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## Identification and functional characterization of NifA variants that are independent of GlnB activation in the photosynthetic bacterium *Rhodospirillum rubrum*

Xiaoxiao Zou<sup>1</sup>, Yu Zhu<sup>2,†</sup>, Edward L. Pohlmann<sup>2</sup>, Jilun Li<sup>1</sup>, Yaoping Zhang<sup>1,2</sup>, Gary P. Roberts<sup>2</sup>

<sup>1</sup>Department of Microbiology and Immunology, College of Biological Sciences and State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing 100094, PR China

<sup>2</sup>Department of Bacteriology and the Center for the Study of Nitrogen Fixation, University of Wisconsin–Madison, Madison, WI 53706, USA

### Abstract

The activity of NifA, the transcriptional activator of the nitrogen fixation (*nif*) gene, is tightly regulated in response to ammonium and oxygen. However, the mechanisms for the regulation of NifA activity are quite different among various nitrogen-fixing bacteria. Unlike the well-studied NifL–NifA regulatory systems in *Klebsiella pneumoniae* and *Azotobacter vinelandii*, in *Rhodospirillum rubrum* NifA is activated by a direct protein–protein interaction with the uridylylated form of GlnB, which in turn causes a conformational change in NifA. We report the identification of several substitutions in the N-terminal GAF domain of *R. rubrum* NifA that allow NifA to be activated in the absence of GlnB. Presumably these substitutions cause conformational changes in NifA necessary for activation, without interaction with GlnB. We also found that wild-type NifA can be activated in a GlnB-independent manner under certain growth conditions, suggesting that some other effector(s) can also activate NifA. An attempt to use Tn5 mutagenesis to obtain mutants that altered the pool of these presumptive effector(s) failed, though much rarer spontaneous mutations in *nifA* were detected. This suggests that the necessary alteration of the pool of effector(s) for NifA activation cannot be obtained by knockout mutations.

### Introduction

Biological nitrogen fixation is a tightly controlled energy-demanding process. In all studied nitrogen-fixing bacteria, nitrogen fixation is regulated at the transcriptional level, though other levels of regulation are also involved in some bacteria (Dixon & Kahn, 2004; Zhang *et al.*, 2003). Transcription of the *nif* (nitrogen fixation) operons is activated by a *nif*-specific transcriptional activator, NifA. However, the mechanism for the regulation of NifA activity is quite different in the various nitrogen-fixing bacteria.

Correspondence: Gary P. Roberts, groberts@bact.wisc.edu.

<sup>†</sup>Present address: Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, Rochester, MN 55902, USA.

The regulation of NifA is well studied in *Klebsiella pneumoniae* and occurs at both the transcriptional and post-translational levels. First, the transcription of the *nifLA* operon in *K. pneumoniae* is regulated by a general nitrogen regulation (Ntr) system, which involves many gene products, such as P<sub>II</sub>, NtrB/NtrC and GlnD (Merrick & Edwards, 1995; Minchin *et al.*, 1988; Porter *et al.*, 1995; Reitzer, 2003). *glnD* encodes a bifunctional, uridylyltransferase/uridylyl-removing enzyme (UTase/UR) that senses the intracellular concentration of glutamine in the cell (Jiang *et al.*, 1998). GlnD reversibly controls the activity of the P<sub>II</sub> proteins (GlnB and its homologues) by uridylylation or deuridylylation (Adler *et al.*, 1975). P<sub>II</sub> proteins are some of the most broadly distributed regulatory proteins, and are integrators of signals of nitrogen, carbon and energy status (Arcond'guy *et al.*, 2001; Commichau *et al.*, 2006; Forchhammer, 2004; Leigh & Dodsworth, 2007; Ninfa & Atkinson, 2000; Ninfa & Jiang, 2005; Zhang *et al.*, 2001a, 2006). The products of *ntrB* and *ntrC* belong to the family of two-component regulators (Stock *et al.*, 2000). NtrB is a histidine kinase that phosphorylates NtrC under nitrogen-limiting conditions and can also act as a phosphatase to dephosphorylate NtrC under nitrogen-excess conditions (Kamberov *et al.*, 1994). NtrB activity is regulated by P<sub>II</sub> in response to the carbon/nitrogen balance in the cell (Ninfa & Atkinson, 2000; Ninfa & Jiang, 2005). The phosphorylated form of NtrC acts as a transcriptional activator of *glnA*, *nifLA*, *glnK*, *amtB<sub>1</sub>* and other operons involved in nitrogen fixation and assimilation (Weiss *et al.*, 1991). Under nitrogen-limiting conditions, the UTase activity of GlnD predominates and it uridylylates P<sub>II</sub>. The uridylylation of P<sub>II</sub> prevents its interaction with NtrB. Without interaction with P<sub>II</sub> the kinase activity of NtrB predominates and it phosphorylates NtrC. The phosphorylated NtrC then activates expression of the *nifLA* operon in *K. pneumoniae*. NifA then activates expression of the other *nif* operons. Under nitrogen-excess conditions, the process is reversed and *nif* is not expressed. The *nifLA* operon is also regulated by the Ntr system in *Azoarcus* sp. BH72, *Pantoea agglomerans* (formerly called *Enterobacter agglomerans*) and *Pseudomonas stutzeri* (Desnoues *et al.*, 2003; Egener *et al.*, 2002; Siddavattam *et al.*, 1995), but this is not the case in *Azotobacter vinelandii* (Blanco *et al.*, 1993).

Secondly, in both *K. pneumoniae* and *A. vinelandii*, NifA activity is post-translationally regulated by NifL. In the presence of ammonium or oxygen, NifA activity is inhibited by NifL through direct protein–protein interaction (Martinez-Argudo *et al.*, 2004b). P<sub>II</sub> is also involved in the regulation of the NifL–NifA interaction (He *et al.*, 1998; Jack *et al.*, 1999; Little *et al.*, 2000, 2002; Stips *et al.*, 2004), but probably by different mechanisms in these organisms (Little *et al.*, 2000; Reyes-Ramirez *et al.*, 2001; Stips *et al.*, 2004).

