***nifDK* Clusters Located on the Chromosome and Megaplasmid of *Bradyrhizobium* sp. Strain DOA9 Contribute Differently to Nitrogenase Activity During Symbiosis and Free-Living Growth**

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Bradyrhizobium sp. strain DOA9 contains two copies of the *nifDK* genes, *nifDKc*, located on the chromosome, and *nifDKp*, located on a symbiotic megaplasmid. Unlike most rhizobia, this bacterium displays nitrogenase activity under both free-living and symbiotic conditions. Transcriptional analysis using *gusA* reporter strains showed that both *nifDK* operons were highly expressed under symbiosis, whereas *nifDKc* was the most abundantly expressed under free-living conditions. During free-living growth, the *nifDKp* mutation did not affect nitrogenase activity, whereas nitrogenase activity was drastically reduced with the *nifDKc* mutant. This led us to suppose that *nifDKc* is the main contributor of nitrogenase activity in the free-living state. In contrast, during symbiosis, no effect of the *nifDKc* mutation was observed and the nitrogen-fixation efficiency of plants inoculated with the *nifDKp* mutant was reduced. This suggests that *nifDKp* plays the main role in nitrogenase enzyme activity during symbiosis. Together, these data suggest that *Bradyrhizobium* sp. strain DOA9 contains two functional copies of *nifDK* genes that are regulated differently and that, depending on their lifestyle, contribute differently to nitrogenase activity.

The agronomical and ecological importance of legumes can be largely attributed to their ability to form a nitrogen-fixing symbiosis with specific bacteria known as rhizobia. These bacteria are housed in nodules, usually located on the root system, and are able to reduce atmospheric dinitrogen into ammonia to the benefit of the host plant. Nitrogen fixation involves an enzyme complex called nitrogenase, which is composed of two enzymes, nitrogenase (Mo-Fe protein) and nitrogenase reductase (Fe protein). The Mo-Fe protein is composed of two pairs of different subunits encoded by the *nifD* and *nifK* genes, while the Fe protein is composed of two identical subunits encoded by the *nifH* gene. In *Ensifer* (*Sinorhizobium*) *meliloti* and *Azorhizobium*

caulinodans, the *nifHDK* genes are organized in an operon, whereas, in *Bradyrhizobium diazoefficiens* USDA110 (formerly named *Bradyrhizobium japonicum*, Delamuta et al. 2013), the *nifH* gene is located several kilobases away from the *nifDK* operon (Fischer 1994). In addition to these three structural *nif* genes, other accessory *nif* genes are required for i) FeMo-co biosynthesis (*nifBENXQSUVY*), ii) nitrogenase maturation (*nifZM*), and iii) electron donation (*nifFJ*) (Masson-Boivin et al. 2009). All these *nif* genes are under the control of the activator protein NifA, which acts in association with the RNA polymerase sigma factor RpoN (σ^{54}) (Dixon and Kahn 2004). The way *nifA* transcription is regulated differs markedly among rhizobia (Fischer 1994). Likewise, the accessory *nif* gene content differs among rhizobium species, some species, including *Rhizobium leguminosarum*, contain only five additional accessory genes, while photosynthetic *Bradyrhizobium* strains as well as *A. caulinodans* contain up to 10 additional accessory genes (Masson-Boivin et al. 2009). In particular, photosynthetic *Bradyrhizobium* and *A. caulinodans* strains have the *nifV* gene that enables the synthesis of homocitrate, which is then incorporated into the FeMo cofactor of dinitrogen reductase (Hoover et al. 1988). The unusual presence of a *nifV* gene in these bacteria could be related to their ability to fix nitrogen under free-living conditions, which is an uncommon characteristic of rhizobia. Indeed, during symbiosis, it has been shown that the plant overcomes the lack of *nifV* in rhizobial strains by providing homocitrate to the bacterium (Hakoyama et al. 2009). This explains why, unlike many free-living diazotrophs, most rhizobia display nitrogenase activity only when they are in the host nodule cells.

