Inactivation of the *nodH* gene in *Sinorhizobium* sp. BR816 enhances symbiosis with *Phaseolus vulgaris* L.

Roseline Remans[1], Carla Snoeck[1], Christel Verreth[1], Anja Croonenborghs[1], Ellen Luyten[1], Maxime Ndayizeye[1], Esperanza Martínez-Romero[2], Jan Michiels[1] & Jos Vanderleyden[1]

[1]Centre of Microbial and Plant Genetics, Kasteelpark Arenberg, Heverlee, Belgium; and [2]Centro de Ciencias Genómicas, Cuernavaca, Morelos, Mexico

Correspondence: Jos Vanderleyden, Centre of Microbial and Plant Genetics, Kasteelpark Arenberg 20, B-3001 Heverlee, Belgium. Tel.: +32 16 32 96 79; fax: +32 16 32 19 66; e-mail: jozef.vanderleyden@biw.kuleuven.be

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Abstract

Sulfate modification on *Rhizobium* Nod factor signaling molecules is not a prerequisite for successful symbiosis with the common bean (*Phaseolus vulgaris* L.). However, many bean-nodulating rhizobia, including the broad host strain *Sinorhizobium* sp. BR816, produce sulfated Nod factors. Here, we show that the *nodH* gene, encoding a sulfotransferase, is responsible for the transfer of sulfate to the Nod factor backbone in *Sinorhizobium* sp. BR816, as was shown for other rhizobia. Interestingly, inactivation of *nodH* enables inoculated bean plants to fix significantly more nitrogen under different experimental setups. Our studies show that *nodH* in the wild-type strain is still expressed during the later stages of symbiosis. This is the first report on enhanced nitrogen fixation by blocking Nod factor sulfation.

Introduction

A complex cascade of signaling events mediates the agronomically important legume–*Rhizobium* symbiosis (Limpens & Bisseling, 2003; Riely *et al.*, 2004). As a result, nodules form on the roots of the host plant and cortical cells become infected with highly differentiated nitrogen-fixing bacteria, referred to as bacteroids. This signaling includes the bacterial-derived signals, so-called Nod factors, which are well-characterized secreted products of root-exudate inducible nodulation genes (Lerouge *et al.*, 1990; Dénarié *et al.*, 1996; Long, 1996; Perret *et al.*, 2000). Based on the genetic diversity of its microsymbionts, *Phaseolus vulgaris* is considered to be a promiscuous host. At least five different species belonging to the genera *Rhizobium* and *Sinorhizobium* have been identified from bean nodules (Michiels *et al.*, 1998a; Martinez-Romero, 2003). All species produce different Nod factors with important structural dissimilarities, indicating nonstringent structural requirements for these active molecules in bean symbiosis (Laeremans *et al.*, 1996, 1999; Laeremans & Vanderleyden 1998). For instance, *P. vulgaris* recognizes various Nod factors irrespective of whether sulfated or not.

The tropical broad-host-range *Sinorhizobium* sp. BR816 (Hungria *et al.*, 1993) synthesizes a complex mix of sulfated Nod factor compounds. Nod factor sulfation requires the presence of activated sulfate, and a specific sulfotransferase that transfers the activated sulfate to the Nod factor backbone. Sulfate activation is generally achieved by the ATP-sulfurylase-catalyzed reaction of sulfate with ATP to yield adenosine 5′-phosphosulfate (APS), coupled with GTP hydrolysis. Subsequently, APS is phosphorylated by an APS kinase to produce 3′-phosphoadenosine 5′-phosphosulfate (PAPS). APS and PAPS are important intermediates of the sulfate assimiliation pathway in all organisms. Redundancy of sulfate activation genes seems to be a widespread phenomenon within a range of plant growth-promoting rhizobacteria (Laeremans *et al.*, 1996; Barnett *et al.*, 2001; Snoeck *et al.*, 2003; Vanbleu *et al.*, 2005). Previously, it was found that *Sinorhizobium* sp. BR816 possesses three functional sulfate-activation systems involved in Nod factor biosynthesis (Laeremans *et al.*, 1997; Snoeck *et al.*, 2003). In contrast to the narrow-host-range *Sinorhizobium meliloti* (Ehrhardt *et al.*, 1995; Schultze *et al.*, 1995; Cronan & Keating, 2004), household and symbiotic (P)APS pools in *Sinorhizobium* sp. BR816 are not strictly separated and can be mutually exchanged. Knocking out all three sulfate-activation systems of *Sinorhizobium* sp. BR816 is necessary to abolish Nod factor sulfation and to obtain cysteine auxotrophy. This caused a decreased nitrogen fixation in inoculated bean plants, which could be restored by adding methionine as an organic sulfur source to the plant nutrient solution (Snoeck *et al.*, 2003).

