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The Nodulation of Alfalfa by the Acid-Tolerant *Rhizobium* sp. Strain LPU83 Does Not Require Sulfated Forms of Lipochitooligosaccharide Nodulation Signals[▽]

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The induction of root nodules by the majority of rhizobia has a strict requirement for the secretion of symbiosis-specific lipochitooligosaccharides (nodulation factors [NFs]). The nature of the chemical substitution on the NFs depends on the particular rhizobium and contributes to the host specificity imparted by the NFs. We present here a description of the genetic organization of the *nod* gene cluster and the characterization of the chemical structure of the NFs associated with the broad-host-range *Rhizobium* sp. strain LPU83, a bacterium capable of nodulating at least alfalfa, bean, and *Leucena leucocephala*. The *nod* gene cluster was located on the plasmid pLPU83b. The organization of the cluster showed synteny with those of the alfalfa-nodulating rhizobia, *Sinorhizobium meliloti* and *Sinorhizobium medicae*. Interestingly, the strongest sequence similarity observed was between the partial *nod* sequences of *Rhizobium mongolense* USDA 1844 and the corresponding LPU83 *nod* genes sequences. The phylogenetic analysis of the intergenic region *nodEG* positions strain LPU83 and the type strain *R. mongolense* 1844 in the same branch, which indicates that *Rhizobium* sp. strain LPU83 might represent an early alfalfa-nodulating genotype. The NF chemical structures obtained for the wild-type strain consist of a trimeric, tetrameric, and pentameric chitin backbone that shares some substitutions with both alfalfa- and bean-nodulating rhizobia. Remarkably, while in strain LPU83 most of the NFs were sulfated in their reducing terminal residue, none of the NFs isolated from the *nodH* mutant LPU83-H were sulfated. The evidence obtained supports the notion that the sulfate decoration of NFs in LPU83 is not necessary for alfalfa nodulation.

Leguminous plants can develop a symbiotic interaction with compatible rhizobia that culminates in the formation of root nodules. These structures contain a differentiated form of rhizobia known as bacteroids, which convert atmospheric nitrogen to ammonia that is utilized by the host. Rhizobia belong to diverse phylogenetic taxa included in the alphaproteobacterium subgroup, but they have in common the capacity to induce nodules on the appropriate legume host. Nodulation is a moderately to highly host-specific interaction in which, with few exceptions, specific rhizobial strains infect a limited range of host plants. Legumes secrete flavonoid compounds that are recognized by the compatible rhizobia, resulting in the induction in the latter of nodulation genes that encode enzymes for the synthesis and secretion of substituted lipooligosaccharides, referred to as the nodulation factors (NFs) (23, 36). 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 (14, 22, 32, 33, 41). The substitutions attached to the chitooligosaccharide core structures of the NFs are characteristic of each rhizobial species or strain and contribute to the NF host specificity. All NFs thus far identified consist of a backbone of two to six β-(1→4)-linked *N*-acetyl-D-glucosamine (GlcNAc) residues. Among the substitutions found on the terminal nonreducing GlcNAc are N-methyl, carbamoyl, and acetyl groups, along with various fatty acids. The reducing GlcNAc residue may be decorated with a sulfate group or with either D-arabinose, L-fucose, or 2-*O*-methylfucose, among other sugar residues. For example, the sulfate group attached to the *Sinorhizobium meliloti* chitooligosaccharide reducing end is necessary for activity on the host plant alfalfa, while its absence renders the NFs active on the nonhost plant vetch. This substituent is therefore a major determinant of host specificity for alfalfa (45). Sulfation of NFs at the chitooligosaccharide reducing end is also present in several other rhizobia, including *Mesorhizobium* sp. strain N33 (isolated from *Oxytropis arctobia*), diverse *Acacia*-nodulating fast-growing rhizobia (*Sinorhizobium terangae* bv. *acaciae*, *Me-*

sorhizobium plurifarium, and *Rhizobium* sp. strain GRH2), and *Rhizobium tropici* (20, 29, 30, 38).

A distinctive feature of *Medicago sativa* (alfalfa) is the marked specificity that it shows for its microsymbiont partner *S. meliloti* or *Sinorhizobium medicae*. In spite of this obligate requirement, other rhizobia were found to be able to nodulate alfalfa (4, 9, 10, 55). In both North and South America, wherever alfalfa plants have been introduced, ineffective nodules produced by *Rhizobium* strains have been found (11, 17). Representatives of such rhizobia include *Rhizobium* sp. strain Or191 (a U.S. isolate) (15) and *Rhizobium* sp. strain LPU83 (an Argentine isolate) (11). Unique features shared by these acid-tolerant alfalfa-nodulating rhizobia include their extended host nodulation range, their relatively inefficient nitrogen fixation in association with *M. sativa* and *Phaseolus vulgaris*, and their extremely low genetic diversity independent of geographic origin (57). LPU83 and related isolates are also very competitive for the nodulation of alfalfa in acid soils (46) but inefficient for biological nitrogen fixation with alfalfa (57). These features point to the Oregon-like rhizobia as a detrimental factor in agricultural soils, where they coexist and compete with the efficient symbiont *S. meliloti*.

Because of their peculiar phenotypic and taxonomic characteristics, the Oregon-like isolates have attracted the attention of rhizobiologists ever since their original isolation by Eardly et al. (15). The accumulated evidence strongly supports the concept that these rhizobia are related to both tropical-legume-infecting rhizobia (e.g., the bean-nodulating *Rhizobium etli*) and temperate-legume-infecting rhizobia (i.e., the *Medicago*-nodulating sinorhizobia). The ability of the Oregon-like isolates to nodulate alfalfa makes them an object of special interest in order to compare their symbiotic genes and signals with those of *S. meliloti*.

