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Regulation of Nitrogen Fixation in Bradyrhizobium sp. Strain DOA9 Involves Two Distinct NifA Regulatory Proteins That Are Functionally Redundant During Symbiosis but Not During Free-Living Growth

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The Bradyrhizobium sp. DOA9 strain displays the unusual properties to have a symbiotic plasmid and to fix nitrogen during both free-living and symbiotic growth. Sequence genome analysis shows that this strain contains the structural genes of dinitrogenase (nifDK) and the nifA regulatory gene on both the plasmid and chromosome. It was previously shown that both nifDK clusters are differentially expressed depending on growth conditions, suggesting different mechanisms of regulation. In this study, we examined the functional regulatory role of the two nifA genes found on the plasmid (nifAp) and chromosome (nifAc) that encode proteins with a moderate level of identity (55%) and different structural architectures. Using gusA (β-glucuronidase) reporter strains, we showed that both nifA genes were expressed during both the free-living and symbiotic growth stages. During symbiosis with Aeschynomene americana, mutants in only one nifA gene were not altered in their symbiotic properties, while a double nifA mutant was drastically impaired in nitrogen fixation, indicating that the two NifA proteins are functionally redundant during this culture condition. In contrast, under in vitro conditions, the nifAc mutant was unable to fix nitrogen, and no effect of the nifAp mutation was detected, indicating that NifAc is essential to activate nif genes during free-living growth. In accordance, the nitrogenase fixation deficiency of this mutant could be restored by the introduction of nifAc but not by nifAp or by two chimeric nifA genes encoding hybrid proteins with the N-terminus part of NifAc and the C-terminus of NifAp. Furthermore, transcriptional analysis by RT-qPCR of the WT and two nifA mutant backgrounds showed that NifAc and NifAp activated the expression of both chromosome and plasmid structural nifDK genes during symbiosis, while only NifAc activated the expression of nifDKc during free-living conditions. In summary, this study provides a better overview of the complex mechanisms of regulation of the nitrogenase genes in the DOA9 strain that involve two distinct NifA proteins, which are exchangeable during symbiosis for the activation of nif genes but not during free-living growth where NifAc is essential for the activation of nifDKc.

Keywords: NifA, Bradyrhizobium, symbiosis, legume, nitrogen, nitrogenase, Rhizobium
INTRODUCTION

Rhizobium-legume symbiosis is considered as the major contributor of biologically fixed nitrogen to terrestrial ecosystems. The reduction of atmospheric N\(_2\) is catalyzed by the nitrogenase enzyme complex, which requires high-energy input in the form of ATP and electrons to break the triple bond. In addition, this enzymatic complex is highly sensitive to molecular oxygen, which irreversibly inactivates the enzyme. Diazotrophic bacteria have evolved sophisticated regulatory circuits of their nitrogen fixation (nif) genes in response to oxygen and nitrogen availability to prevent unnecessary energy consumption and permit the synthesis of the nitrogenase complex only during the proper environmental conditions (Burris and Roberts, 1993; Fischer, 1994).

A master regulator of nitrogen fixation is the NifA protein, which acts in association with the RNA polymerase sigma factor RpoN (\(\sigma^{54}\)) to activate the expression of nif genes by binding to an upstream activating sequence (UAS; 5'-TGT-N\(_{10}\)-ACA-3'). The NifA proteins show a typical three-domain structure. The N-terminal GAF domain is a ubiquitous regulatory system in response to low oxygen tension, while in the free-living and symbiotic states (Wongdee et al., 2016). Data from previous research indicated that both nifDK clusters contribute to nitrogenase activity during symbiosis with *A. americana*, while the nifDK cluster found on the chromosome is the major contributor to the nitrogenase activity of the bacteria under free-living conditions (Wongdee et al., 2016). These data indicate that the two nifDK clusters identified in the DOA9 strain should be differentially regulated. This is supported by the fact that the DOA9 display two nifA genes but also two rpoN homologues, both located on each replicon. The simple explanation is that the nifA found on the chromosome (nifAc) and the nifA found on the plasmid (nifAp) specifically regulated the nifDK cluster found on the replicon where nifA is present. However, cross-talk between these two regulatory circuits would also be expected, given that NifDKp proteins require the expression of the accessory nif genes to form a functional nitrogenase. In particular, the nifENX genes whose products are needed for synthesis of the iron-molybdenum cofactor of nitrogenase, exist as a unique copy and are found downstream of the nifDKc cluster.