This study was aimed at identifying the gene(s) encoding the sulfotransferase(s) involved in Nod factor sulfation in BR816 and to unravel its (their) role in the bean symbiosis.

Materials and methods

Bacterial growth conditions

Escherichia coli was grown in Luria–Bertani medium. *Sinorhizobium* strains were grown in liquid trypton-yeast extract medium at 30 °C or maintained on yeast-mannitol agar plates. For expression analysis and Nod factor purification, bacterial strains were grown in acid minimal salt medium (AMS) with 10 mM NH_4Cl and 10 mM mannitol (Michiels *et al.*, 1998b; Snoeck *et al.*, 2003) (Table 1).

DNA techniques

Standard techniques were used for DNA manipulations (Sambrook *et al.*, 1989). Total genomic DNA was isolated using a Genomic DNA Isolation Kit (Gentra Systems). DNA fragments were recovered from agarose gels using the Nucleotrap kit (Macherey-Nagel). Southern blotting and hybridizations were carried out as previously described (Laeremans *et al.*, 1997). Amplification of DNA fragments by PCR was performed using Pwo DNA polymerase (Boehringer, Mannheim, Germany) with 30 cycles of 1 min at 94 °C, 1 min at 54 °C and 1 min at 72 °C.

Cloning procedures and nucleotide sequencing analysis

Ordered series of sequencing clones were obtained via restriction enzyme mapping of BRVIID9 and BRD2 (Fig. 1) (van Rhijn *et al.*, 1993, 1994; Laeremans *et al.*, 1997) through time-dependent ExoIII deletion procedures (Erase-A-Base®, Promega). DNA sequencing of prepared plasmid DNA was performed on a Pharmacia ALF sequencer with fluorescein-labeled universal and synthetic oligonucleotide primers (Amersham Pharmacia Biotech, Uppsala, Sweden). Database searches were performed using the BLAST software (National Center for Biotechnology Information, National Institute of Health). Assignment of coding regions was performed by a combination of similarity searches and computer prediction using FRAMEPLOT (National Center for Biotechnology Information, National Institute of Health). Prediction of possible RpoN-binding sites was performed using the methodology as described by Dombrecht *et al.* (2002a).

Construction of mutants

A 0.9 kb SphI fragment, containing the promoter region and the 5′ end of *nodH*, was blunted with T4 DNA polymerase and subsequently ligated into the SmaI site of the *sacB* suicide vector pJQ200uc1 (Quandt & Hynes, 1993). A unique small SacI restriction fragment of *nodH* was further replaced by a DNA cassette containing the promoterless *gusA* gene and a kanamycin resistance gene from pWM6 (Metcalf & Wanner, 1993), yielding pFAJ1608. The plasmid

