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The *nodS* Gene of *Rhizobium tropici* Strain CIAT899 Is Necessary for Nodulation on *Phaseolus vulgaris* and on *Leucaena leucocephala*

F. Waelkens,[1] T. Voets,[1] K. Vlassak,[2] J. Vanderleyden,[1] and P. van Rhijn[1]

[1]F. A. Janssens Laboratory of Genetics, Willem de Croylaan 42, B-3001 Heverlee, Belgium;
[2]CNPAB/EMBRAPA, Km 47, Itaguai 23851, Rio de Janeiro, Brazil
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***Rhizobium tropici* strain CIAT899 induces nitrogen-fixing nodules on the roots of a wide range of tropical legumes, including *Phaseolus vulgaris* and *Leucaena leucocephala*. Previously, a DNA region of the CIAT899 pSym plasmid containing the common nodulation genes *nodABC* and one of the *nodD* alleles was characterized (P. van Rhijn, B. Feys, C. Verreth, and J. Vanderleyden, J. Bacteriol. 175: 438-447, 1993). As reported here, the region immediately downstream of *nodC* contains the *nodSU* genes. The nucleotide sequence of these genes is presented. CIAT899 *nodS* and *nodU* mutants were constructed. The *nodS* mutant was completely deficient in nodulation on the host plants *P. vulgaris* and *L. leucocephala*. The *nodU* mutation caused a decrease in nodulation on *Leucaena* but resorted no effect on *Phaseolus*. Introduction of the CIAT899 *nodABCSU* region in *R. etli* CE-3, a strain that only nodulates *P. vulgaris*, caused an extension of the host range of strain CE-3 to *L. leucocephala*.**

Additional keywords: host range, Nod factor

Bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* form nitrogen fixing nodules on their leguminous host plant. Nodule formation is initiated by bacterial signal molecules, called Nod factors, that are synthesized by the products of the plant inducible *nod* genes. Nod factors are lipo-oligosaccharides consisting of three to five β-1,4-linked *N*-acetyl glucosamine residues. The sugar residue at the non-reducing end of the molecule is *N*-acylated, and depending on the strain, modifications can occur at different positions (Dénarié *et al.* 1992). The number of *N*-acetyl glucosamine residues, the nature of the acyl group, and the site of decorations determine the host specific activity of Nod factor molecules on the plant (Dénarié and Cullimore 1993). For example, in *Rhizobium meliloti* Nod factors, the presence of a sulphate group seems to be necessary for nodule induction on the host plant alfalfa.

R. tropici strain CIAT899 was originally isolated from root nodules of bean plants (*Phaseolus vulgaris*). Besides bean, strain CIAT899 can effectively nodulate several other tropical legumes like *Macroptilium* and *Leucaena*. The *nodSU* genes, which seem to be involved in nodulation of *Leucaena* trees, were originally isolated in the broad host range *Rhizobium* sp. NGR234 (Lewin *et al.* 1990). A NGR234 *nodS* mutant has lost the ability to nodulate *Leucaena*, whereas a *nodU* mutant is delayed in *Leucaena* nodulation. In *Bradyrhizobium japonicum*, the *nodSU* genes are also present, but no phenotype has been described for mutants in these genes (Göttfert *et al.* 1990). Introduction of *B. japonicum nodSU* genes into the NGR234 *nodSU* mutants failed to restore the wild-type phenotype of the latter (Lewin *et al.* 1990). *nodSU* genes have also been cloned from *R. fredii* (Krishnan *et al.* 1992) and from *A. caulinodans* (Geelen *et al.* 1993). By hybridization of 35 rhizobia on genomic DNA, a good—although not perfect correlation—was demonstrated between the presence of *nodSU* homologous DNA and nodulation on *Leucaena* (Krishnan *et al.* 1992).

The biochemical function of the NodS protein has been studied by Geelen *et al.* (1993). They present evidence that the *A. caulinodans* NodS is probably responsible for *N*-methylation of the Nod factor. The fact that Nod factors of *Rhizobium* sp. NGR234 and *A. caulinodans* ORS571 are also *N*-methylated (Price *et al.* 1992; Mergaert *et al.* 1993) corroborates this view.

In this paper, we describe the characterization of *nodSU* genes from *R. tropici* strain CIAT899. Mutations in *nodS* and *nodU* of *R. tropici* strain CIAT899 cause a Nod$^-$ and a decreased Nod$^+$ phenotype on *Leucaena*, respectively. It was also shown that *nodS* but not *nodU* is required for nodulation of common bean in *R. tropici* strain CIAT899, *Rhizobium* sp. NGR234 and *A. caulinodans* ORS571. Weak complementation of the NGR234 *nodS* mutant with the CIAT899 *nodS* gene could be demonstrated on both *P. vulgaris* and *L. leucocephala*.

RESULTS

Nucleotide sequence of the CIAT899 *nodSU* region.

A 6.2-kb *Eco*RI fragment from *R. tropici* strain CIAT899, containing part of *nodD1* and the complete *nodABC* operon was cloned in pUC19. A physical map of the resulting plasmid pCD17 was constructed (Fig. 1). Small subclones suitable for sequencing were constructed in the vector pUC18 and the nucleotide sequence of the region downstream of *nodC*

was determined on both strands (Fig. 2). Strong homology was found with previously identified *nodSU* operons from *A. caulinodans*, *B. japonicum*, *R. fredii,* and *Rhizobium* sp. NGR234. The 5′ region of *nodS* overlaps with the *nodC* coding region, and the 3′ end of *nodU* overlaps with the start of *nodI*. This organization resembles that of the *nod(Y)ABCSUIJ* operons in *B. japonicum* and *A. caulinodans* (Göttfert *et al.* 1990; Geelen *et al.* 1993).

