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Identification and Characterization of a *nodH* Ortholog from the Alfalfa-Nodulating Or191-Like Rhizobia

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Nodulation of *Medicago sativa* (alfalfa) is known to be restricted to *Sinorhizobium meliloti* and a few other rhizobia that include the poorly characterized isolates related to *Rhizobium* sp. strain Or191. Distinctive features of the symbiosis between alfalfa and *S. meliloti* are the marked specificity from the plant to the bacteria and the strict requirement for the presence of sulfated lipochitooligosaccharides (Nod factors [NFs]) at its reducing end. Here, we present evidence of the presence of a functional *nodH*-encoded NF sulfotransferase in the Or191-like rhizobia. The *nodH* gene, present in single copy, maps to a high molecular weight megaplasmid. As in *S. meliloti*, a *nodF* homolog was identified immediately upstream of *nodH* that was transcribed in the opposite direction (local synteny). This novel *nodH* ortholog was cloned and shown to restore both NF sulfation and the Nif$^+$Fix$^+$ phenotypes when introduced into an *S. meliloti nodH* mutant. Unexpectedly, however, *nodH* disruption in the Or191-like bacteria did not abolish their ability to nodulate alfalfa, resulting instead in a severely delayed nodulation. In agreement with evidence from other authors, the *nodH* sequence analysis strongly supports the idea that the Or191-like rhizobia most likely represent a genetic mosaic resulting from the horizontal transfer of symbiotic genes from a sinorhizobial megaplasmid to a not yet clearly identified ancestor.

Bacteria of the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium,* and *Azorhizobium* associate with legume plants to form a novel plant organ, the nodule, in which intracellular bacteria reduce atmospheric nitrogen to ammonia that can be used by the plant (Fisher 1994; Geurts and Bisseling 2002; Oldroyd 2001). In most cases, a given rhizobia is able to associate with a small number of plant genera and, conversely, one plant species accepts a limited number of rhizobia as symbiotic partners. In some cases, however, the degree of selectivity from the plant to the bacteria or vice versa may be highly relaxed (Perret et al. 2000).

One of the distinctive features of the symbiosis between *Medicago sativa* (alfalfa) and rhizobia is the marked specificity from the plant to *Sinorhizobium meliloti* and *S. medicae*. In spite of this, other rhizobia were able to nodulate alfalfa (Barran et al. 2002; Cloutier et al. 1996a and b; Villegas et al. 2006), including a rare type of acid-tolerant rhizobial isolate first recovered from a soil in Oregon, United States (Eardly et al. 1985), and later found in central Argentina and Uruguay (Del Papa et al. 1999). Distinctive features of these alfalfa-nodulating rhizobia (hereafter designated as Or191-like rhizobia) are acid tolerance, extended host range for nodulation, inefficiency for nitrogen fixation in *M. sativa* and *Phaseolus vulgaris*, and extremely low genetic diversity regardless of the geographic origin (Wegener et al. 2001). Representatives of such rhizobia include *Rhizobium* sp. strains Or191 (an isolate from the United States) (Eardly et al. 1985) and LPU83 (an isolate from Argentina) (Del Papa et al. 1999). The ability of the Or191-like isolates to nodulate alfalfa makes them of special interest in comparing their symbiotic genes and signals with *S. meliloti*.

As has been demonstrated by several independent laboratories, early root nodule induction by rhizobia has a strict requirement for the secretion of symbiosis-specific lipooligosaccharide signals, called the Nod factors (NFs) (Dénarié et al. 1992; Geurts and Bisseling 2002; Spaink 2000). When the NFs reach the host plant, they induce a series of biochemical and morphological changes accompanied by significant modifications in the pattern of expressed genes (Dénarié and Cullimore 1993, Roche et al. 1992). The substitutions attached to the chitooligosaccharide core structure of the NF are dependent on each rhizobial species or strain and contribute to NF host specificity. Though the genetics and chemical structure of the NFs produced by *S. meliloti* have been characterized extensively, there is, unfortunately, no available information on the type of NF produced by the Or191-like rhizobia. Nodulation of alfalfa by these bacteria raise the question of whether the NFs that they produce are similar to the type of NF synthesized by *S. meliloti*. The NFs produced by *S. meliloti* consist of a chitooligomeric core of β-1,4-N-acetyl-D-glucosamine (three to five units) which is *N*-acylated by unsaturated C16 fatty acids or by a series of C18 to C26 (ω-1) hydroxylated fatty acids. This basic structure synthesized by the translation products of the *nodABC* genes (Dénarié and Cullimore 1993; Fisher and Long 1992) is decorated with a sulfate group at the C6 position of the reducing end and may be O-acetylated on the non-

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reducing end (Demont et al. 1993; Lerogue et al. 1990; Roche et al. 1991 Schultze et al. 1992; Truchet et al. 1991). Modifications made to the basic structure by the products of specific *nod* genes are a major factor in determining which legume species will be nodulated by a given rhizobial strain. In *S. meliloti*, sulfation of NFs has been shown to be essential to elicit root hair curling, infection thread formation, and nodule development on alfalfa (Faucher et al. 1988). This sulfation is dependant on the NodP-NodQ proteins (homologous to ATP sulfurilase and APS kinase, respectively, the enzymes involved in the generation of activated sulfate) and on the sulfotransferase NodH protein (Dénarié and Cullimore 1993; Fisher and Long 1992; Schwedock and Long 1992). In the *Sinorhizobium* sp. strain BR816, an independent CysDN sulfate activation system has been described (Snoeck et al. 2003). It has been shown that the NodH protein has an in vitro sulfotransferase activity and catalyzes the transfer of sulfate from 3′-phosphoadenosine-5′-phosphosulfate to the terminal 6-O position of the *S. meliloti* NF (Ehrhardt et al. 1995). Interestingly, sulfation of NF at the chitooligosaccharide reducing end is not restricted to *S. meliloti* but also is present in several other rhizobia that include *Mesorhizobium* sp. strain N33 isolated from *Oxytropis arctobia* and diverse *Acacia*-nodulating fast-growing rhizobia (*S. terangae* bv. *acaciae*, *Mesorhizobium plurifarium*, *Rhizobium* sp. strain GRH2, and *Rhizobium tropici*) (Folch-Mayol et al. 1996; López Lara et al. 1995; Lorquin et al. 1997; Poupot et al. 1993). Another type of NF sulfation at the C3 position of a C6 substituent 2-O-methylfucose has been described in *Rhizobium* sp. strain NGR234 (Hanin et al. 1997; Quesada et al. 1998) and in an *Acacia*-nodulating *Bradyrhizobium* sp. (Ferro et al. 2000). Although NF sulfation (at the C6 position of the reducing end) is a strict requirement for *S. meliloti* to nodulate alfalfa, NF sulfation does not ensure alfalfa nodulation by the rhizobia.

