTGC-3′), AU2-*Bam*H I (5′-TATGGATCCAGCAAGATCCTCGGTTCCTC-3′), AU3-*Bam*H I (5′-TATGGATCCCAGCTTCAGATGC ACCGCGG-3′), AU4-*Bam*H I (5′-TATGGATCCGCGAACGGCCTGTCGAACGA-3′), AU5-*Bam*H I (5′-TATGGATCCATCCTGAAGACCGGCATGCC-3′), AD1-*Sac* I (5′-TTAAGAGCTCATGAAGCGGCGCTCCTCGGC-3′), AD2-*Sac* I (5′-TTAAGAGCTCGGCGACCATGGTCAGGAAGC-3′), AD3-

Table 1 Plasmids used in this work

Plasmids	Characteristic	Reference/source
pET28a	expression vector/T7 promoter; Km^r	Novagen
pET23c	expression vector/T7 promoter; Amp^r	Novagen
pNC11	pGEM-11zf derivative carrying the *nifA* gene (2.4 kb *Sal* I fragment) from *A. brasilense* Yu62; Amp^r	[13]
pLN1zf, pJR4zf	pGEM-11zf derivatives carrying the *nifA* gene (2.4 kb *Sal* I fragment) with mutations in the codons for Tyr18 and 18/53 respectively from *A. brasilense* Yu62; Amp^r	[12]
pTZ18nifAY43F, pTZ18nifAY53F	pTZ18R derivatives carrying the *nifA* gene (2.4 kb *Sal* I fragment) with mutations in the codons for Tyr43 and 53 respectively from *A. brasilense* Sp7; Amp^r	[14]
pETAN	pET28a derivative carrying a 528bp 5′-terminal of *nifA* gene encoding GAF domain (aa 1－176) from *A. brasilense* Yu62; Km^r	This work
pETAN18, 18/53	pET28a derivatives carrying a 528bp 5′-terminal of *nifA* gene with mutations in the codons for Tyr18 and 18/53 respectively from *A. brasilense* Yu62; Km^r	This work
pETAN43, 53	pET28a derivatives carrying a 528bp 5′-terminal of *nifA* gene with mutations in the codons for Tyr43 and 53 respectively from *A. brasilense* Sp7; Km^r	This work
pETAND1, 2, 3, 4, 5, 6, 7	pET28a derivatives carrying the 5′-terminal of *nifA* gene encoding deleted GAF domains (aa 21－176, 44－176, 66－176, 89－176, 1－120, 1－143 and 1－164, respectively) from *A. brasilense* Sp7; Km^r	This work
pETB	*A. brasilense* Sp7 *glnB* in pET28a; Km^r	This work
pETNB	*A. brasilense* Sp7 *glnB* in pET23c; Amp^r	This work

Sac I (5′-TTAAGAGCTCGCGGTCGATGGTCAGC-ACGC-3′), and AD4-*Sac* I (5′-TTAAGAGCTCCTGCT-CGTCCAGGTCCTCGC-3′). The PCR amplified products were digested with *Bam*H I and *Sac* I and cloned into the pET28a vector, yielding various NifA-N deletion expression plasmids pETAN1-pETAN7. All constructs were sequenced to verify the correct reading frame was preserved for each clone.