Three possible start codons for the CIAT899 *nodS* gene were found (Fig. 2). Depending on the start codon, the overlap with the 3′ end of *nodC* extends over 7 to 26 amino acids. It should be noticed that the derived NodC protein is considerably longer at the carboxy terminus (approximately 20 amino acid residues) than the NodC proteins of *R. leguminosarum* bv. *phaseoli* and *R. meliloti* (data not shown). Since NodS proteins of different rhizobia display little homology at the amino terminus, it is not possible to assign the most likely start codon of the CIAT899 *nodS* gene on the basis of homology.

In Figure 3, we have arbitrarily chosen the shortest NodS protein to align with other known NodS proteins. This shortest NodS has approximately the same length as the NGR234 NodS protein, which is the longest known. Homology between the different NodS proteins consists of 15.4% identical and additionally 32.2% similar amino acids. It can be observed from Figure 3A that in the *R. fredii* NodS, the domain that shows similarity with *S*-adenosyl-methionine (SAM) dependent methyltransferases (Geelen *et al.* 1993) is not present. The dendrogram shown in Figure 3B demonstrates that the CIAT899 NodS protein is most similar to its NGR234 counterpart.

The sequence of the NGR234 NodU protein is not completely known. Therefore, NGR234 is not included in the alignment of NodU proteins, shown in Figure 4. Overall homology is much stronger in NodU proteins than in NodS proteins: 40.2% identical and 30.8% conserved amino acids are observed. If the *A. caulinodans* ORS571 NodU protein, which is the most distant, is excluded, these values even attain 58.7% identity and 25% conserved substitutions.

Mutation and complementation analysis: nodulation phenotype.

To confirm the involvement of *nodS* in nodulation on *Leucaena* (Lewin *et al.* 1990), the nodulation phenotype of *Rhizobium* strains CIAT899 and NGR234 on *L. leucocephala* was compared with the nodulation phenotype of the corresponding *nodS* mutants. The results are shown in Table 1. The wild-type *R. tropici* CIAT899 nodulates *Leucaena* well. A less pronounced Nod$^+$ phenotype on *Leucaena* was obtained with *Rhizobium* sp. NGR234. Both *nodS* mutants were completely Nod$^-$ on *Leucaena*. Introduction of pFW17, containing *nodABCSU* from CIAT899, could partially restore the Nod$^+$ phenotype of the CIAT899 mutant. Heterologous complementation of the NGR234 mutant was very weak: An occasional nodule was observed with the strain NGR234Ω25 harboring pFW17, but no nitrogen fixation.

R. tropici strain CIAT899 was originally isolated from bean nodules. To study the effect of a *nodS* mutation on bean nodulation, bean seedlings were inoculated with the same *Rhizobium* strains as above (Table 1). Wild-type *R. tropici* strain CIAT899 induces large numbers of nitrogen-fixing nodules on bean. Wild-type *Rhizobium* sp. NGR234 also nodulates bean, although nodules are delayed and less abundant than observed with strain CIAT899. As on *Leucaena*, the *nodS* mutants showed a Nod$^-$ phenotype on bean, which could be partially restored by pFW17 for the CIAT899 mutant and very weakly for the NGR234 mutant.

For *A. caulinodans* strain ORS571, nodulation on bean or on *Leucaena* was not described before. However, as this strain also contains an active *nodS* gene (Geelen *et al.* 1993), we included the wild-type ORS571 and the *nodS* mutant in our plant experiments. As can be seen from Table 1, strain ORS571 has a Nod$^+$ phenotype on both host plants tested. The

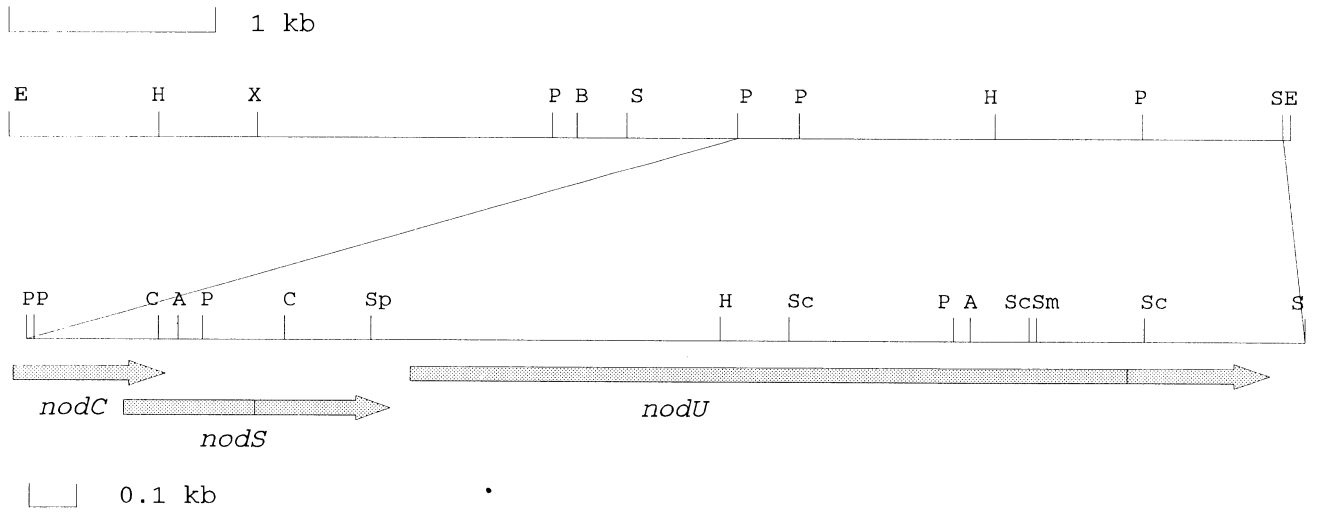

Fig. 1. Physical map of the 6.2-kb *Eco*RI fragment containing the *nodABCSU* genes from *R. tropici* CIAT899. Restriction sites for *Bam*HI = B, *Eco*RI = E, *Hin*dIII = H, *Pst*I = P, and *Sal*I = S are indicated. The sequenced 2.8-kb *Pst*I-*Sal*I fragment (see Fig. 2) is shown below, with additional restriction sites for *Acc*I = A, *Cla*I = C, *Sac*I = Sc, *Sma*I = Sm, and *Sph*I = Sp. Arrows indicate the position of *nodC* (3′ end), *nodS,* and *nodU*.

