Sulfation of Nod Factors via nodHPQ
Is nodD Independent in Rhizobium tropici CIAT899

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A cosmid from the Rhizobium tropici CIAT899 symbiotic plasmid, containing most of the nodulation genes described in this strain, has been isolated. Although this cosmid does not carry a nodD gene, it confers ability to heterologous Rhizobium spp. to nodulate R. tropici hosts (Phaseolus vulgaris, Macroptilium atropurpureum, and Leucaena leucocephala). The observed phenotype is due to constitutive expression of the nodABCUIJ operon, which has lost its regulatory region and is expressed from a promoter present in the cloning vector. Thin-layer chromatography (TLC) analysis of the Nod factors produced by this construction shows that it is still capable of synthesizing sulfated compounds, suggesting that the nodHPQ genes are organized as an operon that is transcribed in a nodD-independent manner and is not regulated by flavonoids.

Se ha aislado un cósmido del plásmido simbiótico de Rhizobium tropici CIAT899 que contiene la mayoría de los genes de nodulación descrito para esta estirpe, menos el gen regulador nodD. La introducción de este cósmido en una estirpe curada del plásmido simbiótico de R. tropici CIAT899 permite la nodulación en las plantas ensayadas (Phaseolus vulgaris, Macroptilium atropurpureum, y Leucaena leucocephala). El fenotipo observado se debe a la expresión constitutiva del operón nodABCUIJ bajo el promotor del gen de resistencia a la kanamicina, que lleva el vector donde se ha clonado el fragmento de ADN. Análisis por cromatografía de capa fina demuestran que esta construcción es capaz de sulfatar el extremo reductor del factor nod. Estas evidencias sugieren que los genes nodHPQ constituyen un operón, y que su expresión es independiente del gen regulador nodD.

Nodule formation in the Rhizobium-legume interaction is triggered by low molecular weight lipo-chitin oligosaccharides produced by the bacteria, in response to plant regulators (Spaink and Lugtenberg 1994). The bacterial nod genes responsible for the synthesis of these lipo-chitin oligosaccharides are regulated by the nodD gene product, which interacts with flavonoids exuded by the plant to activate transcription of the nod operons. For each Rhizobium sp., different groups of flavonoids determine an optimal transcriptional activation rate, depending on the specificity of the nodD gene product for different sets of flavonoids. This is one of the main mechanisms that determine host specificity, as the nod genes will only be transcribed when the right flavonoids are present in the root exudates of the host (or nonhost) plant (Györgypal et al. 1988; Spank et al. 1989).

The nodD protein regulates transcription of the nod operons by interacting with a conserved DNA sequence known as the nod box. The nod box is a region of 47 bp that is located from about –21 to –92 bp upstream of the transcription initiation site (Fisher et al. 1987; Mulligan and Long 1989; Goethals et al. 1992). All nod boxes studied so far show a conserved structure consisting of two inverted repeats with the sequence A-T-C-N9-G-A-T. Within this sequence, the motif T-N11-A has been proved to be important for NodD binding (Schell and Faris Poser 1989; Schell and Sukordhaman 1989; Goethals et al. 1992). Such structure favors the hypothesis that the NodD protein interacts as a tetramer with this conserved DNA sequence, to which it binds even in the absence of flavonoids. However, the affinity for this binding is greatly increased by these compounds (Schlaman et al. 1992), and NodD will only activate transcription of the nod operons in the presence of flavonoids.

Up to now, most nod operons studied have been found to possess a nod-box sequence on their regulatory region, with some exceptions such as the nodPQ genes from Sinorhizobium meliloti and the nodVW and nodZ genes from Bradyrhizobium japonicum. The NodPQ proteins are enzymes involved in the synthesis of adenosine 5′-phosphosulfate (APS) and 3′-phosphoadenosine 5′-phosphosulfate (PAPS), activated sulfate donors that participate in general sulfur metabolism. These compounds are used by NodH to transfer the sulfate group to the nod factor(s) (Roche et al. 1991). Little is known about the regulation of these genes, besides the fact that they do not possess a nod box and hence are not regulated by nodD. The nodVW operon encodes proteins of a two-component regulatory system that respond to flavonoids to activate transcription of some genes in B. japonicum (Loh et al. 1997). These genes have not been found in other Rhizobium spp. (Gottfert et al. 1990). nodZ is a gene from B. japonicum that participates in

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Nucleotide and/or amino acid sequence data have been submitted to the EMBL, GenBank, and DDBJ data bases as accession number X87608.
host specificity, since nodZ mutants are unable to nodulate siratro. Its product is involved in the fucosylation of the nodulation signals, and this functional group has been proved to be important for biological activity of the Nod factors produced by this species. Its expression is enhanced in bacteroids, but it is nifA independent, so the existence of a symbiotic plant regulator is postulated (Stacey et al. 1994). A recent report shows that nolW, a gene from S. fredii involved in soybean cultivar specificity, is also not regulated either by nodD or by flavonoids (Gu et al. 1997).

