Effect of point mutations on *Herbaspirillum seropedicae* NifA activity

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### Abstract

NifA is the transcriptional activator of the *nif* genes in Proteobacteria. It is usually regulated by nitrogen and oxygen, allowing biological nitrogen fixation to occur under appropriate conditions. NifA proteins have a typical three-domain structure, including a regulatory N-terminal GAF domain, which is involved in control by fixed nitrogen and not strictly required for activity, a catalytic AAA+ central domain, which catalyzes open complex formation, and a C-terminal domain involved in DNA-binding. In *Herbaspirillum seropedicae*, a β-proteobacterium capable of colonizing Gramineae of agricultural importance, NifA regulation by ammonium involves its N-terminal GAF domain and the signal transduction protein GlnK. When the GAF domain is removed, the protein can still activate *nif* genes transcription; however, ammonium regulation is lost. In this work, we generated eight constructs resulting in point mutations in *H.* *seropedicae* NifA and analyzed their effect on *nifH* transcription in *Escherichia coli* and *H.* *seropedicae*. Mutations K22V, T160E, M161V, L172R, and A215D resulted in inactive proteins. Mutations Q216I and S220I produced partially active proteins with activity control similar to wild-type NifA. However, mutation G25E, located in the GAF domain, resulted in an active protein that did not require GlnK for activity and was partially sensitive to ammonium. This suggested that G25E may affect the negative interaction between the N-terminal GAF domain and the catalytic central domain under high ammonium concentrations, thus rendering the protein constitutively active, or that G25E could lead to a conformational change comparable with that when GlnK interacts with the GAF domain.

Key words: Biological nitrogen fixation; *Herbaspirillum seropedicae*; NifA

### Introduction

Biological nitrogen fixation is a process carried out by some prokaryotes that reduces dinitrogen (N₂) to ammonia (NH₃) in a reaction catalyzed by the nitrogenase complex. It is highly energy-demanding and is thus controlled at both transcriptional and translational levels (1). Transcription of the *nif* genes, which encode the nitrogenase complex and all gene products necessary to assemble an active enzyme, is controlled by NifA in response to ammonium and oxygen levels. NifA is a σ54-dependent transcriptional activator that shows a typical three-domain structure. The N-terminal GAF domain shows the lowest similarity among NifA homologs, and is involved in ammonium control. The central AAA+ domain interacts with the σ54-RNA polymerase and possesses ATPase activity, while the C-terminal domain shows a helix-turn-helix motif involved in DNA-binding. Two linkers connect these domains: the Q-linker connects GAF and central domains, and the ID-linker connects the central and C-terminal domains.

NifA proteins are separated into two classes based on their regulation by ammonium and oxygen (1). One class occurs in γ-Proteobacteria and is regulated by the anti-activator NifL, while the second class is observed in β-Proteobacteria, where NifL is absent. Nitrogen regulation by both mechanisms involves a PII-like protein and interaction with either NifL or NifA (2). In contrast, oxygen control differs between these two mechanisms. In γ-Proteobacteria, NifL senses oxygen levels through a flavin moiety (3), whereas in NifL-independent regulation, oxygen control is hypothesized to involve a putative Fe-S cluster associated with a cysteine tetrad located at the end of the central domain and ID-linker (4).

*Herbaspirillum seropedicae* is a nitrogen-fixing β-proteobacterium associated with important agricultural Gramineae, such as rice, wheat, sorghum, and sugarcane (5). Transcriptional control of nitrogen fixation in *H. seropedicae* relies on a NifL-independent NifA system that is controlled by both nitrogen and oxygen levels (6).
Recently the regulation of nitrogen fixation in this organism has been reviewed (7).