Thus, in the present work, we aimed to investigate in more detail the regulatory functions of the two nifA genes identified for the *Bradyrhizobium* sp. DOA9. In a first approach, we analyzed the expression levels of both nifA genes under different culture conditions using translational fusions to gusA (\(\beta\)-glucuronidase). We then analyzed the contribution of each regulatory protein to the control of bacterial nitrogenase activity under free-living and symbiotic states by constructing single and double nifA mutants. Finally, the expression level of several nif genes in three different backgrounds, the DOA9 wild-type (WT), \(\Delta\)nifAc, and \(\Delta\)nifAp mutant strains, were analyzed to identify which genes are activated by NifAc and NifAp.

MATERIALS AND METHODS

**Bacterial Strains and Culture Media**

The *Bradyrhizobium* sp. DOA9 WT was obtained from the School of Biotechnology, Suranaree University of Technology, Thailand, while all mutants were constructed in the Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), France. These bacterial strains were grown at 28°C for 4 days in Yeast extract-mannitol (YM) medium (Vincent, 1970) or a BNM-8 minimal medium (Renier et al., 2011). *Escherichia coli* strains were grown in LB medium at 37°C. When required, the media were supplemented with the appropriate antibiotics at the following concentrations: 100 \(\mu\)g/ml kanamycin, 200 \(\mu\)g/ml streptomycin, 20 \(\mu\)g/ml nalidixic acid, and 20 \(\mu\)g/ml cefotaxime.
**Construction of the Reporter and Mutant Strains**

All DNA fragments were amplified using the primers listed in **Supplementary Table S1**. To construct the reporter strains, DOA9-\(Pm\)-fix\(R\)\(f\) and DOA9-\(Pm\)-\(nif\)\(A\)\(p\), the 500-bp upstream region of \(fxR\) and the \(nif\)\(A\)\(p\) operon were amplified by PCR and cloned into the plasmid pVO155-\(npt\)\(2\)-\(cefo\)-\(npt\)\(2\)-\(gfp\). This plasmid, which is a derivative of the pVO155 plasmid (Oke and Long, 1999), could not replicate in the *Bradyrhizobium* strains. The plasmid carries the promoterless \(gus\)\(A\) promoter that was added to the \(npt\)\(2\) in the presence of sucrose and the kanamycin-resistance gene in the regions (between 700 and 1000-bp) of each \(npt\)\(2\)-\(cefo\)-\(npt\)\(2\)-\(gfp\) into the plasmid pVO155-\(hybrid\) of \(nif\)\(Ac\)\(1\). Complementation of the DOA9 \(mutant\)s as previously described (Wongdee et al., 2016).

**Cytological Analysis**

To follow the GUS activity in the nodules elicited by the reporter strains, 30- to 40-\(\mu\)-m-thick sections from fresh nodule samples were prepared using a vibratome (VT1000S; Leica, Nanterre, France) and incubated at 37°C in the dark in GUS assay buffer for 1 h, as described in Bonaldi et al. (2010). After staining, the sections were mounted and observed under bright-field illumination with a microscope (Nikon AZ100; Champigny-sur-Marne, France).

**Determination of Nitrogenase Activity Under Free-Living Conditions**

To determine the nitrogenase enzyme activity under free-living conditions, the *Bradyrhizobium* sp. strain DOA9 and derivatives were grown in 10-ml test tubes hermatically closed (BD Vacutainer, Franklin Lakes, NJ, United States) containing 2 ml of semisolid BNMM-\(B\) medium (agar 0.8% w/v). The BNMM-\(B\) medium is a synthetic plant growth medium (Erhardt et al., 1992) supplemented with a carbon (10 mM succinate) and a cocktail of vitamins (riboflavin at 0.2 \(\mu\)g/ml, biotin at 0.12 \(\mu\)g/ml, thiamine-HCl at 0.8 g/ml, myo-inositol at 0.5 \(\mu\)g/ml, p-aminobenzoic acid at 0.1 \(\mu\)g/ml, nicotinic acid at 0.5 \(\mu\)g/ml, calcium pantothenate at 0.8 \(\mu\)g/ml, and cyanocobalamin at 1 ng/ml) to support growth of *Bradyrhizobium* strains (Renier et al., 2011). It is to note that the BNMM-\(B\) medium was not supplemented with a nitrogenous source but the bacteria growth is possible thanks to the dinitrogen and oxygen present in the air constituting the initial headspace of the test tube. Just after closing hermically the tubes, acetylene gas (1 ml) was injected to a final concentration of 10%. The cultures were then incubated at 28°C without shaking, and the gas samples were analyzed at 7 dpi for ethylene production by gas chromatography, as previously described (Renier et al., 2011).