*Rhizobium tropici* is a broad host range species that is able to nodulate several legumes such as *Phaseolus vulgaris* and *Macropollium atropurpureum*, and the nonrelated species *Leucaena leucocephala* (Martínez et al. 1991). *R. tropici* produces a large variety of Nod factors consisting of two families: a hydrophilic family containing a sulfate group, and a neutral family that lacks the sulfate group. Both families contain methylated lipo-chitin oligosaccharides (Poupot et al. 1993; Folch-Mallol et al. 1996). Nod factor sulfation genes were also isolated from *Rhizobium* sp. strains N33 and BRR16 (Cloutier et al. 1996; Laeremans et al. 1997), and from *R. tropici* CFN299 (Laeremans et al. 1996). In *R. tropici* CFN299 and CIAT899, and *Rhizobium* sp. strain N33, the Nod factor sulfation genes are organized in one nodHPQ operon whereas, in *S. meliloti*, nodH and nodPQ are separated by nodEF and nodG (Debellé and Sharma 1986; Faucher et al. 1988; Cervantes et al. 1989).

In this paper, we demonstrate the production of sulfated Nod factors in the absence of nodD and flavonoids by rhizobia carrying a construction in which the common nodABCUSIJ genes of *R. tropici* CIAT899 are expressed from a constitutive promoter in the cloning vector. We propose that in *R. tropici* the nodHPQ genes are organized as an operon that is transcribed in a nodD-independent manner and is not regulated by flavonoids, as are most of the nod genes.

### RESULTS AND DISCUSSION

**Isolation of cosmids pCV61: Nodulation phenotype in different *Rhizobium* spp.**

We have reported previously the isolation and characterization of the nodulation region of the Sym plasmid of *R. tropici* CIAT899 (Vargas et al. 1990). This region was isolated by introducing a total DNA gene library of the wild-type strain CIAT899 into a pSym-cured derivative strain (RSP900). The whole conjugation mass was then inoculated on *P. vulgaris* plants, and bacteria resistant to tetracycline were recovered from the nodules. During the screening of the clones promoting nodulation on *P. vulgaris*, cosmids pCV61 was isolated, among others (Fig. 1A). This cosmids overlaps with cosmids pCV38 (Fig. 1A) in a region of 27 kb from the second HindIII site to the end of pCV38, and carries the common nodABCUSIJ genes, as well as the nodHPQ region (Fig. 1A). However, pCV61 lacks the 2-kb HindIII fragment present in pCV38 that contains a functional nodD gene (Vargas et al. 1990; Sousa et al. 1993).

pCV61 was introduced into several different *Rhizobium* and *Agrobacterium* spp. to assess host range extension on nodulation of *P. vulgaris*, *M. atropurpureum*, and *L. leucocephala* plants. Table 1 shows the nodulation phenotype on *P. vulgaris* plants when cosmids pCV61 was introduced into *R. tropici* RSP900, *R. leguminosarum* bv. *trifolii* R5043 (pSym cured derivative of wild-type strain RS1051), *R. leguminosarum* bv. *trifolii* wild-type strain RS1051, *S. meliloti* wild-type strain 102F34, and *Agrobacterium tumefaciens* GMI9023 (pTi-cured derivative of wild-type strain C58). pCV61 was able to confer host range extension to all *Rhizobium* and *Agrobacterium* strains tested, and even to confer nodulation ability when introduced into pSym-cured strains such as RSP900 and RS1043. Table 1 also shows the nodulation phenotype on *L. leucocephala* and *M. atropurpureum* plants by *R. tropici* CIAT899, and RSP900 strains carrying pCV61. These results indicate that this plasmid contains all the information needed for *R. tropici* to nodulate its three host plants. *L. leucocephala* and *M. atropurpureum* plants were also nodulated by several *R. leguminosarum* bv. *trifolii* and *R. etli* strains carrying pCV61 (Table 1). In summary, all *Rhizobium* strains carrying pCV61 acquired the ability to nodulate the normal hosts of *R. tropici*, although these plants are not nodulated by the parental strains (*R. leguminosarum* bv. *trifolii* and *R. etli*).