In this work, we present genetic and biochemical evidence of the presence of a *nodH* ortholog in the Or191-like rhizobia where it is not required for nodulation of alfalfa. The ability of the novel *nodH* to complement NF sulfation and symbiosis in homolog mutants in *S. meliloti* is demonstrated, and the phylogenetic position of the *nodH* shown.

RESULTS

Identification of *nodH*-like DNA sequences in the alfalfa-nodulating *Rhizobium* sp. strains Or191 and LPU83.

In *S. meliloti,* the presence of the *nodH*-encoded sulfotransferase was shown to be a strict requirement for the nodulation of alfalfa (Lerogue et al. 1990). Because the Or191-like rhizobia also are able to nodulate alfalfa (Eardly et al. 1985), we were interested in investigating whether a *nodH* gene was present in the *Rhizobium* sp. strain Or191 and in the local isolate *Rhizobium sp.* strain LPU83 (Del Papa et al. 1999). To this end, we first performed a polymerase chain reaction (PCR) assay using total DNA from strains LPU83 and Or191 and primers NODHspp-f/r which share consensus sequences with the *nodH* genes from *S. meliloti* strains 1021 and AK631, *Mesorhizobium* sp. strain N33, and *R. tropici* strains CFN 299 and CIAT 899 (corresponding accession numbers are found below). A single PCR product of approximately 360 bp was obtained for strains LPU83 and Or191 (Fig, 1A). The size of the PCR product was indistinguishable from that of the positive control *S. meliloti* 2011. In agreement with the PCR results, we observed a strong hybridization signal of approximately 20 kbp when *Eco*RI-digested DNA of strain LPU83 was Southern blotted against an *S. meliloti nodH* probe (Fig. 1B). Total DNA digestion with the enzyme *Eco*RV also resulted in a single hybridization band supporting the presence of a single *nodH* copy in the genome of strain LPU83. To obtain further information on the genomic location of *nodH* in the Or191-like rhizobia, we performed Eckhardt gels of strains Or191 and LPU83, cut out agarose slices containing each plasmid DNA, and used them as PCR templates with primers NODHspp-f/r. Positive DNA amplification was achieved only with the DNA fraction corresponding to the rhizobial megaplasmids (not shown). We previously had reported that several isolates of Or191-like isolates from Argentina and strain Or191 have at least two megaplasmids and a plasmid of smaller size (Wegener et al. 2001).

Cloning and sequencing of the *nodH* gene from strain LPU83.

To get a deeper insight into the *nodH* in particular and into the genetic structure of nodulation genes in strain LPU83 in general, we constructed a genomic library of strain LPU83 which was further screened to detect *nod*-containing DNA inserts (discussed below). Five positive cosmids could be identified by colony blotting against an *S. meliloti nodD* probe (three different *nodD*-like sequences were partially sequenced but they are not analyzed in this article). One of the cosmids, cosmid pRVD7, contained the *nodH* sequence amplified by PCR with primers NODHspp-f/r as described earlier. Hybridization of the *Eco*RI-digested pRVD7 against a *nodH* probe resulted in a single signal that corresponded to a DNA fragment of approximately 20 kbp (not shown), in concordance with the size of the positive restriction fragment observed in the genomic Southern blot (Fig. 1B). A 2.2-kb nucleotide sequence that included the complete coding regions of *nodH* (759 bp) and *nodF* (249 bp) was obtained by primer walking using cosmid pRVD7 as a template. Alignment of the *nodH* and *nodF* coding regions against their orthologs in *S. meliloti* showed 85 and 88% sequence identity, respectively.

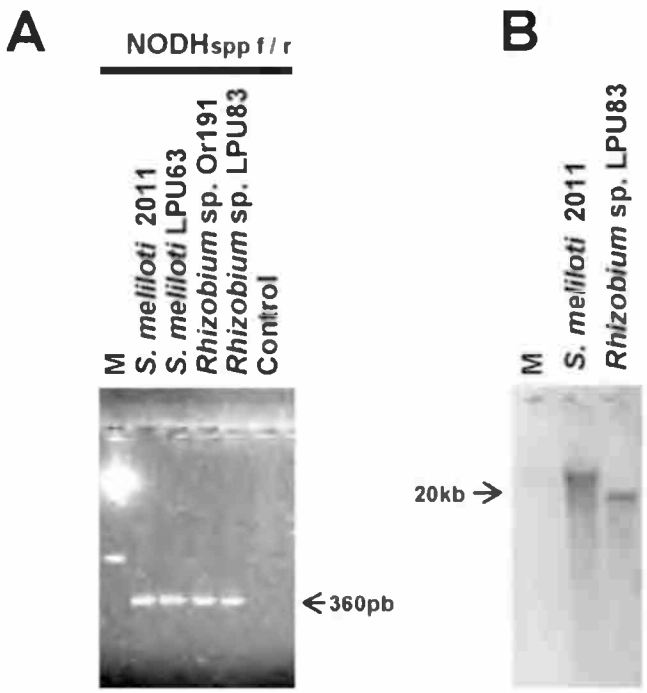

Fig. 1. Polymerase chain reaction (PCR) and hybridization assays to explore for the presence of *nodH* orthologs in the Or191-like rhizobia. **A,** PCR from total DNA of strains LPU83 and Or191 using NODHspp f/r primers. As positive control, the total DNA from *Sinorhizobium meliloti* strain 2011 was used. **B,** Southern blot hybridization of *Eco*RI-digested total DNA of the indicated strains against a digoxigenin-labeled *S. meliloti nodH* DNA probe generated by PCR using primers NODHsm f/r.