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristics	Source
Strains		
Sinorhizobium sp.		
BR816	Broad-host-range *Rhizobium* isolated from *Leucaena leucocephala*	12
FAJ1608	*nodH*::Km-*gusA* mutant of BR816	This study
FAJ1610	*rpoN*::*gusA*-Km mutant of BR816	This study
Escherichia coli		
DH5α	*hsdR*17*endA*1*thi*-1*gyrA*96*relA*1*recA*1*supE*44*ΔlacU*169(ϕ80*lacZ*ΔM15)	21
Plasmids		
pFAJ1608	pJQ200uc1 containing *nodH*::Km-*gusA* fragment, Gm^rKm^r	This study
pFAJ1610	pJQ200uc1 containing *rpoN*::Km fragment, Gm^rKm^r	This study
pFAJ1654	1,2 kb PCR fragment, containing *nodH* and its promoter region, in EcoRI of pTE3	This study
pFAJ1655	pFAJ1654 with *gusA*-Km interposon (pWM6) inserted in the unique SacI site of *nodH*	This study
pFAJ1656	pTE3 with *gusA*-Km interposon (pWM6) inserted in EcoRI	This study
PJQ200uc1	*Bacillus subtilis sacB*-containing suicide vector, Gm^r	25
pTE3	IncP, Tc^r, broad host range vector	27
pWM6	Vector, containing *gusA*-Km interposon, Ap^rKm^r	26
BRVIID9	pLAFR1 carrying *nod* region of BR816	16
Phages		
BRD2	Lambda EMBL3 containing *nodD1..nodJ* region of BR816	22

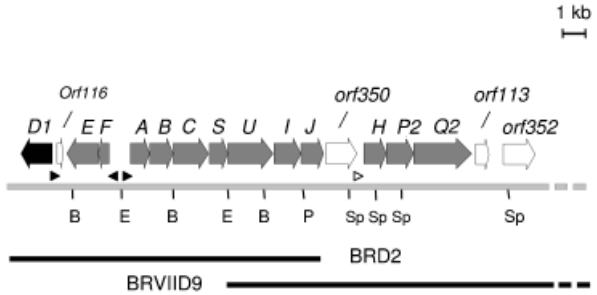

Fig. 1. Physical and genetic map of the *nod* gene cluster of *Sinorhizobium* sp. BR816 (see also Supporting materials). Horizontal arrows indicate the predicted coding sequences of the various ORFs. Nodulation gene homologues are colored in gray. Regulatory genes involved in *nod* gene expression are marked in black. Bold letters refer to the name of the specific *nod* gene. Other putative ORFs, with miscellaneous function, are indicated in white. The positions of *nod*-box sequences are marked with filled arrow heads below the genes. A −24/−12 promoter sequence is indicated with an unfilled arrowhead below the genes. Cosmid BRVIID9 (Laeremans *et al.*, 1997) and phage BRD2 (van Rhijn *et al.*, 1993) are indicated on the map. B, BamHI, E, EcoRI, P, PstI, Sp, SphI.

pFAJ1608 was introduced into *Sinorhizobium* sp. BR816 by triparental mating and double recombinants were selected as described by Snoeck *et al.* (2003). In this way, the *nodH* mutant FAJ1608, containing the *gusA*-Km cassette in opposite orientation of the *nodH* gene orientation, was constructed.

A 580 bp internal *rpoN* homologous fragment of *Sinorhizobium* sp. BR816 was amplified via PCR using primers OJM081 and OJM082 (Michiels *et al.*, 1998b), analyzed by sequencing and inserted into the pJQ200uc1 vector, digested with SmaI. A Km resistance cassette from pWM6 was removed with SalI and ligated into the unique SalI restriction site of the cloned *rpoN* PCR fragment, yielding pFAJ1610. Triparental-conjugation of pFAJ1610 into *Sinorhizobium* sp. BR816 resulted in an *rpoN*::Km mutant FAJ1610.

Cloning of the *nodH* gene region and construction of transcriptional reporter gene fusions

A 1.2 kb DNA fragment containing the complete *nodH* gene as well as its putative promoter region was amplified using the primers 5′-AATGAATTCTCTATCAGCGCTATGG AACG-3′ and 5′ AATGAATTCTGACATGAATGGCTTC AGC-3′ and cloned into the EcoRI site of the pTE3 vector (Egelhoff & Long, 1985), yielding plasmid pFAJ1654.

