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Rhizobial NodL *O*-Acetyl Transferase and NodS *N*-Methyl Transferase Functionally Interfere in Production of Modified Nod Factors

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The products of the rhizobial nodulation genes are involved in the biosynthesis of lipochitin oligosaccharides (LCOs), which are host-specific signal molecules required for nodule formation. The presence of an *O*-acetyl group on C-6 of the nonreducing *N*-acetylglucosamine residue of LCOs is due to the enzymatic activity of NodL. Here we show that transfer of the *nodL* gene into four rhizobial species that all normally produce LCOs that are not modified on C-6 of the nonreducing terminal residue results in production of LCOs, the majority of which have an acetyl residue substituted on C-6. Surprisingly, in transconjugant strains of *Mesorhizobium loti, Rhizobium etli,* and *Rhizobium tropici* carrying *nodL,* such acetylation of LCOs prevents the endogenous *nodS*-dependent transfer of the *N*-methyl group that is found as a substituent of the acylated nitrogen atom. To study this interference between *nodL* and *nodS,* we have cloned the *nodS* gene of *M. loti* and used its product in in vitro experiments in combination with purified NodL protein. It has previously been shown that a chitooligosaccharide N deacetylated on the nonreducing terminus (the so-called NodBC metabolite) is the preferred substrate for NodS as well as for NodL. Here we show that the NodBC metabolite, acetylated by NodL, is not used by the NodS protein as a substrate while the NodL protein can acetylate the NodBC metabolite that has been methylated by NodS.

Rhizobial bacteria have the unique ability to induce formation of nitrogen-fixing nodules on the roots or stems of leguminous plants. The development of legume nodules is largely controlled by reciprocal signal exchange between the symbiotic partners. Legume roots secrete specific flavonoids or isoflavonoids that induce the transcription of many bacterial genes (*nod, nol,* and *noe* genes). Most of these genes are involved in the synthesis and secretion of signal molecules that are essential to trigger nodule formation and that are known as Nod factors. Nod factors from many rhizobial species have been characterized, and their structures have been elucidated. Because their basic structure consists of a chitin oligosaccharide backbone N acylated on the nonreducing terminal residue, they are referred to as lipochitin oligosaccharides (LCOs). The nature of the fatty acid and the combination of diverse chemical substitutions provide host specificity to the LCOs produced by a given rhizobial strain (for reviews, see references 1, 7, 9, and 25).

Although there is a wide variability in LCO structures, two major groups can be distinguished (19, 26, 33). (i) Rhizobia associated with plants of the *Trifolieae, Vicieae,* and *Galegeae* tribes that form indeterminate nodules produce LCOs carrying specific polyunsaturated fatty acids and have either no substitution or a carbamoyl or an *O*-acetyl group on C-6 of the nonreducing terminal *N*-acetylglucosamine (Fig. 1). On the reducing terminal residue, they can have substitutions of sulfate or acetate groups. (ii) Rhizobia associated with plants that form determinate nodules produce LCOs with common fatty acids and, in most cases, the N atom carrying the fatty acid is also replaced with a methyl group. On the nonreducing terminal residue they can also carry carbamoyl groups, while on the reducing terminal residue they can bear sulfate, arabinose, or fucose. Frequently, the fucosyl residue is replaced by acetate, sulfate, and/or methyl groups.

The biochemical functions of most of the nodulation genes involved in the biosynthesis of LCOs have been shown directly or indirectly. The *nodABC* genes are absolutely required for the synthesis of LCOs; NodC produces chitooligosaccharides, NodB removes the *N*-acetyl group from the nonreducing terminal residue, and NodA is involved in the transfer of the acyl chain. Modifications of the LCO core are dependent on *nod* genes that are strain specific. Some nodulation proteins have been purified to homogeneity, and in vitro analysis of their substrate specificities has provided information about their positions in the LCO biosynthesis pathway (for reviews, see references 7 and 14). NodL and NodS are both involved in modifications of the nonreducing terminal residue and are among the best-characterized Nod proteins. The NodL protein has transacetylating activity in vitro with acetyl coenzyme A (CoA) as the acetyl donor, and it is responsible for the presence of one *O* acetyl group on C-6 of the nonreducing terminal residue of LCOs (3). NodS is an *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase involved in N methyl-

