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Isolation and Characterization of the *Rhizobium tropici* Nod Factor Sulfation Genes

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Rhizobium tropici **produces a mixture of sulfated and nonsulfated Nod factors. The genes responsible for the sulfation process in *R. tropici* strain CFN299 were cloned and sequenced. These genes are homologous to the *nodP*, *nodQ*, and *nodH* genes from *R. meliloti*. The identity among the two species is 75% for *nodP*, 74% for *nodQ*, and 69% for *nodH*. NodH resembles sulfotransferases in general and NodQ has the characteristic purine-binding motifs and the PAPS (3′-phosphoadenosine 5′-phosphosulfate) motif. Mutants of NodP and NodH were obtained by site-directed mutagenesis. They are no longer able to synthesize the sulfated Nod factor, as was demonstrated in high-pressure liquid chromatography and thin-layer chromatography assays. The NodP⁻ mutant had a decreased nodulation capacity in *Phaseolus vulgaris* Negro Xamapa bean plants. In contrast, NodH⁻ and NodP⁻ mutants acquired an increased capacity to nodulate the high-nitrogen-fixing bean cultivars N-8-116 and BAT-477. Nodulation was restored to normal levels when the mutants were complemented with a 16-kb clone carrying the wild-type genes. The role of the sulfate on Nod factors in *R. tropici* was dependent on the bean cultivar and the conditions assayed.**

Additional keywords: APS kinase, ATP sulfurylase, *nod* genes.

Bacteria of the genus *Rhizobium* and legume plants can establish a symbiosis that results in the formation of new plant-organs, the nodules. Modified forms of the free living bacteria, the bacteroids, present in these organs fix N_2 into NH_4^+, a plant-assimilable form of combined nitrogen. The formation of nodules requires a bi-directional signal exchange between the symbiotic partners (Fisher and Long 1992; van Rhijn and Vanderleyden 1995). Flavonoid components of the legume root exudates initiate the chemical dialogue (Peters et al. 1986) by triggering the activation of the nodulation (*nod*) genes through the transcriptional activator NodD (for review see Martínez et al. 1990; Schultze et al. 1994). *nod* genes encode enzymes involved in the production of the Nod factor (Dénarié and Cullimore 1993). The *nodABC* nodulation genes produce the enzymes necessary for construction of the Nod factor lipo-oligosaccharide backbone bearing three to five β(1-4) linked *N*-acetyl-glucosamine sugar units (Carlson et al. 1994). The specific modifications are encoded by the host-specific nodulation (*hsn*) genes and are encountered on both the nonreducing and reducing ends of the oligomer. An example of a nonreducing-end side chain modification is a methyl group. The transfer of this group from an activated substrate to the Nod factor backbone is mediated by a methyltransferase that is encoded by *nodS* (Geelen et al. 1993; Waelkens et al. 1995). Other important *hsn* genes are *nodEF*, whose gene products are responsible for a specific acyl chain on the nonreducing end of the backbone, which may be necessary for the Nod signal to cross the plant membrane (Spaink et al. 1994). Other modifications can occur at the terminal-reducing glucosamine, e.g., a 2-O-methylfucose residue found in *Bradyrhizobium japonicum* (Sanjuan et al. 1992; Carlson et al. 1993), and in the broad host strain *Rhizobium* sp. NGR234 (Price et al. 1992), which is required for soybean nodulation.

The presence of sulfate on the signal molecule has been demonstrated in several *Rhizobium* spp., first in *R. meliloti* (Lerouge et al. 1990), subsequently in *Rhizobium* sp. NGR234 (Price et al. 1992), and in *R. tropici* (Poupot et al. 1993). In *R. meliloti*, the sulfate on the Nod factor is required for alfalfa nodulation (Roche et al. 1991). The biological function of this sulfate group remains unclear in the other *Rhizobium* spp. There is evidence that the sulfate group protects the Nod factor against plant-chitinase degradation (Schultze et al. 1993; Staehelin et al. 1994b).

From *R. meliloti*, Cervantes et al. (1989) and Schwedock and Long (1989) isolated the genes whose products are necessary for the formation of precursors for the sulfate modification. *nodPQ* genes encode ATP sulfurylase (Schwedock and Long 1990) and APS (adenosine 5′-phosphosulfate) kinase (Schwedock et al. 1994), which are required to convert the inorganic sulfate source into PAPS (3′-phosphoadenosine 5′-phosphosulfate) via APS. Subsequently, the NodH protein catalyzes the transfer of the activated sulfate from PAPS to the Nod factor core structure (Schultze et al. 1995; Ehrhardt et al. 1995). *R. meliloti nodH* mutants are Nod⁻ in alfalfa (Debellé et al. 1986; Horvath et al. 1986; Swanson et al. 1987).

Previously, the *Phaseolus vulgaris*–nodulating *R. tropici* strain CFN299 was found to produce a mixture of sulfated and nonsulfated Nod factors (Poupot et al. 1993). The aim of this

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work was to isolate and characterize the genes involved in the sulfation of the Nod factor in *R. tropici* type A reference strain CFN299 (Martínez-Romero et al. 1991), and to test the role of the sulfate substitution in the nodulation process of the bacteria with bean by obtaining mutants in *nodP* and *nodH* genes.

RESULTS

Isolation of R. tropici *nodH, nodP,* and *nodQ* genes.