All the *nif* genes are generally found in a DNA region that contains other symbiotic genes, such as the *nod* genes required for the synthesis of Nod factors. In *Rhizobium* and *Ensifer* species, this symbiotic region is located on a plasmid, whereas in *Mesorhizobium* and *Bradyrhizobium* strains this symbiotic region is integrated into the chromosome (MacLean et al. 2007). However, a *Bradyrhizobium* strain, DOA9, was recently reported to harbor a large (700 kb) symbiotic plasmid containing both *nif* and *nod* genes (Okazaki et al. 2015). This nonphotosynthetic *Bradyrhizobium* strain was isolated from rice field soil, using *Aeschynomene americana* as trap legume (Noisangiam et al. 2012), and was shown to be able to induce nitrogen-fixing nodules on a broad range of legume hosts (Teamtisong et al. 2014).

Interestingly, genome sequence analysis revealed that both the chromosome and the plasmid of strain DOA9 harbor a full

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set of *nifHDK* genes that are split into two clusters, *nifH* and *nifDK* (Fig. 1A) (Okazaki et al. 2015). Similarly, DOA9 contains two copies of the *nifA* regulatory gene located on the chromosome and plasmid, respectively. A further survey revealed that this bacterium contains a *nifV* gene and that this gene and all (other) accessory *nif* genes are located on the chromosome (Supplementary Table S1).

Genomic data suggest that the DOA9 strain is able to fix nitrogen under both symbiotic and free-living conditions and possesses two functional nitrogenase complexes that are regulated differently. As this would be a unique case among rhizobia, we examined whether the DOA9 strain displays nitrogenase activity under free-living conditions and, through the construction of reporter and mutant strains, analyzed the expression and role of the two nitrogenase enzyme complexes under different experimental conditions.

RESULTS

The nonphotosynthetic *Bradyrhizobium* sp. strain DOA9 displays nitrogenase activity under free-living conditions.

The presence of a *nifV* gene on the DOA9 chromosome suggests that this strain is able to fix nitrogen under free-living conditions. To test this possibility, we analyzed the nitrogenase activity of bacterial cells grown in closed tubes containing soft agar medium. Soft agar allows the creation of an oxygen gradient that, at a certain depth, is compatible with nitrogenase enzyme activity. Under these conditions and in a nitrogen-free minimal medium, rhizobia like *A. caulinodans* ORS571 and photosynthetic *Bradyrhizobium* strains are able to reduce acetylene, this being indicative of nitrogen fixation (Alazard 1990). Using these experimental conditions, we observed that the amount of ethylene produced by DOA9 cells after 7 days of incubation was less than the amount produced by *A. caulinodans* but more than the amount produced by photosynthetic *Bradyrhizobium* strains (ORS278 and ORS285) (Fig. 1B). In contrast, almost no acetylene reduction activity was observed with the *B. diazoefficiens* USDA110 cells used as the negative control. These data show that the nonphotosynthetic *Bradyrhizobium* sp. strain DOA9 forms an active nitrogenase complex in free-living conditions.

Both *nifDK* operons are highly expressed under symbiosis but regulated differently in free-living conditions.

To analyze the expression of the two *nifDK* operons found on the chromosome (*nifDKc*) and the plasmid (*nifDKp*), two reporter strains, named DOA9-PnifDKc and DOA9-PnifDKp, were constructed by integrating the pVO155-npt2-cefo-npt2-gfp

plasmid just downstream of the promoter region of the corresponding *nifDK* operon. This plasmid carries a promoterless *gusA* gene that can be used to monitor *nifDK* expression and contains a constitutively expressed *gfp* gene and cefotaxime cartridge that is used to select bacterial clones containing the construction (Okazaki et al. 2016). To test the symbiotic expression of the *nifDK* operons, we selected *Aeschynomene americana* as the host plant, as it has been used to isolate the DOA9 strain (Noisangiam et al. 2012). Observations made 14 days postinoculation (dpi) showed that the two reporter strains were able to nodulate *Aeschynomene americana* and fix nitrogen like the wild-type (WT) strain, indicating that the integration of the pVO155 plasmid did not disturb their symbiotic performance (Fig. 2A to F). Cytological analysis revealed that the nodules elicited by the two reporter strains displayed high β -glucuronidase (GUS) activity, in contrast to the WT nodules, in which no activity was detected (Fig. 2G to I). This suggests that the two *nifDK* operons are expressed during symbiosis with *Aeschynomene americana*. Although X-gluc staining is a qualitative measurement of gene expression, it should be noted that no difference in the color of the nodules elicited by the two reporter strains could be detected by eye.