To define the region involved in the observed nodulation phenotypes, a 16-kb EcoRI fragment was deleted from pCV61. The derivative cosmids, pJF611, still carries the nodABCUSIJ genes but lacks the nodHPQ region (Fig. 1A). This cosmis was introduced into strains RSP900 and RS1051 and the nodulation phenotypes of the resulting transconjugants were tested on *P. vulgaris*, *L. leucocephala*, and *M. atropur-
Cosmid pCV61 lacks a nodD gene.

*R. tropici* CIAT899 has been shown to possess four copies of the *nodD* gene. Only one of the copies is known to activate transcription of the *nod* genes in response to flavonoids or root exudates (Sousa et al. 1993; van Rhijn et al. 1993). pCV61 lacks the 2.4-kb *HindIII* fragment that carries the *nodD* gene present in pCV38 (Fig. 1A). This *nodD* gene is the one proved to be functional (Sousa et al. 1993; van Rhijn et al. 1993, 1994). In order to understand the nodulation phenotypes conferred by pCV61 to other *Rhizobium* spp. (specially in the cured derivatives RSP900 and RS1043, which lack the symbiotic plasmid and therefore *nodD*), the possibility of the existence of another functional copy of a *nodD* gene in this plasmid was tested.

Southern blot analysis was used to search for a *nodD* homolog in pCV61. Two different *nodD* probes were hybridized with pCV61: (i) a heterologous *S. meliloti* probe that hybridizes with the *nodD* gene present in pCV38 (Vargas et al. 1990), and (ii) a homologous *R. tropici* probe (521-bp *HindIII-* EcoRI fragment of pCV38) that also hybridizes with the other copies of *nodD* present in CIAT899 (see Figure 1A; Sousa et al. 1993). Neither the heterologous *S. meliloti* probe nor the homologous probe hybridized with pCV61 (data not shown).

To discard the possibility that DNA identity could be too low to detect a homolog of *nodD* we tested whether pCV61 or its deleted derivative pJF611 were capable of inducing a *nodA-lacZ* fusion in plasmid pMP154 (Zaat et al. 1987). This plasmid has been extensively used to explore inducing activities of homologous as well as heterologous *nodD* genes (Spaink et al. 1989; Sousa et al. 1993). Flavonoids that proved to induce the *R. tropici nodD* gene (Sousa et al. 1993), as well as root exudates from *P. vulgaris* and *L. leucocephala*, were used. pMP154 was transferred to strain RSP900 containing pCV61 or pJF611, and the induction of β-galactosidase activity was measured in response to added flavonoids or root exudates (Fig. 2). When apigenin or naringenin was added to the culture media, a 10-fold or six- to eightfold induction of β-galactosidase activity was observed in the control strain (RSP900) containing cosmids pCV38 and pMP154, respectively. Genistein is not an inducer of the *R. tropici nodD* gene present in pCV38 (Fig. 2), although it is a good inducer of the *R. etli nodD* gene (Sousa et al. 1993). However, no induction was detected with pCV61 or pJF611 in the presence of the flavonoids used (Fig. 2). When root exudates of *P. vulgaris* and *L. leucocephala* plants were tested for nodD-inducing activity, pCV38 also showed an eightfold induction, but neither pCV61 nor pJF611 responded to any of the root exudates tested (Fig. 2).

Taken together, the hybridization data and the lack of inducing activity mediated by cosmids pCV61 and pJF611 strongly suggest that there is no functional *nodD* gene in this region of the *R. tropici* CIAT899 symbiotic plasmid.

Production of Nod factors by a strain carrying pCV61.

*R. tropici* CIAT899 produces a large variety of Nod factors that consist of two families: a hydrophilic family of factors containing sulfate and methyl groups, and a neutral family of factors that are nonsulfated but carry a methyl group. In the hydrophilic family, we have previously found several different fatty acyl moieties on the nonreducing end of the molecule (C16:0, C16:1, C16:2, C18:1, and C18:2), while the neutral