The _H. seropedicae_ NifA N-terminal GAF domain comprises the first 184 amino acids (Figure 1), and although it is involved in negative regulation by ammonium, it is not strictly required for activation of _nif_ gene transcription (6,8). This N-terminal GAF domain interacts with GlnK in response to the fixed nitrogen concentration (9). The N-terminal GAF domain is linked to the central domain by the 18 amino acids of the Q-linker. The _H. seropedicae_ NiF central domain comprises 236 amino acids and contains the catalytic site. It also interacts with the ОНГ RNA polymerase subunit (4). The central region of _H. seropedicae_ is linked to the C-terminal domain by a 58-amino acid region named the ID-linker. A conserved cysteine motif located at the end of the central domain and the ID-linker (positions 414, 426, 446, and 451) is suggested to be involved in the regulation of NifA by O2. Mutation of these cysteine residues produces inactive proteins (10). Finally, the last 43 amino acids of the NifA primary sequence form the C-terminal domain, which is responsible for DNA binding (11).

In this work, site-directed mutagenesis was used to determine amino acid residues in _H. seropedicae_ NifA that are important for its control.

**Material and Methods**

**Reagents**

All chemicals were analytical or molecular biology grade and were purchased from Merck (Germany), Sigma (USA), J.T. Baker (Netherlands), or Invitrogen (USA). Restriction enzymes were obtained from Fermentas (Lithuania) or Invitrogen. Oligonucleotides were synthesized by IDT (USA).

**Bacterial strains and growth conditions**

_E. coli_ strains TOP10 (Invitrogen) and S17.1 (12) were used for cloning and conjugation procedures. _E. coli_ were cultured at 37°C in Luria-Bertani broth, terrific broth, super optimal broth (SOB), or SOB with catabolite repression (SOC) broth media (13). _H. seropedicae_ strains (Table 1) were grown in NFbHP medium at 30°C (14) with 37 mM malate and 20 mM NH₄Cl or 0.5 mM glutamate.

**Site-directed mutagenesis**

Point mutations were introduced into the _H. seropedicae_ _nifA_ gene using mutagenic primers (Supplementary Table S1) as described previously (15). The mutated genes were then cloned into pET-29a, using Ndel and BamHI restriction sites, or into pLAFR3.18 (XbaI/HindIII restriction sites) for activity analyses in _E. coli_ or _H. seropedicae_, respectively. These plasmids are listed in Table 1.

**Construction of _H. seropedicae_ mutants**

The _nifA_ gene, cloned into pRAM177 as an Ndel/BamHI fragment, was digested with _Ec_RI to remove 750 bp from the central region of _nifA_. Following re-ligation, the resulting plasmid (pBA3) was digested with _BamHI_ and a _sacB::Km_ cassette, obtained as a _BamHI_ fragment from pHM1701, was introduced into pBA3, producing pBA4. This plasmid was then introduced into _H. seropedicae_ SmR1 (wild type) and LNglnKdel, a _glnK_ mutant (16), by electroporation (10 kV/cm, 4 μF, 330 μF, using a Gibco Cell-Porator, USA). Transformed cells were first selected by growth in NFbHP medium with 1 mg/mL kanamycin, and then by survival in NFbHP medium with 15% (w/v) sucrose to obtain mutants with a second recombination. The _nifA_ mutation was confirmed by DNA amplification using _1U Taq DNA Polymerase_ (Fermentas) in _Taq_ buffer with (NH₄)₂SO₄, 3 mM MgCl₂, 0.8 mM dNTP and 0.4 μM of primers HS_nifA1 and HS_nifA2a (Supplementary Table S1) and the following parameters: one step for 5 min at 95°C and 30 cycles of 30 s at 95°C, 30 s at 45°C and 2 min at 72°C. These strains were named NifAdel and NifAdel/GlnKdel, respectively.

_H. seropedicae_ mutants carrying a chromosomal _nifHlacZ_ fusion were generated by introducing the pTZnifH::lacZ plasmid by electroporation, followed by selection for kanamycin resistance. The pTZnifH::lacZ plasmid was constructed by cloning a _lacZ::Km_ cassette at the _BamHI_ site located downstream from the 5.3-kb fragment containing part of _H. seropedicae_ _nifH_ and its promoter region in the pLAU1 plasmid. The _lacZ::Km_ cassette was obtained as a _BamHI_ fragment from the pKO6.1 plasmid.