Different culture conditions were tested to analyze in vitro expression of the two *nifDK* operons: i) with and without a nitrogen source in the culture medium and ii) growth under aerobic or anaerobic conditions. As shown in Figure 3, only high GUS activity was detected in cultures from the DOA9-PnifDKc reporter strain grown under low oxygen tension and without nitrogen, i.e., the conditions previously used to detect nitrogenase activity. Under the same conditions, cultures containing the DOA9-PnifDKp reporter strain showed very low GUS activity (<1% of the activity measured with the DOA9-PnifDKc strain) (Fig. 3). Taken together, these data show that both *nifDK* operons are expressed during symbiosis with *Aeschynomene americana*, whereas *nifDKc* is the most abundantly expressed operon under N-fixing conditions in the free-living state.

Both *nifDKc* and *nifDKp* contribute to nitrogenase activity in symbiosis with *Aeschynomene americana* but *nifDKp* plays the main role.

To determine the respective contribution of the two *nifDK* operons to nitrogenase activity during symbiosis, we constructed two insertional mutants (DOA9 Ω nifDKc and DOA9 Ω nifDKp). In these mutants, a pVO155 plasmid derivative is integrated into the 5' region of the corresponding *nifD* gene. Observations at 21 dpi, also using *Aeschynomene americana* as host plant, revealed dramatic differences between the two *nifDK* mutants.

Fig. 1. *Bradyrhizobium* sp. strain DOA9 displays nitrogenase activity under free-living conditions. **A**, Genetic organization of the *nifH*, *nifD*, and *nifK* genes (in black) as found on the chromosome and megaplasmid of *Bradyrhizobium* sp. strain DOA9. Note the presence in the chromosome of a *nifV* gene indicative of the ability of the strain to fix nitrogen under free-living conditions. In white, the genes found in synteny in both replicons and, in gray, the genes specific to each replicon. CHP = conserved hypothetical protein. **B**, Occurrence of nitrogenase activity in *Azorhizobium caulinodans* and various *Bradyrhizobium* strains, determined by the ability of the bacteria to reduce acetylene into ethylene cultivated under free-living conditions.

Plants inoculated with the DOA9 Ω *nifDKc* mutant displayed typical symptoms of nitrogen starvation, including foliage chlorosis and reduced plant growth compared with plants inoculated with the WT strain (Fig. 4A and B). This correlated with the total absence of nitrogenase activity measured in the acetylene reduction assay (Fig. 4H). In addition, the DOA9 Ω *nifDKc* mutant resulted in more nodules per plant. Cytological analysis of the nodules showed that they were all hollow inside and were white in color, indicating the absence of leghemoglobin (Fig. 4C and F). All these data demonstrate a strictly *fix*⁻ phenotype for this mutant. In contrast, the effect of the *nifDKp* mutation was less dramatic but remained significant in comparison with plants

inoculated with the WT strain. Plants inoculated with the DOA9 Ω *nifDKp* mutant fixed nitrogen slightly less efficiently (<30%) than those inoculated with the WT strain (Fig. 4H). This lower nitrogenase activity had a direct impact on plant growth (Fig. 4A and B). Like the DOA9 Ω *nifDKc* mutant, the DOA9 Ω *nifDKp* mutant resulted in a higher number of nodules per plant but the nodules looked normal and displayed no signs of senescence (Fig. 4C and E).

To explain the differences between the two *nifDK* mutants, we considered the possibility of a polar effect of the *nifDKc* mutation on the downstream localized accessory *nif* genes (*nifENX*), which are found in single copy on the genome (Fig. 1A). To test