An *R. tropici* CFN299 genome library, cloned in the *Eco*RI site of cosmid pSUP205, was hybridized under low stringency conditions with a polymerase chain reaction (PCR) fragment synthesized from primers P1 (5′-CTGTACTCGATCGGCAA GGAC-3′) and P2 (5′-GGTCCAGTTTGATAGCGGGAAGA C-3′). P1 and P2 were designed by identifying the 100% conserved amino acids, LYSIGKDS and VFPLSNWT, in the aligned nucleotide sequences from the *R. meliloti* 1021 *nodP*, *Azospirillum brasilense* Sp7 *nodP,* and *E. coli* K-12 *cysD* genes. The synthesized DNA, used as a probe, was a 450-bp fragment obtained with total DNA from CFN299 as the template; no fragment was obtained when the DNA from the pSym deleted strain CFN299-10 (Poupot et al. 1993) was used instead. Three positively hybridizing cosmids were selected, all containing a 16-kb *Eco*RI fragment. One of these clones, pSP33, was retained for further analysis. We also identified other cosmids that carried a 1.3-kb *Eco*RI fragment with a weaker hybridization signal. *Eco*RI digests of total DNA from CFN299 showed a single 16-kb restriction fragment that was not observed with the total DNA of CFN299-10 when blots were hybridized to the 450-bp probe (Fig. 1). Under low stringency hybridization conditions, an additional *Eco*RI 1.3-kb restriction fragment was revealed both in the wild type and in CFN299-10 (data not shown). Identical results were obtained when hybridization was performed using as a probe the *R. meliloti nodPQ* genes obtained from pGMI515 (Truchet et al. 1985).

A physical restriction map of the 16-kb fragment was defined (Fig. 2A) and a 4-kb *Pst*I DNA fragment (Fig. 1) that hybridized to the *R. meliloti nodP* and *nodQ* genes was subcloned. When blots of Eckhardt gels, obtained with *R. tropici* strain CFN299, were hybridized either to the 4-kb *Pst*I or to the 1.3-kb *Eco*RI fragment, only the 4-kb probe hybridized to plasmids in a band corresponding to the symbiotic plasmid. The 6.5-kb *Eco*R1 adjacent to the 16-kb fragment showed homology to *nodD* (P. Mavingui, personal communication), *nodABC*, and *nodI* genes (data not shown).

Sequence determination and analysis.

The nucleotide sequence obtained from the three open reading frames shows high homology to *nodH, nodP,* and *nodQ* genes from *R. meliloti*. We localized the putative *nodH* start codon at position 414 (see Fig. 2B) together with a putative Shine-Dalgarno sequence GGA. A sequence of 5 nucleotides (nt) separates the *nodH* stop codon and the *nodP* start codon at position 1169. A possible Shine-Dalgarno sequence AAGGA at position 1156 precedes the *nodP* putative start codon. We identified an overlap of 1 nt between the *nodP* stop codon and the *nodQ* start codon in TGATG at 2068, as has been reported for other *nodP and nodQ* genes by Cervantes et al. (1989) and by Schwedock and Long (1989) for *R. meliloti,* by Vieille and Elmerich (1990) for *A. brasilense,* and for *R. tropici* strain CIAT899 (GenBank accession number X87608). The stop codon of *nodQ* was encountered at position 3966. The number of amino acids of NodH, NodP, and NodQ are 249, 299, and 632, respectively. In addition to the Shine-Dalgarno sequences, we identified other possible sites of interaction with 16S rRNA for *nodH* (TTC), *nodP* (TCTT), and *nodQ* (CTT) genes (Petersen et al. 1988). The GC contents of *nodH*, *nodP,* and *nodQ* were calculated to be 54, 57, and 56%, respectively, and these values are similar to other reported *R. tropici* pSym localized genes, such as *pcsA* (Pardo et al. 1994) or *nodD1* (van Rhijn et al. 1993). The amino acid identity between CFN299 NodH and the homologous polypeptide of *R. meliloti* (Debellé and Sharma 1986) was 67%. We found an overall identity of 78% for NodP and 77% for NodQ. Compared with the NodP and NodQ deduced sequences of *A. brasilense,* we obtained a lower percentage of identical amino acid residues: 66 and 58%, respectively. The amino acid identity of *R. tropici* NodP with CysD, a part of the *E. coli* ATP sulfurylase (Leyh et al. 1988), was 62%. NodQ is homologous to both CysN and CysC, with an overall identity of 50%.

No significant homology to other genes was detected upstream of *nodH*, nor did we find any sequence homology to the *nod* box consensus sequence (Rostas et al. 1986) in 413 nt upstream of *nodH*. At around 135 bp upstream of the *nodH* translation initiation site, a possible –12/–24 RNA polymerase

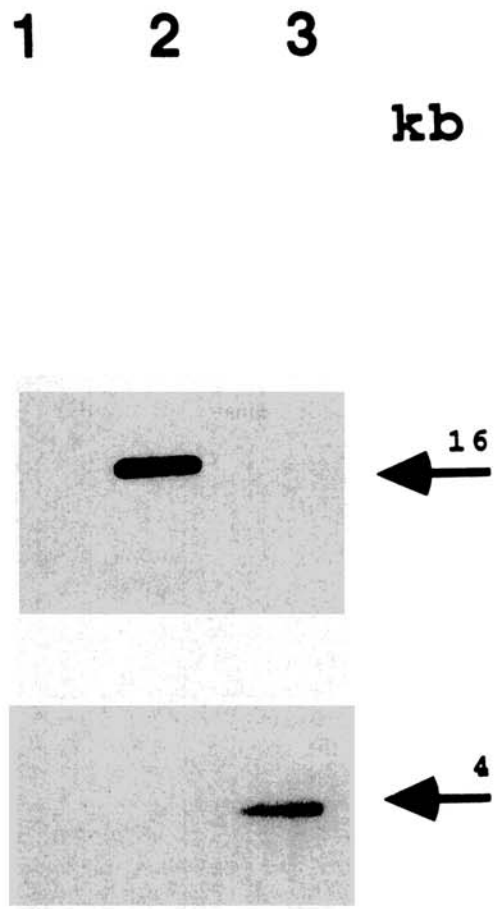

Fig. 1. Autoradiograms of Southern blots of total digested DNA hybridized to an internal *nodP* polymerase chain reaction fragment. Lanes 1 and 2: DNA digested with *Eco*RI; lane 3: DNA digested with *Pst*I. *Rhizobium tropici* strains: lane 1, CFN299-10; lanes 2 and 3, CFN299.