**Plant Cultivation and Analysis Under Symbiotic Condition**

The symbiosis efficiency of the *Bradyrhizobium* DOA9 strain and its derivatives were tested with *A. americana* No. 281 collected from the LSTM greenhouse. The seeds were surface sterilized by immersion in sulfuric acid under shaking for 45 min. Seeds were thoroughly washed with sterile distilled water and incubated overnight in sterile water. Seeds were transferred for 1 day at 37°C in the darkness on 0.8% agar plates for germination. Plantlets were transferred onto the top of the test tubes and covered by aluminum paper for hydroponic culture in buffered nodulation medium (BNM) (Ehrhardt et al., 1992). Plants were grown in a 28°C growth chamber with a 16-h light and 8-h dark regime and 70% humidity. Seven days after transfer, each seedling was inoculated with 1 ml of cell suspension resulting from a 5-day-old bacterial culture washed in BNM and adjusted to an optical density of one at 600 nm. For nodulation and the nitrogen fixation assay, 10 to 20 plants per condition were taken at 20 days post-inoculation (dpi) and analyzed for the number of nodules and nitrogenase activity as previously described (Bonaldi et al., 2010). The experiments were performed in duplicate.
RNA Purification, cDNA Synthesis, and qRT-PCR

The expression of genes involved in nitrogen fixation of strain DOA9 was determined from cells grown under free-living conditions and bacteroids obtained from nodules of *A. americana* under symbiotic conditions. For free-living conditions, the bacterial cells were grown in 150-ml bottles as described just above. For harvesting, cultures were added to a 1:10 volume of "stop solution" [10% Tris-HCl-buffered phenol (pH 8) in ethanol], and cells removed from the liquid medium by centrifugation for 10 min (10,000 rpm, 4°C). The cell pellets were frozen in liquid nitrogen and stored at −80°C. Analysis under symbiotic conditions and RNA isolation from bacteroids were processed from approximately 1 g of frozen nodules by homogenization with a tungsten carbide bead (3 mm; Qiagen, Hilden, Germany) in 2-ml microcentrifuge tubes. Total RNA was isolated from the free-living bacterial cells, and the nodules were disrupted with a hot (65°C) phenol-extraction procedure that was previously described (Babst et al., 1996).

RNA was purified and treated with DNase using mini-prep kits (Qiagen, Valencia, CA, United States). Then, the cDNA was synthesized with iScript TM Reverse transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, United States). Then, 10–50 ng of each cDNA sample was added to PowerUp™ SYBR™ Green master mixed buffer (Applied Biosystems, United States, Canada), and the appropriate amount of specific primers (listed in Supplementary Table S2) were used in the qRT-PCR analyses using an annealing temperature at 55°C for all reactions. The expression of target genes was relatively compared with the expression of the housekeeping gene, *dnak*, using QuantStudio Design & Analysis Software from Applied Biosystems.

RESULTS

**Bradyrhizobium sp. Strain DOA9 Displays Two Distinct nifA Genes Located on Both Chromosome and Mega-Plasmid (pDOA9)**

Two *nifA* homologous genes can be identified in the *Bradyrhizobium* DOA9 strain. One copy found on the chromosome, termed *nifAc*, is located approximately 6 kb from the *nifDKENX* operon and found just downstream of the *fixR* gene (Figure 1A). In *B. japonicum*, *nifA* is also found downstream of *fixR*. It has been shown that the two genes are part of the same transcript (Thöny et al., 1987), suggesting that *fixR nifAc* also forms an operon in the DOA9 strain. Downstream of this operon, a gene (*fer*) encoding a 4Fe-4S ferredoxin and a *suf* operon composed of four genes (*sufB, sufC, sufD, and sufS*) were identified and have been shown to function in the assembly of iron-sulfur clusters (Takahashi and Tokumoto, 2002). The other copy found on the plasmid, known as *nifAp*, is surrounded by genes of unknown function, and no known *nif* or *fix* genes are found in the vicinity. The two corresponding NifA proteins are clearly distinct and are of different lengths; *NifAc* (579(aa) and *NifAp* (503 aa) display only 52% identity. A Pfam analysis to identify functional domains showed that *NifAc* displays a classical NifA architecture with a N-terminal GAF domain, a central sigma 54 interaction domain and a C-terminal HTH domain. *NifAp* shows a less classical structure with only the presence of the central and HTH domains (Figure 1B).