In many other nitrogen-fixing bacteria belonging to the  $\alpha$ -subgroup Proteobacteria, such as *Azospirillum brasilense* and *Rhodospirillum rubrum*, *ntrC* is not essential for *nifA* expression, but NifA activity is tightly controlled in response to ammonium (NH<sub>4</sub><sup>+</sup>) (Liang *et al.*, 1992, 1993; Zhang *et al.*, 1995b, 2000). In these bacteria, no NifL homologue has been found, and NifA activity is regulated directly by one of the P<sub>II</sub> proteins (GlnB). In *R. rubrum* NifA is activated directly by the uridylylated form of GlnB, and the other P<sub>II</sub> homologues, GlnK and GlnJ, are unable to activate NifA (Zhang *et al.*, 2001a, 2004). Similarly, in *A. brasilense* the activation of NifA requires only GlnB, and the other P<sub>II</sub> homologue (Pz or GlnZ) is not involved (Araújo *et al.*, 2004; de Zamaroczy *et al.*, 1993, 1996; Liang *et al.*, 1992). The detailed mechanism for NifA activation in these organisms is still unknown, but

it has been suggested that GlnB binds to the N-terminal GAF domain of NifA to prevent its interaction with the central catalytic domain (Arsene *et al.*, 1996). Consistent with this hypothesis, interaction between NifA and GlnB has been detected in *A. brasilense* and *R. rubrum* by the yeast two-hybrid system (Chen *et al.*, 2005; Zhu *et al.*, 2006).

Nitrogenase activity in *A. brasilense*, *R. rubrum* and several other nitrogen-fixing bacteria is also tightly regulated at the post-translational level by a reversible mono-ADP ribosylation of dinitrogenase reductase (Nordlund & Ludden, 2004; Zhang *et al.*, 1997). Under  $\text{NH}_4^+$  - excess or energy-limiting conditions (such as shifting cells from light to dark for *R. rubrum* or shifting cells from microaerobic to anaerobic conditions for *A. brasilense*), dinitrogenase reductase is ADP-ribosylated and thereby inactivated by DRAT (dinitrogenase reductase ADP-ribosyl transferase, the gene product of *draT*). However, when  $\text{NH}_4^+$  is exhausted or cells are shifted back to light (for *R. rubrum*) or microaerobic conditions (for *A. brasilense*), the ADP ribose group can be removed by DRAG (dinitrogenase reductase activating glycohydrolase; the gene product of *draG*), restoring nitrogenase activity. The activities of DRAT and DRAG are themselves subject to post-translational regulation, in which P<sub>II</sub> proteins are involved (Huergo *et al.*, 2006, 2007; Klassen *et al.*, 2001, 2005; Wang *et al.*, 2005; Zhang *et al.*, 2001a, b, 2006).

Although no structure of a NifA homologue is available, searching the Pfam protein families database (Finn *et al.*, 2006; Sonnhammer *et al.*, 1998) indicates that it has at least three domains: a GAF domain in the N-terminal region, a central  $\sigma^{54}$  interaction domain, and a helix–turn–helix (HTH) domain in the C-terminal region (Martinez-Argudo *et al.*, 2004b; Morett & Segovia, 1993; Studholme & Dixon, 2003). GAF domains are ubiquitous small-molecule-binding domains present in cGMP-regulated cyclic nucleotide phosphodiesterases, adenylyl cyclases, the bacterial transcription factor FhlA, and many other signalling and sensory proteins from all three kingdoms of life (Aravind, 1997; Martinez *et al.*, 2002). The GAF domain of *A. vinelandii* NifA binds  $\alpha$ -ketoglutarate ( $\alpha$ -KG) and controls its interaction with NifL (Little & Dixon, 2003; Martinez-Argudo *et al.*, 2004a). The central domain of NifA, for  $\sigma^{54}$  interaction, is homologous with ATPases of the AAA+ family that are associated with diverse cellular activities, and is involved in ATP hydrolysis and interaction with  $\sigma^{54}$  (Zhang *et al.*, 2002). The HTH motif of the C-terminal domain is involved in DNA binding (Morett *et al.*, 1988).

To further investigate the mechanisms for NifA activation, we used two different approaches to obtain NifA variants that could be activated in the absence of GlnB. First, we obtained two variants, NifA-M173I and NifA-M173V, that interact better with GlnB in the yeast two-hybrid system. Both NifA variants showed GlnB-independent activity in *R. rubrum*. Second, a group of NifA variants was obtained as spontaneous Nif<sup>+</sup> revertants of a *glnB* mutant. We also found that wild-type NifA expressed from a multicopy plasmid can be activated in a GlnB-independent manner under certain growth conditions. We then hypothesized that there might be another gene whose elimination might provide the same phenotype, perhaps by altering the pool of small molecular effectors in the cell to allow NifA to be activated in the absence of GlnB. However, we were unable to generate such mutants by Tn5 insertion, though spontaneous mutations were found in *nifA*. This reinforces the potential for different

NifA substitutions to have this phenotype and suggests that knockout mutations cannot significantly alter the level of effector(s) to cause NifA activation.

## Methods

### Bacterial strains and growth conditions

*Escherichia coli* was grown in LC medium (similar to Luria–Bertani medium, but with 5 g NaCl l<sup>-1</sup>). *R. rubrum* was grown in Supplemental Minimal plus NH<sub>4</sub><sup>+</sup> (SMN) (rich) medium (Fitzmaurice *et al.*, 1989). Antibiotics were used as necessary at the levels described previously (Zhang *et al.*, 2000).

### Whole-cell nitrogenase activity assay

*R. rubrum* was grown in SMN medium, and then inoculated into MG (a nitrogen-limiting, malate/glutamate minimal medium with glutamate as nitrogen source), MN (minimal medium plus NH<sub>4</sub><sup>+</sup>) or MN<sup>-</sup> (NH<sub>4</sub><sup>+</sup>-free minimal medium with N<sub>2</sub> gas) as described previously (Fitzmaurice *et al.*, 1989; Lehman & Roberts, 1991; Zhang *et al.*, 2001a). Whole-cell nitrogenase activity was monitored by the acetylene reduction assay as described previously (Zhang *et al.*, 1995a).