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**Table 1. Nodulation phenotypes on different *Rhizobium tropici* hosts conferred by cosmid pCV61 to *Rhizobium* and *Agrobacterium* spp.**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Phaseolus vulgaris</th>
<th>Leucaena leucocephala</th>
<th>Macroptilium atropurpureum</th>
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<tbody>
<tr>
<td><em>R. tropici</em></td>
<td></td>
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<tr>
<td>CIAT899</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>RSP900</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>RSP900/pCV61</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>RSP900/pJF611</td>
<td>++</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>R. leguminosarum</em> bv. trifolii</td>
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<tr>
<td>RS1043</td>
<td>–</td>
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<tr>
<td>RS1043/pCV61</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>RS1051</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>RS1051/pJF611</td>
<td>++</td>
<td>+</td>
<td>–</td>
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<tr>
<td>R. meliloti</td>
<td></td>
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</tr>
<tr>
<td>102F34</td>
<td>–</td>
<td>–</td>
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<tr>
<td>102F34/pCV61</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td><em>R. etli</em></td>
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<tr>
<td>CE3</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CE3/pCV61</td>
<td>++</td>
<td>+</td>
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<tr>
<td>A. tumefaciens</td>
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</tr>
<tr>
<td>GM19023/pCV61</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND = not determined; ++ = wild type size and amount of nodules; + = size and amount of nodules decreased in comparison with ++; – = negative nodulation.
family only bears C18:1 fatty acyl moieties. In this family, we have also found a novel lipo-chito oligosaccharide that carries a mannose as the reducing sugar (Folch-Mallol et al. 1996).

As cosmid pCV61 does not carry a functional nodD gene, it is not expected to direct the induction of the nod genes of recipient strains (either homologous or heterologous) and therefore no Nod factor production should be expected. Besides, heterologous nodD genes are not expected to respond to R. tropici host root exudates. Nevertheless, this cosmid confers a positive nodulation phenotype to all Rhizobium strains tested (Table 1). One possible explanation for the nodulation phenotype conferred by cosmid pCV61 is that its genes are capable of directing the synthesis of R. tropici Nod factors in a nodD-independent manner, thus allowing the recipient strains to nodulate even nonhost plants. Therefore, nod factor production in strains RSP900(pCV61) and RSP900(pJF611) was investigated.

Figure 3A shows a reversed phase thin-layer chromatography (TLC) analysis of the 14C-glucosamine-labeled Nod factors synthesized by strains CIAT899, RSP900(pCV61), and RSP900(pJF611). Strain RSP900 carrying cosmids pCV61 or pJF611 produced Nod factors even in the absence of naringenin (Fig. 3A, lanes 3–6), while the wild-type strain CIAT899 only produced Nod factors when the culture was induced by the flavonoid (Fig. 3A, lanes 1 and 2). The pattern of Nod factors produced by strain CIAT899, with both sulfated and nonsulfated factors present. In contrast, cosmid pF611 only directed the synthesis of the nonsulfated Nod factors (Fig. 3A, lanes 5 and 6). This is due to the lack of the nodHPQ region, which is essential for Nod factor sulfation (Folch-Mallol et al. 1996) (see Figure 1A). The production of Nod factors by bacteria carrying cosmid pCV61 was tested with L-[(methyl-14C) methionine, to assess if they are methylated as in the wild-type strain. Figure 3B shows that RSP900 carrying pCV61 was able to perform the methylation of Nod factors, even in the absence of an inducer (Fig. 3B, lanes 3 and 4). This result indicates that the nodS gene present in pCV61 is functional.

These analyses suggest that the Nod factors produced by the cured strain carrying pCV61 are very similar to those produced by the wild-type strain CIAT899, thus presumably being efficient in promoting nodulation on normal hosts of R. tropici. It also shows that cosmid pCV61 is able to direct the synthesis of both sulfated and nonsulfated Nod factors, as well as methylated Nod factors, in a nodD-independent manner that allows heterologous recipient strains to produce R. tropici Nod factors without the need of plant regulators. This would explain the host range extension observed in heterologous Rhizobium spp.

**nodABCSUIJ genes in pCV61 are expressed constitutively.**

The reason why pCV61 expresses its nodABCSUIJ genes in the absence of nodD was investigated. Since the nodABCSUIJ promoter, including the nod box, must lie within the inter nodD-nodA region, which is incomplete in pCV61, this region was sequenced in an attempt to identify putative sequences involved in the expression of the nodABCSUIJ genes.

Figure 4 shows the sequence of the 297-bp inter nodD-nodA region of the R. tropici nod region present in pCV38. The HindIII site in which pCV61 is cloned, i.e., the 5’ end of the nod region, lies 73 bp from the 3’ end of the nod-box sequence. These data suggest that the entire nodABCSUIJ promoter is located upstream of the HindIII site, and therefore absent from pCV61. The absence of the nod box in pCV61 therefore explains the loss of inducibility by flavonoids of the nodABCSUIJ genes, and the fact that they are not regulated by nodD.