1.3 Partial purification of the *E. coli* GlnD protein

The expression and partial purification of GlnD was performed according to previous method[16]. *E. coli* BL21 (DE3) cells harboring plasmid pDOP1 were grown overnight at 30℃ in LB medium. This culture was used to inoculate 10 mL LB medium (1/100), followed by 12 h incubation at 30℃. After growth, the whole culture was diluted in 1 L of LB and incubated for 3 h at 30℃ and then at 42℃ for 4 h. Cells were harvested by centrifugation and resuspended in 10 mL buffer D1 (50 mmol/L Tris-HCl, pH 7.5, 200 mmol/L KCl, 0.1 mmol/L DTT, 10% glycerol). Lysozyme was added (50 μg/mL) and the cell suspension was kept on ice for 30 min. Cells were lysed by sonication and the supernatant was treated on ice with 2% streptomycin sulfate for 30 min. After centrifugation at 15000×*g* for 30 min at 4℃, the supernatant was diluted 4 times with buffer D2 (50 mmol/L Tris-HCl, pH 7.5, 0.1 mmol/L DTT, 10% glycerol) and loaded on a 30 mL DEAE-Sepharose fast flow column equilibrated in buffer A (50 mmol/L Tris-HCl, pH 7.5, 50 mmol/L KCl, 0.1 mmol/L DTT, 10% glycerol). The GlnD protein was eluted with a KCl gradient (50 mmol/L－1 mol/L) in buffer A. Fractions showing a high percentage of the GlnD protein, as detected by SDS-PAGE, were pooled. This pool was precipitated using 35% ammonium sulfate. After centrifugation, the precipitate was dissolved and dialyzed against 50 mmol/L Tris-HCl, pH 7.5, 0.1 mmol/L DTT and 30% glycerol. Samples were immediately cooled in liquid nitrogen and kept frozen at –70℃.

1.4 *In vitro* uridylylation assay

Uridylylation assays of purified proteins were performed in 50 μL reaction mixtures with 3 μmol/L GlnB or serial NifA-N mutants, 0.5 mmol/L ATP, 1 mmol/L UTP, 10 mmol/L 2-ketoglutarate, and 0.1－0.5 μmol/L GlnD in buffer: 100 mmol/L Tris-HCl, pH 7.5, 100 mmol/L KCl, 25 mmol/L $MgCl_2$, 5 mmol/L $MnCl_2$, 0.3 mg/mL BSA and 1 mmol/L DTT. Samples were incubated at 30℃ for 60 min and then 1 mmol/L EDTA was added to end the reaction. Samples were analyzed by gel electrophoresis. The effect of each ingredient in reaction mixture was determined by taking each out of the reaction mixture.

1.5 Tricine SDS-PAGE

For the uridylylation assays, samples were loaded on a Tricine SDS-PAGE system[17] with modifications. The stacking gel was prepared with 4% polyacrylamide (stock solution was 48% acrylamide and 1.5% bis-acrylamide) in gel buffer (500 mmol/L Tris-HCl, pH 8.45, 0.05% SDS). The spacer gel was 10% polyacrylamide in gel buffer. The separating gel was 25% polyacrylamide with 12.5% glycerol in gel buffer. The 25% separating gel and the overlaid 10% spacer gel can be polymerized together. Protein samples were diluted in sample buffer (12 mmol/L Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 2 mmol/L 2-mercaptoethanol, 0.02% bromophenol blue) and heated at 65℃ for 10 min before loading. Electrophoresis was carried out at 4℃ using 30 V for 1.5 h followed by 15 mA for 20 h. Running buffers were 200 mmol/L Tris-HCl, pH 8.9, for the anode and 100 mmol/L Tris base, 100 mmol/L Tricine, and 0.1% SDS for the cathode.

1.6 His-tag pull-down assay

Bacterial cells coexpressing serial His6-NifA-N proteins and non-fusion GlnB were grown in a rich medium containing 100 μg/mL of ampicillin and 50 μg/mL of kanamycin. The induction of the T7-*lac* promoter was initiated by addition of 1 mmol/L IPTG at an optical density at 600 nm (A_{600}) of 0.6, and the culture was incubated at 30℃ for 30 min. To obtain nitrogen-free conditions, the culture was then washed 3 times with M9 minimal medium with NH_4Cl omitted and incubated for 3 h at 30℃ in minimal medium supplemented with 18 mmol/L 2-ketoglutarate and 1 mmol/L UTP. To obtain nitrogen-excess conditions, the culture was washed 3 times with M9 minimal medium with NH_4Cl omitted and incubated for 3 h at 30℃ in minimal medium supplemented with 20 mmol/L NH_4Cl. The cells expressing non-fusion GlnB were treated as negative control. Cells were harvested by centrifugation at 5000×*g* for 5 min at 4℃. The cell pellet was resuspended in 1/10 volume of binding buffer (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 10 mmol/L imidazole, pH 8.0), sonicated and clarified by centrifugation at 15000×*g* for 20 min at 4℃. His-tagged proteins were pulled-down from the cell extract

using Ni-NTA Agarose (Novagen) following the manufacturers' instructions. The imidazole concentration was 20 mmol/L in the wash buffer and 250 mmol/L in the elution buffer. Samples were analyzed by Western blotting with appropriate antibodies.