### Random PCR mutagenesis and a yeast two-hybrid selection for *nifA* mutants

Random mutations in *nifA* were generated with the GeneMorph II Random Mutagenesis kit (Stratagene) using pUX686 (a pGAD-C1 derivative carrying *R. rubrum nifA* cloned into the *Bam*HI and *Pst*I sites, AD–NifA fusion) (Zhu *et al.*, 2006) as a template. The mutagenized DNA was digested with *Bam*HI and *Pst*I, and ligated with pGAD-C1. As in our previous report on screening *glnB* mutants, the mutated plasmids were transformed into *Saccharomyces cerevisiae* strain pAJ.69 harbouring pUX679 (BD–GlnB fusion) (UY1) by the lithium acetate method (Gietz *et al.*, 1995; Schiestl & Gietz, 1989). The transformants were selected on synthetic defined (SD; a yeast minimal medium) plates lacking leucine, uracil and histidine, and containing 5 mM 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the His3 protein (Fields, 1993). Starting with a culture carrying the library of mutated *nifA* alleles, several hundred colonies appeared on the selection plates, while very few colonies were seen with the unmutagenized control. Plasmids were recovered from some of colonies and *nifA* was sequenced using the Big Dye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems) to identify mutations. Some plasmids were then reintroduced into UY1 and shown to support growth on SD plates lacking leucine, uracil and histidine, and containing 1.5 mM 3-AT, thus verifying causality.

### Expression of NifA variants in *R. rubrum*

A 3.8 kb fragment carrying wild-type *nifA* was subcloned into a suicide vector, pUX19 (Lies, 1994), yielding pUX201. Two *nifA* alleles identified in the yeast two-hybrid analysis were reconstructed with pUX201 by the QuikChange Site-Directed Mutagenesis kit (Stratagene): pUX202 encodes NifA-M173I and pUX203 encodes NifA-M173V. These plasmids were transformed into *E. coli* strain S17-1 (Simon *et al.*, 1983), then conjugated into UR717 ( *glnB*) (Zhang *et al.*, 2000) and UR1325 ( *glnD*) (Zhang *et al.*, 2005). The resulting strains contained both the wild-type *nifA* allele and a single integrated copy of the

wild-type or mutant allele. In the *glnB* background, these strains were designated UR1739 (the wild-type NifA merodiploid control), UR1740 (NifA-M173I) and UR1741 (NifA-M173V). In the *glnD* background, they were designated UR1742 (the wild-type NifA merodiploid control), UR1743 (NifA-M173I) and UR1744 (NifA-M173V).

Similarly, wild-type and mutated *nifA* were also integrated into the chromosome of wild-type (UR2) background, yielding UR2501, UR2502 and UR2503, and into *draT* (UR213) (Liang *et al.*, 1991) mutant background, yielding UR2327, UR2328 and UR2329.

### Tn5 random mutagenesis and identification of the affected loci

pRL27 was used for Tn5 random mutagenesis. It contains a gene (*tnp*) under the control of the *tetA* promoter from plasmid RP4 that overproduces Tn5 transposase and a mini-Tn5 element carrying both kanamycin resistance (Km<sup>r</sup>) and the origin of replication from plasmid R6K (*oriR6K*) (Larsen *et al.*, 2002). pRL27 was transferred from *E. coli* into *R. rubrum* UR717 (*glnB*) by bi-parental conjugation, as described previously (Liang *et al.*, 1991). After mating on SMN plates overnight, cells were rinsed from the nitrocellulose filters into SMN containing nalidixic acid (N<sub>x</sub>) and Km, grown aerobically for 2 days for enrichment of the transconjugants, and then inoculated into MN<sup>-</sup> (NH<sub>4</sub><sup>+</sup>-free) medium in gas-tight tubes. After degassing and flushing with N<sub>2</sub>, cultures were grown anaerobically in the light for 5–10 days until they turned pink or red, indicating that some mutations restored the Nif<sup>+</sup> phenotype of the *glnB* mutant. The cultures were diluted and plated on SMN plates with N<sub>x</sub> and Km, and single colonies were purified on plates of the same medium. Nitrogenase activity in these mutants was monitored.

To identify the loci affected by the Tn5 insertions in these Nif<sup>+</sup> mutants, the total DNA was isolated, digested with *Bam*HI, and then self-ligated. The *oriR6K* is able to replicate in *E. coli* strains that contain *pir*, which allowed the cloning and analysis of the sites of the Tn5 insertions (Larsen *et al.*, 2002).

### Protein immunoblotting

A TCA precipitation method was used for rapid protein extraction (Zhang *et al.*, 1993). As described previously (Zhang *et al.*, 1995b), proteins were separated by low cross-linker SDS-PAGE (ratio of acrylamide : bisacrylamide, 172 : 1), electrophoretically transferred onto a nitrocellulose membrane, incubated with polyclonal antibody against *A. vinelandii* dinitrogenase reductase, and then visualized with horseradish peroxidase. Active dinitrogenase reductase is completely unmodified and migrates as a single band, while inactive dinitrogenase reductase migrates as two bands, since only one subunit of dinitrogenase reductase is ADP-ribosylated, and ADP-ribosylation slows the migration of the modified subunit.

## Results

### The yeast two-hybrid screen yields NifA variants that have a better interaction with GlnB

In our previous studies, we used the yeast two-hybrid system to detect some variants of GlnB (termed GlnB\*) that interact better with NifA than does the wild-type GlnB (Zhu *et*

*al.*, 2006). In *R. rubrum*, these GlnB\* variants activated NifA in the presence of  $\text{NH}_4^+$ , whereas  $\text{NH}_4^+$  blocks NifA activity completely in the wild-type. The presence of these GlnB\* variants also allowed NifA activity in *glnD* backgrounds, while the uridylylation of GlnB by GlnD is normally essential for wild-type NifA activation. Our hypothesis is that the role of uridylylation of GlnB is primarily to shift the equilibrium of GlnB from a 'nitrogen-sufficient' form toward a 'nitrogen-deficient' form, and these GlnB variants apparently shift that equilibrium through direct structural changes (Zhu *et al.*, 2006).