Two short, inverted repeats with the sequence TTAC-N6-GTAA, which are present in the S. meliloti inter nod box-nodA region (Rostas et al. 1986) and the Rhizobium sp. strain NGR234 inter nod box-nodS region (Lewin et al. 1990), were found (Fig. 4). A similar sequence has been reported to be important for nodD (auto)regulation in R. leguminosarum bv. viciae (Rossen et al. 1985; Mao et al. 1994). In B. japonicum a similar sequence is needed for the regulation of nodD1 as well as the nodYABC operon (Wang and Stacey 1991). Further studies are in progress to determine if this sequence is relevant for nod gene regulation in R. tropici.

Two regions that might correspond to –10 and –35 sequences of a putative promoter in pCV61 were identified upstream of the Shine-Dalgarno region and the nodA start codon (Fig. 4). To check whether the nodABCSUIJ operon in pCV61

![Fig. 3](image1.png)

**Fig. 3.** Reversed phase thin-layer chromatography analysis of Nod factors produced in the presence of (A) 14C-glucosamine hydrochloride or (B) L-[(methyl-14C) methionine of strains CIAT899 (lanes 1 and 2 in A and B), RSP900(pCV61) (lanes 3 and 4 in A and B), and RSP900(pJF611) (lanes 5 and 6 in A). Odd-numbered lanes without inducer, even-numbered lanes with 2 µM naringenin. S, sulfated Nod factors. NS, nonsulfated Nod factors.

![Fig. 4](image2.png)

**Fig. 4.** Nucleotide sequence of the inter nodD-nodA region of Rhizobium tropici CIAT899. Arrows indicate starting codon of nodD and nodA. Continuously underlined sequence corresponds to nod box that putatively regulates nodABCSUIJ operon. Asterisk indicates HindIII site in which pCV61 was cloned. Putative –10 and –35 sequences for nodA are indicated by an overline. Dots show an inverted repeat sequence that may play a role in nod gene regulation. Box indicates a Shine-Dalgarno consensus sequence.
was expressed from this putative promoter, a 1-kb HindIII-EcoRV fragment from pCV61, containing the region downstream of the HindIII site shown in Figure 4 and part of the nodA gene, was cloned into plasmid pMP220, as described in the Materials and Methods. pMP220, which is designed to look for promoters in Rhizobium spp. (Spaink et al. 1987), carries a β-galactosidase reporter gene devoid of its promoter. If a promoter region is cloned into the multiple cloning site, β-galactosidase activity is detected. The resulting plasmid (pJFM2) was introduced into R. tropici RSP900, and β-galactosidase activity was measured in the presence or absence of naringenin. No β-galactosidase activity was detected in either the presence or absence of the flavonoid (data not shown), clearly indicating that the region between the HinII site and nodA in pCV61 lacks a promoter. Therefore, the nodABCSUIJ operon in pCV61 must be transcribed from a promoter present in the vector (pVK102). Sequence of a 0.95-kb HindIII-BglII fragment from pCV61 indicated that the nodABCSUIJ operon in pCV61 is in the same orientation of transcription as the kanamycin-resistance gene present in pVK102 (data not shown). Therefore, this operon in pCV61 is most probably expressed from the kanamycin-resistance gene promoter of the cosmid. However, we cannot rule out the possibility that a hybrid promoter may have been generated with sequences present in the kanamycin-resistance gene and the nod region, when pCV61 was constructed.

nodHPQ genes are not regulated by nodD.