1.7 Western blotting

Polyclonal antibodies against *A. brasilense* GlnB and NifA-N proteins were raised in rabbits as described[18] using purified protein respectively. Total proteins were separated on 12% SDS-PAGE and then electrically transferred onto nitrocellulose membranes. The membranes were blocked for 1 h in Tris-buffered saline (TBS, pH 7.5) containing 5% nonfat dry milk, followed by incubation with appropriate antibody diluted in blocking buffer, and then probed with alkaline phosphatase-conjugated second antibodies. Blots were visualized using the substrate BCIP/NBT detection system (Sigma). Images on the membrane were analyzed using the ImageJ 1.36b software (http://rsb.info.nih.gov/ij/).

2 Results

2.1 *In vivo* and *in vitro* uridylylation of GlnB and serial NifA-N mutants

Figure1 shows the results of *in vivo* uridylylation in *E. coli*. Cell extracts were prepared from the cells incubated in the presence or absence of 20 mmol/L NH_4Cl. The strain *E. coli* BL21(DE3) carrying GlnB expression plasmid was used as a positive control. The uridylylation of target proteins was catalyzed by endogenous GlnD of *E. coli*. We used the Tricine SDS-PAGE system to observe the uridylylation pattern of proteins. This system allows better protein separation of the 5—20 kD range in lower polyacrylamide concentration[19] than the glycine SDS-PAGE system. The uridylylation of GlnB or other low-molecular-weight proteins increases its molecular weight by about 324 Da and this difference can be observed in a Tricine SDS-PAGE system. The band with a higher migration rate corresponds to the non-modified subunit and the second one to the UMP-modified subunit. From Figure 1(b), we can see that GlnB was uridylylated when expressed solely or coexpressed with His6-NifA-N mutant under nitrogen-free conditions and was deuridylylated under nitrogen-excess conditions, which confirmed the validity of this system. However, under the same conditions, no modification of His6-NifA-N or His6-NifA-N-Y18/53F was seen (Figure 1(a)), while GlnB appears to be uridylylated or deuridylylated in response to different nitrogen statuses, suggesting that the uridylylation of GlnB does not lead to the covalent modification of NifA.

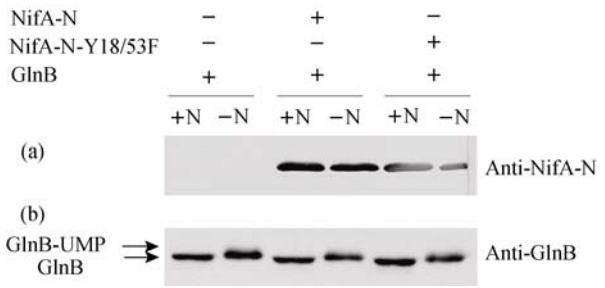

Figure 1 Immunoblots of His6-NifA-N variants and non-fusion GlnB coexpressed in *E. coli* BL21 (DE3) using antibodies raised against NifA-N (a) and GlnB (b). Samples were collected under nitrogen-excess (+N) and nitrogen-free (–N) conditions respectively and analyzed by Tricine SDS-PAGE followed by Western blotting using appropriate antibodies.

In order to perform *in vitro* studies on uridylylation, the His6-GlnB and serial His6-NifA-N mutants were purified on Ni-NTA Agarose columns reparately. The uridylylation of proteins were catalyzed by the uridylyltransferase activity of the GlnD protein (UTase). We used a partially purified *E. coli* GlnD protein in our assays. The uridylylation protocol was performed as described in Sec. 1 and the products were visualized using Tricine SDS-PAGE system. Uridylylation of purified His6-GlnB was carried out with different combinations of ingredients in reaction mixture (Figure 2(a)). The uridylylation of His6-GlnB was dependent on ATP and 2-ketoglutarate. No His6-GlnB-UMP forms were observed in the absence of ATP or 2-ketoglutarate which was in accordance with the results reported previously[20]. Our results also showed that Mn^{2+} was indispensable to the reaction system, for no uridylylated form was observed in the absence of Mn^{2+}. In contrast to the