The divergence of *NifAp* is not limited to the absence of the N-terminal GAF domain, since phylogenetic analysis showed that this protein formed an outgroup that was well separated from the NifA proteins identified in *Bradyrhizobium* strains (Figure 1D).

Interestingly, in both cases, a close examination of the promoter regions of the *fixR*nifAc operon and *nifAp* permitted the identification by manual analysis of a putative NifA and a RpoN binding sites, suggesting that both NifA proteins could autoregulate their own expression level and that of their homolog (Figure 1C).

**Two nifA Genes Identified in DOA9 Strain Are Both Expressed During Symbiosis and Free-Living Growth**

To analyze the expression of the two *nifA* genes identified in the *Bradyrhizobium* DOA9 strain, we constructed two reported strains (DOA9-*Pm-fixRnifAc* and DOA9-*Pm-nifAp*) by integrating the nonreplicative plasmid pVO155-ntp2-cefo-ntp2-gfp, which carries a promoterless *gusA* gene (Okazaki et al., 2016) downstream of the promoter region of the *fixR*nifAc operon and *nifAp* gene. Since the DOA9 strain was isolated using *A. americana* as a trap, we analyzed these two reporter strains in this host plant. Observations at 14 dpi showed that both reporter strains were able to nodulate and fix nitrogen similar to the WT-strain, indicating that the integration of the pVO155 plasmid in these two promoter regions did not alter the symbiotic performance of the strain (Figures 2A–G). Cytological analysis revealed a β-glucuronidase activity in the nodules, which was elicited by the two reporter strains, in contrast to the WT-nodules for which no activity could be detected (Figures 2E–G). Although X-gluc (5-Bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylamine salt) staining is a qualitative measurement of gene expression, the naked eye could observe that the nodules elicited by DOA9-*Pm-nifAp* displayed a more intense blue color than those elicited by the DOA9-*Pm-fixRnifAc* reporter strain (Figures 2F,G), which indicates slight differences in the expression of the two *nifA* genes.

Similar observations were also made during free-living growth under microaerobic conditions and the absence of a combined nitrogen source. Indeed, after 7 days of culture in these conditions, the β-glucuronidase activity measured for DOA9-*Pm-nifAp* (30 Miller unit) was higher than that detected for the DOA9-*Pm-fixRnifAc* reporter strain (22 Miller Unit) (Figure 2H). Taken together, these data indicate that the two *nifA* genes identified in the DOA9 strain are expressed during symbiotic and free-living conditions and that in both conditions, the level of expression of *nifAp* is slightly higher than that of *nifAc*. 
FIGURE 1 | Bradyrhizobium sp. strain DOA9 strain displays two distinct nifA genes. (A) Genetic organization of the two nifA genes (in red) located on both the chromosome and plasmid of the DOA9 strain. nifAc, nifA located on the chromosome; nifAp, nifA located on the plasmid; CHP: Conserved Hypothetical protein, HP: Hypothetical protein, is: integrase and ts: transposase. (B) Predicted domain structure of both NifA proteins. (C) Putative NifA and RpoN boxes identified in promoter region of fixR-nifAc operon and nifAp. Pos: position of 5′ end nucleotide of motif relative to annotated start codon. (D) NifA phylogenetic tree showing relationship between NifA of bradyrhizobia. Sequences were aligned by CLUSTALX, and the tree was generated using the neighbor-joining method (Saitou and Nei, 1987) and displayed using NJPLOT (Perrière and Gouy, 1996). Bootstrap values, expressed as percentages of 1000 replications, are shown at branching points.
nifAc and nifAp Genes in Bradyrhizobium sp. DOA9 Strain Are Functionally Redundant During Symbiosis