A plasmid-borne *nodH-gusA* fusion was constructed by replacing a unique small SacI restriction fragment of *nodH* by the *gusA*-Km reporter of pWM6 (Metcalf & Wanner, 1993), resulting in pFAJ1655. As a control, the *gusA*-Km reporter was directly inserted into the EcoRI digested pTE3 vector, yielding pFAJ1656.

Radioactive labeling of nod metabolites and thin-layer chromatography (TLC) analysis

Nod factors were *in vivo* labeled using the isotopes [^{14}C]-acetate and [^{35}S]-sulfate by a slightly modified version of the protocol of Mergaert *et al.* (1993), as previously described (Laeremans *et al.*, 1997). Nod factors were purified from cells grown in AMS medium.

In vitro plant inoculation assay

Seeds of *P. vulgaris* cultivars BAT477 and Negro Jamapa were surface-sterilized and germinated as described previously (Vlassak *et al.*, 1998). Bean seedlings were planted in nitrogen-free medium and grown in the plant growth room essentially as described by Snoeck *et al.* (2003). The seedlings were inoculated with 10^6 bacteria per plant taken from an overnight diluted culture that was washed with 10 mM $MgSO_4$. Plants were harvested after 3 weeks for determination of nodule number, nodule dry weight, plant dry weight and acetylene reduction activity (ARA) as described previously (Snoeck *et al.*, 2003). Ten plants per strain were tested in each experiment.

Greenhouse inoculation experiments

Beans of cultivar BAT477 were grown in 1-L pots, filled with perlite (hydroponic system) or with soil (Xerosol, pH H_2O 6.61, pH KCl 7.10, 0.80% carbon, 0.014% N, 0.00019% P and 1.38% organic matter) collected from a bean field site in Aguascalientes, Mexico. Initially, the pots filled with perlite were moistened with 100 mL nitrogen-free medium (Snoeck *et al.*, 2003); further 50 mL nitrogen-free medium per plant per week was applied, and distilled water was added at a regular basis. Pots filled with soil were moistened with distilled water only. Surface-sterilized seeds of BAT477 were pregerminated for 2 days on a moist filter paper in the dark at 28 °C. Per pot, one pregerminated seedling was planted. Inoculum was prepared as for the *in vitro* plant inoculation assay. The seedlings were inoculated with 10^6 cells per plant for plants growing in perlite and with 10^8 cells per plant for plants growing in soil. Control plants were not inoculated. For each condition, 12 plants were used and arranged in a completely randomized block design. Plants grown in soil and in perlite-based matrix were harvested at 30 and 40 days after inoculation, respectively, for determination of ARA, nodule number, nodule dry weight, shoot dry weight and root dry weight.

Nodule microscopy

For transmission electron microscopic (TEM) analysis, thin sections of 3-week-old nodules were prepared as described by Xi *et al.* (2000) and analyzed in a Zeiss EM900 electron

microscope. Four independent replicates were analyzed per condition.

β-glucuronidase activity assay

Overnight cultures of the strains tested were diluted 10-fold and grown overnight under shaking. For expression analysis during symbiosis, bacteroids were isolated from the nodules of bean plants 21 days postinoculation and quantitative analysis of β-glucuronidase activity was performed as described by Moris *et al.* (2005). Four independent replicates were analyzed per condition.

Nucleotide sequence accession number

Nucleotide sequence data have been deposited in the GenBank database under the accession number AJ518946.

Results

The *Sinorhizobium* sp. BR816 nodulation gene cluster contains a functional NodH sulfotransferase

The previously described cosmid BRVIID9 and phage BRD2 carry nodulation genes *nodD1*, *nodEF*, *nodABCSUIJ* and *nodP2Q2* of *Sinorhizobium* sp. BR816 (Fig. 1) (Laeremans *et al.*, 1997). Analysis of DNA sequences immediately flanking the *nodP2Q2* genes revealed the presence of an ORF encoding a putative protein of 250 amino acids. The putative protein is similar to several NodH-type Nod factor sulfotransferases such as NodH of *S. meliloti* (68% amino acid identity), *Rhizobium tropici* (66% identity) and *Rhizobium* sp. N33 (69% identity) and was therefore named NodH.

