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

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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 microsymbions, 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; Martínez-Romero, 2003). All species produce different Nod factors with important structural similarities, 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 assimilation 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; Schultz 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 NH₄Cl 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 Smal 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

<table>
<thead>
<tr>
<th>Bacterial strains and plasmids</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tr>
<td>Bacterial strains</td>
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<tr>
<td>Sinorhizobium sp.</td>
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<tr>
<td>BR816</td>
<td>Broad-host-range Rhizobium isolated from Leucaena leucocephala</td>
<td>12</td>
</tr>
<tr>
<td>FAJ1608</td>
<td>nodH::Km-gusA mutant of BR816</td>
<td>This study</td>
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<tr>
<td>FAJ1610</td>
<td>rpoN::gusA-Km mutant of BR816</td>
<td>This study</td>
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<td>Escherichia coli</td>
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<tr>
<td>DH5α</td>
<td>hasR17endA1thi-1gyrA96relA1recA1supE44lacU169(p80lacZΔM15)</td>
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<tr>
<td>Plasmids</td>
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<tr>
<td>pFAJ1608</td>
<td>pJQ200uc1 containing nodH::Km-gusA fragment, Gm'Km'</td>
<td>This study</td>
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<tr>
<td>pFAJ1610</td>
<td>pJQ200uc1 containing rpoN::Km fragment, Gm'Km'</td>
<td>This study</td>
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<tr>
<td>pFAJ1654</td>
<td>1.2 kb PCR fragment, containing nodH and its promoter region, in EcoRI of pTE3</td>
<td>This study</td>
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<td>pFAJ1655</td>
<td>pFAJ1654 with gusA-Km interposon (pWM6) inserted in the unique SacI site of nodH</td>
<td>This study</td>
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<tr>
<td>pFAJ1656</td>
<td>pTE3 with gusA-Km interposon (pWM6) inserted in EcoRI</td>
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<td>PjQ200uc1</td>
<td>Bacillus subtilis sacB-containing suicide vector, Gm'</td>
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<td>pTE3</td>
<td>IncP, Tc', broad host range vector</td>
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<td>pWM6</td>
<td>Vector, containing gusA-Km interposon, Ap'Km'</td>
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<td>pLAFR1 carrying nod region of BR816</td>
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<td></td>
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<tr>
<td>BRD2</td>
<td>Lambda EMBL3 containing nodD1..nodI region of BR816</td>
<td>22</td>
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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 Sall and ligated into the unique Sall 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'-AATGATTCTCTATCAGCGCTATGG AACG-3' and 5' AATGATTCTGCATGAATTGCTC 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 Scl 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 [14C]-acetate and [35S]-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 10^8^ 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 Aquascalientes, 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.
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 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).

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...
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 (pnodH-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/C13.3). 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 σ54 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/C177) as compared with the wild-type strain (182 MU/C47) 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,Q, and
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.

**References**


Nod factor sulfation and common bean symbiosis


**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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