In addition, a close relationship between the *nodH-nodF* region of strain LPU83 and *S. meliloti* was supported by a similar relative location and orientation of both genes (local synteny) which was not present in any of the other identified *nodH*-carrying rhizobia (Fig. 2). The availability of the *nodH* sequence from strain LPU83 and the controversial taxonomy of the Or191-like rhizobia prompted us to reconstruct the phylogenetic position of our cloned sulfotransferase and its translated product (Fig. 3A and B). Both phylogenetic trees resolved in a close relationship between the *nodH*/NodH products from strain LPU83 and their corresponding *S. meliloti* orthologs from strains 1021 and Rm41. A more distant position was identified for the orthologs present in the bean-nodulating rhizobia *R. tropici* CFN 299 and CIAT 899, with an intermediate position for the orthologs from *Sinorhizobium* sp. strain BR816 and *Mesorhizobium* sp. strain N33. This result is supported by previous analyses of other megaplasmidic markers such as *nodC* and *nifH* (Laguerre et al. 2001), which contrasts with the closer phylogenetic position deduced for the Or191-like rhizobia and *R. tropici* after the comparison of their chromosomal 16S rDNAs (Laguerre et al. 2001). Finally, analysis of the central region of both trees (Fig. 3A versus B, nucleotide versus protein phylogenies, respectively) shows an inversion in the relative position of orthologs from strains N33 and BR816. Clearly, the *nodH* sequence from strain N33 compared with that from strain BR816 results in a higher variation at amino acid sequence level relative to the NodH protein from *S. meliloti*.

Symbiotic complementation of a *S. meliloti nodH* mutant and restoration of NF sulfotransferase activity.

In order to investigate whether the *nodH* gene from strain LPU83 can functionally replace the *nodH* gene of *S. meliloti* in symbiosis, cosmid pRVD7 was introduced by conjugation into an *S. meliloti nodH* mutant derived from strain AK631 and the nodulation phenotype evaluated. A month after inoculation, no difference was observed between plants inoculated with the conjugation mix and control plants inoculated with wild-type

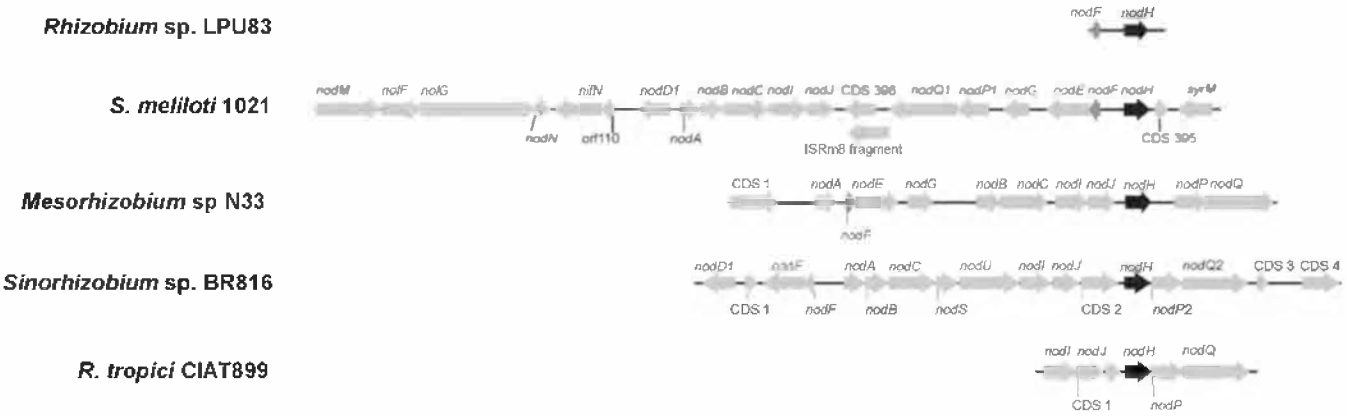

Fig. 2. Genetic disposition of *nodH* orthologs within the nodulation gene clusters of different rhizobia compared with the disposition in *Rhizobium* sp. strain LPU83. The nucleotide sequences used to construct the alignment diagram were obtained from the GenBank under the following accession numbers: *Sinorhizobium meliloti* 1021 (M37417), *Rhizobium* sp. strain LPU83 (DQ212187), *Sinorhizobium* sp. strain BR816 (AJ518946), *Mesorhizobium* sp. strain N33 (U53327), and *Rhizobium tropici* CIAT 899 (X87608). In *S. meliloti* and in *Rhizobium* sp. strain LPU83, the genes *nodH* and *nodF* are contiguous and transcribed in opposite directions.