Recently, Del Papa et al. (12) presented evidence for the presence of a functional *nodH*-encoded NFs sulfotransferase in the strain LPU83 (a well-characterized Oregon-like rhizobium). The *nodH* gene, present in single copy, maps to a high-molecular-weight megaplasmid. As in *S. meliloti*, a *nodF* homolog immediately upstream from *nodH* that was transcribed in the opposite direction was identified. Del Papa et al. (12) also showed that the *nodH* ortholog from LPU83 was able to restore both the NF sulfation and the Nif⁺ Fix⁺ phenotypes when introduced into an *S. meliloti nodH* mutant. *nodH* disruption in LPU83, however, did not abolish the ability of this strain to nodulate alfalfa, but the chemical structures of the NFs were not determined. The sulfation of NFs by other enzymes was proposed by Del Papa et al. (12) as a possible mechanism that would allow the LPU83 *nodH* mutant to nodulate alfalfa. In order to gain a better understanding of the signals used by these rhizobia to induce nodules in *Medicago* spp., the current study was initiated to investigate in detail the genetic structure of the nodulation (*nod*) region in the Oregon-like rhizobia and to elucidate the chemical structures of their NFs.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the strains and plasmids used. *Escherichia coli* was grown on Luria-Bertani (LB) medium (31) at 37°C. Rhizobial strains were grown on TY (7) at 28°C or, alternatively, on modified B⁻ medium as described by Spaink et al. (51) for the analysis and production of NFs.

For solid media, 15 g of agar per liter of medium was added. The final concentrations of antibiotics were either 10 µg ml⁻¹ gentamicin, 25 µg ml⁻¹ kanamycin, and 10 µg ml⁻¹ tetracycline for *E. coli* or 400 µg ml⁻¹ streptomycin, 120 µg ml⁻¹ neomycin, 50 µg ml⁻¹ gentamicin, and 5 µg ml⁻¹ tetracycline for rhizobia.

DNA manipulation and genetic constructs. Plasmid DNA preparation, restriction enzyme analysis, cloning procedures, and *E. coli* transformation were performed by previously established techniques (42). Bacterial matings were performed as described previously (48).

PCR amplifications were carried out in a total reaction volume of 25 µl containing each primer at 0.5 µM, 200 µM deoxynucleoside triphosphates (dNTPs), 3 mM MgCl₂, 2 µl of template DNA, PCR buffer (50 mM KCl, 20 mM Tris HCl, pH 8.0), and 1 U *Taq* DNA polymerase (Invitrogen). The amplifications were effected with an iCycler (Bio-Rad) thermocycler under the following temperature conditions: initial denaturation at 94°C for 1 min; 35 cycles consisting of 10 s of denaturation at 94°C, a 10-s annealing at 53°C, and a 1-min extension at 72°C; and a final 2-min extension at 72°C.

Southern hybridization was carried out with DNA probes labeled with digoxigenin. The probes were synthesized by PCR through the use of digoxigenin-dUTP (Boehringer Mannheim) and appropriate primers for amplifying the region of interest (12). For hybridization, DNA was transferred to nitrocellulose membranes (Hybond N; Amersham) as described by Chomczynski (8). The digoxigenin-labeled DNA probes were hybridized to the membranes at 60°C overnight after blocking nonspecific binding sites for 1 h at 60°C through the use of the solutions and experimental conditions specified by Boehringer Mannheim (catalog no. 1093 657). For the visualization of positive bands, the membranes were incubated with an antibody against the digoxigenin ligand and then washed. The chemiluminescent reaction was initiated at alkaline pH by the addition of CDP-Star (GE Healthcare) as specified by the manufacturer. Images were captured with an HCCD BioChemi System video digital camera (UVP Inc.).

DNA sequencing and sequence analysis. DNAs were sequenced by Macrogen Inc. (Korea) and the reaction products analyzed with an ABI3730XL DNA analyzer. The nucleotide sequence of the nodulation cluster from strain LPU83 was obtained either by means of a sequencing-walking strategy with specific deoxyoligonucleotides and the cosmids pRVD3 and pRDV7 as template DNAs or by subcloning EcoRI restriction fragments of pRDV7 into the high-copy-number vector pG18mob2. In this last approach, the sequencing reaction was also carried out with M13 universal primers. Sequence comparisons and alignments were performed by means of BLAST and ORFinder in the NCBI database through the use of default parameters.

Phylogenetics analysis. The neighbor-joining method was performed by means of MEGA4 (53). Confidence was assessed by bootstrap analysis (1,000 replications).

Construction of the LPU83 reporter strain. A 1.2-kb EcoRI fragment from the cosmid pRDV7 (12) containing a portion of the *nodC* of LPU83 was cloned in the EcoRI site of pK18mob-LK to generate pGATT8. The *nodC* gene has a HindIII site in which we cloned the 4-kb cassette containing *lacZ::aac*C1 from pAB2002 (6) to generate the sense and antisense constructions (plasmids pGATT9 and pGATT10, respectively). Afterwards, pGATT9 and pGATT10 were transferred from *E. coli* S17-1 to LPU83 by conjugation for integration into the *nod* gene cluster of LPU83 in order to generate the LPU83 *nodC::lacZ* sense and LPU83 *nodC::lacZ* antisense strains, respectively. The correctness of plasmid integration was confirmed by PCR with M13 primers and specific oligonucleotides that anneal to the flanking regions of the rhizobial genome.

Construction of stable LPU83 *nodH* mutant. First, a 1.8-kbp DNA fragment, used by Del Papa et al. (12), containing the LPU83 *nodH* gene was interrupted in a single DraI site with the tetracycline resistance cassette from pHP45ΩTc (39). In a second step, the construction was digested with XbaI and PstI and cloned in vector pK18*mobsac*B (44) to generate vector pKnodHTc. A stable *nodH* mutant of *Rhizobium* sp. strain LPU83 was generated by homogenotization of the new mutation within *nodH* gene of strain LPU83; this strain was called LPU83-doubleH. The stable mutant LPU83-doubleH was confirmed by PCR with external primers "nodH-out-Nter" (5′-CCTATCCATTCAAGCCTCCT-3′) and "nodH-out-Cter" (5′-CAAGTCCCTCAGCACCAA-3′) and primers inside the Tcʳ gene, "Tc-out-Nter" (5′-GGCGAGATCACCAAGGTA-3′) and "Tc-out-Cter" (5′-CTCGACCTGAATGGAAGC-3′). The PCR products were digested with restriction enzymes, and the restriction patterns observed confirmed the double crossing-over.

Plasmid profile. Plasmid profiles were visualized by electrophoresis in horizontal agarose gels by a modification of the method of Eckhardt (18).