In S. meliloti, it has been shown that transcription of the nodPQ genes is not regulated by nodD, as they do not possess a nod box in their regulatory region. In this species, the nodH gene has its own nod box and is regulated by NodD protein (Horvath et al. 1986). In a previous paper, we reported the sequence analysis of the R. tropici nodHPQ genes (Folch-Mallol et al. 1996). The spacing between the nodH stop codon and the nodP start codon is very short (5 nucleotides), so it is possible that the nodH gene in R. tropici is forming part of the same transcriptional unit as the nodPQ genes. The fact that pCV61 is able to induce the production of sulfated nod factors in the absence of flavonoids strongly suggests that transcription of the R. tropici nodHPQ genes is independent of nodD and flavonoids. The possibility that these genes are being transcribed from the constitutive promoter of the vector cannot be discarded, but the distance between such promoter and these genes makes this hypothesis unlikely. Two lines of evidence support the idea that transcription of the nodHPQ genes is nodD-flavonoid independent. Firstly, when the upstream region of the R. tropici nodH gene was sequenced, no regions with the conserved structure of the nod boxes located upstream of the nodD-regulated genes were found (accession number X87608). Secondly, some constructions were made with an interposon from plasmid pAB2001, which carries a gentamicin-resistance gene and a promoterless lacZ gene. Different insertions were made into pHM500, which carries the nodHPQ region (including its promoter) as a 6.4-kb EcoRI-KpnI fragment from pCV61 cloned in pMP92 (Fig. 1B). Plasmid pHM560 carries a single insertion in the nodH gene (XhoI site), plasmid pHM550 carries a single insertion in the nodP gene (BamHI site) in the orientation of transcription, while plasmid pHM551 carries the same insertion as pHM550 but in the opposite orientation (Fig. 1B). All these plasmids lacking the functional nodD gene of R. tropici were introduced into strain RSP900 and the resulting transconjugants tested for β-galactosidase activity in the presence or absence of naringenin. Plasmid pHM550 was also introduced into CIAT899. Figure 5 shows that insertions in the nodH and nodP genes in the proper orientation yielded β-galactosidase activity, even without the addition of inducer, while the insertion in nodP in the opposite orientation yielded no activity regardless of flavonoid addition, in a manner similar to plasmid pHM500. It is worth noticing that the highest level of β-galactosidase activity was attained in CIAT899, suggesting that a positive modulator might be present in the pSym of this strain. However, it seems that this system is not regulated by flavonoids, as the induction reaches the same level with or without naringenin. Studies with plasmids to elucidate regulatory circuits do not always correlate with the actual behavior of naturally expressed genes, so the regulation of these genes in the pSym plasmid could be somewhat different. Taken together, these data indicate that nodHPQ genes in R. tropici are not regulated either by nodD or by flavonoids and strongly suggest that these genes are transcribed as an operon.

As mentioned above, there are some examples in which nodulation genes are not regulated by the interaction with the plant via flavonoids. This may not be surprising in the case of nodPQ genes, because these genes are reiterated in the chromosome in S. meliloti (Schwedock and Long 1992) as well as in R. tropici (Folch-Mallol et al. 1996) and could be considered “housekeeping” in the sense that they may participate in general sulfur metabolism.

nodW is also not regulated by nodD or flavonoids (Gu et al. 1997). This gene shows homology with proteins that function as part of the Type III secretion systems of gram-negative bacteria (Cornelis 1994; Salmond 1994), and plays only a minor role in the symbiotic interaction by blocking nodulation only on certain improved soybean cultivars (Chatterjee et al. 1990). In fact, it is not strange that this gene is not induced by
plant regulators because only when the gene is not functional does nodulation proceed on these cultivars.

**nodZ** is an important gene determining host specificity. When transferred to *R. leguminosarum* bv. *viciae*, this strain produces fucosylated Nod signals and acquires the ability to nodulate nonhost plants such as *M. atropurpureum* and *L. leucocephala* (López-Lara et al. 1996). Another report shows that expression of nodZ in some European *R. leguminosarum* strains allows nodulation of Afghan pea, suggesting that fucosylation is equivalent to acetylation in this system (Ovtsyna et al. 1998). These results suggest that nod factor structure may allow a certain degree of flexibility in order to be biologically active in plants.

It is interesting that a homolog of nodZ (ORFB) has been reported in *Rhizobium* sp. strain NGR234 but, in contrast to the *B. japonicum* nodZ, this gene is under the control of a flavonoid-inducible promoter (Fellay et al. 1995). This fact resembles our findings for nodH in the sense that in *S. meliloti* this gene is regulated by nodD and flavonoids but in *R. tropici* it is not.

**nodH** is a host-specificity nodulation gene that plays a major role in nodulation. It has been proven to be essential for *S. meliloti* nodulation on alfalfa, and it is very important for nodulation of *L. leucocephala* plants by *R. tropici*. This gene is not known to participate in any other metabolic route but the sulfation of Nod factors, which in turn are not produced in the absence of inducing flavonoids. It is interesting that some important genes that determine host specificity, such as nodH and nodZ, are not regulated by nodD or flavonoids. These findings suggest the possibility that these genes may participate in other nonsymbiotic metabolic routes in this bacteria.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.**

Bacterial strains and plasmids used in this study are listed in Table 2.