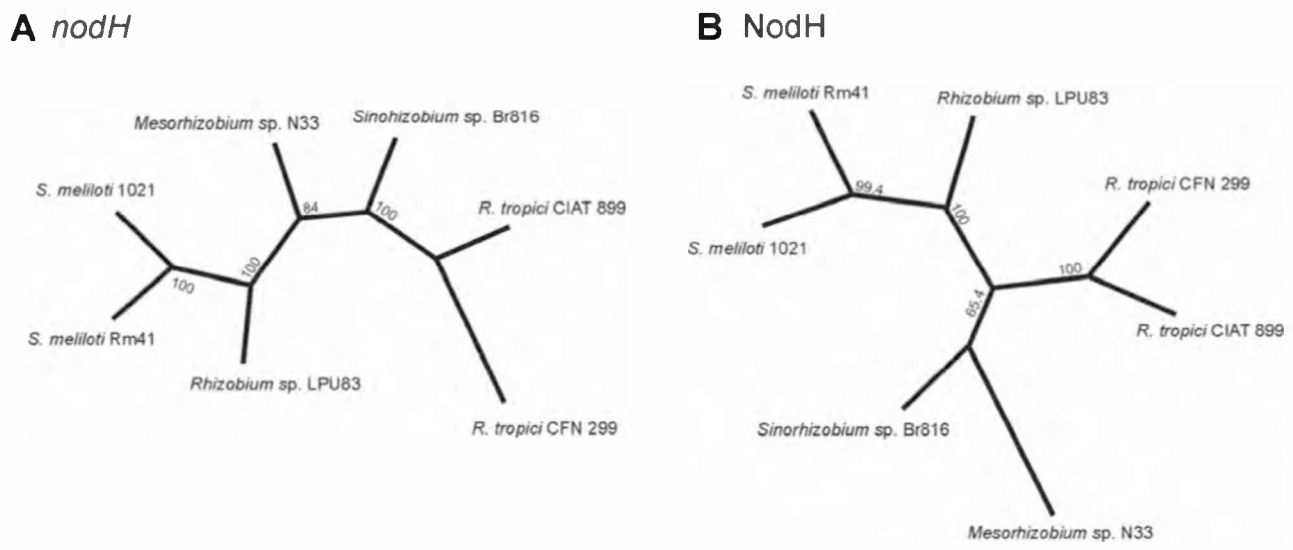

Fig. 3. Phylogenetic (neighbor-joining) trees showing the relationships between different rhizobia as inferred from **A,** their *nodH* aligned DNA sequences and **B,** their translation products. DNA and protein sequences were analyzed using the PHYLIP package with programs and conditions. Bootstrap probability values calculated over 500 replicates are indicated at the branching points. Branches are drawn proportionally to the output values generated by the **A,** dnadist, **B.** protdist, and neighbor-joining analysis. The nucleotide sequences used were obtained from the GenBank under the following accession numbers: *Sinorhizobium meliloti* AK631 (M14052) and *S. meliloti* 1021 (M37417), *Rhizobium* sp. strain LPU83 (DQ212187), *Sinorhizobium* sp. strain BR816 (AJ518946), *Mesorhizobium* sp. strain N33 (U53327), *Rhizobium tropici* CFN 299 (U47272), and *R. tropici* CIAT 899 (X87608).

rhizobia (not shown). The presence of cosmid pRVD7 in bacteria recovered from pink nodules was assessed by a specific PCR assay directed against *bla* sequences from the Hypercos-1. The PCR-positive rhizobia showed the ability to nodulate and fix nitrogen when they were re-inoculated onto alfalfa plants Nodulation of the *S. meliloti nodH* mutant also was complemented when we used a 1.77-kbp DNA fragment containing only the LPU83 *nodH* and its promoter region. One of the clones complemented with the cosmid, designated AK631-*nodH* (pRVD7), was analyzed further by growing the rhizobia in the presence of $Na_2{}^{35}SO_4$ and luteolin to induce the nodulation genes (discussed below). We could recover sulfated NF from the culture supernatants, as is shown in the thin-layer chromatography (TLC) presented in Figure 4. An arrowhead line on the right indicates the position of the strongest sulfated NF component present in supernatants of both the wild-type rhizobia (lanes 1 and 2) and the AK631-*nodH* (pRVD7)-complemented clone (lanes 4 and 6). Control cultures to detect the different molecular forms of NFs also were run using glucosamine hydrochloride ^{14}C as described below. The ability of the *nodH*-carrying cosmid pRVD7 to restore the NF sulfation in *S. meliloti* demonstrates that an active NF sulfotransferase is encoded in *Rhizobium* sp. strain LPU83. Whether this activity is naturally expressed in strain LPU83 has yet to be determined. Unfortunately, we have not yet been able to get clear TLC signals from culture supernatants of the Or191-like rhizobia (using either ^{14}C or ^{35}S labeling).

Symbiotic characterization of a *nodH* mutant of *Rhizobium* sp. strain LPU83.

In view of the key role of the *nodH*-encoded sulfotransferase in the nodulation of alfalfa by *S. meliloti,* we constructed a *nodH* mutant of strain LPU83 (discussed below) and evaluated its nodulation phenotype. The time course of nodule formation in different parts of the root was analyzed (Fig. 5). The pattern of nodulation induced by the *nodH* mutant LPU83-H (Fig. 5B) was compared with those of strain LPU83 (the parent rhizobia) (Fig. 5A) and *S. meliloti* LPU63 (a wild-type symbiont of alfalfa) (Fig. 5C). Nodulation of mutant LPU83-H in primary roots clearly was delayed, showing a lag period that extended until approximately 8 days postinoculation. As a consequence, primary root nodulation by the mutant did not reach a plateau after 4 weeks, as was the case for the parent strain LPU83. In any case, the disruption of *nodH* did not abolish the parent ability to nodulate alfalfa. The size and shape of the nodules induced by LPU83 were comparable with the size and shape of those formed by the mutant LPU83-H. Bacteria recovered from plants inoculated with strain LPU83-H presented, in all cases, the mutant antibiotic resistances (demonstrating that nodulation of alfalfa could not have arisen by genetic reversion). Clearly, the symbiotic consequence of the *nodH* disruption had different effects on *S. meliloti* than on the Or191-like rhizobia. Finally, we explored whether the *nodH* mutation en-