**Protein analyses**

β-galactosidase activity was determined as described previously (17). _E. coli_ strain JM109(DE3), carrying plasmids pRT22 (_Klebsiella pneumoniae_ _nifH::lacZ_) and pET-29a with different _nifA_ mutations, was analyzed as described previously (9). The β-galactosidase activity in _H. seropedicae_ was determined as described previously (16). Nitrogenase activity was determined using cells...
Table 1. Bacterial strains and plasmids.

| Strain/Plasmid          | Characteristics                                                                 | Reference |
|------------------------|---------------------------------------------------------------------------------|-----------|
| **Escherichia coli**   |                                                                                 |           |
| JM109(DE3)             | recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB) F' [traD36 proAB+ lacRI lacZΔM15) ΔDE3 | Promega  |
| TOP10                  | F' mcrA Δ(mvr – hrd RMS – mcrBC) ψ 80 lacZ ΔML5 ΔlacX74 deoR recA1 araD139 Δ(ara – leu) 7697 galU galK rpsL (SmR) endA1 nupG | Invitrogen|
| S17.1                  | SmR, tra+ pro thi recA hsdR (RP4-2 kan::Tn7 tet::Mu)                              | 12        |
| **Herbaspirillum seropedicae** |                                                                  |           |
| SmR1                   | SmR, Nif+                                                                      | 32        |
| LNglnKdel              | SmR, SmR1 strain carrying an in frame deletion of 192 bp of the glnK gene         | 16        |
| NifAdel                | SmR, SmR1 strain carrying an in frame deletion of 750 bp of the nifA gene         | This work |
| NifAdel/GlnKdel        | SmR, LNglnKdel strain carrying an in frame deletion of 750 bp of the nifA gene     | This work |
| **Plasmids**           |                                                                                 |           |
| pBA3                   | AmpR, *H. seropedicae nifA* carrying a 750 bp deletion at its central portion from pRAM1177 plasmid | This work |
| pBA4                   | AmpR, pBA3 plasmid with a sacB::Km cassette                                      | This work |
| pCR 2.1                | AmpR, KmR, cloning vector                                                       | Life Technologies |
| pET29a                 | KmR, T7 promoter expression vector; His-tag fusion                              | Novagen   |
| pET-A215D              | KmR, contains a *H. seropedicae nifA* gene with the A215D mutation cloned into pET-29a | This work |
| pET-G25E               | KmR, contains a *H. seropedicae nifA* gene with the G25E mutation cloned into pET-29a | This work |
| pET-K22V               | KmR, contains a *H. seropedicae nifA* gene with the K22V mutation cloned into pET-29a | This work |
| pET-L172R              | KmR, contains a *H. seropedicae nifA* gene with the L172R mutation cloned into pET-29a | This work |
| pET-M161V              | KmR, contains a *H. seropedicae nifA* gene with the M161V mutation cloned into pET-29a | This work |
| pET-Q216I              | KmR, contains a *H. seropedicae nifA* gene with the Q216I mutation cloned into pET-29a | This work |
| pET-S220I              | KmR, contains a *H. seropedicae nifA* gene with the S220I mutation cloned into pET-29a | This work |
| pET-T160E              | KmR, contains a *H. seropedicae nifA* gene with the T160E mutation cloned into pET-29a | This work |
| pET-ΔN- A215D          | KmR, expresses an N-truncated NifA protein of *H. seropedicae* without its first 203 amino acids and carrying the mutation A215D; pET-29a based plasmid | This work |
| pET-ΔN-Q216I           | KmR, expresses an N-truncated NifA protein of *H. seropedicae* without its first 203 amino acids and carrying the mutation Q216I; pET-29a based plasmid | This work |
| pET-ΔN-S220I           | KmR, expresses an N-truncated NifA protein of *H. seropedicae* without its first 203 amino acids and carrying the mutation S220I; pET-29a based plasmid | This work |
| pKOK 6.1               | AmpR, CmR, KmR, contains a promoter-less lacZ-KmR cassette                       | 33        |
| pLAFR3.18              | CmR, Trc, Mob +, IncP cosmid with the pTZ18R cloning nest                         | 6,34      |
| pLAFR-A215D            | TcR, contains a *H. seropedicae nifA* gene with the A215D mutation cloned into pLAFR3.18 | This work |
| pLAFR-G25E             | TcR, contains a *H. seropedicae nifA* gene with the G25E mutation cloned into pLAFR3.18 | This work |
| pLAFR-K22V             | TcR, contains a *H. seropedicae nifA* gene with the K22V mutation cloned into pLAFR3.18 | This work |

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grown in semi-solid NFbHP medium containing glutamate (0.5 mM). Protein concentration was determined using the Bradford method (18) with bovine serum albumin as a standard.