ORS571 *nodS* mutant, as for the other strains, is completely Nod$^-$ on both host plants.

The *nodU* mutants of *R. tropici* strain CIAT899, *Rhizobium* sp. NGR234 and *A. caulinodans* strain ORS571 were also inoculated on the same host plants. On *Leucaena*, we observed a decreased nodulation phenotype for the CIAT899 *nodU* mutant as compared to the wild-type strain (Table 1). No clear difference in nodulation phenotype between the wild-type strain ORS571 and the *nodU* mutant could be observed, although small differences in kinetics would have escaped detection in our experiment. On bean plants, all three *nodU* mutants formed at least the same number of nodules as compared with the corresponding wild-type strains. This indicates that the Nod$^-$ phenotype of the *nodS* mutants is due to *nodS* deficiency and not to polar effects of the mutations.

R. etli strain CE-3 normally does not nodulate *Leucaena*. We introduced pFW17, carrying the CIAT899 *nodABCSU* operon, into this strain to study the effect on host range. The transconjugant strain CE-3 (pFW17) induced large numbers of nitrogen-fixing nodules on *Leucaena* (Table 1). This indicates that host range limitations of strain CE-3 for *Leucaena* only play at the level of nodule induction, and that the development of nitrogen-fixing bacteroids can be fully supported by the novel host plant. Similarly, introduction of the cosmid pA16, which contains the *nodSU* operon from NGR234, into CE-3 caused an extension of the host range of CE-3 to include *Leucaena* (results not shown).

DISCUSSION

Geelen *et al.* (1993) gave substantial evidence that the biochemical function of NodS is the *N*-methylation of the *N*-acetyl glucosamine residue at the non-reducing end of the Nod factor. The structure of Nod factors from *R. tropici* strain

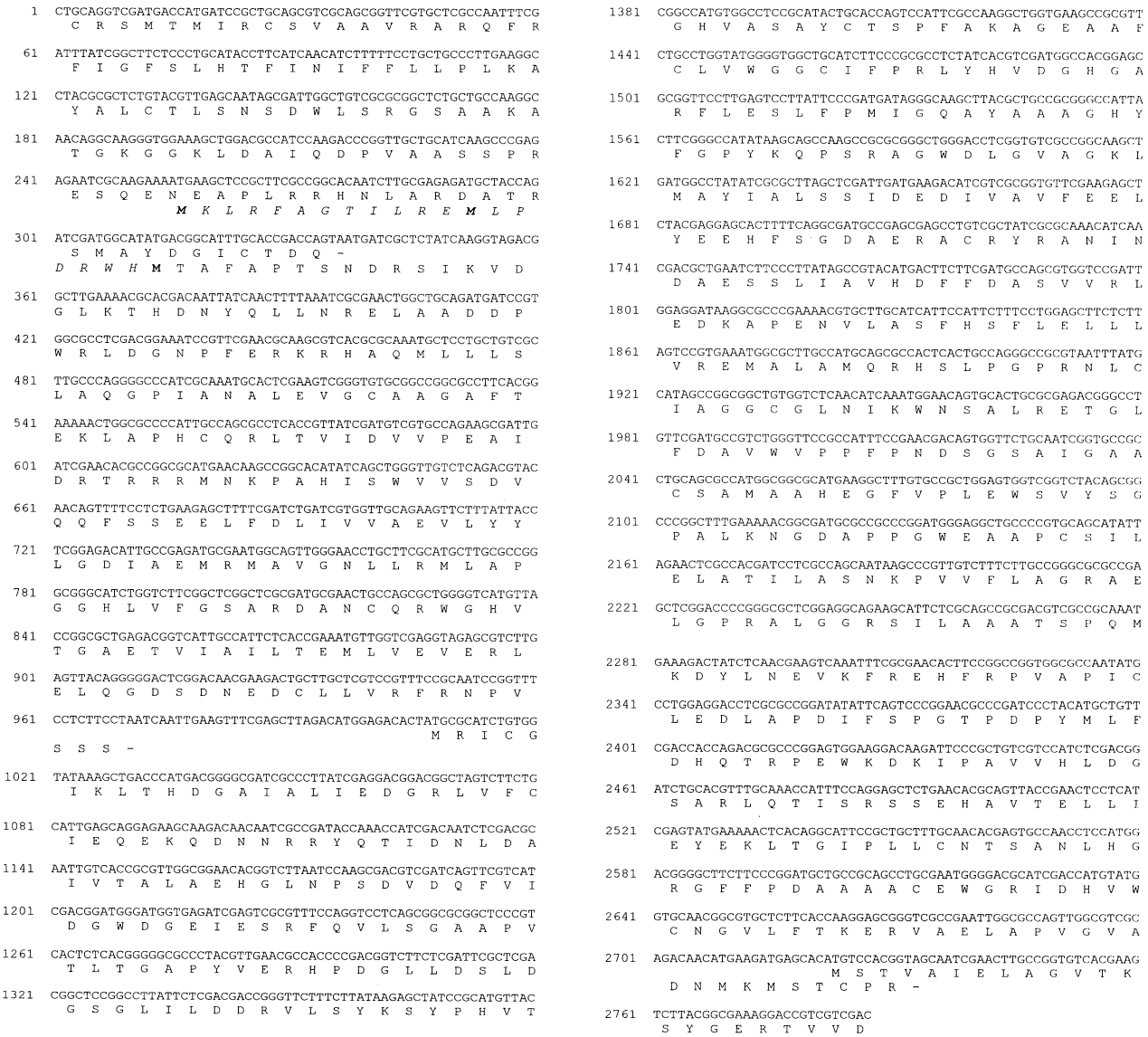

Fig. 2. Complete nucleotide sequence of the 2.8-kb *Pst*I–*Sal*I fragment containing the 3′ end of *nodC* (position 1–337), *nodS* (position 255–971), *nodU* (position 1007-2734), and the 5′ end of *nodI* (position 2722–2787). Derived amino acid sequences are indicated under the nucleotide sequence. Three methionine residues that each can be the amino terminal residue of the NodS protein are indicated in bold character, the amino acid sequence that is not included in the alignment in Fig. 3 is indicated in italics.