The NifA protein has been shown to be essential for symbiotic nitrogen fixation in several rhizobia (Szeto et al., 1984; Schetgens et al., 1985; Fischer et al., 1986; Iismaa and Watson, 1989). To appreciate the relative importance of each nifA gene identified in DOA9 during symbiosis, we constructed various nifA mutants, single nifA mutants, either by insertion (DOA9ΔnifAc and DOA9ΩnifAp) or deletion (DOA9ΔnifAc and DOA9ΔnifAp), and a double nifA mutant (DOA9ΔnifAp:ΩnifAc). As shown in Figures 3A–C and Supplementary Figure S1, the plants inoculated with the different single mutants displayed no significant difference from those inoculated with the WT strain in terms of their growth, the number of nodules formed or the measured nitrogenase activity indicating that the single mutation of the nifAc or nifAp gene had no impact on the symbiotic performance of the strain. In contrast, the plants inoculated with the double nifA mutant (ΔnifAp:ΩnifAc) displayed a strict fix minus phenotype (Figure 3B), and the growth of the plants was similar to that of the non-inoculated plants. Notably, the double nifA mutant induced nodules that were smaller and displayed symptoms of senescence (they were white instead of pink, indicating the absence of leghemoglobin and the central tissue was digested) (Figures 3D–G). Taken together these data suggest that the two NiFA proteins identified in the DOA9 strain are functionally redundant during symbiosis and that at least one functional NiFA protein is absolutely required for symbiotic nitrogenase activity, as observed in other rhizobia.

NifAc Is Essential for Nitrogen Fixation Under Free-Living Conditions

To determine whether the two nifA genes were also exchangeable during free-living conditions, we analyzed the nitrogenase activity of the different constructed nifA mutants after 7 days of culture in semisolid BNM medium. The nifAc mutants including DOA9ΩnifAc and DOA9ΔnifAc were obviously unable to fix nitrogen in their free-living state (Table 1). In contrast, DOA9ΩnifAp and DOA9ΔnifAp mutants displayed nitrogenase activity similar to the WT strain. These data indicate that NifAc is essential for nitrogenase activity during the free-living condition, while NifAp does not play a significant role in this condition. As expected, it was found that the double nifA mutant was not able to fix nitrogen in the free-living state (Table 1).

### Table 1 | Nitrogenase activity in Bradyrhizobium sp. DOA9 (WT) and nifA mutant strains grown under free-living conditions as described in Material and Methods.

| Treatments* | Acetylene reduction (nmol/h/culture) | SD |
|-------------|-------------------------------------|----|
| Non-inoculation | ND | ND |
| WT | 3,643.55 | ±18.01 |
| ΩnifAc | ND | ND |
| ΔnifAc | ND | ND |
| ΔnifAp | 3,588.72 | ±74.99 |
| ΔnifAp:ΩnifAc | ND | ND |
| ΔnifAc::pMG103:nifAc | 3,535.37 | ±137.4 |
| ΔnifAc::pMG103:nifAp | ND | ND |
| ΔnifAc::pMG103:nifAp hybrid1 | ND | ND |
| ΔnifAc::pMG103:nifAp hybrid2 | ND | ND |

*Nitrogen-starved cells were inoculated into glass vials containing 2 mL of medium and 10 mL of gas headspace and incubated at 30°C for 7 days in semisolid culture. SD, standard deviation determined from three replicates; ND, non detected.
FIGURE 3 | The two nifA genes in Bradyrhizobium sp. DOA9 strain are functionally redundant during symbiosis with Aeschynomene americana. (A) Comparison of plant growth (aerial part) non-inoculated (NI) or inoculated with WT and mutant strains DOA9ΔnifAc, DOA9ΔnifAp and DOA9ΔnifAp::ΔnifAcDOA9 (at 20 dpi). (B) Amount of acetylene-reducing activity (ARA) in A. americana plants inoculated with WT and mutant strains. (C) Number of nodules per plant inoculated by WT and nifA mutant strains. (D–F) Transversal sections of nodules elicited by WT (D), DOA9ΔnifAc (E), DOA9ΔnifAp (F), and DOA9ΔnifAp::ΔnifAc (G) mutants. Scale bars are 1 mm for (D–G). In (B,C), error bars represent standard error (n = 10). Different letters above error bars indicate significant differences at P < 0.05 (Tukey’s HSD test).