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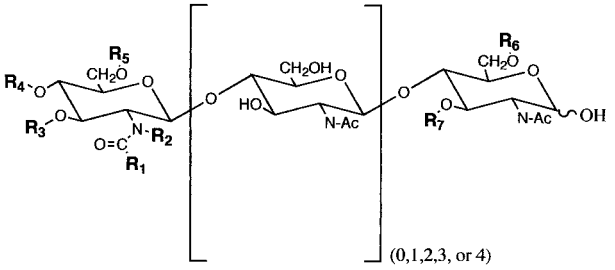

R_1 = fatty acyl
R_2 = H or CH_3
R_3 = H or NH_2CO
R_4 = H or NH_2CO
R_5 = H, CH_3CO or NH_2CO
R_6 = H, CH_3CO, SO_3, Ara, Fuc, 2-O-methylFuc, 4-O-acetyl(2-O-methylFuc), or 3-O-sulfo-2-O-methylFuc
R_7 = H, Ara, or Fuc

FIG. 1. Scheme of LCO structures in rhizobia.

ation of LCOs (12, 13). NodS uses N-deacetylated chitooligosaccharides, the products of the NodBC proteins (the so-called NodBC metabolites), as its methyl acceptors (11, 18). Although the NodL protein appears to be able to acetylate various substrates, such as LCOs, chitin fragments, and *N*-acetylglucosamine, the NodBC metabolite is the preferred substrate and is probably the in vivo acetyl-accepting substrate (2, 3).

We have introduced the *nodL* gene of *Rhizobium leguminosarum* bv. viciae into four different rhizobial species (all producing LCOs of type 2) and have studied the LCO structures produced by the recombinant strains. *Mesorhizobium loti* carrying *nodL* produces LCOs which are acetylated but, surprisingly, in contrast to the wild-type LCOs, have no *N*-methyl group on the nonreducing terminal residue. In order to study in more detail the apparent interference between the *nodL* and *nodS* genes, we have cloned *nodS* of *M. loti,* and its product has been overproduced in *Escherichia coli*. In vitro studies have shown that NodS is unable to N methylate the NodBCL metabolite.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. Broad-host-range plasmids were mobilized from *E. coli* to rhizobia using pRK2013 as a helper plasmid (8). Rhizobia were grown in B^- medium (27). Antibiotics were added, when required, to the following final concentrations (micrograms per milliliter): tetracyline, 2 (10 for *Rhizobium etli*); spectinomycin, 200. For induction of LCOs, naringenin was added to the medium to a final concentration of 1.5 μM.

DNA sequence analysis. DNA sequencing was achieved by the dideoxy chain termination reaction method (24) with a T7 sequencing kit (Pharmacia). The sequencing primers were M13/lac-Z forward and reverse (Perkin-Elmer), and the sequencing vectors were pGEM-T (Promega) and pBluescript SK (Stratagene). The comparison of primary structures was carried out using the Genetics Computer Group (University of Wisconsin, Madison) software package.

Cloning of *M. loti nodS*. DNA manipulations were carried out using standard procedures (23). Plasmids were isolated with a Qiaprep spin plasmid kit (Qiagen). The *nodS* gene from *M. loti* E1R was obtained by PCR using genomic DNA from the bacterium as the template and primers (see Fig. 5A): NB, GATCCA AACAATCAATTTTACCCAAT; U3 (oMP124), CC(A/G)TC(A/G)TGIGTIA (A/G)(T/C)TTIATICC(A/G)C; S1 (oMP119), CGCGACCC(G/A)TGGCG(C /G)(C/A)TCGAC; S2 (oMP120), CTCCGCACCGGC(C/A)(A/G)CATGCCCC CA; C1 (oMP118), GTGTACATTGAGCAATAGCGATTGG (obtained from Isogen, Maarssen, The Netherlands).