CIAT899 has not been published yet, but the *R. tropici* strain CFN299, that has the same host range as CIAT899, was shown to produce *N*-methylated Nod factors (Poupot *et al.* 1993). We therefore postulate that in *R. tropici* strain CIAT899, the presence of *nodS* will correlate with the presence of an *N*-methyl group in the Nod factors, as it was observed for *Rhizobium* sp. NGR234 and *A. caulinodans* ORS571.

The assumption that the NodS protein is a methyltransferase is primarily based on the presence of a conserved domain that shows similarity with a presumed *S*-adenosyl methionine (SAM) binding domain in SAM-dependent methyltransferases (Geelen *et al.* 1993). However, as we show in the alignment of deduced NodS proteins, the *R. fredii* NodS protein does not contain this conserved region. Krishnan *et al.* (1992) invoke poor expression of the *R. fredii nodS* gene to explain lack of *Leucaena* nodulation, but it could also be that the *R. fredii* NodS protein is not functional as a methyltransferase.

Our experiments demonstrate that *nodS* mutations in *R. tropici* CIAT899, *Rhizobium* sp. NGR234, and *A. caulinodans* ORS571 result in a Nod$^-$ phenotype on the host plants bean and *Leucaena*.

Introduction of the wild-type *nodABCSU* operon from CIAT899 on a broad host range plasmid could only partially restore the Nod$^-$ phenotype in the homologous strain, and to an even lesser extent in the heterologous NGR234 mutant. The reason for this poor complementation is not clear and several hypotheses can be considered.

Considering the fact that *nodU* mutants still nodulate the

3A

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RT   MTAFAPTSNDRSIKVDGLKTHDNYQLLNRELAADDPWRLDGNPFERKRHAQMLLLSLAQGPIANA-LEVGCAAGAFTEKLAPHCQRLTVIDVVPEAIDRT   99
NGR  MCK-SLCRSVHGVSEANLTQVNNYHLLHRELAAEDPWRLDANAFEQERHSQMLRLSLSQGPITNA-LEVGCAAGAFTEKLAPYCKRLTVIDVVPRAIART   98
RF   M------RSSRS----------------------DIRRCFGCRFPK-------VLSPTHSKSGAR-FAI-------HGKLAPYCKRLTVIDVVPRAIART   57
BJ   M-----------------TQDENHQLLERELAVDDPWRLDTSAFEQQEYAQMLRMSRRDGDAASAALEVGCAAGAFTEMLAPLCERLTVVDVMPQAIERT   83
ORS  M-------EDPDLVATNLSPAGVKDLLRKDLDARDPWNISSCEYEQKRLHAIMELCLG-GRTLGDVLEIGCAAGALTERLSIYSQSLTVVELMTEAIAKA   92
     *                                * .. . . .       .. . . ...       ..*. ...***..... **...

RT   RRRMNKPAHISWVVSDVQQFSSEELFDLIVVAEVLYYLGDIAEMRMAVGNLLRMLAPGGHLVFGSARDANCQRWGHVTGAETVIAILTEMLVEVERLELQ  199
NGR  RQRMKESSHINWIVADVRQFSTQQLFDLIVVAEVLYYLEDVAAIRTAVHNLVSMLAPSGHMVFGSAIDANCRRWGHVAGAETVIAMLNETLIEVERLYCR  198
RF   RQRMKESSHINWIVADVRQFSTQQLFDLIVVAEVLYYLEDVAAIRTAVHNLVSMLAPSGHMVFGSAIDANCRRWGHVAGTETVIAMLNETLIEVEQLYCR  157
BJ   RLRTGKWSHISWVTCDIQRFSTTEQFDLIVVAEVLYYLRDVVEMHAAIRNLVSMLAPDETLIFGSARDEICQRWGHAAGAETVIALFNESLSEIERRHCC  183
ORS  QERVNN-SDIIWHEQDVCQMDLNAKYDTIICTEVLYYIHEKERLRGALENIVQLLRSDGQFIFGSPRNAVCRSWGHLFGAEAVLELAQSRLRIVDLRRIN  191
     . *  . ..* *   *. ...  . .* *. .*****. .   .. *. *.. .* ... ..***. .. *..***  *.*.*...  . *  ..

RT   GDSDNEDCLLVRFRNPVSSS-------  219
NGR  GASVNEDCLLSRFQK--STT-------  216
RF   GASVNEDCLLSRFQK--STT-------  175
BJ   TGSASEDCLIVRFLKPGGASAQSNAGH  210
ORS  GGLPGQDCVIAKLTR----------R  207
     ..  ..**.. .. .
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3B