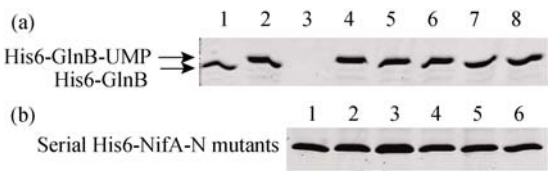

Figure 2 *In vitro* uridylylation assay. (a) *In vitro* uridylylation assay of His6-GlnB. 1, Uridylylation reaction in the absence of GlnD; 2, uridylylation reaction of His6-GlnB; 3—8, uridylylation reaction in the absence of GlnB, BSA, ATP, 2-KG, Mn^{2+} and UTP, respectively. (b) *In vitro* uridylylation assays of serial His6-NifA-N variants. 1, Uridylylation reaction in the absence of GlnD; 2—6, *in vitro* uridylylation assays of His6-NifA-N, His6-NifA-N-Y18F, His6-NifA-N-Y43F, His6-NifA-N-Y53F and His6-NifA-N-Y18/53F, respectively.

above results, when the serial His6-NifA-N mutants were incubated in the same reaction mixtures, no uridylylated forms were observed (Figure 2(b)), indicating that the serial His6-NifA-N mutants did not have the potentiality to be uridylylated.

From these results we can conclude that the presence of uridylylated GlnB did not lead to the covalent modification of NifA. This means that it is very unlikely that the activity of NifA can be regulated by GlnB through covalent modification.

2.2 GlnB interacts with the N-terminal domain of NifA

Control cells expressing non-fusion GlnB solely and cells coexpressing both non-fusion GlnB and serial His-tagged NifA-N mutants were induced under nitrogen-free conditions. His6-NifA-N mutants were pulled-down from the cell extract using Ni-NTA Agarose and were recovered only in the elution fraction of cells carrying the His6-NifA-N plasmids. GlnB was detected in the elution fraction of cells expressing serial His6-NifA-N mutants (Figure 3(a)), indicating that GlnB interacts with N-terminal of NifA under nitrogen-free conditions.

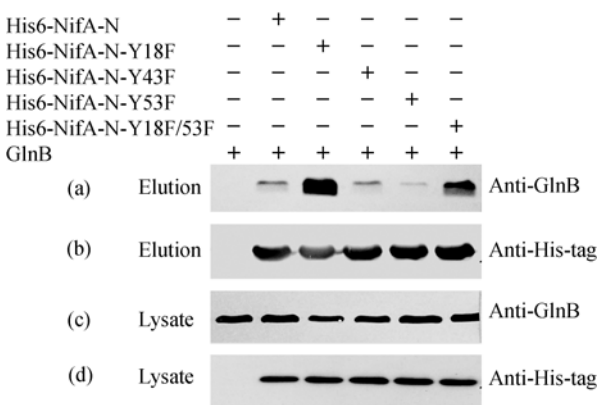

Figure 3 Interactions between GlnB and serial His6-NifA-N mutants using His-tag pull-down assays under nitrogen-free conditions. Non-fusion GlnB was expressed with or without serial His6-NifA-N mutants in *E. coli* BL21 (DE3) and serial His6-NifA-N mutants were pulled-down from each cell lysate. The presence of GlnB and His6-NifA-N mutants in elution fraction of cells was examined by Western blotting with anti-GlnB or anti-NifA-N antibodies, respectively ((a) and (b)). The expression of non-fusion GlnB and His6-NifA-N mutants in cell lysates was examined by Western blotting with anti-GlnB or anti-NifA-N antibodies, respectively ((c) and (d)).