Results

In this work, we generated eight constructs that introduced NifA point mutations (K22V, G25E, T160E, M161V, L172R, A215D, Q216I, and S220I) and analyzed their effects on transcriptional activation activity. Four mutations were based on described NifA mutations from *Rhodospirillum rubrum* (19) and *Sinorhizobium meliloti* (20), while the other four amino acids were chosen from among conserved residues (Supplementary Table S2). Residues were selected for mutagenesis by aligning NifA proteins from *K. pneumoniae*, *Azoarcus* sp., and *Azotobacter vinelandii*, which are regulated by NifL, and from *H. seropedicae*, *R. rubrum*, *S. meliloti*, *Rhodobacter capsulatus*, *Bradyrhizobium japonicum*, and *Azospirillum brasilense*, which are regulated in a NifL-independent manner (Supplementary Table S2). The mutations were located in the N-terminal GAF domain (K22V, G25E, T160E, M161V, and L172R) and the central domain (A215D, Q216I, and S220I) of *H. seropedicae* NifA (Figure 1). The secondary structure of each mutant protein was predicted using the Psipred tool (21), which indicated no major differences in secondary structure between the mutants and wild-type NifA (data not shown).

The ability of the *H. seropedicae* NifA mutants to activate *nif* promoters was determined in *E. coli* JM109 (DE3) carrying plasmid pRT22 (*K. pneumoniae nifH::lacZ* fusion) (Figure 2). Full-length NifA, expressed from plasmid pRAM1, showed no β-galactosidase activity, consistent with previous descriptions, mainly because of lower expression of endogenous *E. coli* PII, which is necessary to relieve the negative control of the N-terminal GAF domain on the catalytic domain of NifA (7). In contrast, the N-terminal truncated NifA protein (∆N-NifA) expressed from pRAM2 was fully functional in *E. coli* regardless of the ammonium concentration (22). These results confirmed the regulatory role of the N-terminal GAF domain on *H. seropedicae* NifA that has been described previously: in the presence of ammonium or the absence of PII, the N-terminal GAF inhibits NifA transcriptional activity (6,23). The constructed NifA point mutants

| Strain/Plasmid | Characteristics | Reference |
|---------------|-----------------|-----------|
| pLAFR-L172R   | Te<sup>R</sup>, contains a *H. seropedicae* nifA gene with the L172R mutation cloned into pLAFR3.18 | This work |
| pLAFR-M161V   | Te<sup>R</sup>, contains a *H. seropedicae* nifA gene with the M161V mutation cloned into pLAFR3.18 | This work |
| pLAFR-Q216I   | Te<sup>R</sup>, contains a *H. seropedicae* nifA gene with the Q216I mutation cloned into pLAFR3.18 | This work |
| pLAFR-S220I   | Te<sup>R</sup>, contains a *H. seropedicae* nifA gene with the S220I mutation cloned into pLAFR3.18 | This work |
| pLAFR-T160E   | Te<sup>R</sup>, contains a *H. seropedicae* nifA gene with the T160E mutation cloned into pLAFR3.18 | This work |
| pLAU1         | Amp<sup>R</sup>; pTZ18R-based plasmid contains a 5.3 kb fragment of *H. seropedicae* including part of the nifH gene and its promoter region | 35 |
| pMH1701       | Km<sup>R</sup>, expresses an N-truncated NifA protein of *H. seropedicae* without its first 185 amino acids upon its own promoter; pLAFR3.18 based plasmid | 36 |
| pnnifAN185    | Km<sup>R</sup>, *H. seropedicae* nifA into pET29a vector | Stefanello A.A. (unpublished) |
| pRAM1         | Km<sup>R</sup>, *H. seropedicae* nifA into pET29a vector | 8 |
| pRAM1T7       | Amp<sup>R</sup>, *H. seropedicae* nifA removed as a Ndel/BamHI fragment from pRAM1 and cloned into pT7-7 vector | 37 |
| pRAM2         | Km<sup>R</sup>, expresses an N-truncated NifA protein of *H. seropedicae* without its first 203 amino acids; pET29a based plasmid | 8 |
| pRAMM1        | Te<sup>R</sup>, *H. seropedicae* nifA into pLAFR3.18 vector | 16 |
| pRT22         | Cm<sup>R</sup>, *K. pneumoniae nifH::lacZ* | 38 |
| pT7-7         | Amp<sup>R</sup>, T7 promoter expression vector | Biolabs |
| pTZ18R        | Amp<sup>R</sup>, cloning vector carrying lac promoter | 39 |
| pTZnifH::lacZ | Amp<sup>R</sup>, Km<sup>R</sup>, contains a *H. seropedicae* nifH::lacZ fusion; pLAU1 based plasmid | This work |