We supposed that it might be possible to find GlnB-independent (and therefore  $\text{NH}_4^+$  - constitutive) NifA variants using a similar approach. That is, we reasoned that if binding of GlnB-UMP to NifA stabilizes the transcriptionally active form of NifA, then NifA variants that are equilibrium-shifted toward that form for structural reasons might have a higher affinity for GlnB-UMP and/or GlnB. Because wild-type NifA has a very low affinity for the non-UMP form of GlnB in the yeast system (Zhu *et al.*, 2006), we supposed that NifA variants with improved affinity for GlnB should have a better interaction in the yeast.

We therefore mutagenized *nifA* in the yeast expression vector by error-prone PCR, and screened for NifA variants with improved interaction with GlnB. We initially tried 30 cycles of PCR with the GeneMorph II Random Mutagenesis kit from Stratagene. The mutagenized plasmid pool was transferred into a yeast strain producing wild-type GlnB (UY1), and selection for growth on minimal medium (SD) plates lacking leucine, uracil and histidine, but containing 5 mM 3-AT, a competitive inhibitor of His3 protein, was performed (Zhu *et al.*, 2006). Several clones were purified, and plasmids from these yeast strains were isolated and the *nifA* genes were sequenced. Eight out of nine plasmids had multiple substitutions in *nifA*, though two were siblings (first screening in Table 1). Intriguingly, four of six non-identical clones had substitutions at Met-173, suggestive of causation. Because of the high mutation frequency, we remutagenized *nifA* with the following modifications: increasing the amount of template by fivefold and decreasing the number of PCR cycles from 30 to 12. Twenty-four plasmids containing the *nifA* alleles were isolated from yeast strains that grew well on the selection medium, and 19 plasmids were shown to be causative of the phenotype after they were reintroduced into UY1. Again, M173I and M173V substitutions predominated among non-identical plasmids (second screening in Table 1). We then reconstructed single M173I and M173V substitutions in the yeast plasmid and verified that these were both able to support growth of yeast on selective medium.

### **NifA-M173V and NifA-M173I variants are active in *R. rubrum* mutants lacking GlnB-UMP or GlnB**

The above results show that NifA-M173V and NifA-M173I interact better with GlnB in yeast than wild-type. We then tested the physiological effect of their presence at normal levels in *R. rubrum*. Appropriate mutations were constructed and cloned into a pUX19 suicide vector, yielding pUX2022 (NifA-M173I) and pUX2023 (NifA-M173V). These plasmids, together with pUX2021 carrying wild-type *nifA*, were integrated into *glnB* (UR717) or *glnD* (UR1325) backgrounds. The nitrogenase activity in these strains is shown in Table 2 under *nif*-derepressing conditions in MG (a nitrogen-limiting, minimal medium with glutamate as nitrogen source). In both *glnB* and *glnD* backgrounds, the

altered NifA variants caused substantial nitrogenase activity, while little NifA activation was seen with wild-type NifA. This indicates that these substitutions not only allow NifA to interact better with GlnB in yeast, but also cause NifA to be activated independently of the GlnB uridylylation state (based on the *glnD* result) and in fact of any GlnB interaction at all (based on the *glnB* result). This result is consistent with the original hypothesis of the conformational change in NifA, though it obviously does not prove it. For unknown reasons these NifA variants showed higher nitrogenase activity in the *glnB* mutant background than in the *glnD* mutant background. One simple explanation is that the NifA activity in these variants can be slightly inhibited by interaction with unmodified GlnB, since these NifA variants were shown to interact well with unmodified GlnB in yeast.

### Nitrogenase activity is still regulated by $\text{NH}_4^+$ in these NifA variants

As mentioned in the Introduction, nitrogenase activity in *R. rubrum* is regulated post-translationally by the DRAT–DRAG regulatory system: DRAT modifies dinitrogenase reductase to inactivate nitrogenase activity, while DRAG removes the covalently modified ADP ribose group from dinitrogenase and restores its activity. A screen/selection for mutants of *Rhodopseudomonas palustris* has been reported recently in which several mutants were found that produced hydrogen in the presence of  $\text{NH}_4^+$ , and four of these had mutations in *nifA* (Rey *et al.*, 2007). Interestingly, the nitrogenase activity in these *nifA* mutants is no longer regulated by  $\text{NH}_4^+$ , even though *Rhodopseudomonas palustris* appears to have *draTG* homologues (Rey *et al.*, 2007). We investigated whether substitutions in our NifA variants also altered the DRAT–DRAG regulation in *R. rubrum*. We found that although mutants with NifA-M173I and M173V variants possessed GlnB-independent NifA activity, and NifA activity was no longer regulated by  $\text{NH}_4^+$ , the nitrogenase activity in these mutants was still regulated by  $\text{NH}_4^+$ . As shown in Table 3, when grown in MG (nitrogen-limiting) medium, strains carrying wild-type *nifA* (UR2501), NifA-M173I (UR2502) or NifA-M173V (UR2503) showed high nitrogenase activity, similar to that seen in UR2 (wild-type). However, little or very low nitrogenase activity was seen in these strains when they were grown in MN (minimal medium plus  $\text{NH}_4^+$ ). To test the hypothesis that nitrogenase activity in UR2502 and UR2503 was tightly regulated by the DRAT–DRAG regulatory system, we examined the behaviour of these NifA variants in a *draT* background. This *draT* mutant (UR213) lacks the DRAT activity that ADP-ribosylates dinitrogenase reductase, so nitrogenase activity is no longer regulated in response to either darkness or addition of  $\text{NH}_4^+$  (Liang *et al.*, 1991). *draT* mutants with NifA-M173I or M173V (UR2328 or UR2329) showed high nitrogenase activity in both MG- and MN-grown cultures, while *draT* mutants with wild-type NifA (UR2327) showed little nitrogenase activity in MN-grown cultures, since in this strain NifA activity is still regulated by  $\text{NH}_4^+$ .

The protein level and the modification status of dinitrogenase reductase are completely consistent with the nitrogenase activity seen in these mutants in Table 3. As shown in Fig. 1, when grown in MN ( $\text{NH}_4^+$ -excess) medium, little dinitrogenase reductase accumulated in UR2, UR2501, and UR2327, while high levels of dinitrogenase reductase accumulated in mutants with the NifA-M173I or M173V variant (UR2502, UR2503, UR2328 and UR2329). The difference is that the dinitrogenase reductase was modified in UR2502 and UR2503, but was completely unmodified in UR2328 and UR2329, since these two strains lack DRAT.