From Figure 3(a), we can see that the intensity of GlnB signal in the elution fraction from cells expressing His6-NifA-N-Y18/53F was several-fold greater than His6-NifA-N. This is consistent with the previous results determined in *A. brasilense* that the double mutant protein NifA-Y18/53F displayed a higher activity than the wild type protein[12]. Surprisingly, the most strengthened GlnB signal is in the elution fraction from cells expressing His6-NifA-N-Y18F. The reason for this is not completely clear. An explanation could be that the intensity of interaction between GlnB and NifA-N partially reflects the activity of NifA. Maybe some other factors are also involved in the process. Further tests need to be done to allow us to draw further conclusions. A problem worthy to be noted is that most GlnB is in uridylylated form when it was pulled-down with serial His6-NifA-N mutants under nitrogen-free conditions, and this is consistent with the notion that GlnB-UMP, rather than GlnB interacts with NifA[21].

In contrast to the result above, GlnB was not detected in the elution fraction of cells coexpressing His6-NifA-N-Y18/53F and non-fusion GlnB under nitrogen-excess conditions (Data not shown), indicating that the excess nitrogen stimulates the UR activity of GlnD to de-uridylylate GlnB, resulting in its poor interaction with NifA. This is in accordance with the results from yeast two-hybrid system in earlier report that no interaction was observed between GlnB and the double mutant NifA-Y18/53F [12].

2.3 Amino acid residues 66-88 and 165-176 regions of NifA-N play a critical role in interaction with GlnB

To investigate the molecular sites involved in the GlnB-NifA-N interaction, a series of deletion mutants of NifA-N had been constructed. Then the His-tag pull-down assay was carried out again under nitrogen-free conditions. As shown in Figure 4(c), the serial His6-NifA-N deletion mutants were pulled-down from extracts of cells carrying the His6-NifA-N deletion mutants plasmids. GlnB was not detected in the elution fraction of cells expressing His6-NifA-N-D4 and His6-NifA-N-D7 (Figure 4(b)), suggesting that they do not interact with GlnB *in vivo*. Collectively, these data indicate that the amino acid residues 66—88 and 165—176 regions of NifA-N play a critical role in interaction with GlnB and the amino acid 144—164 region of NifA-N inhibits the interaction between GlnB and NifA-N.

3 Discussion

In *A. brasilense*, the activity of NifA depends on GlnB since NifA is synthesized but inactive in a *glnB* mutant[22—24]. Moreover, GlnB was found to interact with

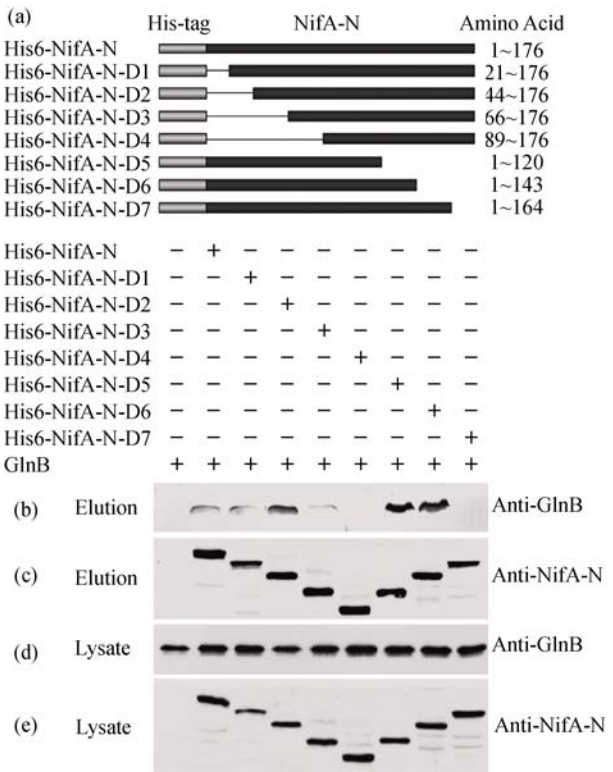

Figure 4 Interactions between GlnB and His6-NifA-N deletion mutants. (a) His-tagged NifA-N and its serial deletion mutants used in this work are schematically represented. (b)—(e) Interactions between GlnB and His6-NifA-N deletion mutants using His-tag pull-down assays under nitrogen-free conditions. Non-fusion GlnB was expressed with or without serial His6-NifA-N deletion mutants in *E. coli* BL21 (DE3) and serial His6-NifA-N deletion mutants were pulled-down from each cell lysate. The presence of GlnB and His6-NifA-N deletion mutants in elution fraction of cells was examined by Western blotting with anti-GlnB or anti-His-tag antibodies, respectively ((b) and (c)). The expression of non-fusion GlnB and His6-NifA-N mutants in cell lysates was examined by Western blotting with anti-GlnB or anti-His-tag antibodies, respectively ((d) and (e)).