Fig. 2. The chromosomal and plasmid *nifDK* operons of *Bradyrhizobium* sp. strain DOA9 are expressed during symbiosis with *Aeschynomene americana*. **A**, Comparison of the growth of aerial parts of plants not inoculated (NI) or inoculated with wild type (WT) and reporter strains DOA9-*PnifDKc* and DOA9-*PnifDKp* (at 14 days postinoculation). **B** to **D**, Root nodules observed with a fluorescent stereomicroscope equipped with a green fluorescent protein (GFP) filter. **B**, Nodules elicited by WT, **C**, by DOA9-*PnifDKc*, and **D**, by DOA9-*PnifDKp*. **E**, Acetylene-reducing activity in *Aeschynomene americana* plants inoculated with WT and reporter strains DOA9-*PnifDKc* or DOA9-*PnifDKp*. **F** Number of nodules per plant elicited by WT and DOA9-*PnifDKc* and DOA9-*PnifDKp*. **G** to **I**, Expression of the *gusA* reporter gene revealed on 40- μ m nodule sections stained with X-Gluc. **G**, Nodules elicited by WT, **H**, by DOA9-*PnifDKc*, and **I**, by DOA9-*PnifDKp*. Scale bars are 1 mm for (B to D) and 250 μ m (G to I). In panels E and F, error bars represent standard error ($n = 10$). Different letters above the error bars indicate significant differences at $P < 0.05$ (Tukey's highly significant difference test).

this hypothesis, we introduced in the DOA9 Ω nifDKc mutant, a plasmid harboring either the *nifDKc* genes or the *nifENX* genes placed under the control of the *nifDKc* promoter. As shown in Figure 4A, B, C, and H, introduction of a plasmid containing the *nifENX* genes complemented the nitrogenase activity of the DOA9 Ω nifDKc mutant whereas a plasmid containing *nifDKc* did not, which supports the previous hypothesis. For a more detailed analysis of the importance of the *nifDKp* genes during symbiosis, we constructed a deleted *NifDKc* mutant (DOA9 Δ nifDKc) that is expected to have no, or a less dramatic, effect on the expression of the *nifENX* genes. To this end, the chromosomal encoded *nifDK* genes were replaced by a cefotaxime cartridge that did not contain a translational terminator. As shown in Figure 4A, B, C, G, and H, DOA9 Δ nifDKc behaved like the WT strain, no effect of the mutation was observed in any of the symbiotic parameters (plant growth, number of nodules, nitrogenase activity, and the histological features of the nodules). This suggests that NifDKp alone is sufficient to enable full nitrogenase activity whereas NifDKc is apparently not, given the lower symbiotic efficiency previously observed for the DOA9 Ω nifDKp mutant. Taken together, these data suggest that both *nifDK* operons are functional during symbiosis but that NifDKp is the main contributor to the measured nitrogenase activity.

The *nifDKc* operon is the major contributor to the nitrogenase activity of the DOA9 strain under free-living conditions.

We also examined the contribution of each *nifDK* operon to the nitrogenase activity of the DOA9 strain cultivated in vitro. As expected, in this condition, the DOA9 Ω nifDKc mutant did not fix nitrogen (Fig. 4I). In contrast, the DOA9 Ω nifDKp mutant displayed nitrogenase activity similar to the WT strain, showing that the *nifDKp* operon did not play a significant role in these culture conditions. In contrast to the symbiotic conditions,

the DOA9 Δ nifDKc displayed a very low nitrogenase activity (Fig. 4I). Similar results were obtained with the DOA9 Ω nifDKc mutant complemented with a plasmid containing the *nifENX* genes. These observations strongly suggest that the weak nitrogenase activity detected with these last two mutants is not the result of altered *nifENX* expression but of a lack of complementation by NifDKp. This last result is in line with the very low-level expression of the *nifDKp* operon found in this condition (Fig. 3). Taken together, these results support the hypothesis that the *nifDKc* operon is the major contributor to the measured nitrogenase activity under free-living conditions.

DISCUSSION

Several reports in the literature indicate the reiteration of some structural nitrogenase genes in some rhizobium strains. For example, *A. caulinodans* and several photosynthetic *Bradyrhizobium* strains (BTAi1, ORS278, S58) contain two *nifH* genes (Giraud et al. 2007; Norel and Elmerich 1987), whereas *R. etli* CFN42 (originally named *R. phaseoli*) contains two complete *nifHDK* operons (copies a and b) and a truncated *nifHD* operon (copy c) (Quinto et al. 1982, 1985). In all these cases, the reiterated genes are located on the same replicon (chromosome for the *Bradyrhizobium* strains and the symbiotic plasmid for the *Rhizobium* species) and they are 100% identical or only differ by one or two amino acid mismatches. This suggests that these multiples copies are most probably the result of one or more recent duplication events. The *Bradyrhizobium* sp. strain DOA9 is an exceptional case among rhizobia because the duplicated *nifHDK* genes are found on both the plasmid and the chromosome and are not perfectly conserved (the two copies of *nifH*, *nifD*, and *nifK* genes display, respectively, 94, 92.6, and 91.5% nucleotide identity). However, a phylogenetic analysis conducted on NifH, NifD, or NifK using sequences from different *Bradyrhizobium* strains showed that the identified chromosomal and plasmid copies in strain DOA9 are the closest neighbors (Supplementary Fig. S1). It is, thus, also possible that a more ancestral duplication event occurred, followed by the transposition of one copy on the plasmid, each gene copy subsequently evolving independently by accumulating different mutations. The absence of accessory *nif* genes on the plasmid supports this scenario.