There is also a third band between the unmodified band (indicated as U) and the modified band (indicated as M). Since it is absent under  $\text{NH}_4^+$ -excess conditions and can cross-react with the antibody against *A. vinelandii* dinitrogenase reductase, it might be the alternative (Fe-only) dinitrogenase reductase or another unmodified form of dinitrogenase reductase. When grown in MG medium, all strains had similar amounts of unmodified dinitrogenase reductase (data not shown). These results indicate that the activity of NifA-M173I or NifA-M173V is no longer regulated by  $\text{NH}_4^+$ , although nitrogenase activity in these variants is still regulated by  $\text{NH}_4^+$  through the DRAT/DRAG system.

We noticed a high nitrogenase activity in UR2328 and UR2329 (*draT* mutants with NifA-M173I and NifA-M173V variants) when grown MN medium. This seems to be inconsistent with the hypothesis that we proposed above that NifA activity in these variants can be slightly inhibited by interaction with unmodified GlnB, since GlnB should be unmodified in MN-grown cells. We suggest two possible explanations. (i) Cell growth and nitrogenase activity vary in different media. However, we were unable to perform the best comparison of nitrogenase activity in *glnB draT* and *glnD draT* mutants with these NifA\* variants in MN medium, since *glnD* mutants fail to grow in MN medium (Zhang *et al.*, 2005). (ii) Alternatively, the protein level of GlnB is much lower in MN-grown cells than in MG-grown cells, since one of its promoters is repressed under N-excess conditions (Cheng *et al.*, 1999). This lower level of GlnB may be insufficient to inhibit NifA activity in MN-grown cells.

#### **Wild-type NifA can be activated in the absence of GlnB under $\text{N}_2$ -fixing conditions when expressed from a multicopy plasmid**

As we reported previously, GlnB is essential for NifA activation and little nitrogenase activity is seen in a *glnB3* mutant (UR717) in MG (nitrogen-limiting) medium (Zhang *et al.*, 2000) (Table 4). When wild-type *nifA* was expressed from a multicopy plasmid in the *glnB3* mutant background, a low level of nitrogenase activity was detected in UR744 when it was grown in MG medium (Zhang *et al.*, 2000) (Table 4). This result is consistent with the equilibrium hypothesis: when elevated amounts of NifA are present due to the multicopy plasmid, higher levels of NifA (inactive form) should accumulate, and the equilibrium should produce a small population of the active form of NifA, leading to some low level of nitrogenase activity. Surprisingly, we found dramatically different nitrogenase activity in these strains when they were grown in  $\text{MN}^-$  ( $\text{NH}_4^+$ -free) medium with  $\text{N}_2$  gas. As shown in Table 4, a *glnB3* mutant (UR717) failed to grow in  $\text{MN}^-$  medium, and little nitrogenase activity was detected. However, UR744 (*glnB3* mutant with multicopy *nifA*) grew reasonably well and had a moderate nitrogenase activity in  $\text{MN}^-$  medium, similar to that seen in the wild-type control (UR741). For unknown reasons, nitrogenase activity in  $\text{MN}^-$ -grown cultures of the wild type strains was always lower than that in MG-grown cultures, as has been reported previously in both wild-type (UR2) and other strains (Zhang *et al.*, 2001a, 2005). These results support the notion that in the absence of GlnB, some other effector(s) (either proteins or small molecules) can also affect NifA activation.



## Tn5 random mutagenesis of *glnB* mutant and screening for mutants with high nitrogenase activity in this background

As shown above, there is GlnB-independent NifA activation, which is affected in some way by nitrogen status. To identify the effector(s) involved in NifA regulation, we performed Tn5 mutagenesis on a *glnB* mutant and sought mutants that would allow NifA to be activated in a GlnB-independent manner. Our reasoning was that some Tn5 insertions might alter the pools of the effector molecules, and such mutants would provide useful information about these effector(s).

After Tn5 mutagenesis and enrichment in MN<sup>-</sup> medium, many mutants displayed a Nif<sup>+</sup> phenotype in a *glnB* mutant background. The sites of the Tn5 insertion in about 50 strains were identified by sequencing, and several strains showed identical loci of the Tn5 insertion in ORFs named Rru\_A3654/3555, A2353, A1638, A1681, A0934 and B0039 in the *R. rubrum* genome, indicating that these are siblings (Table 5). When selected Tn5 insertions were subcloned into a suicide vector and moved back into the *glnB* background, the strains remained Nif<sup>-</sup>. Thus, the Tn5 insertions were not causative of the Nif<sup>+</sup> phenotype.

Because our PCR mutagenesis of *nifA* revealed GlnB-independent variants, we reasoned that some of these Nif<sup>+</sup> mutants found after Tn5 mutagenesis might also have spontaneous mutations in *nifA*. We amplified and sequenced *nifA* from 17 Nif<sup>+</sup> strains obtained by Tn5 mutagenesis. As shown in Table 5, all strains but one had mutations in *nifA*, and some had the identical M173I or M173V substitution mentioned above. To confirm that these *nifA* mutations were causative of the GlnB-independence, we reconstructed several single substitutions in *nifA*, integrated them into the chromosome of a *glnB* mutant and measured the nitrogenase activity. As shown in Table 6, these new NifA variants with M173L, L184R, A243T, G36E and T38P substitutions caused moderate nitrogenase activity in a *glnB* mutant background, similar to that seen in NifA-M173I and M73V variants. All of these NifA variants have substitutions in the N terminus, strongly suggesting the important role of this GAF domain in the regulation of NifA activity.