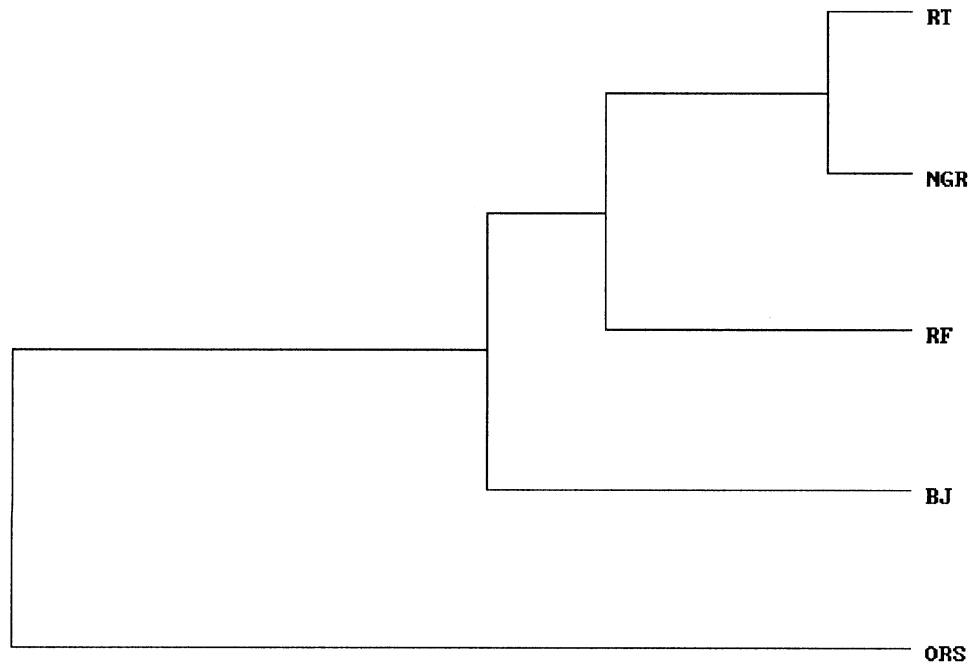

Fig. 3. Alignment of NodS proteins from *Rhizobium tropici* (RT), *Rhizobium* sp. NGR234 (NGR), *R. fredii* (RF), *Bradyrhizobium japonicum* (BJ), and *Azorhizobium caulinodans* ORS571 (ORS). **A,** Alignment of amino acid sequences. Perfectly conserved positions are indicated by an asterisk, conserved substitutions by a dot. The conserved domain that has similarity to methyltransferases is indicated in bold character. **B,** Dendrogram of NodS proteins, showing the relative distances.

host plants, the hypothesis that the nodulation phenotype is due to downstream effects of the *nodS* mutation, not covered by the complementing *nodABCSU* plasmid, can be ruled out. Problems with stability of the plasmid pFW17 could be invoked. However, it is not likely that a *Rhizobium* strain needs to proliferate much before sufficient amounts of Nod factor are produced, so that plasmid loss would not affect early stages in the symbiosis.

4A

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RT   M-----------------------RICGIKLTHDGAIALIEDGRLVFCIEQEKQDNNRRYQTIDNLDAIVTALAEHGLNPSDVDQFVIDGWDGEIESRF   76
BJ   M-----------------------RICGIKLTHDGAIAVVEDGRRLFCVEQEKRGNGPRYQSVDNLDAVVFALAEHGLNPRDIDQFVIDGWDGENESQF   76
RF   MKTACFPVFRNRLPDLDRDLDRDTKRVCGIKLTHDGAIAVVEDGRLVFCTEQEKRNNNSRYQEINNLDAVVAALAENGVNARDVDQFVIDGWDGEAESRF  100
ORS  MKR-------------------------CGLKLTHDGGVAVLDGRDLVACIEMEKLTNNERYRRIEHTDEIALALHRSGFQPSDIDEYIIDGWDGEVDAWV   76
     *                           **.******..*....   . *.* ** .*. **. ... *... **   * ...*.*...******* ..

RT   QVLSGAAPVTLTGAPYVERHPDGLLDSLDGSGLILDDRVLSYKSYPHVTGHVASAYCTSPFAKAGEAAFCLVWGGCIFPRLYHVDGHGARFLESLFPMIG  176
BJ   QLLSGAVPVALKGAPYVERHAEGLLDSVDGYGLLLGGEEFPYKSYPHVTGHVASAYSTSPFASAGKPALCLVWDGCIFPRLYYVEPQGARLIGSLFPMIG  176
RF   KVLSGETPVILRGAPYVERHAEGLLDWIGGSGLTLGDRVFSYRSYPHVTSHVASAYCTSPFAKSGDPALCLVWDGCIFPQLYHVEGKRASFVKSLFPVTG  200
ORS  ELLGAAGRVQLKVAPYVEKEPDRANEFTQGFGLNILGRDYTYKSAPHVMGHIASVYCTSPFAIFKQKALCLVWDGSIWPRLYEISDGGIRFINTLPFMIG  176
     ..*... .* *  *****....   .   * ** . .   .*.* *** .*.**.*.*****   . *.****.*.*.*.** ..    ... .*  ..*

RT   QAYAAAGHYFGPYKQPSRAGWDLGVAGKLMAYIALSSIDEDIVAVFEELYEEHFSGDAERACRYRANINDAESSLIAVHDFFDASVVRLEDKAPENVLAS  276
BJ   HAYAAAGLHFGPYRQPNRSSWDLGIAGKLMAYIELGSVDESIVEVFQGLYETRSAADTEQARRYRENINNAEASLAVIHDFFESSALRLKAKRAEDVLAS  276
RF   QAMAAAGHYFGPYKQTSRGGWDLGVAGKLMAFVALGSVHVRIVAVFQKLYQEHFAGDTALACAFRANINNSESSLAAVHDFFAASALQLGQRRPKTCLHR  300
ORS  HAYACAGHHFGPYKNADRTSWKLDLAGKLMSYMSTGTVDSRITAAIQTSYQNNLAGMSPNALSYRRMSANTSVALIQTHRFFEEIGVLVAGAPEHDILAT  276
     .* * ** .****....*..*.*..*****.... ....  *.....  *... .. .  *  .*   .... .*  .* **..  . .     .. *