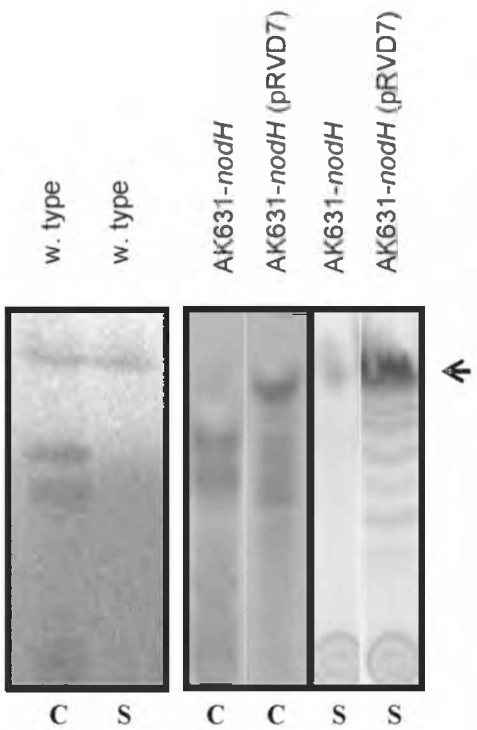

Fig. 4. Autoradiogram of reverse-phase thin-layer chromatography of ^{14}C- or ^{35}S-labeled lipochitooligosaccharides produced by wild-type *Sinorhizobium meliloti* and the *nodH* mutant complemented with the *nodH* gene from *Rhizobium* sp. strain LPU83. **A,** Wild-type *S. meliloti* and **B,** AK631-nodH with (AK631-*nodH* [pRVD7]) and without plasmid pRVD7 that bears the *nodH* gene of strain LPU83. C and S denote nod factor (NF) samples labeled with ^{14}C and ^{35}S, respectively. Arrows denote the inferred sulfated NF species in the ^{14}C-labeled samples.

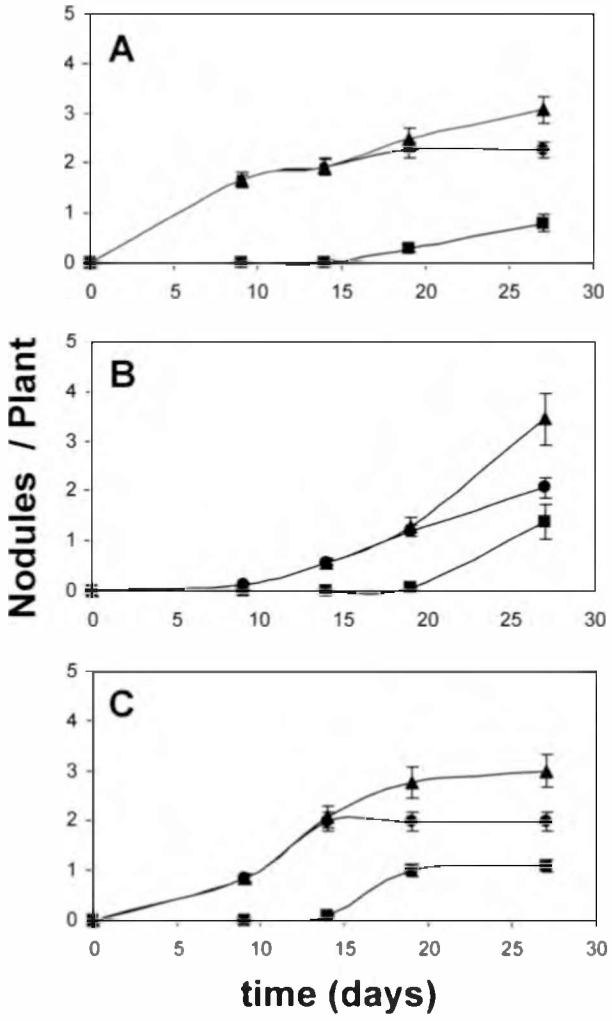

Fig. 5. Nodulation kinetics in alfalfa roots of *Rhizobium* sp. LPU83 and its *nodH* mutant LPU83-H. **A,** *Rhizobium* sp. LPU83, **B,** *Rhizobium* sp LPU83-H, and **C,** wild-type *S. meliloti* LPU63. Sets of 30 plants were inoculated with approximately 5×10^4 bacteria per plant of each strain. Nodules on the whole root (▲), primary root (●), and secondary roots (■) were scored at the indicated times. The results are given as the average number of nodules per plant. Error bars indicate standard deviations. Results are taken from a representative experiment among a set of three.

ables the mutant to nodulate *Vicia* spp., as was the case in *S. meliloti nodH* mutants (Faucher et al. 1988). Inoculation of mutant LPU83-H in *Vicia hirsuta* did not result in nodule induction (data not shown).

DISCUSSION

We present the identification, isolation, and characterization of a *nodH* gene present in the broad host range *Rhizobium* sp. strain LPU83. Previous reports from our laboratory had shown that *Rhizobium* sp. strain LPU83 belongs to a rare group of alfalfa-nodulation rhizobia present in soils of Argentina and Uruguay (Del Papa et al. 1999). *Rhizobium* sp. strain LPU83 is genetically related to the promiscuous *Rhizobium* sp. strain Or191 originally recovered from an acid soil of Oregon (Eardly et al. 1985, 1992, 1995). As is largely documented in the literature, the presence of a sulfate group at the (reducing) C6 position of the NFs synthesized by *S. meliloti* is a strict requirement for these rhizobia to nodulate alfalfa (Roche et al. 1991; Truchet et al. 1991). Based on this requirement and on the known ability of the Or191-like rhizobia to nodulate alfalfa, we investigated the presence of *nodH* orthologs in the local isolate LPU83. Cloning and sequencing the complete *nodH* gene from strain LPU83 allowed us to perform a sequence comparison with NF-associated sulfotransferases from other rhizobia. Sequence alignment revealed the closer sequence relationship with the *S. meliloti nodH* ortholog. In agreement with this, a contiguous location of *nodH-nodF* was observed in both *Rhizobium* sp. strain LPU83 and in *S. meliloti* 2011 (local synteny), contrasting with a different *nodH* neighborhood present in *R. tropici*, *Sinorhizobium* sp. strain BR816, and extended-host-range *Mesorhizobium* sp. strain N33. In these last three rhizobia, the *nodH* gene clusters with *nodPQ*, which also is involved in sulfate metabolism. Immediately downstream of *nodH*, however, there are differences in gene structure between *S. meliloti* and the Or191-like rhizobia. Although *syrM* and the promoter of *nodD3* follow *nodH* in *S. meliloti*, *syrM* was not present in strain LPU83 at this location. Only a *nodD3*-like promoter was present 259 bp downstream of *nodH*, preserving the previously described consensus sequences associated with *syrM*-regulated promoters (Barnett et al. 1996). Within a 68-bp promoter signature, we found 85% identity when compared with the same region in *S. meliloti* 1021. The *nodD3* promoter in strain LPU83 also showed 57% identity when compared with the *S. meliloti syrA* promoter which, like the *nodD3* promoter, is positively regulated by *syrM*. Such observations raise the question of whether the sole presence of the *nodD3* promoter is the reminiscence of an ancestral sinorhizobial sequence or whether it is functionally associated with a *syrM* homolog present at other genomic location in the Or191-like rhizobia.