The putative function of NodH as a Nod factor sulfotransferase was confirmed after analysis of the Nod metabolites produced by the *Sinorhizobium* sp. BR816 wild type and its derived *nodH* mutant FAJ1608 using TLC (Fig. 2a and b). The *nodH* mutant is no longer producing sulfated Nod factors, upon induction with apigenin (the strongest known *nod* gene inducer in *Sinorhizobium* sp. BR816 (van Rhijn *et al.*, 1994) (Fig. 2b). This indicates that a single *nodH* gene copy is operative, as observed for other rhizobial strains (Roche *et al.*, 1991; Laeremans *et al.*, 1996). Construct pFAJ1654, constitutively expressing the *nodH* gene of BR816 (see Materials and methods), was used to complement for the *nodH* mutant phenotype. Nod factor sulfation was restored after complementing the *nodH* mutant with pFAJ1654 (Fig. 2c). Based on the local genetic organization (Fig. 1), insertion of the *gusA*-Km cassette into *nodH* most likely has a polar effect on *nodP2Q2*. However, it was previously shown that the function of *nodP2Q2* is compensated by *nodP1Q1* and even *cysDN* (Snoeck *et al.*, 2003).

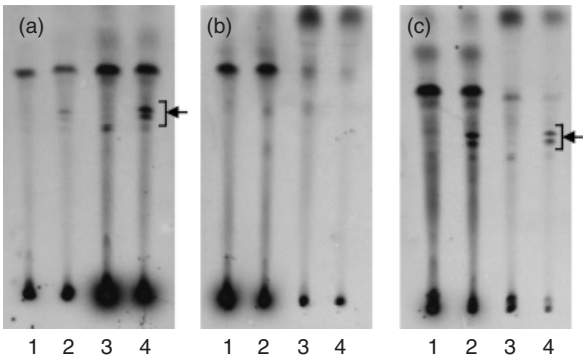

Fig. 2. Autoradiogram of reverse-phase TLC profiles of butanol extracts of radioactively labeled *Sinorhizobium* sp. BR816 wild type (a), FAJ1608 (b), FAJ1608 (pFAJ1654) (c). Lanes 1 and 2, ^{14}C-labeled; lanes 3 and 4, ^{35}S-labeled. Lanes 1 and 3, noninduced lanes; lanes 2 and 4, apigenin induced. The numbering of lanes applies to each TLC plate. Spots representing sulfated Nod factors are indicated with an arrow.

Both are intact in the *nodH* mutant FAJ1608. Moreover, the mutation in FAJ1608 can be complemented for Nod factor sulfation by introducing only the *nodH* gene (pFAJ1654).

Symbiotic phenotype of *nodH* mutants

The *nodH* mutant FAJ1608 was used to study the role of Nod factor sulfation in symbiosis between common bean and the broad-host-range strain BR816.

It was found that the symbiotic nitrogen fixation capacity increased by *c.* 85% in BAT477 bean nodules infected with the *nodH* mutant strain FAJ1608 compared with the wild-type rhizobia when plants were grown under controlled conditions in the growth chamber (Fig. 3a). The mutant FAJ1608 induced a significantly higher nodule dry weight when compared with the wild-type rhizobia. No significant differences in nodule number were observed. Interestingly, TEM analysis of sections of bean nodules revealed that symbiosomes are consistently more densely packed in plant cells colonized by the *nodH* mutant compared with the wild type (Fig. 4). When bean plants were inoculated with the complemented *nodH* mutant pFAJ1654/FAJ1608, the increase in plant symbiotic parameters compared with plants inoculated with wild-type BR816 was no longer observed (data not shown).

A similar phenotype was observed when the *nodH* mutant FAJ1608 was inoculated on the roots of the Negro Jamapa bean cultivar (Fig. 3a).