Compounds used and their preparation. The flavonoids used in this study were naringenin (4′,5,7-trihydroxyflavanone), apigenin (4′,5,7-trihydroxyflavone), chrysin (5,7-dihydroxyflavone), luteolin (3′,4′,5,7-tetrahydroxyflavone), genistein (4′,5,7-trihydroxyisoflavone), daidzein (4′,7-dihydroxyisoflavone),

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant properties[a]	Reference or source
Sinorhizobium meliloti 2011	Sm^r, Nod$^+$ Fix$^+$ in alfalfa, derived from strain SU47	J. Dénarié, France
Rhizobium sp. strains		
LPU83	Sm^r, Nod$^+$ Fix$^{+/-}$ in alfalfa	11
LPU83-H	LPU83 *nodH*::pGnod83, Sm^r Gm^r	12
LPU83(pMP604)	LPU83 harboring pMP604, Sm^r Tc^r	This work
LPU83-H(pMP604)	LPU83-H harboring pMP604, Sm^r Gm^r Tc^r	This work
LPU83 *nodC*::*lacZ* sense	Derivative of *Rhizobium* sp. strain LPU83, Sm^r Gm^r, sense transcriptional fusion on *nodC* gene with pGATT9	This work
LPU83 *nodC*::*lacZ* antisense	Derivative of *Rhizobium* sp. strain LPU83, Sm^r Gm^r, antisense transcriptional fusion on *nodC* gene with pGATT10	This work
LPU83-double H	Derivative of *R.* sp. strain LPU83 harboring the Tc^r gene from pHP45Ω-Tc interrupting *nodH*	This work
Escherichia coli strains		
DH5α	*recA* Δ*lacU169* φ80d*lacZ*ΔM15	Bethesda Research Laboratories
S17-1	*E. coli* 294 RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	48
Plasmids		
pK18mob-LK	Cloning vector, Km^r, pK18mob with a modified multiple-cloning site	This work
pG18mob2	Cloning vector, Gm^r	24
pAB2002	Ap^r, promoterless *lacZ-aacC1* cassette conferring Gm^r	6
pGATT8	pK18mob-LK with a 1,25-kb EcoRI fragment of LPU83 *nodCI* genes, Km^r	This work
pGATT9	HindIII *lacZ-aacC1* cassette from pAB2002 in HindIII site of pGATT8, sense orientation	This work
pGATT10	HindIII *lacZ-aacC1* cassette from pAB2002 in HindIII site of pGATT8, antisense orientation	This work
pK18*mobsacB*	Mobilizable vector, allows for selection of double crossover, Km^r	44
pKnodHTc	Derivative of pK18*mobsacB* with a Tc^r gene from pHP45Ω-Tc interrupting the *nodH* gene of LPU83	This work
pHP45Ω-Tc	Ap^r, carrying a DNA cassette for Tc^r flanked by transcription and translation terminators	39
pMP604	Tc^r, FITA plasmid	49
pRDV3	Hypercos I containing a *nod* region of *Rhizobium* sp. strain LPU83	This work
pRDV7	Hypercos I containing a *nod* region of *Rhizobium* sp. strain LPU83	12

[a] Sm^r, Tc^r, Km^r, Ap^r, and Gm^r, streptomycin, tetracycline, kanamycin, ampicillin, and gentamicin resistance, respectively.

isoliquiritigenin (4,2′,4′-trihydroxychalcone), fisetin (3,3′,4′,7-tetrahydroxyflavone), flavanone (2,3-dihydroflavone), kaempferol (3,4′,5,7-tetrahydroxyflavone), morin (2′,3,4′,5,7-pentahydroxyflavone), flavones (2-phenyl-4H-1-benzopyran-4-one), umbelliferone (7-hydroxycoumarin), 6-hydroxyflavanone, 4,7-dihydroxyflavanone, quercetin (3,3′,4′,5,7-pentahydroxyflavone dihydrate), 7-hydroxyflavone, 5-hydroxyflavones, hesperedin (hesperetin 7-rhamnoglucoside), and coumesterol (7,12-dihydroxycoumestan). Flavonoid solutions were prepared as 1-mg ml^{-1} stocks in ethanol, and the final concentration in cultures was 1 μg ml^{-1}.

For the preparation of crude exudates, seeds of *Medicago sativa* (alfalfa cv. Monarca) and *P. vulgaris* (bean cv. Negro Jamapa) were surface sterilized for 10 min with 20% (vol/vol) commercial bleach (equivalent to 55 g/liter active Cl$_2$), followed by six washes with sterile distilled water. Seeds were then placed in 20 ml of water. Two days later, the exudates were filtered (0.2-mm pore size) and stored.

Determination of β-galactosidase activity. The β-galactosidase activity was determined by the sodium dodecyl sulfate-chloroform method as described by Miller (31). The β-galactosidase activities were measured at least twice.

TLC analysis of nodulation factors. The thin-layer chromatography (TLC) analyses were performed according to Spaink et al. (51). TLC (HPTLC Silica Gel 60; Merck) was carried out with 50% (vol/vol) acetonitrile in water as the mobile phase. For the radiolabeling of lipochitooligosaccharides (LCOs), 0.4 μCi of [^{14}C]GlcNAc (specific activity, 55 Ci/mol; Amersham) was used. The TLC plates were visualized with a phosphorimager after 7 days of exposure.

LCO purification and chemical structure determination. To obtain large quantities of LCOs, 2 liters of luteolin-induced bacterial culture was extracted with 0.6 liter of *n*-butanol and the extract evaporated to dryness under vacuum. The residue containing the LCOs was then suspended in 10 ml of 60% (vol/vol)

acetonitrile in water with shaking overnight and the solvent adjusted to 20% (vol/vol) acetonitrile in water. A prepurification step was then performed by passing the crude extract through a C$_{18}$ cartridge (Resprep). The solid-phase extraction column was first washed with 20% (vol/vol) acetonitrile in water to remove salts and polar compounds, and the NFs were then eluted with methanol.

Chromatographic separation was performed with a Perkin-Elmer series 200 high-pressure liquid chromatography (HPLC) system (Wellesley, MA) coupled to an Applied Biosystems QTRAP liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Foster City, CA) consisting of a triple-quadrupole linear ion trap mass spectrometer equipped with an electrospray ion source.

