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

Jorge Luis Folch-Mallol,[1,2] Hamid Manyani,[1] Silvia Marroquí,[1] Carolina Sousa,[1] Carmen Vargas,[1] Noreide Nava,[2] José Manuel Colmenero-Flores,[2] Carmen Quinto,[2] and Manuel Megías[1]

[1]Department of Microbiology and Parasitology, University of Seville, 41012, Seville, Spain; [2]Department of Plant Molecular Biology, Institute of Biotechnology, U.N.A.M., Cuernavaca, Morelos 62271, Mexico

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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 *nodABCSUIJ* 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 *nodABCSUIJ* bajo el promotor del gen de resistencia a la kanamicina, que lleva el vector donde se ha clonado el fragmento de ADN. Análisis por cromatografia 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; Spaink 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

Corresponding author: Manuel Megías, Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, Apdo. Postal 874, 41012-Seville, Spain; Telephone: +34-95-4556766; Fax: +34-95-4628162; E-mail: megias@cica.es

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 *Macroptilium 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 BR816 (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 *nodABCSUIJ* 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 cosmid 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*, cosmid pCV61 was isolated, among others (Fig. 1A). This cosmid overlaps with cosmid pCV38 (Fig. 1A) in a region of 27 kb from the second *Hin*dIII site to the end of pCV38, and carries the common *nodABCSUIJ* genes, as well as the *nodHPQ* region (Fig. 1A). However, pCV61 lacks the 2-kb *Hin*dIII 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 cosmid pCV61 was introduced into *R. tropici* RSP900, *R. leguminosarum* bv. *trifolii* RS1043 (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 *Eco*RI fragment was deleted from pCV61. The derivative cosmid, pJF611, still carries the *nodABCSUIJ* genes but lacks the *nodHPQ* region (Fig. 1A). This cosmid 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-*

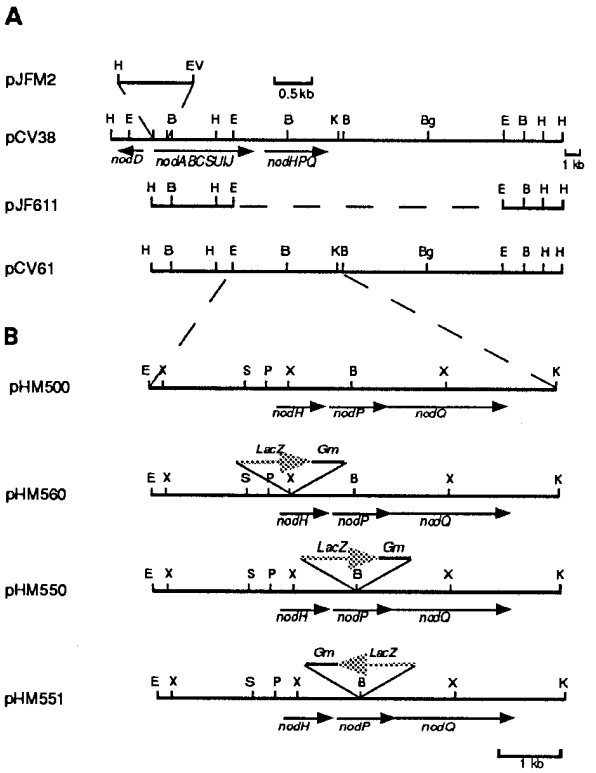

Fig. 1. A, Location of *Rhizobium tropici* CIAT899 nodulation genes in cosmids pCV38 and pCV61 and other constructions used in this study. Size and position of genes are approximate. Arrows indicate direction of transcription. **B,** Reporter gene insertions in *nodHPQ* region. Light arrows indicate direction of transcription of *lacZ* gene; bold arrows indicate direction of transcription of *nodHPQ* genes. Gm, gentamicin resistance. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; and X, *Xho*I.

pureum plants (Table 1). Cosmid pJF611 was still capable of conferring host range extension to the *R. leguminosarum* bv. *trifolii* wild-type strain RS1051, and nodulation ability to the *R. tropici* cured derivative RSP900, although the size and number of nodules formed in *L. leucocephala* plants were severely reduced, compared with the wild-type strain CIAT899, or strain RSP900 (pCV61). Similar results were obtained when nodulation was tested on *M. atropurpureum* plants. This is probably due to the fact that this construction lacks the *nodHPQ* region, which has been shown to be important for efficient nodulation of *L. leucocephala* plants by *R. tropici* (Folch-Mallol et al. 1996). This region, or another absent in pJF611 and present in pCV61, must also be necessary for optimal nodulation of *M. atropurpureum* by *R. tropici*.