RT   FHSFLELLLVREMALAMQRHS-LPGPRNLCIAGGCGLNIKWNSALRETGLFDAVWVPPFPNDSGSAIGAACSAMAAHEGFVPLEWSVYSGPALKNGDAPP  375
BJ   FHVFLERLLVKEIAMVLLRHSSLPGARNLCIAGGCGLNIKWNSALRATGLFDDVWVPPFPNDSGSAIGAACGAMAAQDGFEPLEWSVYSGPALQESEVPP  376
RF   L-IFSSNVSSLTKWRTLQHHP-LPGARNLCIAGGCGLNIKWNSALRQTGLFDSVWVPPFPNDSGSAIGAACCEIVAQQGFVPLDWSVYSGPSLQDRQVPA  398
ORS  FHYFVERLLIETLRHELARAGR-NMSRNLCISGGCGLNIKWNSALRSSGLFRDVWVSPFPNDSGSAIGAACSALVANDGLVPINWSVFSGPHLVKSTPDA  375
     .  * . .   .   . .      .******.*************** .*** .***.************** ...*..*. *..***.*** * .    .

RT   GWEAAPCSILELATILASNKPVVFLAGRAELGPRALGGRSILAAATSPQMKDYLNEVKFREHFRPVAPICLEDLAPDIFSPGTPDPYMLFDHQTRPEWKD  475
BJ   DWEAAPCSLPELASILADNKPVIFLSGCAGLGPRALGGRSILAAPTSPEMKDHLNDIKRREHFRPVVPICLEDRAPEIFSPGTPDPYMLFDHQTRANWRD  476
RF   GWHASPCSISEVAAILASNKPVVFLSGRTELGPRALGGRSILAAATSPEMKDHLNEIKFREHFRPVAPICLEDRAPDIFSPGTPDPYMLFDHQTKMPWQD  498
ORS  NWRGSACELSELAALLADGEPVVFLAGRAELGPRALGARSILAPASDRSMKDRLNAAKQREYFRPVAPICLEDRAPEIFEPGSNDRYMLYDHKVREGWRD  475
     .* ...*.. *.*..**...**.**.* ..******.*****..... *** **. * **.****.****** **.**.**. *.***.**...  *.*

RT   KIPAVVHLDGSARLQTISRSSEHAVTELLIEYEKLTGIPLLCNTSANLHGRGFFPDAAAACEWGRIDHVWCNGVLFTKERVAELAPVGVADNMKMSTCPR  575
BJ   KIPAVVHLDGSARLQTISRNSPHKIAALLIEFEQLTGIPLLCTTSANLHGRGFFPDAAAACQWGRVEHVWCEGMLWSKTVIKKSSPT----ERLLSA---  569
RF   KVPAVVHLDGSARLQTISRNSQHKVAEVLVEYEKLTGIPLLCNTSANYHGRGFFPSAAAACEWGRVEHVWCDGMLYRKPSATA----------------  581
ORS  RVPAIMHLDGSARVQTIARTSAHPVAKLLVEYEKLTNIPLLCNTSANALGRGFFPDVASACTWGRIAKVWAENVLWSNDVDARIP--------------  560
     ..**..*******.***.*.* * .. .*.*.*.**.*****.****  ******..*.** ***...** ...*. .
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4B

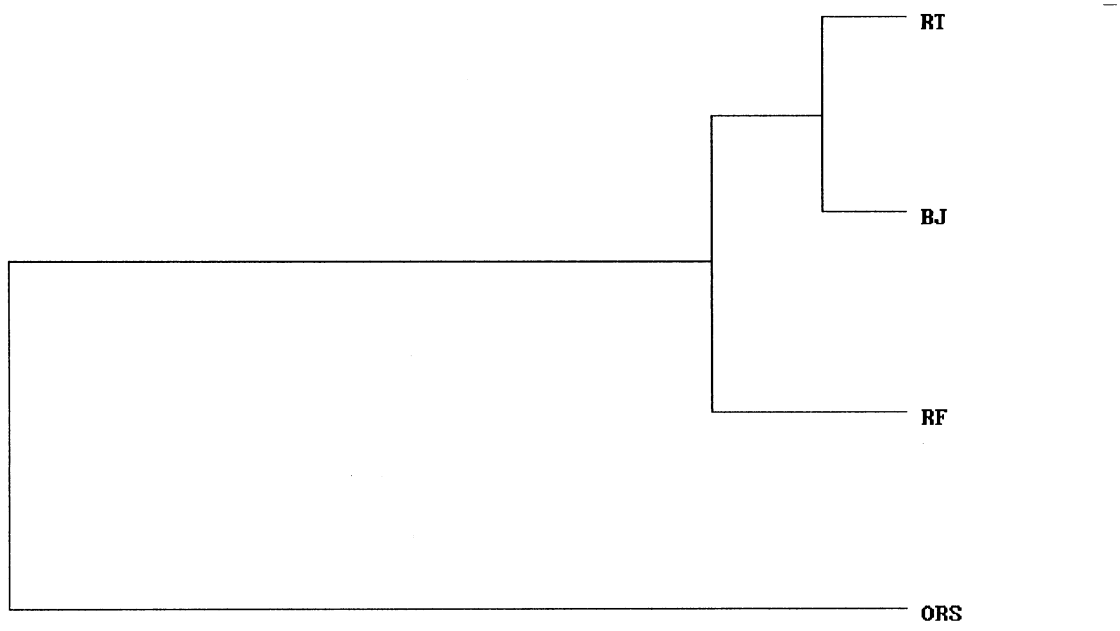

Fig. 4. Alignment of NodU proteins from *Rhizobium tropici* (RT), *R. fredii* (RF), *Bradyrhizobium japonicum* (BJ), and *Azorhizobium caulinodans* ORS571 (ORS). **A,** Alignment of amino acid sequences. Perfectly conserved positions are indicated by an asterisk, conserved substitutions by a dot. **B,** Dendrogram of NodU proteins, showing the relative distances.