binding site was located, although there is no complete match with the proposed consensus sequence (Barrios et al. 1995). No stable transcription stop signal was identified in the entire sequenced region. The three motifs (GxxxxGK, DxxG, and NKxD) characteristic of GTP-binding sites (Dever et al. 1987) that exist in *R. meliloti* (Cervantes et al. 1989) and *A. brasilense* NodQ (Vieille and Elmerich 1990) were also found in *R. tropici,* separated by 40 to 80 amino acids. In addition, at the C terminal part of NodQ, we identified an ATP-binding motif (GxxxxGK, Fig. 2B) as well as a PAPS motif (K(A/G)xxGxxx (N/E)x(0 or 1)FT; Fig. 2B) (Satishchandran et al. 1992).

In a concurrent study with *R. tropici* type B reference strain CIAT899, the sequence of the *nodHPQ* genes was also determined (GenBank accession number X87608) and by comparing the respective sequences we only found two nucleotides to be different, one at position 335, upstream of the NodH coding region, and the other at 1356, with only the latter resulting in an amino acid substitution from arginine into proline.

Characterization of *nodH* and *nodP* mutants and genetic complementation analysis.

The Nod metabolites from CFNE200 and CFNE201 were analyzed both by thin-layer chromatography (TLC) and by high-pressure liquid chromatography (HPLC). A low constitutive production of Nod factors was always observed in the absence of flavonoids. In all cases, upon induction the mutants showed a drastic decrease or complete abolishment of the sulfated molecules (Fig. 3). For the genetic complementation of the mutants, we cloned the 16-kb *Eco*RI DNA fragment (Fig. 2A) containing the *nodHPQ* operon in the broad host range vector pLAFR3, resulting in cosmid pTL3, and introduced this construct into the mutants. The 4-kb *Pst*I fragment (Fig. 2A) only partially complemented the mutants (data not shown), while pTL3 restored the production of Nod metabolites by the mutants to wild-type levels, as observed on TLC autoradiograms (Fig. 3A). Cosmid pTL3 was used to introduce *nodHPQ* genes into the wild-type strain to investigate the effect of additional copies of these genes on Nod factor production. The TLC pattern showed only a small increase in the