Our data suggest that both copies of *nifDK* genes code for a functional nitrogenase during symbiosis but that *nifDKp* plays the main role in the measured nitrogenase activity. In contrast, more drastic differences were observed when cells were grown under free-living conditions, in which the measured nitrogenase enzyme activity was mainly the result of *NifDKc* expression. These data recall those reported for *A. caulinodans*. In this bacterium, the two *nifH* genes appear to be functionally redundant during symbiosis while the *nifH1* gene was found to be the main contributor of nitrogenase activity in free-living conditions (Iki et al. 2007).

In addition to the difference in the transcriptional level of the two *nifDK* clusters observed under free-living conditions, two other observations led us to suppose that *nifDKc* and *nifDKp* are regulated differently in the DOA9 strain. First, this strain contains two *nifA* genes distributed on the two replicons. In the same way, strain DOA9 contains two *rpoN* genes also distributed on the two replicons. In contrast to the NifHDKc and NifHDKp proteins, which are very well conserved, the two NifA and RpoN copies differ markedly, with respectively 52 and 55% of amino acid identity. In particular, NifAp lacks part of the N-terminal region corresponding to a sensory input domain belonging to the GAF family, which has been shown to influence stability and oxygen sensitivity of the protein (Screen et al. 1994). Second, the putative NifA binding sites in the

Fig. 3. Expression of chromosomal and plasmid *nifDK* operon of *Bradyrhizobium* sp. DOA9 in free-living conditions. **A** and **B**, β -galactosidase activity of DOA9-PnifDKc (black bar) and DOA9-PnifDKp (white bar), cultivated in buffered nodulation medium B, with the addition of a nitrogen source (+N) or without a nitrogen source (-N), under aerobic (A) or microaerobic (B) conditions.

Fig. 4. Role of the plasmid and chromosomal *nifDK* operon of *Bradyrhizobium* sp. DOA9 during symbiosis and free-living states. **A**, Comparison of the growth of the aerial parts of plants not inoculated (NI) or inoculated with wild type (WT) and its mutant derivatives (at 21 days postinoculation [dpi]). **B**, Fresh mass of the plants inoculated with WT and its mutant derivatives (at 21 dpi). **C**, Number of nodules per plant elicited by WT and its mutant derivatives (at 21 dpi). **D**, Cross sections of nodules elicited by WT and **E**, the mutants DOA9 $\Omega nifDKp$, **F**, DOA9 $\Omega nifDKc$, and **G**, DOA9 $\Delta nifDKc$. The red arrow in **F** indicates senescence of the central tissue of the nodule. Scale bars are 250 μ m for (**D** to **G**). **H**, The occurrence of acetylene-reducing activity in *Aeschynomene americana* plants inoculated with WT and its mutant derivatives. **I**, Occurrence of nitrogenase activity in WT and its mutant derivatives cultivated in free-living conditions. In **B**, **C**, **H**, and **I**, error bars represent standard error ($n = 20$). Different letters above the error bars indicate significant differences at $P < 0.05$ (Tukey's highly significant difference test).

upstream regions of *nifDKp* and *nifHp* differ by one nucleotide from the canonical motif (TGT-N10-ACA), whereas this motif is perfectly conserved in the upstream regions of *nifDKc* and *nifHc* (Supplementary Figs. S2 and S3). It is, thus, possible that the regulatory NifA proteins behave differently in response to different environmental factors and regulate expression of distinct regulons by recognizing slightly different DNA motifs.