Because NifAp lacks the N-terminal GAF domain, a simple hypothesis would be to postulate that NifAp protein is not active under free-living conditions, due to the absence of this functional domain. To check this hypothesis, we constructed two chimeric NifA hybrid proteins, one corresponding to the almost complete NifAp, to which has been added the first 70 AA of NifAc, the second corresponding to the sigma 54 interaction domain and HTH domain of NifAp (from the AA 150 to 503), to which was added the complete GAF domain of NifAc (the first 220 AA). These constructs were cloned into the pMG103 plasmid under the constitutive npt2 promoter and reintroduced into the DOA9ΔnifAc mutant (Supplementary Figure S2). As controls, we also reintroduced the complete nifAc or nifAp gene using the same plasmid and npt2 promoter. As shown in Table 1, only the reintroduction of the complete nifAc gene completely restored the nitrogenase activity of the DOA9ΔnifAc mutant. No gain of function was observed for all other constructs. This suggests that simple addition of the GAF domain is not sufficient to rebuild functional activity in NifAp protein under free-living conditions.

NifAc and NifAp Activate Differently the Expression of Chromosome and Plasmid nifHdk Genes According to the Culture Conditions

The analysis of nifA mutants on plants suggested that the two NifA proteins are functionally redundant, but considering that a functional redundancy of the nifHDK genes found on the chromosome and the plasmid was also reported (Wongdee et al., 2016), we cannot completely exclude the possibility that the absence of phenotype observed for the single nifA mutants results in fact to this last redundancy. In other words, we can ask whether
FIGURE 4 | Expression of nitrogen-fixing genes (nifDKc, nifDKp, nifHc, and nifHp) and nifAc, nifAp genes in Bradyrhizobium sp. DOA9 (WT) and nifA mutant strains grown under symbiosis (A) or free-living conditions (B). Bacteroid cells were obtained from A. americana nodules at 20 days post-inoculation (A), and bacterial cells grown under free-living conditions were obtained after 7 days of culture in BNM-B medium without glutamate (B). Total RNA was extracted and subjected to quantitative reverse transcription-PCR (qRT-PCR) with an internal standard of dnaK. All data were from one representative experiment that was repeated three times. Error bars indicate standard deviation.

Each NifA protein activates only one specific set of nifHDK genes, or both sets found on the chromosome and the plasmid.

To answer this question, we used quantitative reverse transcription PCR (qRT-PCR) to analyze the level of expression of the structural nif genes (nifDKc, nifHc, nifDKp, nifHp) as well as controls, namely, the two regulatory nifA genes (nifAc, nifAp) in both the WT and the two ΔnifAc and ΔnifAp mutants. As shown in Figure 4A, during symbiosis, the expression profiles of all structural nif genes is well conserved in the WT and the two nifA mutants. This clearly demonstrates that in this condition, the two NifA proteins can activate the expression of the two nifHDK sets, definitively confirming their functional redundancy in this condition. In contrast, during free-living growth, we observed that the ΔnifAc mutant differed drastically from the WT and the ΔnifAp mutants. Indeed, in the ΔnifAc mutant, no expression of nifDKc and p was detected, and the expression levels of nifHc and p were also extremely low. These data, which were in concordance with the absence of nitrogenase activity detected in this mutant, confirms the essential role of NifAc protein in the activation of nif genes under free-living conditions. In addition, for this mutant, no expression of nifAp could be detected, indicating that NifAc also activates the expression of nifAp which is in accordance with the presence of putative NifA and RpoN binding sites found in the nifAp promoter region.

In contrast, the pattern of expression of the nif genes remains very similar between the WT strain and the ΔnifAp mutant during free-living conditions, confirming that NifAp is dispensable during this condition. Interestingly, in these culture conditions, nifDKc was expressed at a far higher level than nifDKp. These data are in agreement with our previous study, which showed that NifDKc was the major contributor to the bacterial nitrogenase activity under free-living conditions (Wongdee et al., 2016).

Taken together, these data confirm that the two NifA proteins are exchangeable in the activation of nif genes during symbiosis, but not during free-living conditions, where NifAc is essential.