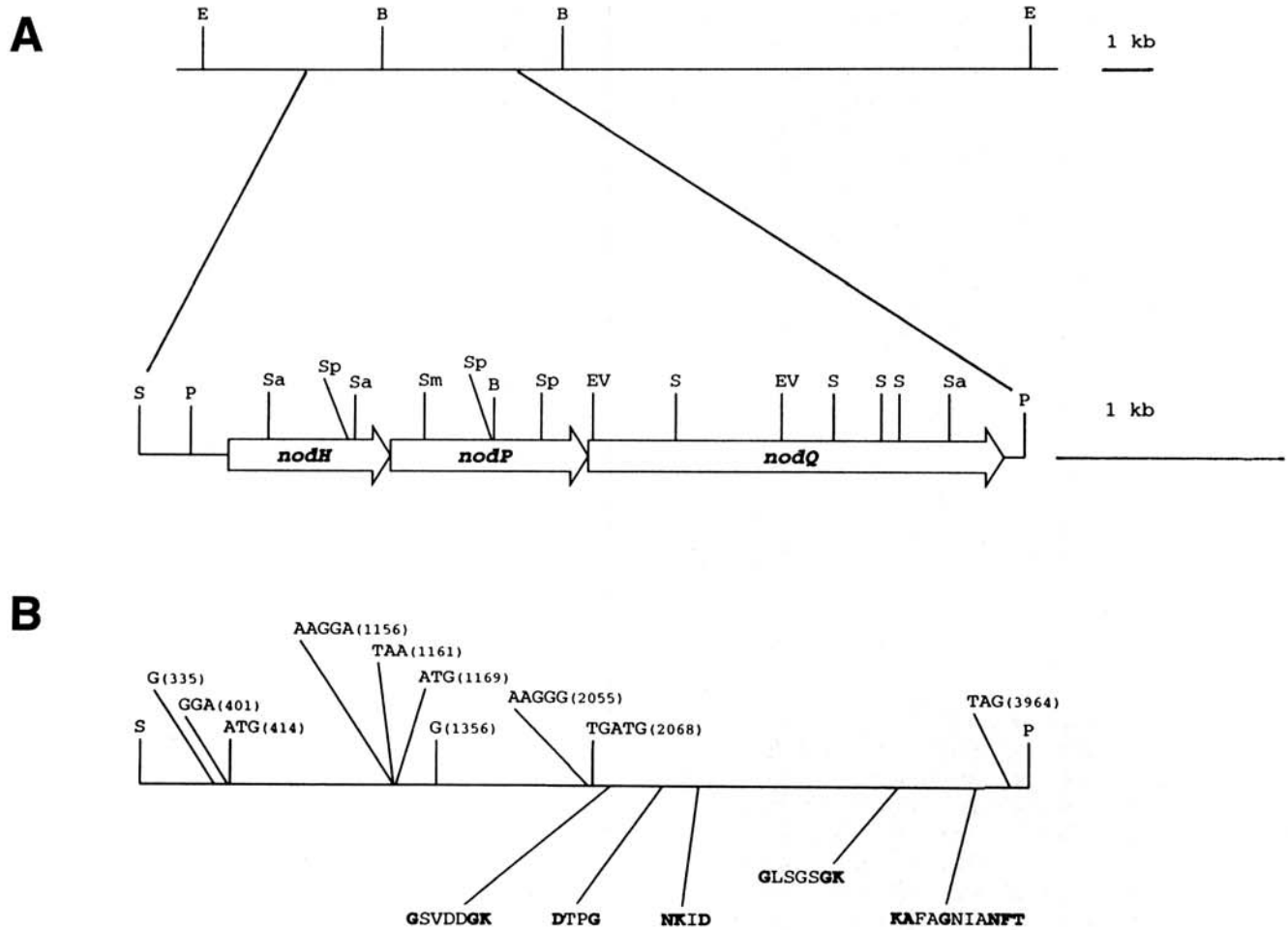

Fig. 2. A, Physical and genetic map of the *nodHPQ* region. Restriction sites are marked: *Eco*RI (E), *Bam*HI (B), *Sal*I (S), *Pst*I (P), *Sac*I (Sa), *Sph*I (Sp), *Sma*I (Sm), *Eco*RV (EV). **B,** Horizontal line represents *Rhizobium tropici* strain CFN299 *nodHPQ* zone, depicted in **A**. Extreme *Sal*I (S) and *Pst*I (P) restriction sites are indicated. Above horizontal line: translation signal sequences (see Results) followed by their respective positioning as submitted to GenBank. Numbers refer to first letter of corresponding sequences, except for position 2068, which indicates the 'A' in the overlap of the *nodP* stop-codon and the *nodQ* startcodon. The two guanines are the nucleotides that are different from the *R. tropici* strain CIAT899 *nodHPQ* region. Under the horizontal line: amino acid sequences of the GTP-, ATP-, and PAPS (3′-phosphoadenosine 5′-phosphosulfate)–binding motifs. Conserved amino acid residues shown in bold (see Results).