## Discussion

We previously proposed a model in which GlnB has at least two forms: a ‘nitrogen-sufficient’ form and a ‘nitrogen-deficient’ form. The uridylylation of GlnB is primarily to shift the equilibrium of GlnB from a nitrogen-sufficient form to a nitrogen-deficient form (Zhu *et al.*, 2006). Similar to GlnB, NifA appears to exist in at least two forms, dependent on the nitrogen status of the cell. Under nitrogen-excess conditions GlnB is deuridylylated, and this unmodified form of GlnB is probably unable to interact well with NifA under normal physiological conditions. Under nitrogen-limiting conditions, GlnB is uridylylated and this modified GlnB might have a much better affinity for NifA. The interaction with GlnB–UMP causes a conformational change in NifA, which activates its activity.

We report here that several substitutions in the N-terminal GAF domain of NifA, derived using two independent methods, allow NifA to be activated in the absence of GlnB. We believe that these substitutions also cause a conformational change in NifA, similar to that caused by the interaction with GlnB–UMP. The NifA-M173I or M173V variants interact

strongly with GlnB in yeast. Because GlnB is presumably unmodified in yeast, this indicates that conformational change in the T-loop of GlnB by uridylylation is not necessary for its interaction with these NifA variants. Because the inhibition of NifA activity by  $\text{NH}_4^+$  is mediated by  $\text{P}_{\text{II}}$  and these NifA variants showed GlnB-independent activity, the activity of these NifA variants is also no longer subject to regulation in response to  $\text{NH}_4^+$  (Table 3). However, because of the DRAT–DRAG regulatory system, the nitrogenase activity in these NifA variants is still regulated by  $\text{NH}_4^+$ , and high nitrogenase activity was only seen in *draT* mutants with altered *nifA* under nitrogen-excess (MN) conditions (Table 3). This is quite different from a recent report that nitrogenase activity in some *Rhodopseudomonas palustris* *nifA* mutants is not regulated by  $\text{NH}_4^+$ , even though *Rhodopseudomonas palustris* appears to have *draTG* homologues (Rey *et al.*, 2007). However, the DRAT–DRAG regulatory system has not been studied in *Rhodopseudomonas palustris*, and it is not known if it is actually functional.

Another important observation that we report here is that some other effector(s) could also activate NifA activity in a GlnB-independent manner. When cells were grown in  $\text{MN}^-$  ( $\text{NH}_4^+$ -free) medium, UR744 (*glnB* mutant with a multicopy *R. rubrum nifA*) showed a nitrogenase activity similar to that seen in UR741 (wild-type with a multicopy *R. rubrum nifA*) (Table 4). One possibility is that some effector(s) could also interact with and activate NifA, similar to the role of GlnB–UMP interaction. The difference in NifA activation between MG- and  $\text{MN}^-$ -growing cells suggests a dramatic change in the pools of some effector(s) under these different growth conditions. It is unknown whether the effector is a small molecule or protein. As mentioned in the Introduction, small molecules, such as ATP, ADP and  $\alpha$ -KG, apparently play important roles in the regulation of  $\text{P}_{\text{II}}$  function in *E. coli*, *R. rubrum* and other bacteria and archaea (Dodsworth & Leigh, 2006; Dodsworth *et al.*, 2005; Forchhammer, 2004; Jiang & Ninfa, 2007; Johansson & Nordlund, 1997; Jonsson & Nordlund, 2007; Ninfa & Jiang, 2005; Wolfe *et al.*, 2007; Zhu *et al.*, 2006). These small molecules or other unidentified effector(s) might also be able to bind *R. rubrum* NifA to modulate its activity directly. It has been reported that  $\alpha$ -KG binds the N-terminal GAF domain of *A. vinelandii* NifA to prevent its inactivation by NifL (Little & Dixon, 2003). NifA belongs to the AAA+ superfamily of ATPases (Zhang *et al.*, 2002), so ATP is also a candidate effector. We also noticed previously that  $\text{NH}_4^+$  addition causes a more rapid loss of nitrogenase activity in  $\text{MN}^-$ -grown cells than in MG-grown cells (Zhang *et al.*, 2000, 2005). These results suggest that changes in pools of these effector(s) might affect the DRAT–DRAG regulatory system, which is also regulated by  $\text{P}_{\text{II}}$  (Huelgo *et al.*, 2006, 2007; Zhang *et al.*, 2001b, 2003). Our unsuccessful attempt to use Tn5 to find other genes whose elimination allowed NifA to be activated in the absence of GlnB suggests that knockout mutations cannot alter the level of effector(s) significantly to cause the activation of NifA.

Previous studies indicated that the N-terminal GAF domain of NifA in *A. brasilense* and *Herbaspirillum seropedicae* plays an important role in regulation. The N-terminal GAF domain of *A. brasilense* NifA interacts directly with GlnB, as detected using a yeast two-hybrid system (Chen *et al.*, 2005). We also found interaction between the N-terminal GAF domain of *R. rubrum* NifA and GlnB by this method (data not shown). Deletion of this GAF domain in *A. brasilense* and *H. seropedicae* NifA altered NifA regulation, and the truncated

NifA of *A. brasilense* showed significant NifA activity in *glnB* mutant backgrounds that prevent wild-type NifA activation (Arsène *et al.*, 1999). The activity of truncated NifA of *H. seropedicae* was no longer regulated by  $\text{NH}_4^+$  (Souza *et al.*, 1999). Several tyrosine residues in the N-terminal region of *A. brasilense* NifA have also been suggested to be involved in NifA regulation (Arsène *et al.*, 1999; Chen *et al.*, 2005). However, *R. rubrum* NifA with a Y29F substitution (Y29 is the only conserved tyrosine residue in the N-terminal domain of *R. rubrum* NifA) was inactive in *glnB* and *glnD* backgrounds (data not shown), indicating that this residue is unimportant in *R. rubrum* NifA regulation. When we constructed five different truncations of the GAF domain in *R. rubrum* NifA (residues 1–57, 1–106, N–172, 2–243, and 2–220) NifA activity was completely abolished, but we cannot rule out the possibility of indirect effects on protein stability (data not shown).

In summary, we have identified several substitution mutations in the GAF domain of *R. rubrum* NifA, allowing NifA to be activated in the absence of GlnB. The activities of two NifA variants, NifA-M173I and NifA-M173V, are no longer responsive to  $\text{NH}_4^+$ . With the addition of the mutation in *draT*, which abolishes the post-translational regulation of nitrogenase activity, *nifA draT* double mutants were able to fix nitrogen and produce hydrogen under  $\text{NH}_4^+$ -excess conditions, and thus would be potentially useful for the production of biohydrogen.