DISCUSSION

In this study, we showed that the two nifA genes present in the Bradyrhizobium sp. DOA9 strain are expressed during both symbiotic and free-living growth and encoded functional proteins. In particular, we observed that these two NifA are
perfectly exchangeable for the regulation of nitrogen fixation during symbiosis. These data were unexpected, given the moderate level of identity between these two proteins and the difference observed in their architectural organization (see Discussion below). Furthermore, in the only other example rhizobial strain reported to contain two nifA genes, *Mesorhizobium loti*, there was no functional redundancy observed between these two NifA proteins since the ΔnifA2 mutant gave a Fix− phenotype, while no symbiotic defect was observed for the ΔnifA1 mutant (Sullivan et al., 2001; Nukui et al., 2006). In fact, the regulatory role of NifA1 in this last bacterium is unclear, since the expression of NifA-regulated genes, i.e., those containing NifA and RpoN binding sites boxes in their promoter, were drastically impacted in the ΔnifA2 mutant but not in the ΔnifA1 background (Sullivan et al., 2013).

The origin of these two nifA genes in the DOA9 strain is puzzling. Their localization on different replicons and their moderate level of identity suggest that they were separately acquired, rather than via duplication of a single gene. In both cases, a Blast search using the amino acid sequence of NifAp or NifAc returned as best hits *Bradyrhizobium* NifA homologs, suggesting that both nifA genes derived from a common ancestor. However, while the percentage of identity of NifAc with the other bradyrhizobial NifA ranges between 91 to 76 %, this percentage drops to 55-50% for NifAp. In accordance, a phylogenetic analysis (Figure 1D) clearly showed that NifAp forms an outgroup from the bradyrhizobial NifA proteins. Furthermore, nifAp is found in an unusual genomic context, as no known nif or fix genes are found in the vicinity, in contrast to the other rhizobial nifA genes that were always found associated with genes involved in nitrogen fixation (Fischer, 1994). The presence of insertion sequence elements belonging to the IS3 family surrounding nifAp suggests the possibility that nifAp could have been separated from nif genes by a transposition event (Supplementary Text S1).

In all of the rhizobia in which nifA has been studied, it has been shown that NifA is absolutely required to activate nitrogen fixation during symbiosis (Szego et al., 1984; Schetgens et al., 1985; Fischer et al., 1986; Issmaa and Watson, 1989). If the plant perceives that the nodules are ineffective, a sanctioning program is rapidly triggered (Westhoek et al., 2017), such as observed in the absence of NifA (Westhoek et al., 2007). On the other hand, although nifAp has strongly diverged from the other nifA, NifAp remains functional, as it can activate both nifHDKc and p during symbiosis in the absence of NifAc. This suggests that maintaining two functional NifA proteins in DOA9 strain may be selectively advantageous even if there is an overlap in their regulatory function.

The major striking difference between NifAp and NifAc or the other bradyrhizobial NifA is the lack of an N-terminal GAF domain. There are several reports in various diazotrophic bacteria indicating that the GAF domain plays a key role in the modulation of NifA activity. For example, in *Azotobacter vinelandii*, the GAF domain binds 2-oxoglutarate, a key metabolic signal of carbon status, the presence of which influences the interaction with the antiactivator protein NifL (Martinez-Argudo et al., 2004). In the same vein, in *Herbaspirillum seropedicae*, NifA regulation by ammonium involves its N-terminal GAF domain and the signal transduction protein GlnK (Aquino et al., 2015). In contrast, to our knowledge, no functional role has been attributed to the N-terminal GAF domain of the rhizobial NifA proteins. It does not play an obvious role, since its deletion in the NifA of *S. meliloti* or *B. diazoefficiens* does not impair the ability of the protein to activate nif genes (Beynon et al., 1988; Fischer et al., 1988). Furthermore, it exists among rhizobia, one example (*R. leguminosarum*), for which NifA naturally lacks this GAF domain and which, despite this, maintains its essential role in the activation of nif genes during symbiosis indicating that this domain is dispensable, at least under this culture condition (ismaa and Watson, 1989). Therefore, it is not so surprising that NifAp maintains a regulatory role during symbiosis, despite the lack of a N-terminal GAF domain. It is more surprising that NifAp is not functional during free-living growth. Our RT-qPCR analysis clearly showed that in free-living conditions, nifAp was expressed, which excludes the possibility that the lack of complementation of the ΔnifAc mutant is due to the absence of NifAp synthesis. It is possible that the GAF domain, which is dispensable during symbiosis, plays a more prominent role in NifA activity under in vitro conditions. We tested this hypothesis by constructing two chimeric NifA hybrid proteins containing the N-terminal part of NifAc and the C-terminal part of NifAp, but these constructs did not restore the free-living nitrogen fixing deficiency of the ΔnifAc mutant. Nevertheless, we cannot completely reject this hypothesis because it is possible that these hybrid NifA proteins did not have the correct conformation. Further studies at the protein level remain necessary to better understand the function and mode of action of both NifA homologs under free-living conditions.