This increase in nitrogen-fixation capacity by inactivation of *nodH* was further confirmed under different experimental settings. In greenhouse trials using a hydroponic system with a perlite-based matrix or using soil with bean-planting history from Aguascalientes (important bean cultivating area in Mexico), plants inoculated with the *nodH* mutant

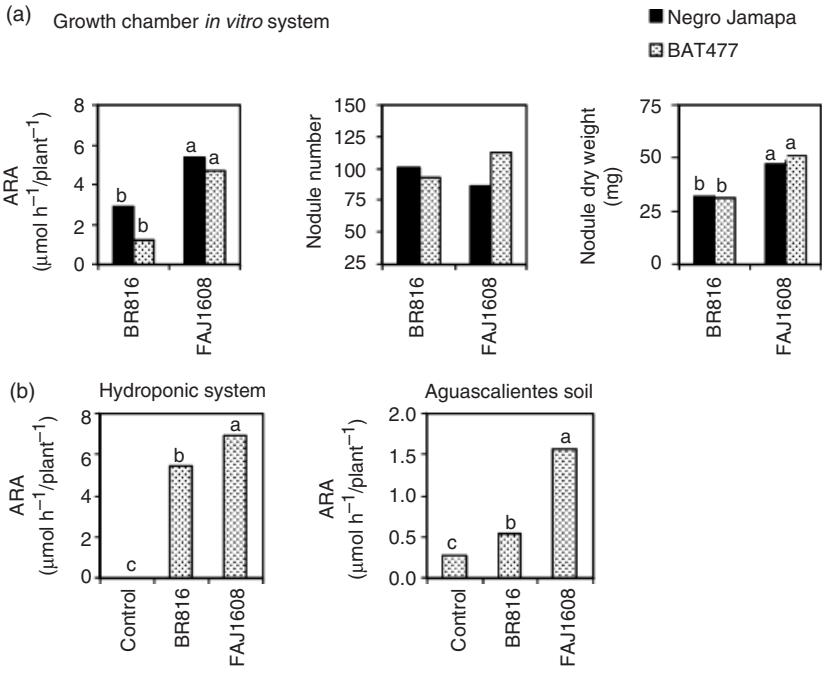

Fig. 3. Symbiotic response of *Phaseolus vulgaris* to inoculation with wild-type strain *Sinorhizobium* sp. BR816 and *nodH* mutant (FAJ1608). Bean plants were grown in a growth chamber *in vitro* plant assay (a) or in greenhouse trials using a hydroponic culture system and soil from Aguascalientes bean field sites in Mexico (b). ARA, acetylene reduction activity. Bars represent the average of ten to twelve independent measurements. Only significantly different values per measured plant parameter at $P = 0.05$ (Tukey's test) carry corresponding different letters above the bar or data point.

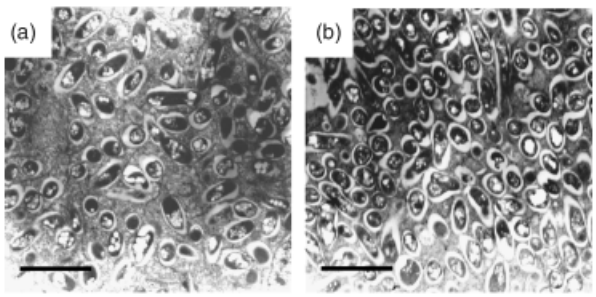

Fig. 4. Nodule anatomy. Representative TEM of sections of 3-week-old nodules, formed by the wild-type strain BR816 (a), *nodH* mutant FAJ1608 (b) on BAT477 bean plants. The scale of the bar is 3 μm.

fixed significantly more nitrogen than plants inoculated with BR816 wild type (Fig. 3b). The increase in symbiotic performance by knocking out *nodH* was associated with a strong increase (48%) in shoot dry weight for plants grown in Aguascalientes soil.