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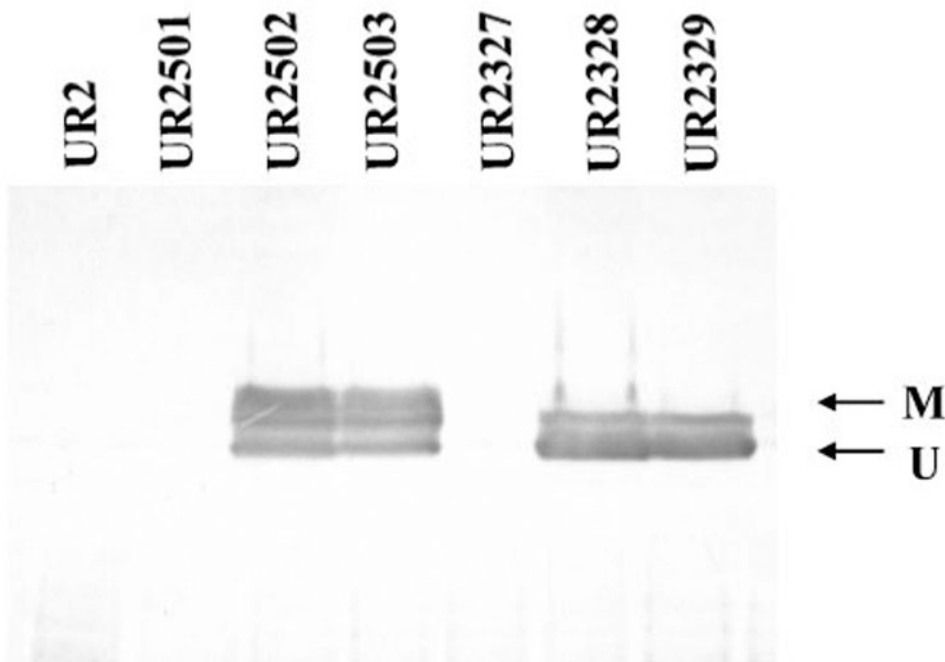
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## Abbreviations

<b>3-AT</b>	3-amino-1,2,4-triazole
<b>α-KG</b>	α-ketoglutarate



**Fig. 1.**

Immunoblot of the dinitrogenase reductase in UR2 (wild-type), UR2501 (wild-type NifA in wild-type background), UR2502 (NifA-M173I in wild-type background), UR2503 (NifA-M173V in wild-type background), UR2327 (wild-type NifA in *draT* mutant background), UR2328 (NifA-M173I in *draT* mutant background) and UR2329 (NifA-M173V in *draT* mutant background) when cells were grown in MN ( $\text{NH}_4^+$ -excess) medium. Proteins were extracted quickly with TCA, separated by SDS-PAGE, and immunoblotted with antibody against *A. vinelandii* dinitrogenase reductase. As described in Methods, active dinitrogenase reductase is unmodified and migrates as a single band (indicated by U), while inactive dinitrogenase reductase migrates as two bands with the upper band representing the modified subunit (indicated by M). A third band between the unmodified and the modified bands might be an alternative dinitrogenase reductase or another unmodified form of dinitrogenase reductase.



**Table 1**  
**Location of substitution(s) in *nifA***

Plasmid	Location of residues (substitutions) in NifA *
<b>First screening:</b>	
pMNA-2-1	<b>M173I</b> (ATG→ATT), I196F, I217I, L418L, T516M
pMNA-5-1	No mutation
pMNA-11-1 and 19-1	<b>M173V</b> (ATG→GTG), D126Y, K208R, E413E
pMNA-12-1	<b>M173V</b> (ATG→GTG), V48G, T64S, R156H, P282T, E324A, R438H, G524D
pMNA-14-5 and 23-4	L41I, V96I, V132M, S241P, A360A, A376V, V473A
pMNA-24-1	<b>M173I</b> (ATG→ATA), P116L, A482V, M428L
pMNA-26-4	D126D, P146P
<b>Second screening:</b>	
pMNA-2-2	V96A, S183R
pMNA-3-2	<b>M173V</b> (ATG→GTG), S16P, A376A
pMNA-4-1 and 7-1	<b>M173I</b> (ATG→ATT), A200A, R481F
pMNA-8-6	<b>M173I</b> (ATG→ATC), Y380Y
pMNA-9-2	<b>M173I</b> (ATG→ATT), V75A, K275K
pMNA-10-1	S32T, L486P
pMNA-12-2	E321N, F329F
pMNA-14-4 and 26-3	<b>M173V</b> (ATG→GTG), S22S, A243T, P511T
pMNA-15-4 and 16-4	<b>M173I</b> (ATG→ATA)
pMNA-17-5	<b>M173I</b> (ATG→ATA), T370A
pMNA-18-2	<b>M173V</b> (ATG→GTG), G126N, P207L
pMNA-20-3	<b>M173I</b> (ATG→ATT), Y522C
pMNA-24-2	<b>M173V</b> (ATG→GTG)
pMNA-27-1	<b>M173I</b> (ATG→ATT), K591N
pMNA-28-1	<b>M173I</b> (ATG→ATT), V86A
pMNA-30-1	<b>M173I</b> (ATGAATC), Y380Y, G556V

\* The substitutions and codon changes at the M173 residue are shown in bold type. Base substitutions that did not change the encoded amino acid are also indicated (e.g. I217I).