Specifically concerning the very poor complementation of the NGR234 *nodS* mutant, it is possible that the expression level of the CIAT899 *nodSU* genes is insufficient. In NGR234, *nodSU* has its own promoter and *nod* box, and expression levels may be much higher than in a *nodABCSU* operon, where transcripts are very long and where the overlap between *nodC* and *nodS* may hinder efficient translation. An alternative explanation for the lack of complementation in the NGR234 mutant may be that the CIAT899 *nodS* gene product poorly recognizes the Nod factor intermediates available in the NGR234 *nodS* mutant, because of other modifications present. This would mean that *nodS* is a host specific *nod* gene in the strict sense, i.e., that interspecies complementation can not occur.

A possible explanation for the incomplete complementation in the CIAT899 background may be that the balance between levels of different *nod* gene products is disturbed, due to the presence of multiple copies of *nodABCSU*.

Introduction of either the CIAT899 *nodABCSU* operon or the NGR234 *nodSU* operon into *R. etli* strain CE-3 extended the host range to *Leucaena*. This is somewhat unexpected, since bean is the natural host of strain CE-3 and our results demonstrate that *nodS* is necessary for nodule formation on bean. However, hybridization with a NGR234 *nodSU* probe on total DNA of strain CE-3 revealed no homology (unpublished results). Krishnan *et al.* (1992) performed DNA hyridizations on a large number of *Rhizobium* strains using the *R. fredii nodSU* region as a probe. They included seven strains isolated from bean, and only in the strains that also nodulate *Leucaena*, homology with the *nodSU* probe was detected. It seems that for bean, like for *Leucaena* (Krishnan *et al.* 1992), several genetic strategies for nodulation are possible.

Our results raise the question as to the role of *N*-methylation in the determination of host range. It is possible that in the strains CIAT899, NGR234, and ORS571, *N*-methylation is inispensable for nodulation on bean and *Leucaena*. Strain CE-3 also forms at least some *N*-methylated Nod factors (E. Martinez, personal communication), although no *nodS* homologous DNA is present, but it does not nodulate *Leucaena*. Possibly *Leucaena* nodulation requires a higher concentration of methylated Nod factors than bean. Possibly in CE-3 only a minor fraction of the Nod factors is methylated by a household methyltransferase or a poorly conserved NodS, so that the concentration of methylated Nod factors is sufficient for bean but not for *Leucaena* nodulation. Introduction of heterologous *nodSU* genes could improve methylation, or in the case of the complete CIAT899 *nodABCSU* operon, the total amount of Nod factors produced could be increased. Alternatively, the CE-3 Nod factors may carry an additional modification that makes them competent for bean nodulation together with, or independently of the presence of a methyl group. We favor the idea of an additional modification, since CE-3 nodulates only bean, and is thus expected to be ideally equipped for nodulation on its natural host.

Leucaena nodulation may thus require a higher concentration of methylated Nod factor, or alternatively it may require a different additional modification. This would then be achieved upon introduction of *nodU*. The hypothesis that host range extension could be due to the introduction of *nodU* seems less probable, because the *nodU* mutants of NGR234 (Lewin *et al.* 1990) and those tested in this study are only marginally affected in *Leucaena* nodulation, which indicates that the role of *nodU* in *Leucaena* nodulation is limited. However, it could be that in the presence of an active NodU protein, the distribution of Nod factor molecules is shifted towards competence for *Leucaena* nodulation. Our observations that *nodU* mutants of CIAT899, NGR234, and ORS571 nodulate bean at least as well as the wild-type strains, would indicate that in the absence of NodU, the balance is shifted in the direction of bean nodulation. Elucidation of the biochemical function of the NodU protein would certainly help to confirm this hypothesis.

MATERIALS AND METHODS

Strains, plasmids and media.

Strains and plasmids used in this study are listed in Table 2. *Rhizobium* and *Azorhizobium* strains were grown on TY

Table 1. Nodulation phenotype of rhizobia on *Leucaena* and *Phaseolus*

	Phaseolus	*Leucaena* (plastic pots)	*Leucaena* (Leonard jars)	
Inoculated strain	**Nodules per plant[a]**	**Nodules per plant[a]**	**Nodules per plant[a]**	**ARA[a] (nmoles/h. plant)**
Not inoculated	0	0	0	0
CIAT899	50	5	8	1,200
CIAT899 *nodS*	0	0	0	0
CIAT899 *nodS* (pFW17)	32	ND	2	200
CIAT899 *nodU*	61	2	ND	ND
NGR234	5	ND	6	600
NGR234 *nodS*	0	ND	0	0
NGR234 *nodS* (pFW17)	0.2	ND	0.3	0
NGR234 *nodU*	10	ND	ND	ND
ORS571	75	2	4	ND
ORS571 *nodS*	0	0	0	ND
ORS571 *nodU*	66	0.7	3.5	ND
CE-3	ND	ND	0	0
CE-3 (pFW17)	ND	ND	7	1,350

[a] Mean values for four to six plants. ARA = acetylene reduction activity; ND = not determined.

(Beringer 1974) or YEM medium (Hooykaas *et al.* 1977), supplemented when necessary with (per milliliter) kanamycin (50 µg), neomycin (40 µg), tetracycline (10 µg), and nalidixin (60 µg). Sucrose (5%) was added to the medium for selection of double homologous recombinants. Medium for *E. coli* was LB medium (Miller 1972), supplemented when necessary with (per milliliter) ampicillin (100 µg), kanamycin (50 µg), and gentamycin (25 µg).

DNA manipulation and sequencing.

Standard protocols were used for cloning of DNA restriction fragments (Maniatis *et al.*1992). Sequencing reactions on double-stranded DNA were performed with the AutoRead Sequencing kit (Pharmacia-LKB) and sequencing gels were run on an ALF automatic sequencer (Pharmacia-LKB). Processing and analysis of sequence data was done with the PCGENE software package (Intelligenetics).