The mechanism of regulations involving NifA in the DOA9 strain are certainly more complex than expected, considering that this strain also contains two rpoN homologous genes found on both the plasmid and chromosome. NifA activates nif gene expression and other genes by forming a complex with RpoN (Gong et al., 2007; Hauser et al., 2007). We can ask whether each NifA protein can form a complex with both RpoN proteins and depending on the NifA and RpoN composition, whether the activity of this complex and its affinity for a DNA binding motif differs. Intriguingly, while the promoter regions of nifDKc and nifHc contain a perfectly conserved NifA binding site (5'-TGT-N10-ACA-3'), only a nonconserved site differing by one nucleotide has been identified in the upstream regions of nifDKp, nifHp (Wongdee et al., 2016). Analysis of the expression of these nif genes suggests that these slight variations do not impact the ability of the two NifA proteins to activate the nifDK and nifH genes on both the chromosome and plasmid during symbiosis, since a similar level of expression of these different nif genes was observed for the 3 bacterial backgrounds tested (WT and the two nifA mutants). However, at the same time,
under free-living growth, nifDKc was more highly expressed than nifDKp. Therefore, we cannot exclude the hypothesis that these slight variations in the upstream activating sequence differentially impact the affinity of NifA (at least for NifAc) according to the environmental conditions. Behind the control of nif genes, the NifA protein influences various cellular processes in rhizobia (Fischer et al., 1986; Gong et al., 2007; Hauser et al., 2007). Thus, it is attractive to speculate that the presence of multiple NifA and RpoN proteins in the DOA9 strain facilitates the switch from a free-living to a symbiotic lifestyle and vice-versa, allowing better control of the expression of various sets of genes.

AUTHOR CONTRIBUTIONS

JW, NT, NB, PT, and EG conceived the experiments. JW, NT, PT, and EG conducted the experiments. JW, NT, PT, and EG analyzed the results and wrote the paper. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01644/full#supplementary-material

FIGURE S1 | The two nifA genes in Bradyrhizobium sp. DOA9 strain are functionally redundant during symbiosis with Aeschynomene americana. (A) Comparison of plant growth (aerial part) non-inoculated (NI) or inoculated with WT and insertion mutant strains DOA9ΔnifAc and DOA9ΔnifAp (at 20 dpi). (B–D) Root nodules observed with a fluorescent stereomicroscope equipped with a green fluorescent protein (GFP) filter. (B) Nodules elicited by WT; (C) Nodules elicited by DOA9ΔnifAc; (D) Nodules elicited by DOA9ΔnifAp. (E) Acetylene-reducing activity (ARA) in A. americana plants inoculated with WT and insertion mutant strains DOA9ΔnifAc and DOA9ΔnifAp. (F) Number of nodules per plant elicited by WT and DOA9ΔnifAc and DOA9ΔnifAp. (G–I) Cross section of nodule elicited by WT (G) and mutants DOA9ΔnifAc (H) and DOA9ΔnifAp (I). Scale bars are 250 μm for (D–G). (E,F) error bars represent standard error (n = 10). Different letters above error bars indicate significant differences at P < 0.05 (Tukey’s HSD test).

FIGURE S2 | Bradyrhizobium sp. strain DOA9 strain displays two distinct nifA genes. (A) Sequence alignment of NifAc and NifAp. Arrows and boxes indicate different portions of NifAc and NifAp used to form chimeric NifA proteins. (B) The color boxes indicate NifA protein domains including GAF (blue), sigma factor 54 (red) interaction (purple), and HTH (green) domains. (B) Schematic representation of different versions of nifA introduced into plasmid pHMG103-npt2-cefo under control of the constitutive npt promoter. Each constructed plasmid was transferred into DOA9ΔnifAc cells for complementation experiments (see Table 1).

TABLE S1 | Primers used in this study.

TEXT S1 | Analysis of insertion sequences in the nifAp surrounding region.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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