**Media and bacterial growth conditions.**

*Rhizobium* strains were grown at 28°C in TY (Beringer 1974) or in minimal B- medium as described by Spank et al. (1992), for the production of Nod factors. *Escherichia coli* cultures were grown in Luria broth medium (Miller 1972) at 37°C. Antibiotics were added to the following final concentrations (in µg · ml⁻¹): rifampicin (Rif), 50; tetracycline (Tc), 10; gentamicin (Gm), 35; spectinomycin (Sp), 80; ampicillin (Ap), 100; kanamycin (Km), 30; and streptomycin (Sm), 100. Flavonoids were diluted in ethanol and used at a final concentration of 2 µM.

**Genetic techniques.**

In order to mobilize pVK102 derivatives from *E. coli* to *Rhizobium* spp., triparental matings were carried out as described by Kondorosi et al. (1982) with pRK2073 as a helper plasmid. Selection was made on TY medium with the appropriate antibiotics.

**DNA manipulations and sequencing.**

Total genomic DNA, large- and mini-scale plasmid, and cosmid DNA preparations were done as described by Sambrook et al. (1989). DNA manipulations, including subcloning, restriction digests, ligations, transformations, and electrophoresis were performed according to Sambrook et al. (1989).

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**Table 2. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizobium tropici</em></td>
<td>Wild type, Rif', Cm', Ap'</td>
<td>Martínez et al. 1985</td>
</tr>
<tr>
<td>CIAT899</td>
<td>PSym cured derivative of CIAT899</td>
<td>Vargas et al. 1990</td>
</tr>
<tr>
<td>RSP900</td>
<td>Derivative of wild-type RS800, Rif', Sp'</td>
<td>Rodríguez-Quiriones et al. 1989</td>
</tr>
<tr>
<td><em>R. leguminosarum</em> bv. <em>trifoli</em></td>
<td>PSym cured derivative of strain RS1051, Rif', Sp'</td>
<td>Rodríguez-Quiriones et al. 1989</td>
</tr>
<tr>
<td>RS1051</td>
<td>Wild type</td>
<td>Dylan et al. 1986</td>
</tr>
<tr>
<td>RS1043</td>
<td>R' derivative of wild-type CFN42</td>
<td>Noel et al. 1984</td>
</tr>
<tr>
<td><em>R. etli</em></td>
<td>PSym cured derivative of CE3, Rif'</td>
<td>Palacios et al. 1983</td>
</tr>
<tr>
<td>CE3</td>
<td>PSym cured derivative of CE3, Rif'</td>
<td>Rosenberg and Huguet 1984</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td>Cloning vector, IncP, Tc'</td>
<td>Spaink et al. 1987</td>
</tr>
<tr>
<td>GMI9023</td>
<td>Cloning vector, IncP, Tc'</td>
<td>Spaink et al. 1987</td>
</tr>
<tr>
<td>Plasmids</td>
<td>pSym cured derivative of CS8, Rif'</td>
<td>Rosenberg and Huguet 1984</td>
</tr>
<tr>
<td>pCV38</td>
<td>28.8-kb HindIII fragment of the PSym of CIAT899 cloned in pVK102</td>
<td>Vargas et al. 1990</td>
</tr>
<tr>
<td>pCV61</td>
<td>26.4-kb HindIII fragment of PSym of CIAT899 cloned in pVK102</td>
<td>Vargas et al. 1990</td>
</tr>
<tr>
<td>pVKK12</td>
<td>Cosmid derivative of pRK290, Km', Tc', Ap'</td>
<td>Knauf and Nester 1982</td>
</tr>
<tr>
<td>pBS.SK</td>
<td>Cloning vector, Ap'</td>
<td>Stratagene, La Jolla, CA</td>
</tr>
<tr>
<td>pMP154</td>
<td>nodA::lacZ cloned in pMP190</td>
<td>Zaat et al. 1987</td>
</tr>
<tr>
<td>pMP220</td>
<td>Derivative of plasmid pKT214, carrying lacZ gene without a promoter, Tc'</td>
<td>Spaink et al. 1987</td>
</tr>
<tr>
<td>pMP92</td>
<td>Cloning vector, IncP, Tc'</td>
<td>Spaink et al. 1987</td>
</tr>
<tr>
<td>pJF611</td>
<td>EcoRI fragment deletion from pCV61</td>
<td>This work</td>
</tr>
<tr>
<td>pFM1</td>
<td>1-kb HindIII-EcoRV fragment of pJF611 cloned in pBS.SK</td>
<td>This work</td>
</tr>
<tr>
<td>pFPM2</td>
<td>KpnI-PstI fragment of pF111 cloned in pMP220</td>
<td>This work</td>
</tr>
<tr>
<td>pF1V1</td>
<td>0.95-kb BglII-HindIII fragment of pHK102, cloned in pBS.SK</td>
<td>This work</td>
</tr>
<tr>
<td>pSM302</td>
<td>0.24-kb PstI-SalI fragment of pHM500 in pBS.SK</td>
<td>This work</td>
</tr>
<tr>
<td>pHM500</td>
<td>6.4-kb EcoRI-KpnI fragment of pCV61 carrying nodDHPQ genes cloned in pMP92</td>
<td>This work</td>
</tr>
<tr>
<td>pHM550</td>
<td>BamHI insertion of lacZ-Gm' cassette in nodP gene</td>
<td>This work</td>
</tr>
<tr>
<td>pHM551</td>
<td>Same as pHM550 in opposite orientation</td>
<td>This work</td>
</tr>
<tr>
<td>pHM560</td>
<td>SalI/XhoI insertion of lacZ-Gm' cassette in nodH gene</td>
<td>This work</td>
</tr>
</tbody>
</table>
To construct plasmid pJFM2, a 1-kb HindIII-EcoRV from pHF611 was subcloned in pSKII° to give the intermediate plasmid pJFM1. Subsequently, the 1-kb region was inserted, as a PstI-PstI fragment, in pMP220 to give pJFM2.