HPLC analyses were performed on a Tracer Spherisorb ODS2 C$_{18}$ reverse-phase column (250 by 2.1 mm) with a particle size of 5 μm (Teknokroma, Barcelona, Spain). The flow rate was 0.3 ml min^{-1}. The chromatographic separation was performed through the use of a binary gradient consisting of water (A) and acetonitrile (B), with both components containing 0.1% (vol/vol) formic acid. The elution profile was isocratic for 5 min at 30% B, then with B linearly increasing from 30% to 100% for 30 min, and finally isocratic again for 3 min. Mass spectrometric detection was performed in the positive mode. For HPLC-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas, 35 lb/in^2; ion spray potential, 5,500 V; source temperature, 300°C; source gas, 60 lb/in^2; declustering potential, 50 V; and entrance potential, 10 V. MS/MS experiments were performed with a collision energy of 35 V. Only those precursor ions with an *m/z* of between 800 and 1,600 and intensities higher than 1,000 cps were selected for MS/MS analysis.

Plant nodulation tests. Nodulations test were performed as described previously (54).

Nucleotide sequence accession numbers. The complete *nod* gene cluster in plasmid pLPU83b was deposited in GenBank under accession number

GU123168. For the construction of the intergenic region *nodEG* (IGS$_{NOD}$) tree, one of each of the representative isolates described by Bailly et al. (2) was used. Those isolates, their GenBank accession number, and the IGS$_{NOD}$ group were as follows: STM2778, DQ405467.1, AH; STM2782, DQ405471.1, AF; STM2797, DQ405486.1, AE; STM2811, DQ405540.1, G; STM2825, DQ405510.1, S; STM2832, DQ405517.1, R; STM2833, DQ405518.1, U; STM2835, DQ405520.1, V; STM2836, DQ405521.1, W; STM2837, DQ405522.1, Z; STM2840, DQ405525.1, X; STM2841, DQ405526.1, Y; STM2861, DQ405570.1, L; STM2867, DQ405576.1, D; STM2874, DQ405583.1, E; STM2876, DQ405585.1, A; STM2878, DQ405587.1, C; STM2879, DQ405588.1, B; STM2883, DQ405592.1, K; and STM2889, DQ405492.1, T. The IGS$_{NOD}$ from *Rhizobium mongolense* USDA 1844 (DQ406575.1), the pSymA from *S. meliloti* (AE006469), and the pSMED02 from *S. medicae* (CP000740) were also included in the analysis. For the construction of *nodD* phylogenetic trees, the respective locus tags of *S. meliloti* SMa0870, SMa0757, and SMa0840 for *nodD1*, *nodD2*, and *nodD3* were analyzed along with the corresponding respective tags of *S. medicae* Smed6183, Smed5941, and Smed6212 for *nodD1*, *nodD2* and *nodD3*.

RESULTS

Identification of pSym in LPU83. In many rhizobial species most of the essential genes required for the symbiotic process are located in plasmids that have been traditionally designated symbiotic plasmids or pSyms. Del Papa et al. (12) recently mapped a *nodH*-positive signal by PCR on the megaplasmid region of modified Eckhardt gels using the Oregon-like strain LPU83. Unfortunately, in the work by Del Papa et al. (12), the two high-molecular-weight megaplasmids previously reported by Wegener et al. (57) could not be properly resolved in order to unequivocally map the *nod* signal to one of the two megaplasmids. To obtain further information on the genomic location of *nodH* in the Oregon-like rhizobia, we used here a modified Eckhardt (18) protocol with which we separated three plasmid bands in strain LPU83 with estimated sizes of 130 kbp (pLPU83a), 900 kbp (pLPU83b), and 1.8 Mbp (pLPU83c). Southern hybridization analysis revealed that *nodH* was located on plasmid pLPU83b (data not shown), demonstrating that this replicon is a likely carrier of other *nod* genes (pSym).

Physical map of the complete *nod* gene cluster in plasmid pLPU83b. To gain insight into the genetic structure of the nodulation genes in strain LPU83, we partially sequenced the clones pRDV7 and pRDV3 obtained from a genomic library previously constructed by Del Papa et al. (12). A 15.5-kb contig was assembled and annotated. It was found to carry 13 open reading frames, designated *nodABCD1D3EFGHIJPQ*, whose predicted translation products are similar to known Nod proteins of a variety of rhizobial species. A comparative analysis of the *nod* sequences in strain LPU83 against the corresponding gene orthologs present on the genomes of rhizobia for which complete sequences are available revealed a significant similarity with the *nod* genes of the alfalfa-nodulating rhizobia *S. meliloti* 1021 and *S. medicae* WSM419. Interestingly, partial DNA sequences (0.5 to 1.5 kbp) of *nodA*, *nodB*, *nodC*, *nodE*, and *nodG* of *R. mongolense* USDA1844 showed the highest identities with the corresponding genes of strain LPU83 (92, 94, 97, 96, and 93%, respectively). It was noted that the *nodG* gene of strain LPU83, a 3-oxoacyl–acyl carrier protein reductase, was found to be truncated, with only the 52 N-terminal amino acids being present. Lopez-Lara and Geiger (28) suggested that *nodG* is a duplication of the housekeeping gene *fabG*, thus implying that NodG in LPU83 might likely be func-

tionally replaced by another ortholog function(s) present in this rhizobium.

The location and orientation of *nod* genes showed a quite remarkable synteny (Fig. 1A) with the homologous clusters from pSymA of *S. meliloti* 1021 (3) and from pSmed02 of *S. medicae* WSM419 (http://genome.jgi-psf.org/sinme/sinme.info .html). In the *nod* region we found three conserved *nodD*-dependent promoter elements (*nod* boxes). No major differences were found between the consensus sequence derived for the *nod* boxes of LPU83 and those present in other alfalfa-nodulating rhizobia (Fig. 1B).

A remarkable difference between the *nod* clusters present in *S. meliloti* and in *S. medicae* was that the transcriptional regulator *syrM*, a symbiotic regulatory gene mediating bacterial responses to plant signals (13), was absent in LPU83. Other evolutionary differences between *nod* clusters of the three rhizobia concern the kind and distribution of insertion sequence (IS) elements within these genetic regions (Fig. 1A).