PCR was performed using ULTma DNA polymerase (Perkin-Elmer) in a Stratagene Robocycler gradient 40 thermocycler. The amplification conditions were 1 min at 94°C, 4 min at 37°C, and 1 min at 72°C for 30 cycles. The product of the PCR amplification with primers NB and U3 was cloned into the pGEM-T vector (Promega) to yield construct pMP4603. The insert was partially sequenced, and the regions around the start and stop codons were analyzed. Based on the sequences thus determined for the two ends of the open reading frame (ORF), homogeneous primers (oMP179 and -180) were synthesized, with restriction sites introduced at the 5′ ends of the primers in order to facilitate

TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
XL1 Blue	Host for cloning	Stratagene (?)
BL21(DE3)	Host for expression	130
Rhizobia		
E1R	Rif^r derivative of wild-type *M. loti* isolate E1	15
CE3	Sm^r derivative of wild-type *R. etli* CFN42	20
CIAT899	Wild-type *R. tropici*	17
GRH2	Wild-type *Rhizobium* sp. isolated from *Acacia*	N. Toro, Granada
Plasmids		
pRK2013	IncColE1 Tra^+ Km^r	8
pBlueScript SK	Cb^r sequencing vector	Stratagene
pGEM-T	Cb^r cloning vector	Promega
pET3a	Cb^r expression vector	Novagen
pET16b	Cb^r expression vector	Novagen
pMP4603	pGEM-T containing *nodS* of *M. loti*	This work
pMP4601	pET16b containing *nodS* of *M. loti*	This work
pMP4600	pET3a containing *nodS* of *M. loti*	This work
pMP280	IncP; contains *nodD* of *Rlv;* Tc^r	28
pMP2112	IncW; contains *nodD* of *Rlt;* Spt^r	15
pMP2107	IncP; contains *nodD* of *Rlt* and pA-*nodL* of *Rlv;* Tc^r	3
pMP2109	IncW; contains *nodD* of *Rlt* and pA-*nodL* of *Rlv;* Spt^r	22

[a] Abbreviations: Rif^r, Sm^r, Km^r, Cb^r, Tc^r, and Spt^r, rifampin, streptomycin, kanamycin, carbenicilline, tetracycline, and spectinomycin resistance, respectively; Inc, plasmid incompatibility group; Tra^+, region of conjugation transfer; *Rlt, R. leguminosarum* bv. trifolii; *Rlv, R. leguminosarum* bv. viciae; pA, promotor of *nodA* gene of *R. leguminosarum* bv. viciae.

cloning into an expression vector: oMP179, GGAATTCATATGGGTGCGAA TTTGACC (the *Nde*I restriction site is underlined); oMP 180, GGACGTCGA CTCAGGAAGCGGAAATC (the *Sal*I restriction site is underlined) (both obtained from Isogen). These primers were used in a PCR with *M. loti* E1R genomic DNA (the PCR conditions were as described above except for the annealing temperature of 54°C). The resulting fragment was purified over an agarose gel, digested with *Nde*I-*Sal*I, and ligated into pET16b that had been digested with *Nde*I and *Xho*I, yielding plasmid pMP4601. Plasmid pMP4600 was obtained by subcloning the *Nde*I-*Bam*HI fragment of pMP4601 containing the *nodS* gene into pET3a. For sequencing of *nodS*, the *Xba*I-*Bam*HI insert of pMP4601 was subcloned into pBluescript SK.

As the source of *nodL,* plasmids pMP2107 (IncP) and pMP2109 (IncW) were used. Both plasmids carry, in addition to the *nodL* gene under the control of the *nodA* promoter, *nodD* of *R. leguminosarum* bv. trifolii, which provides in all cases induction of LCOs after the addition of naringenin. In order to compare the production of LCOs under the same conditions but in the absence of *nodL,* plasmids carrying only *nodD* of *R. leguminosarum* bv. viciae(pMP280) or *nodD* of *R. leguminosarum* bv. trifolii(pMP2112) were transferred into the different rhizobia. The IncW plasmids pMP2112 and pMP2109 were used in the case of *M. loti* E1R because IncP plasmids are difficult to maintain in that strain (15).

Overproduction of *M. loti* NodS in *E. coli*. *E. coli* strain BL21(DE3) (30), which expresses the T7 polymerase under the control of the *lac* promoter, was transformed with either pMP4601 or pMP4600. NodS was produced from BL21(DE3), carrying pMP4601, as a translational fusion with a histidine tag that can be used for purification of the fusion protein with a Ni column. Because it was impossible to isolate an active NodS protein using a Ni column (see Results), NodS preparations were obtained from BL21(DE3)(pMP4600) as follows. One-liter cultures were grown on Luria-Bertani medium containing 25 μg of carbenicillin/ml at 37°C to an optical density at 620 nm of approximately 0.4, and then IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.1 mM in order to induce *nodS* expression from pMP4600. After 4 h of induction, the cells were harvested by centrifugation. The cell pellet was suspended in 10 mM phosphate buffer and lysed by sonication. The suspension was centrifuged at 6,800 × *g* for 15 min to obtain the cell extract. The supernatant containing the soluble NodS protein was used in the in vitro experiments. As a negative control, cell extracts were obtained in the same way from strain BL21(DE3) carrying the empty pET3a vector (without the *nodS* gene).