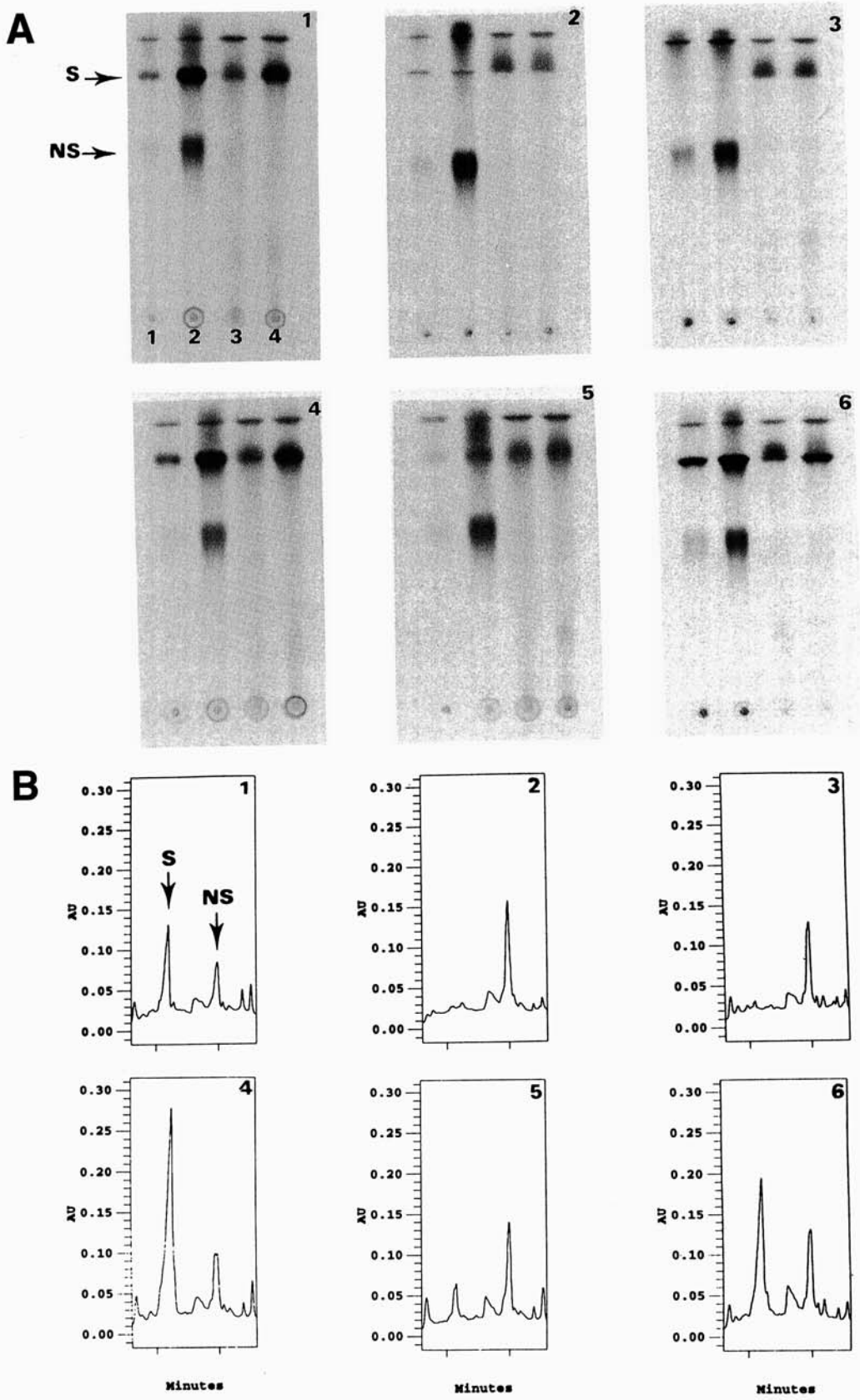

Fig. 3. A, Reverse-phase thin-layer chromatography patterns of *Rhizobium tropici* wild-type and mutant strains. ^{14}C-labeled: lanes 1 (noninduced) and 2 (induced); ^{35}S-labeled: lanes 3 (noninduced) and 4 (induced). **B,** High-pressure liquid chromatography profiles. *Y* axis: absorption units; *X* axis: retention time in minutes, only molecules eluting between minutes 8 and 18 are shown (minute 10 and 15 marked on the proportional time scale). 1: CFN299; 2 CFNE200; 3: CFNE201, 4: CFN299/pTL3, 5: CFNE200/pTL3, 6: CFNE201/pTL3. Sulfated Nod metabolites marked by S, nonsulfated by NS.

sulfated Nod factor and a decrease of nonsulfated Nod factor (Fig. 3A). The HPLC profile, however, showed a more drastic increase of sulfated Nod factor in the transconjugants compared with the wild-type HPLC pattern (Fig. 3B).

Plant phenotype determination.

In Negro Xamapa beans, the *nodP* mutant CFN200 formed around 70% of the nodules, compared with the wild-type strain. The high-nitrogen-fixing bean cultivar N-8-116 was also used to test the nodulation abilities of the mutants and the complemented *R. tropici* strains. From day 12, both mutants CFNE200 and CFNE201 showed a significant increase in nodule number per plant ($P < 0.05$, Student's t test) and no significant difference between them appeared at the 95% level of probability (Fig. 4). Introduction of the wild-type CFN299 *nodHPQ* operon into the mutants to complement their mutation did result in a decrease of nodule numbers per plant, although only for the NodH$^-$ transconjugant is this change likely to be significant at the 95% level. Although additional copies of *nodHPQ* were introduced into the wild-type strain and changes in the ratio of sulfated and nonsulfated Nod factor were recorded (Fig. 3B), no significant differences in nodule numbers were found compared with CFN299. We never observed differences in nodule morphology.

DISCUSSION

Nod metabolites are key compounds in eliciting nodules in legume roots. These metabolites with particular chemical substitutions have been found to be produced by the different *Rhizobium* and *Bradyrhizobium* spp. and by *Azorhizobium*. The Nod factors from *R. tropici* resemble those of *R. meliloti* in having a sulfate moiety at the reducing glucosamine. In *R. meliloti, nodP, nodQ,* and *nodH* genes were reported to encode the enzymes for the generation of the activated forms of sulfate (Schwedock et al. 1994) and for the transfer of this moiety to the lipo-oligosaccharide backbone, respectively (Ehrhardt et al. 1995; Schultze et al. 1995). In this study we isolated the corresponding *R. tropici* genes present in a single copy on the symbiotic plasmid. The *R. tropici* NodQ protein (Fig. 2B) possesses two purine-binding motifs and a PAPS-binding consensus in support of its role in ATP sulfurylase and APS kinase activity. The carboxy terminal polypeptide domain, homologous among sulfotransferases (Roche et al. 1991), was also found to be conserved in *R. tropici* CFN299 NodH (data not shown). The *nodHPQ* gene sequences of *R. tropici* strains CFN299 (type A reference strain) and CIAT899 (type B reference strain) are practically identical. *R. tropici* strains were divided into two types that share low (36%) DNA-DNA homology (Martínez-Romero 1994). Differences in repetitive DNA (REP)–PCR patterns, in multilocus enzyme electrophoresis, in phenotypic traits, in ribosomal gene sequences, and in megaplasmids also allow the distinction of the two groups in such a way that the status of the two types as two different species has been discussed (Martínez-Romero et al. 1991; van Berkum et al. 1994; Géniaux et al. 1995). Nevertheless, our results showed almost identical *nodHPQ* gene sequences and an identical physical map for CIAT899 (Vargas et al. 1990) and CFN299 in the *nod* gene region analyzed. It may be proposed that there occurred a lateral transfer of the symbiotic plasmid among the two subtypes, thus the two *R. tropici* subtypes would constitute a genospecies (Jones and Sneath 1970).

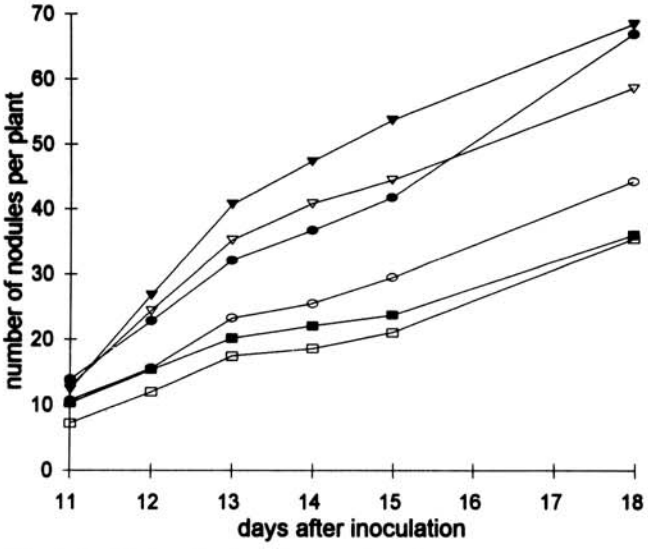

Fig. 4. Nodulation kinetics of *Phaseolus vulgaris* cultivar N8-116 inoculated with *Rhizobium tropici* wild-type and mutant strains. The experiment was conducted three times. One experiment is represented. CFN299 (filled squares), CFN299/pTL3 (open squares), CFNE200 (filled triangles), CFNE200/pTL3 (open triangles), CFNE201 (filled bols), CFNE201/pTL3 (open bols).