Amp<sup>R</sup>, Cm<sup>R</sup>, Km<sup>R</sup>, Te<sup>R</sup>: resistance to ampicillin, chloramphenicol, kanamycin or tetracycline, respectively.
were analyzed under the same conditions and showed no activity, except for NifA G25E, which partially activated nifH transcription in E. coli. G25E also appeared to retain some nitrogen control, as activation of transcription was higher under low ammonium concentrations. This result indicated that the G25E substitution affected the need for PII for NifA activity in H. seropedicae.

Considering that A215D, Q216I, and S220I are located in the central domain of NifA, and that the full-length protein is inactive in E. coli (6) (Figure 2), these three mutations were also tested using an N-truncated form (GAF truncated protein) (Figure 3). The removal of the first 203 amino acids of NifA yields an active protein in E. coli (8), as shown using protein expressed from plasmid pRAM2. The N-truncated protein (ΔN-NifA) was active regardless of the nitrogen level, but only under low O₂, reinforcing its sensitivity toward O₂. The N-truncated ΔN-Q216I and ΔN-S220I mutants showed lower β-galactosidase activity than that expressed by pRAM2, indicating that these mutations negatively affect transcriptional activity, while retaining O₂ responsiveness. In contrast, ΔN-A215D was inactive under all tested conditions, suggesting that a negatively-charged amino acid at position 215 affects the catalytic activity of the protein. These proteins were expressed under all conditions tested, as determined by gel electrophoresis (data not shown). The three disrupted amino acids are close to the ATP-binding site, which is located at positions 231-238.

In contrast to H. seropedicae, a NifA strain with a mutation in this region (M217I) in S. meliloti was oxygen tolerant (20).

The point mutations were also analyzed in an H. seropedicae background. Assuming that a functional NifA variant leads to nif gene transcription, nitrogenase activity can be determined. However, for these assays it was necessary to construct two H. seropedicae mutant strains: a nifA mutant strain and a double mutant nifA/ glnK, both of which were obtained by partial gene deletion. These strains were named NifAdel and NifAdel/GlnKdel, respectively. The nifA/glnK double mutant allowed detection of a NifA mutant that does not require GlnK for activity, as this PII protein is responsible for relieving the nitrogen-regulated negative control of NifA (16). These H. seropedicae mutant strains showed no nitrogenase activity (acetylene reduction method; Table 2) (24, 25). However, the nitrogenase activity was restored in the pRAMM1-carrying NifAdel strain, which expresses the full-length NifA, and in the NifAdel/GlnKdel strain carrying pppN185, which expressed an N-truncated form of NifA (Table 2).

To analyze the effect of NifA mutations on nitrogenase activity, each construct was cloned into the pLAFR3.18 vector, which is stable in H. seropedicae, and transformed into both the NifAdel and NifAdel/GlnKdel strains. Assays performed with NifAdel showed that the G25E mutant was fully active, Q216I was partially active, and the other mutants showed no significant nitrogenase activity (Table 2). However, NifA levels similar to those of the wild type were expressed from pRAMM1, while the Q216I mutant did not show any nitrogenase activity in the absence of GlnK (assay in NifAdel/GlnKdel strain). In contrast, the G25E mutant demonstrated nitrogenase activity, which implied that G25E was active and does not require GlnK for activity.