Enzyme assays, radiolabeling, and TLC analysis of the products. The activity of NodS was assayed by following incorporation of a radioactive methyl group into the $GlcNH_2$-$GlcNAc_3$ tetrasaccharide (NodBC metabolite), which had been chemically synthesized. Radiolabeled *S*-[*methyl*-^{14}C]adenosyl methionine (0.025 μCi; Amersham) was used as the methyl donor. Enzyme activity was assayed in 20 mM Tris-HCl, pH 7.0, for 2 h at room temperature. The resulting modified chitin oligosaccharides were extracted into chloroform and analyzed on silica thin-layer chromatography (TLC) plates (Merck), which were developed in butanol-ethanol-water (5:3:3 [vol/vol/vol]). Radioactive spots were visualized with a Molecular Dynamics PhosphorImager using ImageQuant software after overnight exposure.

NodL protein was purified from *E. coli* BL21(DE3) harboring pMP3401 as previously described (3). NodL assays were performed as described previously (3).

Detection of LCOs by TLC. In vivo labeling of LCOs was carried out in 1-ml cultures using either 0.1 μCi of D-[1-^{14}C]glucosamine (54 mCi/mmol; Amersham) or 0.1 μCi of *L*-[*methyl*-^{14}C]methionine (55 mCi/mmol; Amersham). In all cultures, LCO production was induced with naringenin, and after overnight growth, the LCOs were isolated from the cultures by *n*-butanol extraction. Samples were concentrated by evaporation and chromatographed on reversed-phase C_{18}-coated silica plates (Sigma) using a mobile phase of acetonitrile-water (1:1 [vol/vol]). Radiolabeled components were detected with a PhosphorImager using ImageQuant software.

Purification of LCOs. LCOs were extracted from 1-liter cultures and purified by reversed-phase high-performance liquid chromatography (HPLC) as described by López-Lara et al. (15) for the LCOs of *M. loti*(pMP2109) and *R. etli*(pMP2107) and as described by López-Lara et al. (16) for the LCOs of *Rhizobium* sp. strain GRH2(pMP2107) and *R. tropici*(pMP2107).

FAB-MS and CID-MS-MS. Positive-ion fast-atom bombardment mass spectra (FAB-MS) were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at 10 kV accelerating voltage. The FAB gun was operated at 6 kV accelerating voltage with an emission current of 10 mA and using xenon as the bombarding gas. The spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used and were recorded and averaged on a Hewlett Packard 9000 data system running JEOL COMPLEMENT software. Collision-induced dissociation mass spectrum-mass spectrum (CID-MS-MS) data were recorded using the same instrument, with helium as the collision gas in the third field-free region collision cell at a pressure sufficient to reduce the parent ion to one-third of its original intensity. The HPLC fractions were redissolved in 15 to 30 μl of dimethyl sulfoxide, and 1.5-μl aliquots of sample solution were loaded into a matrix of monothioglycerol. De-O-acetylation was carried out by incubating 10 to 35% of each fraction overnight at room temperature in 300 μl of methanol–25% aqueous ammonium hydroxide (1:1 [vol/vol]). The reagent was removed under vacuum, and the product was redissolved in 20 μl of dimethyl sulfoxide for analysis.

Nucleotide sequence accession number. The amino acid sequence of the *M. loti nodS* gene product was deduced from the nucleotide sequence of the gene (GenBank accession number AF290510).