Gene expression of *nodH* and *nifH* in wild type versus *nodH* mutant bacteroids

Although NodH is an enzyme involved in the biosynthesis of Nod factors, and therefore with a role during the early stages of the legume–rhizobia communication, the drastic effect of knocking-out *nodH* on the symbiosis in terms of nitrogen fixation and nodule dry weight prompted us to analyze *nodH* gene expression in bacteroids. Therefore, plasmid pFAJ1655 (p*nodH-gusA*) was introduced into BR816 and its corresponding *rpoN* mutant FAJ1610, and *nodH* gene expression was quantitatively measured in bacteroids of mature bean nodules (21 days postinoculation) inoculated with *Sinorhizobium* species BR816 and the *rpoN* mutant strain, both containing pFAJ1655 (p*nodH-gusA*). Plasmid pFAJ1656 was used as a control.

It can be seen from Fig. 5 that *nodH* expression occurs in bacteroids and is higher compared with free-living conditions (11.9 MU ± 1.3). Furthermore, *nodH* expression is dependent on RpoN. This is in agreement with the *in silico* analysis of the 5′ upstream region of *nodH*, revealing a σ^{54} box (GG-N10-GT). We therefore speculate that NodH is still active as a sulfotransferase in nodules and uses activated sulfate from the APS/PAPS pool. To further substantiate the higher ARA observed in nodules of BR816 *nodH* mutant (FAJ1608) versus wild type, we measured expression of a translational p*nifH-gusA* fusion (pFAJ21) (Van de Broek *et al.*, 1992) in isolated bacteroids from bean nodules, induced by the wild type and *nodH* mutant FAJ1608. *nifH* encodes a subunit of the nitrogenase enzyme. FAJ1608 shows a significantly (Tukey $P < 0.05$) higher *gusA* expression per bacteroid (445 MU ± 177) as compared with the wild-type strain (182 MU ± 47) in 21-day-old bean nodules.

Discussion

We have identified the *nodH* gene of *Sinorhizobium* sp. BR816, encoding a protein with sulfotransferase activity for sulfation of Nod factors produced by BR816. The activated sulfate pool (APS/PAPS) is the donor substrate in the sulfotransferase-catalyzed reaction. In BR816, at least three redundant enzyme complexes, encoded by $nodP_1Q_1$,

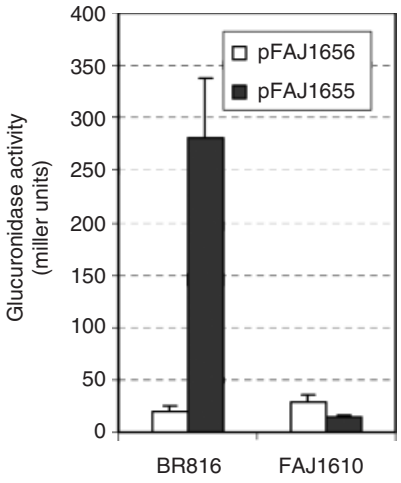

Fig. 5. Expression of the *Sinorhizobium* sp. BR816 *nodH–gusA* gene fusion during symbiosis. Expressions were determined using plasmid-borne *nodH-gusA* (pFAJ1655) and control *gusA* fusion (pFAJ1656) in *Sinorhizobium* sp. BR816 wild type and *rpoN* mutant FAJ1610 background. Bars represent the means of four independent measurements ± SD.

nodP$_2$*Q*$_2$ and *cysDN*, contribute to this activated sulfate pool, as a triple mutant is required to abolish sulfation of BR816 Nod factors (Snoeck *et al.*, 2003). In contrast, transfer of activated sulfate to Nod factors is due to a single *nodH* gene, as a *nodH* mutant no longer produces sulfated Nod factors.

A striking observation from this study is the enhanced nitrogen-fixation capacity of bean plants nodulated with the BR816 *nodH* mutant. This was observed in three independent settings. Further, expression analysis of *nodH* in the bacteroids indicates a role for NodH in the later stages of symbiosis. Usually, nodulation gene expression is strongly reduced during the late stages of the symbiosis (Schlaman *et al.*, 1998). However, some examples of *nod* gene expression during the later stages have been described previously (D'Haeze *et al.*, 1998; Barnett *et al.*, 2004).