**Table 2**  
**Nitrogenase activity in the *glnB* or the *glnD* mutant with wild-type or altered *nifA* integrated into the chromosome when grown in MG (nitrogen-limiting) medium**

UR strain	<i>nifA</i> integrated into DglnB or DglnD background	Nitrogenase activity*
UR1739	Wild-type <i>nifA</i> integrated into <i>glnB</i> mutant (UR717)	<10
UR1740	<i>nifA11</i> (encoding NifA-M173I) integrated into <i>glnB</i> mutant (UR717)	470
UR1741	<i>nifA12</i> (encoding NifA-M173V) integrated into <i>glnB</i> mutant (UR717)	450
UR1742	Wild-type <i>nifA</i> integrated into <i>glnD</i> mutant (UR1325)	<10
UR1743	<i>nifA11</i> (encoding NifA-M173I) integrated into <i>glnD</i> mutant (UR1325)	200
UR1744	<i>nifA12</i> (encoding NifA-M173V) integrated into <i>glnD</i> mutant (UR1325)	230

\* Each unit of nitrogenase activity is expressed as nanomoles ethylene produced per hour per millilitre of cells at an OD<sub>600</sub> of 1. Each activity value is from at least five replicate assays from different individually grown cultures. SD, 5–15 %.

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**Table 3**  
**Nitrogenase activity in *R. rubrum* wild-type or *draT* mutant with wild-type or altered *nifA* integrated into the chromosome when grown in MG (nitrogen-limiting) and MN (NH<sub>4</sub><sup>+</sup>-excess) media**

Strain	<i>nifA</i> integrated into wild-type or <i>draT</i> background	Nitrogenase activity*	
		MG (nitrogen-limiting)	MN (NH <sub>4</sub> <sup>+</sup> -excess)
UR2	Wild-type	800	<10
UR2501	Wild-type with wild-type <i>nifA</i>	750	<10
UR2502	Wild-type with <i>nifA11</i> (encoding NifA-M173I)	660	30
UR2503	Wild-type with <i>nifA12</i> (encoding NifA-M173V)	770	30
UR2327	<i>draT</i> mutant with wild-type <i>nifA</i>	610	<10
UR2328	<i>draT</i> mutant with <i>nifA11</i> (encoding NifA-M173I)	660	450
UR2329	<i>draT</i> mutant with <i>nifA12</i> (encoding NifA-M173V)	500	540

\* Units of nitrogenase activity are expressed as in Table 2. Each activity value is from at least five replicate assays from different individually grown cultures. SD, 5–15 %.

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**Table 4**  
**Nitrogenase activity in *R. rubrum* strains containing single or multiple copies of *R. rubrum nifA* when grown in MG (nitrogen-limiting) or MN<sup>-</sup> (NH<sub>4</sub><sup>+</sup> -free) media**

Strain	Plasmid (gene)	Nitrogenase activity <sup>*</sup>	
		MG (nitrogen-limiting) <sup>†</sup>	MN <sup>-</sup> (NH <sub>4</sub> <sup>+</sup> -free) <sup>‡</sup>
UR2 (wild-type)	None (single copy of <i>R. rubrum nifA</i> )	800	400
UR717 ( <i>glnB3</i> )	None (single copy of <i>R. rubrum nifA</i> )	<10	<10
UR741 (wild-type)	pYPZ239 (multicopy of <i>R. rubrum nifA</i> )	650	300
UR744 ( <i>glnB3</i> )	pYPZ239 (multicopy of <i>R. rubrum nifA</i> )	50	280

<sup>\*</sup> Units of nitrogenase activity are expressed as in Table 2.

<sup>†</sup> Nitrogenase activities of these strains in MG medium are similar to those recorded previously (Zhang *et al.*, 2001a).

<sup>‡</sup> UR717 grew very slowly in MN<sup>-</sup> medium, with a final OD<sub>600</sub> of 0.3 after 2 days of growth, while UR2 and UR741 had a final OD<sub>600</sub> of ~3. UR744 grew slightly slower in MN<sup>-</sup> medium, with a final OD<sub>600</sub> of 1.7.

**Table 5**  
**Loci of Tn5 insertion and substitutions in *nifA* in *R. rubrum* mutants**

Tn5 insertion locus*	ORF*	Substitution in <i>nifA</i>
4194344 (9) <sup>†</sup>	Between Rru_A3654 and Rru_A3654	A243T (2) <sup>‡</sup>
40371 (3)	Rru_B0039	M173I (2)
2738176 (5)	Rru_A2353	L184R (2)
1929051 (3)	Rru_A1638	M173I
1974362 (3)	Rru_A1681	G36E
1113948 (21)	Rru_A0934	M173I
3073513 (1)	Rru_A2643	G36E
2087876 (1)	Rru_A1795	No mutation in <i>nifA</i>
3405657 (1)	Rru_A2956	M173I
3047404 (1)	Rru_A2619	M173L
3533303 (1)	Rru_A3067	M173V
3336134 (1)	Rru_A2892	T38P
1264071 (1)	Rru_A1073	T38P
184684 (1)	Rru_A0158	M173V

\* The Tn5 insertion loci and its ORF are based on the *R. rubrum* genomic sequence at [http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Overview&list\\_uids=19059](http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Overview&list_uids=19059).

<sup>†</sup>The numbers in parentheses indicate the number of strains that were sequenced and had identical loci of Tn5 insertion.

<sup>‡</sup>The numbers in parentheses indicate the number of strains in which *nifA* was sequenced.

**Table 6**  
**Nitrogenase activity in *R. rubrum* *glnB* mutants with NifA variants when grown in MG (nitrogen-limiting) medium**

Strain	<i>nifA</i> integrated into <i>glnB</i> mutant (UR717)	Nitrogenase activity*
UR2	Wild-type	800
UR1739	Wild-type <i>nifA</i> integrated into <i>glnB</i> mutant	<10
UR1740	<i>nifA11</i> (encoding NifA-M173I) integrated into <i>glnB</i> mutant	470
UR1741	<i>nifA12</i> (encoding NifA-M173V) integrated into <i>glnB</i> mutant	450
UR11742	<i>nifA23</i> (encoding NifA-M173L) integrated into <i>glnB</i> mutant	450
UR11743	<i>nifA24</i> (encoding NifA-L184R) integrated into <i>glnB</i> mutant	400
UR11744	<i>nifA25</i> (encoding NifA-A243T) integrated into <i>glnB</i> mutant	500
UR11745	<i>nifA26</i> (encoding NifA-G36E) integrated into <i>glnB</i> mutant	220
UR11746	<i>nifA27</i> (encoding NifA-T38P) integrated into <i>glnB</i> mutant	310

\* Units of nitrogenase activity are expressed as in Table 2.

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