To sequence the inter nodD-nodA region, a 1.6-kb, HindIII-BamHI fragment of pCV38 was subcloned in pSKII° and the universal M13 primer was used. The 50-30 chain was sequenced by the Sanger procedure (Sanger et al. 1977).

To sequence the region upstream of nodH, a 0.24-kb SalI-PstI fragment of pHM500 was subcloned in pSKII°, resulting in the plasmid pSM302. Both strands were sequenced by the dideoxy chain termination method (Sanger et al. 1977), in an automatic sequencer with the universal M13 (~20) and reverse primers.

DNA hybridization analysis was carried out according to Southern (1975). Hybridizations were done in 50% formamide at 42°C for the homologous R. tropici probe, while heterologous probes were hybridized in 30% formamide at 42°C. Post-hybridization washes of the Southern blots were in 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) at 50°C.

Computer methods.

DNA sequences were analyzed with the following programs: DNA Strider (C. Mark, Dept. Biologie, Centre d’etudes Nucléaires de Saclay, 91190 Gif-sur-Yvette, France), Brujene (J. Vara, Centro Nacional de Biotecnologia, Madrid, Spain), and FASTA, GAP, and PRETTY of the GCG Sequence Analysis software package version 8.0 (Devereaux et al. 1984).

Determination of β-galactosidase activity.

Assays of β-galactosidase were carried out as described by Zaat et al. (1987). Naringenin (2 lM), apigenin (2 lM), genistein (2 lM), or L. esculenta and P. vulgaris root exudates were added, as indicated. The cultures were concentrated 10-fold in 10 mM MgSO4, and 0.1 ml was subsequently used for β-galactosidase activity determinations (Miller 1972). β-Galactosidase activities were measured at least three times and all standard deviations were less than 20%.

Preparation of P. vulgaris and L. esculenta root exudates.

Root exudates were prepared as reported by van Brussel et al. (1986) and Zaat et al. (1987). Sterile exudates were concentrated 10-fold by vacuum evaporation at 0°C, and were used as 10-fold stocks in induction growth conditions for the determination of β-galactosidase activities.

TLC analysis of Nod factors.

TLC analyses were performed according to Spaink et al. (1992). Briefly, Rhizobium strains were grown on minimal B-medium supplemented with the appropriate antibiotics and flavonoids, as indicated. Cultures were grown to saturation and the supernatant was extracted with 0.5 ml of water-saturated n-butanol. The n-butanol was evaporated to dryness and the resulting powder resuspended in 40 ml of n-butanol. Five microlitters of this solution was applied to the TLC plate (ODS:100% octadecyl silanization; Sigma, St. Louis, MO), where the Nod factors were separated with 50% Acetonitrile/H2O (vol/vol) as the mobile phase.

For the radiolabeling of lipo-chitin oligosaccharides the following compounds and quantities were used: 1 mCi of L-(methyl-14C) methionine (specific activity 55 mCi/mmol; Amersham, Iberica SA, Madrid), or 0.5 mCi of glucosamine hydrochloride 14C (specific activity 52 mCi/mmol; Amersham). TLC plates were exposed to Kodak X-Omat R film for 15 days, and the film was developed with Kodak reagents according to the manufacturer’s instructions.

Plant nodulation tests.

Nodulation tests and reisolation of bacteria from nodules were performed as described by Vargas et al. (1990).

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