There currently are five *nodH* homologs deposited in the GenBank, two from *S. meliloti* (Fisher et al. 1987; Horvath et al. 1986), two from *R. tropici* (Folch-Mallol et al. 1996; Laeremans et al. 1996), and one from *Mesorhizobium* sp. strain N33 isolated from *O. arctobia* nodules (Cloutier et al. 1996a and b). Though other NF sulfotransferases have been identified in acacia-nodulating rhizobia (Ferro et al. 2000), they are active on structurally different NFs (i.e., fucosylated signal molecules) and are highly divergent in sequence when compared with the NodH protein family. The close relationship between the symbiotic genes from *S. meliloti* and strain LPU83 also is supported by analysis of the *nodC* (Laguerre et al. 2001). The available *nod* phylogeny supports a close evolutionary position for the symbiotic information carried by *S. meliloti* and the Or191-like rhizobia. Interestingly, a different view arises upon analysis of the 16S rDNA chromosomal region of strain Or191, which shows closer sequence similarity with the 16S region of the bean-nodulating rhizobia *R. etli*, *R. leguminosarum* bv. *phaseoli*, and *R. tropici*; as well as to the 16S of *R. leguminosarum* bvs. *viciae* and *trifolii* (Laguerre et al. 2001). Similarly, a close relationship between partial sequences of the 16S rDNA of strain Or191 and those of the bean-nodulating strains Olivia-4 and FL-27 has been reported by Eardly and associates (1995). Thus, it is most likely that the Or191-like rhizobia might represent a genetic mosaic resulting from the horizontal transfer of symbiotic genes from a sinorhizobial megaplasmid to a not yet clearly identified ancestor with 16S related to those of current rhizobia that associate with the common bean, pea, and *Trifolium* spp. Interestingly, Laguerre and associates (2001) have reported that *Rhizobium* sp. strains HT2a2 and HT4c1, isolated in Europe, have 16S rDNA restriction fragment

Table 1. Bacterial strains and plasmids used in this work

Bacterial strains	Relevant information[a]	Source
Sinorhizobium meliloti		
LPU63	Wild-type *S. meliloti*	Del Papa et al. 1999
2011	Wild-type *S. meliloti*	J. Denarié, Toulouse, France
AK631	*S. meliloti exoB*, *lpsZ*$^+$, Nod$^+$, Fix$^+$	Kondorosi, France
AK631-*nodH*	*S. meliloti* AK631 *nodH*::Tn*5* Str^r, Nm^r	Kondorosi, France
AK631-*nodH* (pRVD7)	*S. meliloti* AK631-*nodH* (pRVD7)	This work
Rhizobium sp.		
Or191	Oregon, United States	Earldly et al. 1985
LPU83	Castelar, Argentine	Del Papa et al. 1999
LPU83-H	LPU83 *nod*H::pGnod83	This work
Escherichia coli		
DH5α	*end*A1, *hsd*R17, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, Δ*lac*U169 (Φ80 *lac*ZΔM15)	Gibco BRL.
S17-1	*E. coli 294, RP4-2 Tc:: Mu-Km::Tn7 integrated into the chromosome*	Simon 1983
NM554	F$^-$, *recA*, *araD139*, Δ(ara, leu),7696, Δ*lacY*74, *galU*$^-$, *galK*$^-$, *hsr*$^-$, *hsm*$^+$, *strA*, *mcrA*(–), *mcrB*(–)	Stratagene
Plasmids		
pRK2013	Helper plasmid for mobilization of IncP and IncQ plasmids, Km^r	Ditta et al.1980
pVRD	Hypercos I (Supercos + *oriT* from RP4)	A. Pühler-M. Drogue, Bielefeld, Germany
pRVD7	Hypercos I containing a nod region that includes *nodHF* of *Rhizobium* sp. LPU83	This work
pG18nod83	pG18mob2 containing an internal fragment of *nodH* from LPU83 amplified with primers NODHlpu83 f/r	This work
pCR 2.1-Topo	Ap^r, Km^r, *lacZ*	Invitrogen
pG18mob2	Gm^r	Kirchner and Tauch 2003

[a] Str^r, Nm^r, Km^r, Ap^r, Gm^r = streptomycin, neomycin, kanamycin, ampicillin, and gentamycin resistance, respectively.

length polymorphism type Or191 and their *nodC* closely related to that of *R. tropici*. Unfortunately, we do not have any information on the *nifH* type of these rhizobia to explore its relationship with the homolog from strain Or191 which, according to previous data, forms its own linage (Eardly et al. 1985; Laguerre et al. 2001). Regardless of the type of genetic contributions that resulted in current genome structure of the Or191-like bacteria, they were able to induce nodulation on *Medicago sativa*, *Phaseolus vulgaris* (Eardly et al. 1992), and *Leucaena leucocephala* (Del Papa et al. 1999). However, in *M. sativa* and *P. vulgaris,* the Or191-like rhizobia displayed a defective symbiosis in terms of nitrogen-fixation and bacteroid development (Eardly et al. 1995, Wegener et al. 2001). Several other legumes from classical inoculation groups were not nodulated by the Or191-like rhizobia (Eardly et al. 1992). Thus, current observations taken together suggest that either i) the Or191-like rhizobia have not yet acquired the complete set of functions to became a full legume symbiont (this also is supported by structural data of deficiently infected nodules) or ii) they are a residual form of formerly active symbionts, which is unlikely in terms of the coevolution of rhizobia with their host plants. Whatever the case, the biology and the interaction of these bacteria with the environment severely conditioned the genetic diversity of the Or191-like germ plasm irrespective of their distant genetic location. The absence of a known true host plant for these bacteria, their capacity to induce inefficient root nodules in different legumes (temperate and tropical), and the unusual absence of detectable genetic polymorphisms among independent isolates (Wegener et al. 2001) make them an interesting target of analysis to improve our knowledge on the rhizobial evolution strategies.