From these observations, a simple regulatory model could be proposed, in which NifAc and RpoNc specifically control the *nifDKc* and *nifHc* genes, and vice versa, NifAp and RpoNp specifically control the *nifDKp* and *nifHp* genes. However, crosstalk 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, which appear to be cotranscribed from the *nifDKc* promoter. In addition, a perfect NifA and RpoN binding site is located in the promoter region of *nifAp*, suggesting that expression of NifAp is under the direct control of NifAc.

Taken together, our results show that the *Bradyrhizobium* sp. strain DOA9 has a highly unusual *nif* gene content and is able to fix nitrogen in both free-living and symbiotic states, in which the two *nifDK* copies contribute differently. Thus, *Bradyrhizobium* sp. DOA9 is an excellent model strain to study the function and regulation of rhizobial nitrogenases. Experiments are currently underway to determine if the two *nifH* copies behave like the two *nifDK* copies and to identify the specific regulons of the NifA and RpoN proteins in this bacterium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media.

Bradyrhizobium sp. strain DOA9 was obtained from the School of Biotechnology, Suranaree University of Technology, Thailand. This bacterium and derivatives, as well as ORS278, ORS285, and *B. diazoefficiens* USDA110, were grown at 28°C in yeast mannitol (YM) medium (Vincent 1970) or in buffered nodulation medium B (BNM-B) minimal medium. BNM-B medium is a synthetic plant growth medium (Ehrhardt et al. 1992) supplemented with a carbon (succinate) and a nitrogen source (glutamate) and various trace elements (Renier et al. 2011). *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C. When required, the media were supplemented with the appropriate antibiotics at the following concentrations: kanamycin, 100 µg/ml; streptomycin, 200 µg/ml; nalidixic acid, 25 µg/ml; and cefotaxime, 20 µg/ml.

Construction of the reporter and mutant strains.

Standard molecular biology techniques were used for all cloning work. All the primers used to clone DNA fragments are listed in Supplementary Table S2. For the construction of the reporter strains DOA9-P*nifDKc* and DOA9-P*nifDKp*, the 500-bp upstream region of each *nifDK* operon was amplified by polymerase chain reaction (PCR) and was cloned into plasmid pVO155-npt2-cefo-npt2-gfp. This plasmid, which is nonreplicative in *Bradyrhizobium* strains, is a derivative of the plasmid pVO155 (Oke and Long 1999) and carries the promoterless *gusA* gene, a constitutively expressed *gfp*, and kanamycin- and cefotaxime-resistance genes (Okazaki et al. 2015). For the construction of the two *nifDK* insertional mutants (DOA9Ω*nifDKc* and DOA9Ω*nifDKp*), 300- to 400-bp internal fragments of each *nifD* gene were amplified by PCR and were cloned into the plasmid pVO155-Sm-npt2-gfp harboring a streptomycin/spectinomycin-resistance gene. The resulting plasmids were introduced into *E. coli* S17-1 by electroporation (15 kv/cm, 100 Ω, and 25 µF) and were transferred into the *Bradyrhizobium* sp. strain DOA9 by biparental mating, using the protocol described by Giraud et al. (2010). Transconjugants were selected on YM

plates supplemented with (per milliliter) 25 µg of nalidixic acid, 20 µg of cefotaxime, and 100 µg of kanamycin or 25 µg of nalidixic acid and 200 µg of streptomycin.

For the construction of the *nifDKc* deletion mutant (DOA9Δ*nifDKc*), the 750-bp upstream and downstream regions of the *nifDKc* gene were obtained by PCR. The two regions were merged by overlap extension PCR. The overlap PCR fragment obtained was then digested by *Bgl*II/*Xba*I and was subsequently cloned in pNPTS129, after *Bam*HI/*Xba*I digestion. This plasmid cannot replicate in *Bradyrhizobium* spp., it carries the *sacB* gene that induces bacterial death in the presence of sucrose and the kanamycin-resistance gene (Tsai and Alley 2000). The cefotaxime cartridge from the pVO155-npt2-cefo vector (Okazaki et al. 2016) was liberated by *Hind*III digestion and was introduced between the upstream and downstream regions previously cloned in the pNPTS129 plasmid. The resulting plasmid was then transferred into DOA9 by biparental conjugation, as described above. Single recombinant clones were obtained by antibiotic selection and were verified by PCR. Double recombinant clones were then obtained by growth on sucrose in the presence of cefotaxime but without the addition of kanamycin. Candidate clones were checked for the loss of kanamycin resistance from the pNPTS129 plasmid, and the deletion of the *NifDKc* gene was verified by PCR.