Phylogenetic position of LPU83 within the *Medicago*-nodulating rhizobia inferred from the intergenic region *nodEG* (IGS$_{NOD}$). Bailly et al. (2) obtained a Bayesian supertree of different rhizobia based on the partial sequences of their *nodABC-nodEG* gene clusters. A clade including several strains of *R. mongolense* appeared as one of the first emerging branches among the *Medicago* symbionts. A second clade of the same supertree included strains of *S. medicae*, *S. meliloti* bv. *medicaginis*, and *S. meliloti* bv. *meliloti* (2). A similar tree structure resulted when separated sequences of either *nodA*, *nodB*, *nodC*, *nodE*, *nodG*, or IGS$_{NOD}$ were used (2). Previous work by others (26) and from our laboratory (12) supported the idea that the Oregon-like rhizobia carried symbiotic information related to *Medicago*-nodulating rhizobia. Similarly, *R. mongolense* carries symbiotic information related to *Medicago*-nodulating rhizobia (47). Thus, in order to ascertain the phylogenetic position of the symbiotic genes of the Oregon-like rhizobia, we performed a neighbor-joining analysis using the IGS$_{NOD}$ region, including representative rhizobia from the clusters described by Bailly et al. (2). The results showed that strain LPU83 clustered together with the representative strain *R. mongolense* USDA 1844 (Fig. 2).

Induction of *nod* genes in strain LPU83 and structural analysis of its NFs. Since no data regarding the conditions required for NF production in the Oregon-like rhizobia were available, we first evaluated the effects of different flavonoids on both the transcription of *nod* genes and NF production. Induction of common *nod* genes by 20 different flavonoids was evaluated through the use of a *nodC-lacZ* transcriptional fusion in strain LPU83, as described in Materials and Methods. As a positive control of *nod* induction, a plasmid that contained the flavonoid-independent transcription activation *nodD*$_{FITA}$ gene (plasmid, pMP604) (49) was introduced into strain LPU83 *nodC::lacZ*. A 20-fold increase in β-galactosidase activity was observed compared with that of the isogenic strain without plasmid pMP604. None of the 20 flavonoids assayed showed any significant induction of β-galactosidase activity in strain LPU83 *nodC::lacZ*. Alfalfa and bean seed exudates were also tested, and neither of them was found to be an effective inducer of the *nod* genes in strain LPU83. The results indicate that while the NodD variant provided by pMP604 is able to activate *nod* transcription, none of the flavonoids tested were
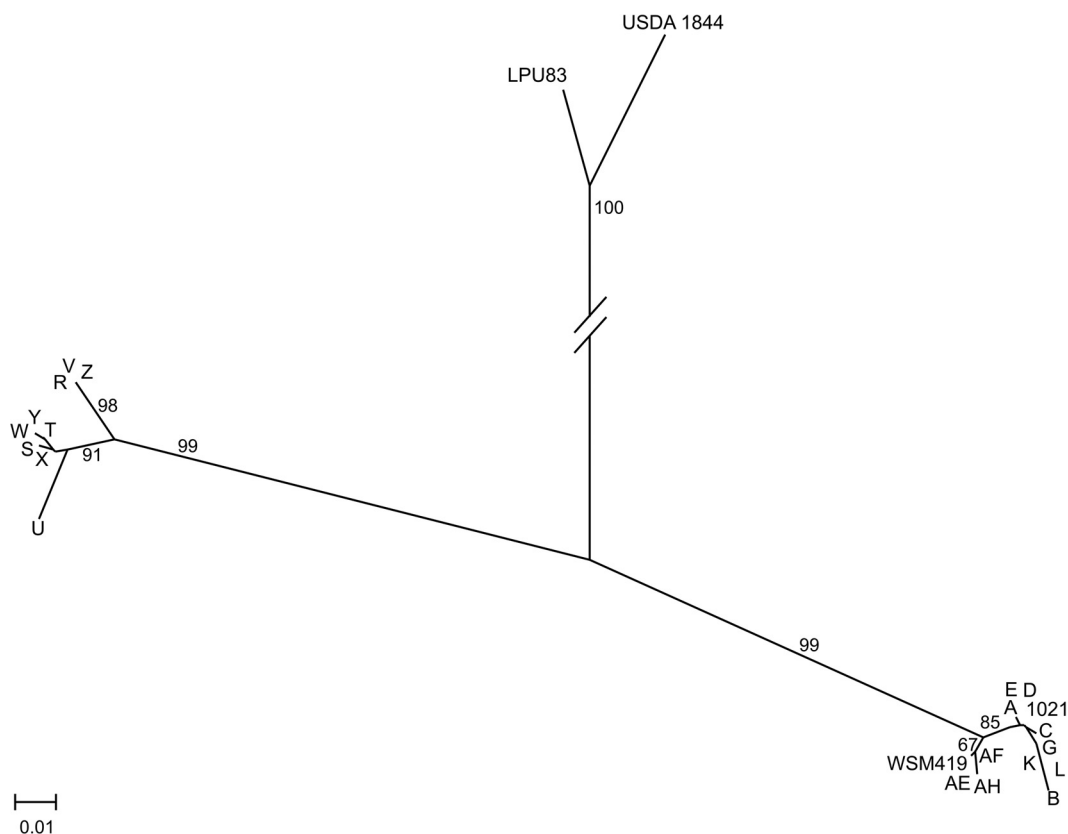
FIG. 1. Genetic organization of the nodulation clusters in LPU83. (A) Genetic organization of the nodulation cluster of LPU83 compared with the same regions in *S. meliloti* and *S. medicae*. The color combination indicates the organization of ortholog genes within these clusters. (B) Analysis of the conserved *nod* boxes within the *nod* cluster region. DNA sequences were obtained from GenBank under accession numbers AE006469 for pSymA of *S. meliloti* 1021 and CP000740 for pSMED02 of *S. medicae* WSM419. The accession number of the *nodD2* gene of LPU83 is AJ578434. Red on yellow, invariant nucleotides; blue on light blue, strongly conserved.

able to mediate a proper interaction of the LPU83 NodD with the existing *nod* boxes. The lack of detectable *nod* transcription correlated with the absence of radiolabeled *N*-acetyl-^{14}C-NFs in reverse-phase TLC when any of the previously tested 20 flavonoids were assayed as putative inducers. On the other hand, consistent with the observed *nodC-lacZ* induction in strain LPU83(pMP604), NF production was detected in this strain. This strain allowed us to prepare extracts containing enough NF to be suitable for structural analysis by HPLC/MS-MS. NFs were isolated as described in Materials and Methods from luteolin-induced cultures of both LPU83(pMP604) and LPU83-H(pMP604). HPLC and MS/MS analysis were performed as described previously (10a). By this means, we could identify 9 different types of NF variants in LPU83 and 24 types in mutant LPU83-H. Table 2 summarizes the chemical structures of the different NFs found in strains LPU83 and

LPU83-H. The NFs identified were trimers, tetramers, and pentamers of GlcNAc and showed different decorations on their reducing or nonreducing terminal residues. The N-acyl chains on the nonreducing GlcNAc residues showed different lengths and degrees of unsaturation (Table 2). Remarkably, while in strain LPU83 most of the NFs were sulfated in their reducing terminal residue, none of the NFs isolated from the *nodH* mutant LPU83-H were sulfated. The absolute lack of sulfate in all NFs produced by mutant LPU83-H strongly suggests that no functional homologs of *nodH* are present in these rhizobia.