As common bean can be efficiently nodulated by *Rhizobium* strains producing either sulfated or nonsulfated Nod factors, we postulate that the effect of the *nodH* mutation on symbiotic performance is likely not due to the nonsulfated Nod factors per se, but rather due to the fact that the activated sulfate pool is no longer used by the NodH sulfotransferase and therefore is better available as a substrate in other biosynthetic pathways. It seems plausible to postulate that there is a limiting pool of activated sulfate.

The ubiquitous role of the sulfate-activation pathways in maintaining substituents of bacterial determinants for symbiosis, together with its primary function in sulfate assimilation and cysteine-derived metabolism, is complex. Lipopolysaccharides are such bacterial determinants for which recently a role as a communication signal toward plant cells, consecutive to Nod factors, in nodule differentiation has been elucidated (Mathis *et al.*, 2005). Sulfation of *S. meliloti* lipopolysaccharide requires functional NodPQ sulfate-activating enzymes (Cedergren *et al.*, 1995). Both NodH and LpsS sulfotransferase activities seem to compete for a limiting pool of intracellular activated sulfate (Poupot *et al.*, 1995; Cronan & Keating, 2004; Gressent *et al.*, 2004). An *lpsS* mutant of *S. meliloti*, showing reduced lipopolysaccharide sulfation, provokes an increased number of nodules on the roots of alfalfa plants (Cronan & Keating, 2004).

A metabolic link between sulfur metabolism and nitrogen fixation may be explained by a high requirement for inorganic sulfur obtained from cysteine for the synthesis of metallo-sulfur clusters (Zheng *et al.*, 1993). The nitrogenase complex requires Fe–S clusters for the maturation and correct functioning of the structural proteins NifH and NifDK, and for the proteins of the electron cascades that confer reductive power to the nitrogenase. The genes encoding proteins for Fe–S cluster biosynthesis, e.g. *Rhizobium etli iscN-nifSU*, fulfill key roles in symbiotic nitrogen fixation (Dombrecht *et al.*, 2002b). Transcriptional profiling in *E. coli* indicates that sulfur limitation can impede iron–sulfur (Fe–S) cluster formation (Gyaneshwar *et al.*, 2005). Alternative or parallel pathways for cysteine biosynthesis may boost the provision of sulfur for biosynthesis of nitrogenase often present in high levels. Barnett *et al.* (2004, supporting materials) reported an increased expression of *nodQ1* in *S. meliloti* nitrogen-fixing bacteroids compared with the expression in nodule bacteria of a fix$^-$ mutant, indicating a role for the sulfate-activating enzyme NodQ1 in nitrogen fixation. The presence of additional copies of the *nodPQ* genes on the 144-kb accessory plasmid pSmeSM11a isolated from a dominant *S. meliloti* strain underlines the importance of sulfate-activation genes in the *Sinorhizobium-Medicago* symbiosis (Stiens *et al.*, 2006).

This study is a first example of fine-tuning of the *Rhizobium*-bean symbiosis by inactivation of *nodH*. Our results indicate that, besides their function in early signaling, it is conceivable that Nod factors might have additional roles during symbiosis. Alternatively, the NodH enzyme or its substrates might modulate the activity or the amount of other rhizobial molecules implicated in symbiosis.

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Authors' contribution

R.R. and C.S. contributed equally to this work.

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Supplementary material

An additional data file listing all ORFs in the nodulation gene cluster of *Sinorhizobium* sp. BR816 is available with the online version of the paper. The best BLASTP hit of each putative protein in the nonredundant GenBank is shown. The data were obtained from DNA sequencing and *in silico* analysis as described in Materials and methods and the literature (van Rhijn *et al.*, 1993, 1994; Laeremans *et al.*, 1997).

The following supplementary material is available for this article:

Table S1. Proposed function of open reading frames (ORFs) located in symbiotic region of *Sinorhizobium* sp. BR816.

This material is available as part of the online article from:http://www.blackwellsynergy.com/doi/abs/10.1111/j.1574-6968.2006.00521.x (This link will take you to the article abstract).

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