The preliminary suspicion of the presence of a *nodH* in the Or191-like bacteria was confirmed, as well as its functional relationship with the sinorhizobial ortholog (complementation experiments). Unexpectedly, however, the *nodH* knockout in the Or191-like bacteria did not abolish their ability to nodulate alfalfa, resulting in a severely delayed nodulation. Though the *nodD3* promoter is located downstream and reading in the same direction, polar effects cannot be neglected. The observed behavior is clearly different from that observed in *S. meliloti* and in the unrelated *Mesorhizobium* sp. strain N33, both of which fail to nodulate alfalfa upon the *nodH* mutation (Cloutier et al. 1996a and b). In other rhizobia, such as *R. tropici*, the consequences of the lack of NF sulfation are dependent on the host plant cultivar, and on the conditions assayed (Laeremans et al. 1996). Though NF sulfation by other enzymes in strain LPU83 cannot be disregarded, our observations show that the roots of alfalfa are still responsive to the nodulation signals produced by the Or191-like rhizobia in the absence of an active *nodH*. Though we demonstrated the ability of *nodH* from LPU83 to restore NF sulfation in the *S. meliloti nodH* mutant, we did not find adequate conditions to induce (and label) NFs in the Or191-like rhizobia. Such limitation, likely due to improper *nod* induction, reflects, once again, the particular behavior of the Or191-like rhizobia at the time they are expressing their symbiotic-related traits.

Due to their peculiar phenotypic and taxonomic characteristics, the Or191-like isolates have attracted the attention of rhizobiologists since their original isolation by Eardly and associates (1985) in Oregon. Twenty years later, the collected evidence strongly supports the idea that the Or191-like rhizobia are genetically connected to both tropical-legume-infecting rhizobia (i.e., the bean-nodulating *R. etli*) and temperate-legume-infecting rhizobia (i.e., *Medicago*-nodulating sinorhizobia). Such a "patched" genetic constitution obviously has imposed severe restrictions on the classical taxonomy of these rhizobia (Eardly et al. 1992; Laguerre et al. 2001). With a genetic structure that preserves clear evidences of horizontal gene transfer events (likely via a sinorhizobial symbiotic megaplasmid), the Or191-like isolates represent a valuable system to better understand the underlying mechanisms of rhizobial diversification and evolution. How do the Or191-like rhizobia preserve their extremely low genetic variation in such a genomic context? (Wegener et al. 2001). The molecular (and functional) characterization of new genetic markers from the Or191-like rhizobia such as the one presented in this work, together with upcoming information from new, fully sequenced rhizobial genomes, will be key elements toward a deeper tracing and reconstruction of the Or191 genomic structure and genealogy.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Rhizobia and *Escherichia coli* strains used in this work are listed in Table 1. Rhizobia were grown at 28ºC on TY medium (Beringer 1974) or YEM (Vincent 1970). *E. coli* were grown at 37ºC on LB medium (Sambrook et al. 1989). For solid media, 15 g of agar per liter of medium were added. Bacterial matings were performed as described by Simon and associates (1983) using *E. coli* S17-1. When necessary, the final concentrations of antibiotics were neomycin at 100 μg/ml, streptomycin at 400 μg/ml, ampicillin at 200 μg/ml, kanamycin at 25 μg/ml, and gentamycin at 10 μg/ml for *E. coli* and 50 μg/ml for rhizobia.

DNA manipulations.

Plasmid DNA preparations, restriction enzyme analysis, cloning procedures, and *E. coli* transformation were performed according to previously established techniques (Sambrook et al. 1989). Total genomic DNA was prepared as described by Simon and associates (1991). Southern hybridizations were carried out using DNA probes labeled with digoxigenin. The probes were synthesized by PCR using digoxigenin-dUTP and appropriate primers to amplify the region of interest. For hybridizations, DNA extracted from bacteria was digested and transferred to nitrocellulose membranes (Hybond N; Amersham Iberica SA, Madrid) as described by Chomczynski (1992).

PCR amplifications.

PCR primers and conditions used were as follows. Primers NODHsm (NODHsm-f 5′-TGATGTTGCCATTCGCCACC-3′ and NODHsm-r 5′-CTCTTGACGCCGAAGAATAC-3′) were designed based on consensus sequences between the *nodH* genes of *S. meliloti* AK631 (GenBank M14052) and *S. meliloti* 1021 (GenBank M37417) to generate a 212-bp amplicon. Primers NODHsme (NODHsme-f 5′-GCAGGCAAGGCAAAC-3′ and NODHsme-r 5′-TCAAAGAAGCTCGCG-3′) were designed based on consensus sequences among the *nodH* genes of *S. meliloti* AK631 and 1021, *Mesorhizobium* sp. strain N33 (GenBank U53327), *R. tropici* CFN 299 (GenBank U47272), and *R. tropici* CIAT 899 (GenBank X87608) to generate an approximately 360-bp amplicon. Primers NODH-lpu83 (NODH-lpu83-f 5′-TGTGGGCTGCAAGATCAA-3′ and NODH-lpu83-r 5′-AAATCGTCGGCAGCTTTG-3′) were designed to amplify 250 bp of the *nodH* gene from *Rhizobium* sp. strain LPU83. Primers NODHout (NODHout-f 5′-GCCTCACCATGCCATAAGAT-3′ and NODHout-r 5′-GCTGCAAGATCAACGAACCT-3′) were designed to border the amplification product of primer set NODH-lpu83. PCR amplifications were performed in a total reaction volume of 25 μl containing PCR reaction buffer (50 mM KCl, 20 mM Tris HCl, pH 8.0), 200 μM each dNTPs, 3 mM $MgCl_2$, 2 μl of template DNA, and 1 U of *Taq* DNA polymerase (Gibco, BRL). All amplifica-

tions were performed with an Idaho capillary air thermocycler using the following temperature conditions: initial denaturation at 94°C for 1 min.; followed by 35 cycles of 10 s of denaturation at 94°C, 10 s of annealing at 52°C, and 30 s of extension at 72°C; and a final extension of 1 min at 72°C. The PCR reaction (10 μl) was separated in 1.5% agarose gels containing ethidium bromide at 0.5 to 1 μg/ml and photographed by using a Kodak DC290 digital camera.