Due to the relative positions of the genes and the lack of an internal transcription termination signal, *nodHPQ* appear to be organized in one operon. But the translation appears to be independent for the three genes since we found both *nodP* and *nodQ* having a putative ribosome binding site. It remains to be established whether the –12/–24 resembling motif has a role in gene regulation. We also found that the *nodP* mutation was restored by a low copy cosmid vector containing the 4-kb *Pst*I *nodHPQ* fragment (Fig. 2A), although not completely to the wild-type levels. This indicates that an additional transcription regulation element may exist upstream of *nodH*, within the borders of the *Eco*RI sites but out of the 4-kb *Pst*I *nodHPQ* segment (Fig. 2A). We could not locate a *nod* box consensus sequence in the 413 bp upstream of NodH. A 0.6-kb *Sal*I-*Sac*I fragment (Fig. 2A), containing the upstream zone and part of the *nodH* gene, was fused to a promoterless *gusA* reporter gene. No significant differences in the expression of the reporter gene were obtained upon induction by flavonoids (data not shown). Possibly, the *nodHPQ* operon is not regulated by flavonoids, although a *nod* box motif could be located farther upstream out of the cloned fragment.

The *nodP* insertion mutant showed a drastically reduced production of sulfated Nod factors (Fig. 3A and B), so apparently there is no alternative efficient provider of the activated sulfate source, PAPS, in the nodulation pathway. Theoretically, PAPS could be provided by a second *nodPQ* copy, as in *R. meliloti* (Schwedock and Long 1992), or by (a) housekeeping gene(s) (noted as the saa locus by the same authors). We demonstrated that there was only one *nodPQ* gene copy, located on the pSym. In *R. tropici* CFN299, the isolated 1.3-kb *Eco*RI DNA fragment (see Results) showed low homology to the *nodPQ* probe and, moreover, after partial sequencing, the deduced amino acid sequence of this fragment showed

some homology to *E. coli* CysDNC, APS and PAPS generating enzymes for the amino acid sulfation pathway. This indicates that the 1.3-kb *Eco*RI fragment probably contains (parts of) housekeeping genes involved in the sulfation pathway of amino acids.

The overexpressing *nodHPQ* strain CFN299(pTL3) shifted the balance of sulfated and nonsulfated Nod factor toward the former but there was still an important production of nonsulfated Nod metabolite. In contrast, when the *R. meliloti nodPQ* genes, cloned in pGMI515, were introduced in *R. tropici*, the transconjugant produced only sulfated Nod factors (Poupot et al. 1995b). The difference could be caused by different expression levels due to different regulation of the *R. tropici* and *R. meliloti nodPQ* genes. Otherwise it may be speculated that there exists a repressor on the CFN299 16-kb *Eco*RI DNA fragment. Further mapping of this region would help to clarify this.

The NodH$^-$ and NodP$^-$ insertion mutants produce only nonsulfated Nod factor (Fig. 3), but it cannot be excluded that low amounts, undetectable by the methodology presented, could still be present. The effect of these mutants was a 30% decrease in nodule number in Negro Xamapa beans with CFNE200 and a consistent, drastic increase in the number of nodules in bean cultivar N-8-116 with CFNE200 and CFNE201. The same results were obtained with BAT-477, another high-nitrogen-fixing bean cultivar. When we complemented the mutants by introducing the CFN299 *nodHPQ* fragment, the nodule number always decreased, although the pTL3 in CFN200 did not restore the number of nodules to wild-type level. *R. tropici* strains give low numbers of nodules and have a delay in nodulation in the high-nitrogen-fixing cultivars N-8-116 or BAT-477, but form high numbers of nodules in the standard bean cultivar Negro Xamapa in agar medium. (E. Martínez-Romero, unpublished).

In *R. meliloti*, Horvath et al. (1986), Faucher et al. (1988), and Schwedock and Long (1992) demonstrated that *nodH* mutants nodulated no more the normal host alfalfa, while there was a host shift to vetch. For this reason, *nodH* is considered to be a determinant for host-specific alfalfa nodulation. Ogawa et al. (1991) reported that *R. meliloti nodH*::*Tn*5 still showed nodulation in *Melilotus albus* (a normal host plant) even up to levels statistically indistinguishable from inoculation experiments with the wild-type strain, depending on the plant growth conditions. They demonstrated that in *R. meliloti,* the requirement for *nodH* in nodulation changed with plant growth conditions. The necessity for the sulfated Nod factor was not as stringent on vermiculite as on agar slopes and the mutation affected the nodule number on *Medicago sativa* more severely than on *Melilotus albus*.

Martínez et al. (1995) found that the sulfated Nod factors promoted the formation of nodulelike structures in vitro in the absence of bacteria. Meristem induction and a reduced number of bumps were obtained when the nonsulfated factor was tested under the same conditions with Negro Xamapa bean roots. Differences in solubility of the two types of Nod factors could explain why nonsulfated Nod factors failed to induce nodulation in vitro, as they would be less polar and thus less soluble in the aqueous agar-Fahreus medium.