The G25E mutation was also tested in the NifAdel and NifAdel/GlnKdel strains carrying a nifH::lacZ chromosomal fusion, which allowed assessment of transcriptional NifA activity in the presence of high ammonium concentrations (Figure 4). The wild-type H. seropedicae strain (SmR1) carrying the nifH::lacZ fusion only showed β-galactosidase activity at low ammonium concentrations. Conversely, the G25E mutant showed nifH::lacZ transcription in both the NifAdel and NifAdel/GlnKdel strains, regardless of ammonium concentration. However, comparison of β-galactosidase activity at both low and high ammonium concentrations indicated that the G25E mutant protein was partially regulated by ammonium, as transcriptional activity was higher at low ammonium concentrations. This result suggested that the G25E mutant did not depend on GlnK for activity, but could still detect ammonium concentration.

**Discussion**

H. seropedicae NifA is regulated by both ammonium and oxygen (6). The effect of O₂ on the NifA protein is
related to a putative Fe-S cluster involving four cysteine residues located at the end of the central domain and the ID-linker. These conserved cysteine residues are found in all NifA proteins that are directly sensitive to oxygen, but absent in NifA proteins that depend on NifL for oxygen control (4). In *H. seropedicae*, mutation of the conserved cysteine residues rendered inactive proteins (10). Conversely, Krey et al. (20) obtained a *S. meliloti* NifA mutant (M217I) that was active even under high O2 levels. Using sequence alignment, we determined the corresponding amino acid residue in *H. seropedicae* to be serine 220. The NifA S220I mutation resulted in lower activity in *H. seropedicae* (Table 2) and partial activity in *E. coli* (Figure 3), but retained sensitivity toward O2, indicating a difference in behavior compared with *S. meliloti* NifA M217I.

Two further amino acid residues close to S220 in *H. seropedicae* NifA were also mutated and analyzed. The A215D mutation was benign in both *H. seropedicae* and *E. coli*, even if an N-truncated form was used. However, the N-truncated protein form carrying the Q216I mutation showed transcriptional activity dependent on O2. This mutant Q216I *H. seropedicae* NifA strain also produced an active nitrogenase complex dependent on the GlnK protein, similar to the wild type. These results indicate that Q216I and S220I retained regulatory activities similar to the wild type, although with lower activity. Conversely, because no transcription from strains carrying the A215D mutation was observed under any of the conditions tested, the alanine residue at position 215 is likely to be essential for activity. Alternatively, a negative charge at position 215 may be more deleterious for *H. seropedicae* NifA compared with the previous substitutions. Mutations M161V and L172R in *H. seropedicae* NifA correspond to mutations M173V and L184R described previously in *R. rubrum* (19). Analysis carried out using a yeast two-hybrid system showed that RlM173V produced a protein with stronger GlnB interaction, whereas the RlL184R mutant did not require GlnB for activity. In *R. rubrum*, GlnB is the PII protein responsible for controlling NifA activity (26). The *H. seropedicae* NifA M161V mutant was inactive in all conditions tested, while the L172R mutant showed very low nitrogenase activity, indicating that these amino acids are important for the overall NifA activity.

*H. seropedicae* differs from *R. rubrum* in that nitrogen regulation depends on GlnK (16). Among the eight *H. seropedicae* NifA mutations investigated in this work, the G25E mutation rendered an active NifA protein that did not require GlnK. Mutation G25E in *H. seropedicae* corresponds to G36E in *R. rubrum*, which also produced a partial active NifA independent of GlnB (19). The G25E mutation may affect the negative regulatory interaction between the N-terminal GAF domain and the catalytic central domain under high ammonium concentrations, resulting in a constitutively active protein. Furthermore, the glutamate residue at position 25 could lead to a