Overproduction of NFs: effects on the symbiotic behavior of LPU83. The Oregon-like rhizobia have a peculiar nodulation phenotype. The mutant LPU83-H does not require sulfated variants of NFs for the nodulation of alfalfa (12). LPU83-H also kept the capacity to induce nodules in *Phaseolus vulgaris*

FIG. 2. Neighbor-joining tree obtained for IGS$_{NOD}$ of *Medicago*-nodulating bacteria. Alleles belonging to either *S. medicae*, *S. meliloti* bv. *medicaginis*, or *S. meliloti* bv. *meliloti* group in different clades, while LPU83 and *R. mongolense* USDA 1844 group together in an independent clade. The letters indicate the groups mentioned in Materials and Methods. The bar indicates 0.01 substitution/site.

and *Leucaena leucocephala*. We wanted to investigate whether the inability of the Oregon-like rhizobia to induce a fully fixing symbiosis in alfalfa arises from deficiencies in NF production as a consequence of the observed poor *nod* induction (see "Induction of *nod* genes in strain LPU83 and structural analysis of its NFs" above). To approach this question, we compared the symbiotic behavior on alfalfa of strain LPU83 with that of the NF overproducer LPU83(pMP604). Figure 3 shows that strain LPU83(pMP604) started to nodulate 2 to 3 days earlier than strain LPU83 and that strain LPU83(pMP604) induced a higher number of nodules per plant [there is also an increase in the number of nodules when LPU83-H(pMP604) is compared with LPU83-H]. Though such slightly improved nodulation kinetics induced by plasmid pMP604 might be due to an increased production of NFs, no detectable changes in plant dry weight were evident, indicating that the poor nitrogen fixation of the Oregon-like rhizobia in alfalfa derives from deficiencies in a symbiotic signal(s)/determinant(s) different from the NFs.

DISCUSSION

In this report, we present the characterization and genetic organization of the *nod* cluster and the determination of the chemical structure of the Nod factors of the broad-host-range *Rhizobium* sp. strain LPU83. This strain has the ability to nodulate *Medicago sativa* (alfalfa), *M. truncatula*, *M. nigra* var.

nigra, *M. arabica*, *M. falcata*, *M. alba* var. *alba*, *M. indica*, *M. messanensis*, *M. officinalis*, *Trigonella foenum-graecum*, *T. caerulem*, *Phaseolus vulgaris* (the common bean), and *Leucena leucocephala* (11, 57). Strain LPU83 belongs to a rare group of rhizobia initially isolated by Eardly et al. in the United States (15–17). These isolates are acid tolerant, very competitive for the nodulation of alfalfa in acid soils, and inefficient in biological nitrogen fixation with alfalfa. Because of these characteristics, we had a particular interest in the molecular analysis of the *nod* cluster present in these rhizobia and in its relatedness to the homologs from other rhizobia. Here we mapped the *nod* cluster in pLPU83b. This plasmid, of ca. 900 kb, was shown to be smaller than the *nod*-harboring pSyms present in other alfalfa-nodulating rhizobia (3; http://genome.jgi-psf.org/sinme /sinme.info.html). To gain a deeper insight into the genetic structure of the *nod* cluster of an Oregon-like rhizobium, the *nod* region of strain LPU83 was fully sequenced. A high synteny with the *nod* clusters of *S. meliloti* and *S. medicae* was observed, with a very high DNA sequence identity (higher than 82% at the DNA level). A careful analysis of the available sequence data for the *nod* genes of strain LPU83 revealed the presence of three *nodD* paralogs (the two copies *nodD1* and *nodD3* presented here and the *nodD2* previously deposited in GenBank under accession number AJ578434). In *S. meliloti*, *nodD1* is activated by complex plant seed extracts or by the flavonoid luteolin, *nodD2* is activated only by seed extracts, and

TABLE 2. Nodulation factor structures produced by LPU83 and the *nodH* mutant

Structure[a]	LPU83(pMP604)		Structure	LPU83 *nodH*(pMP604)	
	$(M + H)^+$, m/z	Fragment ions (b-ions), m/z		$(M + H)^+$, m/z	Fragment ions (b-ions), m/z
			V(C$_{18:1}$-OH,NMe)	1,286	456, 659, 862
			V(C$_{18:0}$,NMe)	1,272	442, 645, 848, 1,051
V(C$_{18:1}$,NMe,S)	1,350	440, 643, 846, 1,049 [(M − 80)$^+$ = 1,270]	V(C$_{18:1}$,NMe)	1,270	440, 643, 846, 1,049
V(C$_{18:2}$,NMe,S)	1,348	438, 641, 844, 1,047 [(M − 80)$^+$ = 1,268]	V(C$_{18:2}$,NMe)	1,268	438, 641, 844, 1,047
			V(C$_{18:0}$,NMe) dNAc	1,230	442, 645, 848, 1,009
			V(C$_{16:1}$)	1,228	398, 804, 1,007
V(C$_{18:2}$, NMe) dNAc	1,226	641, 844, 1,005	V(C$_{18:2}$,NMe) dNAc	1,226	438, 641, 844, 1,005
IV(C$_{20:0}$, NMe)	1,098	470, 673, 877	IV(C$_{20:0}$, NMe)	1,097	470, 673
IV(C$_{20:1}$,NMe, S)	1,175	468, 671, 874 [(M − 80)$^+$ = 1,095]	IV(C$_{20:1}$,NMe)	1,095	468, 671, 874
			IV(C$_{20:2}$,NMe)	1,093	466, 669, 872
			IV(C$_{18:1}$-OH, NMe)	1,083	456, 649, 862
IV(C$_{18:0}$,NMe,S)	1,149	442, 645, 848 [(M − 80)$^+$ = 1,069]	IV(C$_{18:0}$,NMe)	1,069	442, 645, 848
IV(C$_{18:1}$,NMe,S)	1,147	440, 643, 846 [(M − 80)$^+$ = 1,067]	IV(C$_{18:1}$,NMe)	1,067	440, 643, 846
IV(C$_{18:2}$,NMe,S)	1,145	438, 641, 844 [(M − 80)$^+$ = 1,065]	IV(C$_{18:2}$,NMe)	1,065	438, 641, 844
			IV(C$_{16:0}$,NMe)	1,041	414, 617, 820
			IV(C$_{16:0}$)	1,027	400, 603, 806
			IV(C$_{16:1}$)	1,025	398, 601, 804
			IV(C$_{16:2}$)	1,023	396, 599
			III(C$_{20:1}$)	892	468, 671
			III(C$_{20:2}$,NMe)	890	466, 669, 872
			III(C$_{18:0}$,NMe)	866	442, 645, 848
III(C$_{20:0}$,NMe)	894	470, 673	III(C$_{18:1}$,NMe)	864	440, 643
			III(C$_{18:2}$,NMe)	862	438, 641, 844
			III(C$_{16:2}$)	820	396, 599