Construction of a cosmid gene library of *Rhizobium* sp. strain LPU83.

Total DNA of *Rhizobium* sp. strain LPU83 was prepared according to the method of Simon and associates (1991), partially digested with the restriction endonuclease *Sau*3AI, and treated with alkaline phosphatase. Separately, Hypercos I vector was first digested with *Xba*I, treated with alkaline phosphatase, and afterward digested with the restriction enzyme *Bam*HI. The ligated DNA was packaged into lambda bacteriophage particles using a Gold Pack II kit (Stratagene), and introduced into *E. coli* NM554 by transfection. The screening of the library to search for cosmids that contained the *nod* region from strain LPU83 was carried our using an *S. meliloti nodD* DNA probe available in our laboratory. Those clones that showed a positive hybridization signal were analyzed individually for the presence of *nodH* using the general consensus primers NODH2f/r which proved to give positive *nodH* amplification when using genomic DNA of strain LPU83. A positive cosmid was identified and designed pRVD7.

DNA sequencing and phylogenetic analysis.

The nucleotide sequence of the *nodH–nodF* region from strain LPU83 was obtained by sequencing walking using specific deoxyoligonucleotides and cosmid pRVD7 as template DNA. Reaction products were separated and analyzed with an Automatic Laser Fluorescent DNA Sequencer (Pharmacia). The final sequence was deposited in the GenBank under the accession number DQ212187. Nucleotide and amino acid sequences were aligned with ClustalW (Thompson et al. 1994). The phylogenetic tree of *nodH* was inferred by using the Phylogenetic Inference Package (PHYLIP) (Felsenstein 1989) with dnadist and the neighbor-joining analyses from Kimura's (Kimura 1980) two-parameter nucleotide distances. The phylogenetic tree of the translated product NodH was constructed using protdist and the neighbor-joining method with a Dayhoff PAM distance matrix. Confidence in neighbor-joining trees was assessed by bootstrap analysis (500 data sets) with the Seqboot and Consense programs of PHYLIP.

Construction of a *nodH* mutant of *Rhizobium* sp. strain LPU83.

The *nodH* mutant of *Rhizobium* sp. strain LPU83 was generated by site-directed vector integration mutagenesis. An internal DNA region of the *nodH* coding sequence was amplified by PCR using primers NODH-lpu83. First, the PCR product was cloned into the shuttle vector pCR-Topo (Invitrogen). In a second step, the cloned insert was released with *Eco*RI and subsequently cloned into the *Eco*RI site of plasmid pG18mob2 (gentamycin-resistant). The resulting recombinant plasmid finally was transferred by conjugation from strain S17-1 to *Rhizobium* sp. strain LPU83. Putative *nodH* mutants that had been generated after site-specific integration of the plasmid (single crossover) were selected by their expected streptomycin and gentamycin resistance. Disruption of *nodH* was confirmed in one of the mutant clones by Southern hybridization and by site-specific PCR using M13 primers and primers NODH-out that border the insertion site of the plasmid. The nodH mutant of *Rhizobium* sp. strain LPU83 was designed LPU83-H.

Plant nodulation tests.

Medicago sativa seed (alfalfa, cv. Monarca) and *V. sativa* were surface sterilized for 10 min with commercial bleach 20% (vol/vol; NaClO concentration equivalent to 55 g of active Cl_2 $liter^{-1}$) followed by six washes with sterile distilled water. Surface-sterilized seed were germinated on water agar (1.5%, wt/vol). Seedlings (2 to 4 days old) were transferred to gamma-irradiated sterilized plastic-growth pouches (Mega Minneapolis International, Minneapolis, MN, U.S.A.) containing 10 ml of nitrogen-free Fåhraeus-modified mineral solution, pH 6.7 (Lodeiro et al. 2000). Three days later, primary roots were inoculated with approximately 10^5 rhizobia per root by dripping 100 μl of a bacterial suspension onto the root from the tip toward the base. The rhizobia were obtained from log-phase of growth YEM cultures. The plants were cultured in a growth chamber at 22°C with a 16-h light photoperiod. The CFU contained in the inocula were estimated by plate counts. The nodulation kinetics was evaluated during 4 weeks postinoculation.

TLC of NFs.

TLC analyses were performed according to Spaink and associates (1992) with minor modifications. Briefly, a saturated culture was diluted in fresh minimal medium, supplemented with the corresponding antibiotic to absorbance at 600 nm = 0.02 in 1 ml of final volume. Then, 10 μCi of (^{14}C) glucosamine (56 mCi/mmol) (Amersham Iberica SA) or 10 μCi of (^{35}S) $NaSO_4$ were added, and cells were induced simultaneously with 1 μM luteolin and incubated at 28°C for 24 h. Supernatants of labeled cultures were extracted with water-saturated n-butanol. The n-butanol was evaporated to dryness and the resulting powder resuspended in 40 μl of n-butanol. The amount corresponding to 0.4 ml of the initial culture supernatant was applied on octadecyl silica TLC plates (ODS: 100% octadecyl silanization) (Sigma-Aldrich, St. Louis). TLC plates were exposed to Kodak X-Omat R film for 7 days, and the film was developed with Kodak reagents according to the manufacturer's instructions.

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