Complementation of the DOA9Ω*nifDKc* mutant.

For complementation of the DOA9Ω*nifDKc* mutant with *nifENX* genes, the amplified promoter region of the *nifDKc* operon was first introduced into pMG103-npt2-cefo, harboring a constitutive cefotaxime-resistance gene, leading to the pMG103-npt2-cefo-*pm-nifDKc* plasmid. Next, the amplified *nifENX* genes were cloned downstream of the introduced promoter region. For complementation of the DOA9Ω*nifDKc* mutant with *nifDKc* genes, the *nifDKc* genes, including the 500-bp upstream promoter region, were amplified and cloned directly into pMG103-npt2-cefo. These constructed vectors were introduced in the DOA9Δ*nifDKc* mutant by electroporation (17.5 kv/cm, 100 Ω, and 25 µF). Plasmid-containing strains were selected on YM plates supplemented with (per milliliter) 20 µg of cefotaxime and 100 µg of spectinomycin.

Plant cultivation and symbiotic analysis.

The symbiotic character of the *Bradyrhizobium* sp. strain DOA9 and derivatives was tested with *Aeschynomene americana* No. 281 from the Laboratoire des Symbioses Tropicales et Méditerranéennes collection. The seeds were surface sterilized by immersion in sulphuric acid for 40 min with shaking, were then washed abundantly with sterile distilled water, and were incubated overnight in sterile water. The seeds were then transferred on 0.8% agar plates for 1 day at 37°C in the dark for germination. The resulting plantlets were transferred onto the top of test tubes covered by aluminum paper for hydroponic culture in BNM (Ehrhardt et al. 1992). The plantlets were placed in a growth chamber at 28°C 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 reach an optical density of 1 at 600 nm. For the nodulation and nitrogen fixation assay, 10 to 20 plants per condition were taken at 14 or 21 dpi, the number of nodules was counted, and nitrogenase activity was analyzed as previously described (Bonaldi et al. 2011). All the experiments were carried out in duplicate.

Cytological analysis.

To detect GUS activity in the nodules elicited by the reporter strains, 30- to 40-µm thick sections were cut from fresh nodule samples using a vibratome (VT1000S; Leica, Nanterre, France),

were incubated at 37°C in the dark in a GUS assay buffer for 1 h as described by Bonaldi et al. (2010). After staining, the sections were mounted and were observed with a microscope (Nikon AZ100; Champigny-sur-Marne, France), using bright field illumination.

Determination of nitrogenase activity under free-living conditions.

To determine nitrogenase enzyme activity under free-living conditions, *Bradyrhizobium* sp. strain DOA9 and derivatives as well as *B. diazoefficiens* USDA110 and *Bradyrhizobium* sp. strains ORS278 and ORS285 and *Azorhizobium caulinodans* ORS571 were grown in 10-ml test tubes (BD Vacutainer, Franklin Lakes, NJ, U.S.A.) containing 2 ml of BNM-B medium (without glutamate) soft agar (0.8%), as described by Alazard (1990). Acetylene was injected to reach a final concentration of 10%. The cultures were incubated for 7 days at 28°C, and gas samples were analyzed for ethylene production by gas chromatography, as described in (Renier et al. 2011).

Determination of GUS activity under free-living conditions.

The two DOA9 reporter strains were grown for 4 days in YM medium, were collected, and were washed with BNM-B medium (without glutamate) as described above. Bacterial cells were inoculated in flasks containing BNM-B medium supplemented or not with sodium glutamate (final concentration 6 mM) to obtain an initial optical density at 600 nm at 0.05. Aliquots (55 ml) of culture were injected into 150-ml bottles sealed with rubber stoppers and were incubated at 28°C for 11 days without shaking. Replicas of the cultures were simultaneously incubated in cotton-stoppered flasks under agitation to evaluate growth under aerobic conditions. Samples were taken at different intervals and GUS activity was measured using p-nitrophenyl glucuronide as substrate (Jefferson 1987). GUS activity units were calculated according to Miller (1972).

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