[a] NF structures are represented following the convention (50) that indicates the number of GlcNAc residues in the backbone (roman numeral), the length and degree of unsaturation of the fatty acyl chain, and the other substituents, which are listed in the order they appear, proceeding clockwise from the fatty acid.

nodD3 was proposed to modulate *nod* genes in the absence of any plant factor (31a). The putative assignment of *nodD1* and *nodD3* in strain LPU83 was made based on their synteny with the corresponding *nodD* copies in *S. meliloti*. In spite of this assignment, the *nodD3* of LPU83 has higher sequence identity with *S. meliloti* 1021 *nodD1* than with *S. meliloti* 1021 *nodD3* (Fig. 4). Alleles *nodD1*, *nodD2*, and *nodD3* of *S. meliloti* and *S. medicae* grouped as separates branches. The *nodD2* gene of LPU83 groups with its sinorhizobial orthologs, whereas the *nodD1* and *nodD3* genes of LPU83 group together with the
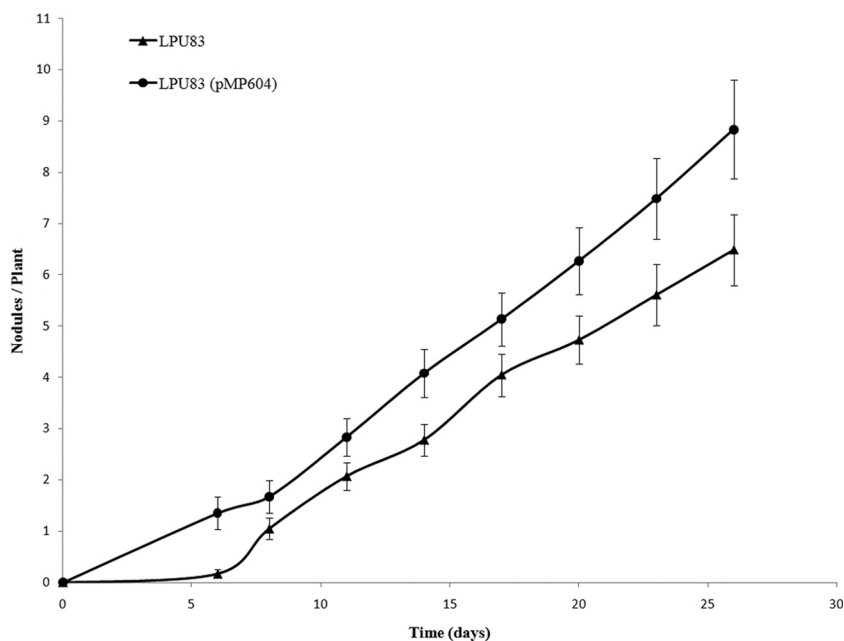

FIG. 3. Nodulation kinetics in alfalfa roots of *Rhizobium* sp. strain LPU83 and NF overproducer LPU83(pMP604). Sets of plants were inoculated with approximately 10^5 bacteria of each strain per plant. Nodules were scored at the indicated times. The results are given as the average number of nodules per plant. Error bars indicate standard deviations.
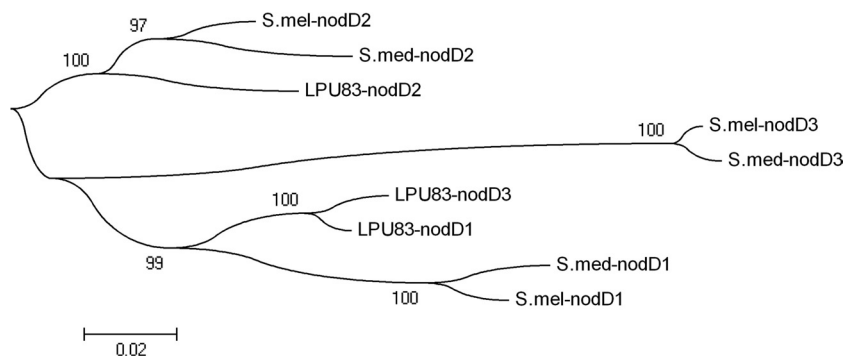
FIG. 4. Neighbor-joining tree obtained for *nodD* genes. A phylogenetic tree of alleles *nodD1*, *nodD2*, and *nodD3* belonging to either LPU83, *S. medicae*, or *S. meliloti* is shown. Alleles *nodD1* and *nodD3* of LPU83 group in their own clade, showing a gene duplication event without a deep differentiation as in *Sinorhizobium* strains. The bar indicates 0.02 substitution/site.

nodD1 genes of *S. meliloti* and *S. medicae.* The present gene structure at the *nodD* loci in LPU83 could be the consequence of an early gene duplication that did not further progress toward a significant sequence differentiation between *nodD1* and *nodD3* in the Oregon-like rhizobia. These observations raise the question of whether the current forms of *nodD3* present in *S. meliloti* and *S. medicae* represent gene variants not (yet?) evolved in the Oregon-like rhizobia. The differences observed at the *nodD* loci and the absence of *syrM* in strain LPU83 may both be related to the observed lack of transcription of the *nodC-lacZ* fusion with either flavonoids or alfalfa seed exudates (the capacity of a yet-unidentified flavonoid to

induce the *nod* genes in the Oregon-like rhizobia cannot be disregarded).