Conjugation.

Triparental conjugations using pRK2013 as a helper for mobilization were performed as described before (van Rhijn *et al.* 1993).

Construction of *nodS* and *nodU* mutants.

A 1.15-kb *Acc*I-*Hin*dIII fragment, covering most of *nodS* and 530 bp of *nodU*, was cloned as a blunt-end fragment in the *Sma*I site of pJQ200-uc1. This vector carries the *sacB* gene from *B. subtilis*, which allows positive selection for loss of the vector in Gram-negative bacteria. The resulting construct contains a single *Pst*I site, located in the 5′ region of *nodS*. In this site, a kanamycin-resistance cassette, isolated from pUC-4K, was inserted. The resulting plasmid, pJQ200::*nodS*::4K, was transferred to *R. tropici* CIAT899 by conjugation. Selection was on TY plates containing nalidixin and neomycin, which yielded clones with the pJQ200::*nodS*::4K construct integrated in the genome. Such a single recombinant was grown in liquid medium with the antibiotics, and plated on TY with nalidixin, neomycin, and 5% sucrose. Colonies obtained by this selection procedure, were all *bona fide* double recombinants, as confirmed by hybridization with a *nodS* containing fragment and with the pUC-4K construct as a probe. One double recombinant, CIAT899 *nodS*::4K, was retained for phenotypic characterization.

For the construction of a *nodU* mutant, a 2-kb *Sph*I-*Sal*I fragment covering the complete *nodU* coding sequence was cloned in pUC18. The resulting construct contains a single *Pst*I site, located in the 5′ region of *nodU*. In this site, the kanamycin-resistance cassette from pUC-4K was inserted. The 3.4-kb *Sph*I-*Sal*I fragment from this construct was cloned as a blunt-end fragment in the *Sma*I site of pJQ200-uc1. The resulting plasmid, pJQ200::*nodU*::4K was used for the construction of a double recombinant, CIAT899 *nodU*::4K, as described for the *nodS* mutant.

Construction of strains for mutation and complementation analysis.

The 6.2-kb *Eco*RI fragment from pCD17, containing the CIAT899 *nodABCSU* operon and the 5′ end of the adjacent *nodD1* copy was cloned on the broad host range plasmid pLAFR1. The resulting plasmid pFW17 was introduced by conjugation in the CIAT899 and NGR234 *nodS* mutants, as well as in the narrow host range *R. etli* strain CE-3. Likewise, plasmid pA16, carrying the NGR234 *nodSU* operon, was introduced into *R. etli* strain CE-3.

Plant experiments.

P. vulgaris and *L. leucocephala* seeds were surface sterilized as described (van Rhijn *et al.* 1993), and germinated on TY agar plates for 2 days at 28° C in the dark. Bean seedlings were then transferred to plastic pots, filled with a 50:50 mixture of sand and vermiculite. *Leucaena* seedlings were planted in plastic pots or in Leonard jars. Subsequently they were inoculated with 1,000 µl (bean) or 250 µl (*Leucaena*) of an overnight culture of the appropriate *Rhizobium* strain. Nutrient solution for the plants was 0.5 × Norris medium (1× Norris medium: $CaSO_4.2H_2O$, 0.35g/L; KCl, 0.149 g/L; K_2HPO_4, 0.05 g/L; KH_2PO_4, 0.1 g/L; $MgSO_4.7H_2O$, 0.493 g/L;

Table 2. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
Rhizobium strains		
CIAT899	Wild- type isolate from *Phaseolus vulgaris*	EMBRAPA, Brazil
CIAT899 *nodS*	*nodS* mutant of CIAT899, Nm^r	This study
CIAT899 *nodU*	*nodU* mutant of CIAT899, Nm^r	This study
NGR234 (Rif^r)	Rif^r derivative of *Rhizobium* sp. NGR234	Lewin *et al.* 1990
NGR234 *nodS*	*nodS* mutant of NGR234, Km^r	Lewin *et al.* 1990
NGR234 *nodU*	*nodU* mutant of NGR234, Sp^r	Lewin *et al.* 1990
ORS571	*Azorhizobium caulinodans* type strain	Dreyfus et al., 1988
ORS571 *nodS*	*nodS* mutant of ORS571, Km^r	Geelen *et al.* 1993
ORS571 *nodU*	*nodU* mutant of ORS571, Km^r	Geelen *et al.* 1993
CE-3	Str^r derivative of *Rhizobium etli* strain CFN42	Noel *et al.* 1984; Segovia *et al.* 1993
Plasmids		
pCD17	pUC19 with 6-kb *Eco*RI fragment containing *nodABCSU* from CIAT899	This study
pFW17	pLAFR1 with 6-kb *Eco*RI fragment from pCD17	This study
pA16	pRK7813 with *nodSU* genes from NGR234, Tc^r	Lewin *et al.* 1990
pUC18, pUC19	Cloning vectors, Ap^r	Yanisch-Perron *et al.* 1985
pLAFR1	Broad host range cosmid, Tc^r	Friedman *et al.* 1982
pUC-4K	Plasmid containing Km^r cassette	Pharmacia Biotech
pRK2013	Helper for triparental conjugation, Km^r	Figurski and Helinski 1979
pJQ200-uc1	Suicide vector containing *sacB* from *B. subtilis*	Quandt and Hynes 1993

$FeSO_4.7H_2O$, 1.25 mg/L; citric acid, 1.25 mg/L; Gibson's spore elements (Vincent 1970), 1 ml/L). Bean plants were harvested after 18–21 days, *Leucaena* plants after 5–6 wk. Parameters evaluated were nodule number and in the case of *Leucaena* plants grown in Leonard jars also acetylene reduction activity. Bacteria were reisolated from nodules and tested for growth on appropriate media and antibiotics. All plant experiments were repeated at least three times; the results shown are from one representative experiment.

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