Hirsch (1992) discussed the existence of specific plant cell receptors for the Nod metabolites based upon the very low amounts in which they were biologically active. Nonleguminous plants such as rice (Shibuya et al. 1993) or tomato (Staehelin et al. 1994a) seem to possess receptors that recognize Nod factors. These receptors function as perception molecules for microbial signals that elicit a subsequent defense response with plant root chitinases hydrolyzing the (lipo-)chito-oligosaccharides. These plant receptor molecules did not make the discrimination between chito-oligosaccharides and Nod metabolites. *R. meliloti* putative Nod factor receptors from roots of *Medicago trunculata* were characterized by Bono et al. (1995), who showed that the binding was not dependent on the *O*-acetyl nor on the sulfate modification. In contrast, sulfate was required for *R. meliloti* Nod factors to provoke plasma membrane depolarization in alfalfa root cells (Felle et al. 1995). The substitutions on the Nod factor oligosaccharide backbone determine the rate of hydrolysis by plant chitinases; sulfate in some cases prevents degradation but in others enhances it (Staehelin et al. 1994b; M. Schultze, personal communication). Our results that show different symbiotic performance of the mutants depending on the cultivar may be explained if different cultivars had chitinases with different specificities; alternatively, bean cultivars may have different Nod factor receptors.

Nodulation of bean is induced by diverse types of Nod factors since *Rhizobium etli* produced only nonsulfated lipo-oligosaccharides presenting substitutions different from those of the *R. tropici* Nod metabolites. Signal molecules from both species are pentamers, possess a methyl group, and have vaccenic acid as the lipid chain on the nonreducing glucosamine. Fucose in *R. etli* Nod factors is located on the same position as the sulfate group in *R. tropici* (Poupot et al. 1995a).

Possibly, the increase in nodule numbers with *R. tropici nodP* and *nodQ* mutants is due to a metabolic link between the sulfation pathway of the Nod factor and another side substitution process. A putative candidate of a metabolically linked substitution could be the methyl group on the nonreducing terminal glucosamine. Introduction of the *R. meliloti nodPQ* genes into CFN299 resulted in a decrease in the rate of methylation, while all lipo-oligosaccharide backbones were sulfated. Waelkens et al. (1995) showed that the *nodS* gene, a putative methyltransferase, was indispensable for bean nodulation on vermiculite. The *nodH* mutation may have provoked an increased PAPS usable in the methylation pathway and thus possibly altered the methylation pattern, resulting in an increased nodulation. Another explanation could be that other not yet chemically characterized *R. tropici* Nod factors, present in only small amounts and metabolically linked to the sulfation pathway, could be responsible for bean nodulation.

Our results provide evidence for the presence of a differentially organized *nodHPQ* operon in *R. tropici*. The individual role of each gene is nevertheless difficult to establish since mutations by the cassette insertion might be polar on downstream genes, perhaps even on a gene located downstream of *nodQ*. We clearly showed that sulfated Nod factors are not the main nodulation-inducing agents on high-nitrogen-fixing bean cultivars; rather, they are detrimental to nodulation of plants grown in agar. It has been postulated that *R. tropici* is an opportunistic nodulator of bean. *R. tropici* is capable of nodulating other legumes as well. The sulfate group on the Nod factor may be critical to nodulate some of the other hosts. We are currently isolating *nodPQ* genes from other *Rhizobium* spp. to get a better understanding of the role of sulfate on Nod metabolites related to host specificity.

MATERIALS AND METHODS

Growth media, strains and plasmids.

Table 1 lists the strains and plasmids used in this study. *Escherichia coli* strains were grown at 37°C in Luria broth medium supplemented with antibiotics when required (mg per liter): ampicillin (Ap, 100), tetracycline (Tc, 10), gentamycin (Gm, 25), kanamycin (Km, 25), and spectinomycin (Sp, 100). *Rhizobium* strains were grown at 29°C on peptone-yeast (PY) medium (Beringer 1974) with nalidixic acid (Nal, 20) and supplemented with other antibiotics when necessary: Km (25), Sp (50), Gm (25), and Tc (2). When selecting for double homologous recombinants, 5% sucrose was added to the medium. Triparental matings, using pRK2013 as a helper plasmid, were performed as described by van Rhijn et al. (1993).

Cloning of DNA fragments and DNA sequencing.

DNA was isolated and fragments were cloned in pUC18 and pUC19 by means of standard protocols described in Sambrook et al. (1989). An ALF DNA sequencer (Pharmacia Biotech, Uppsala, Sweden) was used to determine the nucleotide sequences. Sequencing reactions were performed using alkaline denatured double-stranded DNA as a template, according to the manufacturer's protocol provided with the AutoRead Sequencing kit (Pharmacia Biotech) with the M13 universal or reverse primers. We designed oligonucleotides to complete the nucleotide determination of both strands. The complete sequence was deposited in the GenBank nucleotide database under accession number U47272. The obtained sequences were analyzed with the PCGENE software (Intelligenetics, Mountain View, CA) and the GCG software package (University of Wisconsin, Madison, version 8.0.1-UNIX 1994).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Rhizobium tropici		
CFN 299	Wild-type strain isolated from bean	Martínez-Romero et al. 1991
CFN 299-10	pSym deleted strain from CFN299	Poupot et al. 1993
CFNE 200	Km^r $NodP^-$ mutant of CFN299	This study
CFNE201	Km^r $NodH^-$ mutant of CFN299	This study
Plasmids		
pUC18/19	Ap^r cloning vectors	Norrander et al. 1983
pJQ200SK	*B. subtilis SacB* containing suicide vector, Gm^r	Quandt and Hynes 1993
pRK2013	Km^r helper plasmid for triparental mating	Figurski and Helinski 1979
pLAFR3	Tc^r broad host range cosmid	Staskawicz et al. 1987
pUC-4K	Vector containing Km^r cassette	Pharmacia Biotech, Uppsala, Sweden
pSUP205	Tc^r cosmid vector	Simon et al. 1983
pIC20-R	pSUP205 containing *R. tropici* CFN299 *nodHPQ*	Marsh et al. 1984
pTL3	pLAFR3 with 16-kb *Eco*RI fragment containing CFN299 *nodHPQ* genes, Tc^r	This study
pGMI515	RP4′, Tc^r, Ap^r	Truchet et al. 1985

Polymerase chain reaction.