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 *Hin*dIII 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 *Hin*dIII-*Eco*RI 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 cosmid 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

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

Strains	Plants[a]		
	Phaseolus vulgaris	***Leucaena leucocephala***	***Macroptilium atropurpureum***
R. tropici			
CIAT899	++	++	++
RSP900	–	–	–
RSP900/pCV61	++	++	++
RSP900/pJF611	++	+	+
R. leguminosarum bv. *trifolii*			
RS1043	–	–	–
RS1043/pCV61	++	ND	ND
RS1051	–	–	–
RS1051/pCV61	++	++	++
RS1051/pJF611	++	+	+
R. meliloti			
102F34	–	–	–
102F34/pCV61	++	ND	ND
R. etli			
CE3	++	–	–
CE3/pCV61	++	++	++
A. tumefaciens			
GMI9023	–	–	–
GMI9023/pCV61	++	ND	ND

[a] ND = not determined; ++ = wild type size and amount of nodules; + = size and amount of nodules decreased in comparison with ++; – = negative nodulation.

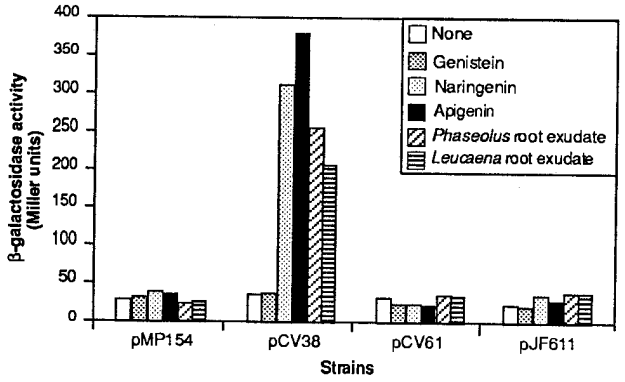

Fig. 2. β-Galactosidase activity of a *nodA-lacZ* fusion in *Rhizobium tropici* RSP900/pMP154 transcojugants carrying cosmids pCV38, pCV61, and pJF611. Flavonoids were added to a final concentration of 2 μM. β-Galactosidase activities were measured at least three times and all standard deviations were less than 20%.

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 ^{14}C-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 RSP900(pCV61) was very similar to that produced by CIAT899, with both sulfated and nonsulfated factors present. In contrast, cosmid pJF611 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-^{14}C) 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.

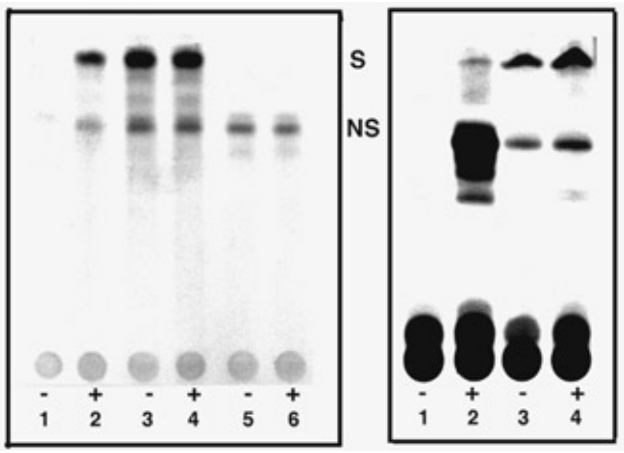

Fig. 3. Reversed phase thin-layer chromatography analysis of Nod factors produced in the presence of **(A)** ^{14}C-glucosamine hydrochloride or **(B)** L-(methyl-^{14}C) methionine of strains CIAT899 (lanes 1 and 2 in **A** and **B**). RSP900/pCV61 (lanes 3 and 4 in **A** and **B**), and RSP900/pJF611 (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.

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 *Hin*dIII 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 *Hin*dIII 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

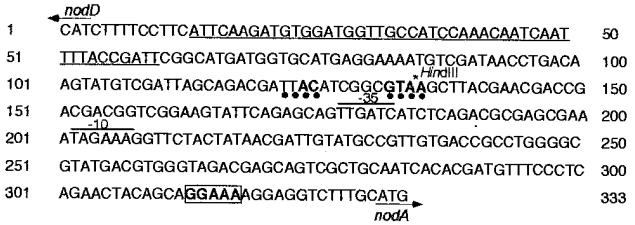

Fig. 4. Nucleotide sequence of the inter *nodD-nodA* region of *Rhizobium tropici* CIAT899. Arrows indicate starting codon of *nodD* and *nodA*. Continuously underlined sequence corresponds to *nod* box that putatively regulates *nodABCSUIJ* operon. Asterisk indicates *Hin*dIII site in which pCV61 was 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 *Hin*dIII-*Eco*RV fragment from pCV61, containing the region downstream of the *Hin*dIII 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 *Hin*dIII 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 *Hin*dIII-*Bgl*II 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 *Eco*RI-*Kpn*I fragment from pCV61 cloned in pMP92 (Fig. 1B). Plasmid pHM560 carries a single insertion in the *nodH* gene (*Xho*I site), plasmid pHM550 carries a single insertion in the *nodP* gene (*Bam*HI 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.