the N-terminal GAF domain of NifA by using yeast two-hybrid system. GlnB can be reversibly uridylylated by GlnD depending on the nitrogen status of the cell and the uridylylated GlnB is essential for NifA activity, because a *glnB* mutant strain in which the Tyr51 residue was replaced by Phe showed a Nif$^-$ phenotype[14]. These data led to the proposal that GlnB is required to activate NifA by antagonizing the inhibitory effect of its N-terminal domain under nitrogen-fixing conditions. However, the physiological importance of the interaction between NifA-N and GlnB detected from yeast two-hybrid system is still unknown. It is unknown whether or not the regulation of NifA activity mediated by GlnB resulted in covalent modification of NifA, nor if GlnB interacted directly on the N-terminal domain of NifA.

In order to clarify the situation, we have now used some experiments to investigate whether NifA can be modified by uridylylated GlnB. Firstly, we determined the uridylylation status of GlnB and NifA-N mutant in *E. coli* under nitrogen-excess and nitrogen-free conditions. We chose the wild type and the double mutant protein NifA-N-Y18/53F for this research, because the mutation of Tyr18 and Tyr53 was known to enhance NifA activity relative to the wild type protein[12]. Secondly, we used the *in vitro* uridylylation assays to observe the uridylylation pattern of GlnB and serial NifA-N mutants. The results indicate that the presence of uridylylated GlnB did not lead to the covalent modification of NifA.

The interaction between NifA and GlnB was observed using yeast two-hybrid system in *A. brasilense*[12], but no further studies have been published as yet. The present work was carried out to investigate the differences of interactions between GlnB and serial NifA-N mutants. Though the yeast two-hybrid system is the most popular technique for analyzing protein-protein interactions, it has some limitations, including high rates of false positives and false negatives. Moreover, it is not favorable to detect prokaryotic protein-protein interactions in eukaryotic yeast cells due to the huge differences between two kinds of organisms. Thus, experimental results from yeast two-hybrid assays are often further confirmed by *in vitro* tag-based pull-down assays. The traditional tag-based pull-down assay is an *in vitro* method used to determine physical interaction between two or more proteins and useful for confirming the existence of a protein-protein interaction predicted by other research techniques (e.g., co-immunoprecipitation, yeast two-hybrid and density gradient centrifugation). The greatest disadvantage of this method is that the binding occurs under potentially non-physiological conditions. In order to enable the investigation of prokaryotic protein-protein interactions in a prokaryotic genetic background and the occurrence of binding under physiological conditions, we used an improved His-tag pull-down assay to detect the interactions between GlnB and serial NifA-N mutants. *E. coli* and *A. brasilense* are prokaryotes that have similar genetic backgrounds and the process of protein synthesis. As far as the small signal transduction protein GlnB is concerned, it can be reversibly uridylylated depending on the nitrogen status of the cell in both cells. Therefore, the results of protein-protein interactions between GlnB and serial NifA-N mutants in *E. coli* can reflect the circumstances of *A. brasilense* to a certain extent.

In conclusion, we have shown that GlnB interacts with N-terminal of NifA in *E. coli* under nitrogen-free conditions and the intensity of interaction between GlnB and NifA-N-Y18/53F is greater than wild type protein, thus confirming the suggestion derived from the higher nitrogenase activity of strains complemented with plasmid carrying mutated *nifA* gene [12]. Our results also indicate that the amino acid residues 66—88 and 165—176 regions of the N-terminal domain of NifA play a crucial role in interaction with GlnB.

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