In vitro synthesized DNA fragments were obtained by means of *Taq* DNA polymerase (Boehringer, Mannheim, Germany), according to the manufacturer's protocol, with 34 cycles of 1 min at 93°C, followed by 3 min at 40°C and 5 min at 72°C on a Hybaid thermal reactor (Hybaid, Middlesex, UK).

DNA hybridization.

Southern blotted DNA fragments on noncharged nylon membranes (Amersham Life Science, Buckinghamshire, England) were hybridized with nonradioactive digoxigenin (DIG)-labeled probes by using the DIG labeling and detection kit (Boehringer), according to the manufacturer's instructions. All hybridizations were performed under high stringency conditions except where otherwise noted.

Plasmid profiles.

Rhizobium tropici megaplasmids were separated and visualized by means of a modified Eckhardt protocol (Géniaux et al. 1995).

Construction of *nodP* and *nodH* mutants.

A 2.2-kb *Pst*I-*Sal*I fragment, carrying *nodH*, *nodP*, and the 5′ end of *nodQ* with a unique *Bam*HI site in *nodP*, was ligated in pJQ200sk, which contains the *Bacillus subtilis sacB* gene, inducible by sucrose to allow positive selection of the double homologous recombinants. A Km-resistance cassette from pUC-4K was introduced into the *nodP* gene in a unique *Bam*HI site. The *Bam*HI site from pJQ200sk was previously eliminated by filling the vector *Bam*HI site with Klenow polymerase. This construct was conjugated into *R. tropici* strain CFN299. The selection was performed on PY medium containing Nal, for counterselection of *E. coli*, Km, and sucrose. All colonies further analyzed were Gm sensitive and carried the Km cassette, as confirmed by hybridizations with the *R. tropici nodP* or with the resistance cassette as probes. One mutant (CFNE200) was used for TLC and HPLC analysis and in plant nodulation assays.

The 2.2-kb *Pst*I-*Sal*I fragment was cloned in pJQ200sk, of which the *Sac*I site was eliminated by deletion of the resulting 3′ ends by the 3′ to 5′ exonuclease activity of nuclease *S1* (Amersham Life Science). The *nodH* mutant was constructed by replacing a 400-bp *Sac*I internal gene fragment by a Km cassette from pUC-4K. Therefore, the cassette was first cloned in the *Bam*HI site from pIC20-R vector and cut out by *Sac*I digestion. The resulting pJQ200sk:: *nodH*::Km construct was used in a triparental mating, obtaining the double recombinant CFNE201.

Complementation of the mutants and construction of an overexpressing strain.

The 16-kb *Eco*RI fragment containing the *nodHPQ* genes was cloned in the broad host range cosmid vector pLAFR3 (pTL3) and mated into CFNE200 or into CFNE201. The same construct was introduced in the wild-type strain CFN299 to provide additional copies of *nodHPQ*.

Radiolabeling of Nod metabolites and TLC analysis.

Fresh strains grown overnight on PY medium with Nal supplement were inoculated in 1 ml of liquid minimal medium

(Poupot et al. 1993) at an OD_{600} of 0.1, with the *nod* gene inducer apigenin at a final concentration of 1.2 μmol per liter when necessary. After 3 h growth, the radioactive label was added: 2 μCi ^{35}[S]-sulfate or 0.1 μCi of D-[^{14}C(U)]-glucosamine hydrochloride. After 12 h of labeling, cells were pelleted for 10 min at 13,000 × *g*. The supernatants were passed through a C_{18} SEP-PAK cartridge (Waters Millipore, Milford, MA). All hydrophilic molecules were washed out with 10 ml of milliQ water and the Nod metabolites were subsequently eluted with 3 ml of methanol. The solution was dried under N_2 gas at 35°C and stored at –20°C. The samples were applied on reverse-phase TLC plates (RP-18F_{254s}, Merck, Darmstadt, Germany). The mobile phase was methanol/ammonia 5.5 N (9:1, vol/vol). Radioactive compounds in the TLC plates were visualized by autoradiography, using Hyperfilm-βmax (Amersham) after 4 days of exposition.

Plant nodulation assay.

Seeds of *Phaseolus vulgaris* Negro Xamapa or N-8-116 were surface sterilized and germinated as described by Martínez et al. (1985). Nodulation experiments were performed according to Martínez et al. (1985). Germinated seeds were transferred to agar flasks and inoculated from an overnight culture with approximately 10^5 bacteria per plant. Ten plants per strain were tested in each experiment. Bacteria were isolated from nodules and their identity was verified by resistance to antibiotics.

HPLC profiles of Nod factors.

Bacterial supernatants from 10-ml cultures were fractionated through SEP-PAK cartridges as described previously and were applied to a reverse-phase C_{18} cartridge (LiChropher 100 RP-18, 5 μm, Merck). The gradient described by Poupot et al. (1993) was used to elute the Nod metabolites and the absorbance of the eluting compounds was monitored at 206 nm. The purified sulfated and nonsulfated Nod factors obtained from Poupot et al. (1993) were used as controls and they eluted at 11 and 15 min, respectively.

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