nolW 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

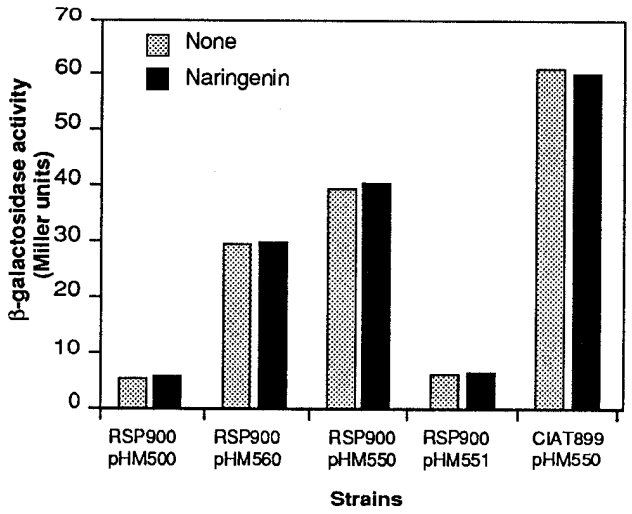

Fig. 5. β-Galactosidase activity of a *lacZ* interposon in *Rhizobium tropici* RSP900 transconjugants carrying plasmids pHM500, pHM550, pHM551, and pHM560, and a CIAT899 transconjugant carrying plasmid pHM500. Naringenin was added to a final concentration of 2 μM. β-Galactosidase activities were measured at least three times and all standard deviations were less than 20%.

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 Spaink 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 $\mu g \cdot ml^{-1}$): 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).

Table 2. Bacterial strains and plasmids

Strains or plasmids	Relevant characteristics	Source or reference
Rhizobium tropici		
CIAT899	Wild type, Rif^r, Cm^r, Ap^r	Martínez et al. 1985
RSP900	pSym cured derivative of CIAT899	Vargas et al. 1990
R. leguminosarum bv. *trifolii*		
RS1051	Derivative of wild-type RS800, Rif^r, Sp^r	Rodriguez-Quiñones et al. 1989
RS1043	pSym cured derivative of strain RS1051, Rif^r, Sp^r	Rodriguez-Quiñones et al. 1989
R. meliloti		
102F34	Wild type	Dylan et al. 1986
R. etli		
CE3	Rif^r derivative of wild-type CFN42	Noel et al. 1984
CFN2001	pSym cured derivative of CE3, Rif^r	Palacios et al. 1983
Agrobacterium tumefaciens		
GMI 9023	pSym cured derivative of C58, Rif^r	Rosenberg and Huguet 1984
Plasmids		
pCV38	28.8-kb *Hin*dIII fragment of the pSym of CIAT899 cloned in cosmid pVK102	Vargas et al. 1990
pCV61	26.4-kb *Hin*dIII fragment of pSym of CIAT899 cloned in cosmid pVK102	Vargas et al. 1990
pVK102	Cosmid derivative of pRK290, Km^r, Tc^r	Knauf and Nester 1982
pBS.SK	Clonig vector, Ap^r	Stratagene, La Jolla, CA
pMP154	*nodA::lacZ* cloned in pMP190	Zaat et al. 1987
pMP220	Derivative of plasmid pKT214, carrying *lacZ* gene without a promotor, Tc^r	Spaink et al. 1987
pAB2001	Plasmid carring *lacZ*-Gm^r cassette, Ap^r	Becker et al. 1995
pMP92	Cloning vector, IncP, Tc^r	Spaink et al. 1987
pJF611	*Eco*RI fragment deletion from pCV61	This work
pJFM1	1-kb *Hin*dIII-*Eco*RV fragment of pJF611 cloned in pBS.SK	This work
pJFM2	*Kpn*I-*Pst*I fragment of pJFM1 cloned in pMP220	This work
pYL01	0.95-kb *Bgl*II-*Hin*dIII fragment of pVK102, cloned in pBS.SK	This work
pSM302	0.24-kb *Pst*I-*Sal*I fragment of pHM500 in pBS.SK	This work
pHM500	6.4-kb *Eco*RI-*Kpn*I fragment of pCV61 carring *nodHPQ* genes cloned in pMP92	This work
pHM550	*Bam*HI insertion of *lacZ*-Gm^r cassette in *nodP* gene	This work
pHM551	Same as pHM550 in opposite orientation	This work
pHM560	*Sal*I/*Xho*I insertion of *lacZ*-Gm^r cassette in *nodH* gene	This work

To construct plasmid pJFM2, a 1-kb *Hin*dIII-*Eco*RV from pJF611 was subcloned in pSKII$^+$ to give the intermediate plasmid pJFM1. Subsequently, the 1-kb region was inserted, as a *Kpn*I-*Pst*I fragment, in pMP220 to give pJFM2.

To sequence the inter *nodD-nodA* region, a 1.6-kb, *Hin*dIII-*Bam*HI 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 *Sal*I-*Pst*I 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 2× SSC (1× 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 Nucleaires de Saclay, 91190 Gif-sur-Yvette, France), Brujene (J. Vara, Centro Nacional de Biotecnología, 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 μM), apigenin (2 μM), genistein (2 μM), or *L. esculenta* and *P. vulgaris* root exudates were added, as indicated. The cultures were concentrated 10-fold in 10 mM $MgSO_4$, 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 microliters 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/H_2O (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-^{14}C) methionine (specific activity 55 mCi/mmol; Amersham, Iberica SA, Madrid), or 0.5 mCi of glucosamine hydrochloride ^{14}C (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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