Despite all these considerations, the NF signals produced by the Oregon-like rhizobia are able to promote the nodulation of alfalfa and other legumes. Thus, to start characterizing the early symbiotic signals of strain LPU83, we isolated the NFs produced by strain LPU83 and analyzed their chemical structures by HPLC-MS/MS (the results are summarized in Table 3). Interestingly, many of the NFs were N methylated at the nonreducing terminal residue, a characteristic typically found in bean-nodulating bacteria (37, 38) as the result of a reaction carried out by the *nodS* gene (21). Krishnan et al. (25) reported

TABLE 3. Comparison of nodulation factors and their decorations

Strain	Fatty acyl chains	R1	R2	R3	R4	R5	R6	n	Reference(s)
Bradyrhizobium japonicum USDA110	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$	H	H	H	MeFuc	H	H	2	43
Rhizobium etli CE3	$C_{18:0}$, $C_{18:1}$	Me	Cb, H	H	AcFuc	H	H	2	37
R. fredii USDA191	$C_{18:1}$, $C_{18:0}$, $C_{16:1}$	H	H	H	Fuc, MeFuc	H	H	0, 1, 2	5
R. leguminosarum bv. *trifolii* ANU843	$C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:3}$	H	H	H, Ac	H	H	H	0, 1, 2	35
R. leguminosarum bv. *viciae* RBL5560	$C_{18:1}$, $C_{18:4}$	H	H	Ac	H	H	H	1, 2	52
R. loti NZP2213	$C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{20:0}$, $C_{20:1}$, $C_{22:1}$	H	Cb	H	Fuc, AcFuc	H	Fuc	−1, 0, 1, 2	34
Rhizobium sp. strain LPU83	$C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:0}$, $C_{20:1}$, $C_{20:2}$	Me	H	H	S, H	H	H	0, 1, 2	This work
Rhizobium sp. strain NGR234	$C_{16:1}$, $C_{18:0}$, $C_{18:1}$	Me	Cb, H	Cb, H	MeFuc, AcMeFuc, SMeFuc	H	H	2	40
R. tropici CIAT 899	$C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{20:0}$, $C_{20:1}$	Me, H	H	H	S, H	H	H	1, 2	20
Sinorhizobium meliloti RCR2011	$C_{16:1}$, $C_{16:2}$, $C_{16:3}$	H	H	H, Ac	S	H	H	1, 2	1, 27

that the *nodS* gene could extend the host range of certain rhizobia. The presence of methylated NFs in Oregon-like strains could explain their ability to nodulate the common bean and *Leucaena leucocephala*, constituting an extended host range (20). Evidence of *nodS* orthologs in the Oregon-like rhizobia was not reported, nor were methylated NFs found in *S. meliloti* (36). The N methylation of the NFs in the Oregon-like rhizobia reflects, at the chemical level, the known relationship between these bacteria and bean-nodulating rhizobia. Table 3 summarize the comparative structures of NFs of bean- and alfalfa-nodulating rhizobia. Clearly, the NFs of LPU83 have the characteristics of both groups. The N-acyl chains and the N-methyl group at position R1 are similar to those of *R. tropici* or *R. etli*, while the substitutions at positions R2 to R6 are similar to those of *S. meliloti* or *S. medicae*.

The most striking observation regarding the NF analysis, however, concerned the fact that no sulfated forms of NFs could be detected in the *nodH* mutant LPU83-H. The strain was previously shown to be able to nodulate alfalfa (12), but we did not know if NF sulfated forms could still be present in the LPU83 *nodH* mutant. We showed here that nodulation of alfalfa by mutant LPU83-H occurs in the absence of detectable sulfated forms of NFs by MS/MS. To rule out the possibility that a reversion of the *nodH* mutation in strain LPU83-H could have generated sulfated NF production during the course of the nodulation assays, an independent and stable mutant was constructed by insertion of a tetracycline resistance cassette by double crossover within the *nodH* gene. As expected, plant assays showed that the new *nodH* mutant also nodulated alfalfa, as observed for mutant LPU83-H. In the context of the accepted essential requirement of sulfated NFs for the nodulation of alfalfa (9, 19, 56), the results presented here open a new context of analysis to explore in mutant LPU83-H other signal forms for rhizobia to nodulate alfalfa.

The central matter underlying the genetic and symbiotic characterization of the Oregon-like rhizobia is the value of these bacteria in helping to unravel the evolutionary history of rhizobia that nodulate *Medicago* spp. and their mechanisms of diversification and evolution. Bailly et al. (2) suggested that the *Sinorhizobium* species acquired the ability to nodulate plants of the *Medicago* genus through a horizontal transfer of *nod* genes from the ancestor of one of the *Rhizobium* species, citing the lack of congruence between symbiotic and housekeeping gene phylogenies, and they further proposed that *R. mongolense* could be this ancestor because this species appeared as the first emerging branch among the *Medicago* symbionts in a *nodABC* and *nodEG* supertree phylogeny. The fact that our analysis of the IGS$_{NOD}$ from strain LPU83 positions this strain in the same branch as the type strain *R. mongolense* 1844 indicates that the Oregon-like rhizobia might represent an early alfalfa-nodulating genotype. A more extended similarity between Oregon-like rhizobia and *R. mongolense* is supported by the close relationship between the 16S rRNA genes of these two rhizobia reported by Laguerre et al. (26). As we already mentioned, the genetic sequence of the *nod* cluster of the LPU83 revealed that this bacterium is closely related to *R. mongolense* and forms an independent cluster within the alfalfa-nodulating rhizobia. The NF characterization revealed a chimeric structure between the alfalfa- and bean-nodulating rhizobia. Unfortunately, no data are yet available on the chemistry of NFs from

R. mongolense. To gain a deeper insight into how the so closely related symbiotic clusters present in *S. meliloti*, *S. medicae*, *R. mongolense*, and the Oregon-like rhizobia evolved to give place to their specific symbioses will require the help of larger-scale sequencing approaches together with classical experiments cross-combining specific pSym determinants in different genomic backgrounds.

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