### Table 2. Effect of NifA mutations on *H. seropedicae* nitrogenase activity.

| Construct         | *Herbaspirillum seropedicae* strains |
|-------------------|--------------------------------------|
|                   | NifAdel | NifAdel/GlnKdel |
| pRAMM1            | 3.3 ± 1.5 | 0.05 ± 0.09 |
| ppnifAN185        | nd      | 10 ± 7 |
| K22V              | 0       | 0 |
| G25E              | 19 ± 6  | 13 ± 2 |
| T160E             | 0       | 0 |
| M161V             | 0       | 0 |
| L172R             | 0.15 ± 0.04 | 0 |
| A215D             | 0       | 0 |
| Q216I             | 2.7 ± 0.6 | 0 |
| S220I             | 0.58 ± 0.07 | 0 |

Nitrogenase activity was determined as described using strains NifAdel and NifAdel/GlnKdel grown in semi-solid medium. Strains carrying plasmids expressing wild-type NifA (pRAMM1), an N-truncated NifA (ppnifAN185), or the indicated NifA mutants expressed from pLAFR3.18-based plasmids were assayed. Data are reported as the mean ± SD of at least 3 independent experiments. Nitrogenase activity is reported as nmol ethylene · mg protein⁻¹ · min⁻¹. The nitrogenase activity in the *H. seropedicae* SmR1 strain (wild type) was 13 ± 1 nmol ethylene · mg protein⁻¹ · min⁻¹. nd: not determined.
allowed us to determine the NifA transcriptional activity
10 mM NH$_4$Cl under aerobic conditions at 30°C. Cells were then
expressing the G25E NifA mutant; (−) indicates absence of plasmid. Cells were grown in NFbHP medium supplemented with
10 mM NH$_4$Cl under aerobic conditions at 30°C. Cells were then
centrifuged (1700 g for 2 min), resuspended in NFbHP (nitrogen-
free) medium, and de-repressed for 7 h under 1.5% oxygen.
β-galactosidase was determined as described. Data are reported
as the mean±SD of at least 3 independent experiments. β-galactosidase activity is reported as Miller units.

![Graph](image)

**Figure 4.** Transcriptional activity of indicated *Herbaspirillum seropedicae* strains carrying a chromosomal nifH:lacZ fusion. + G25E indicates cells carrying a pLAFR3.18-based plasmid expressing the G25E NifA mutant; (−) indicates absence of plasmid. Cells were grown in NFbHP medium supplemented with 10 mM NH$_4$Cl under aerobic conditions at 30°C. Cells were then centrifuged (1700 g for 2 min), resuspended in NFbHP (nitrogen-free) medium, and de-repressed for 7 h under 1.5% oxygen. β-galactosidase was determined as described. Data are reported as the mean±SD of at least 3 independent experiments. β-galactosidase activity is reported as Miller units.

conformational change comparable with that produced when GlnK interacts with the N-terminal GAF domain.

The G25E mutant was also analyzed using a nifH:lacZ chromosomal fusion in *H. seropedicae* (Figure 4). This allowed us to determine the NifA transcriptional activity even in the presence of high ammonium concentrations, a condition where nitrogenase activity is not observed (16). The assay showed that the G25E mutant is active in the absence of GlnK, as observed by nitrogenase activity, but also showed partial regulation by fixed nitrogen, with higher transcriptional activity under low ammonium concentrations than in the presence of high ammonium concentrations (Figure 4). The partial regulation by fixed nitrogen was also observed in assays performed in *E. coli* (Figure 2).

The observed ammonium regulation could be related to the GAF domain, which has been shown to bind small molecules such as cyclic nucleotides and 2-oxoglutarate (27). In *A. vinelandii*, formation of the NifL-NifA complex is prevented by the binding of 2-oxoglutarate to the NifA GAF domain (28). Although it has not been confirmed that the N-terminal GAF domain of *H. seropedicae* NifA binds small molecules, the possibility that a small molecule such as 2-oxoglutarate may interact with the protein, signaling a cellular deficit of fixed nitrogen, cannot be ruled out.

**Supplementary material**

Click here to view [pdf].

**Acknowledgments**

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