RESULTS

Effect of introducing *nod*L into rhizobia. With the initial intention of examining the effect on the host range of modifying the LCOs produced by a range of different rhizobia, the *nodL* gene from *R. leguminosarum* by viciae was introduced into *M. loti*, *R. etli*, *Rhizobium tropici*, and *Rhizobium* sp. strain GRH2. These species were chosen for having either a broad (*Rhizobium* sp. strain GRH2 and *R. tropici*) or narrow (*M. loti* and *R. etli*) host range and producing LCOs with a wide variety of substitutions (LCOs from *M. loti* and *R. etli* are N methylated, carbamoylated, and acetylfucosylated, while those from *R. tropici* and *Rhizobium* sp. strain GRH2 are N methylated and sulfated). Our results showed that *M. loti* E1R(pMP2109) nodulates *Lotus corniculatus* as efficiently as the wild type or the strain carrying pMP2112. The strains CIAT899(pMP2107), CE3(pMP2107), and GRH2(pMP2107) form nodules on *Phaseolus vulgaris* Negro Jamapa at the same rate as their wild-type counterparts. *Rhizobium* sp. strain GRH2(pMP2107) is able to nodulate *A. cacia cyanophylla* and *Acacia melanoxylon* in the same way as does its wild type, which was originally isolated from *A. cyanophylla* (data not shown). Introduction of *nodL*, therefore, apparently does not affect the nodulation abilities of these strains.

In order to demonstrate that *nodL* is expressed in these strain, the LCOs produced by the four strains bearing *nodL* were screened following D-[^{14}C]-glucosamine labeling, using reversed-phase TLC (Fig. 2). In all four cases, the strain bearing *nodL* produced LCOs with lower mobility than those produced by the same strain in the absence of *nodL* (Fig. 2), showing that NodL is able to modify the LCOs in all these different backgrounds and results in LCOs having increased hydrophobicities.

Structures of LCOs produced by rhizobia bearing the *nodL* gene. In order to determine the range of LCO structures produced by the transformed rhizobia, 1-liter cultures were induced with naringenin and the LCOs were extracted into butanol. The butanol extracts from each of the four strains were fractionated on reversed-phase HPLC (15, 16). From each of the four transformed rhizobial strains, fractions corresponding to the major peaks of UV absorbance were collected, and the relevant fractions were pooled. The LCOs in each of the peaks from each of the four rhizobial strains were analyzed using FAB-MS and CID-MS-MS.

The HPLC profile of LCOs isolated from *M. loti* E1R(pMP2112) (*nodD* only) shows two peaks; peak I is a broad peak eluting at 40% acetonitrile, while peak II elutes at 60% acetonitrile (15) (Fig. 3). Consistent with the TLC results, the LCOs isolated from *M. loti*(pMP2109) elute later in the

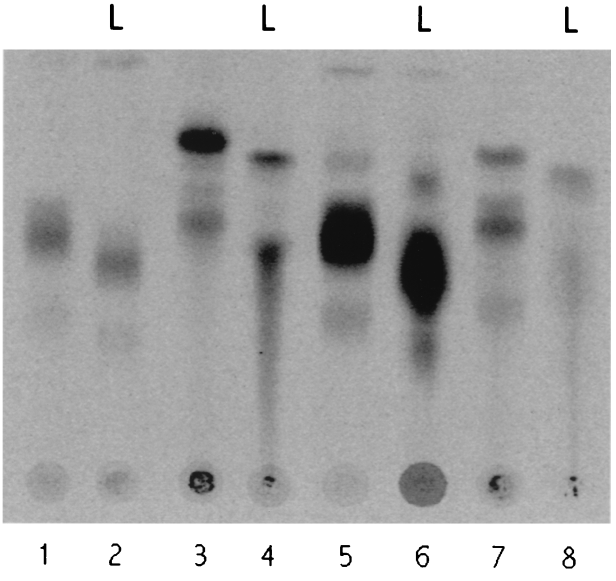

FIG. 2. Reversed-phase TLC separation of [^{14}C]glucosamine-labeled LCOs from different strains in the presence or the absence of *nodL*. Lanes: 1, *M. loti* EIR(pMP2112); 2, *M. loti* EIR(pMP2109); 3, *R. tropici* CIAT899(pMP280); 4, *R. tropici* CIAT899(pMP2107); 5, *R. etli* CE3(pMP280); 6, *R. etli* CE3(pMP2107); 7, *Rhizobium* sp. strain GRH2(pMP280); 8, *Rhizobium* sp. strain GRH2(pMP2107) (all were induced with naringenin). In lanes 1, 3, 5, and 7, the plasmids carry only *nodD,* while in lanes 2, 4, 6, and 8, the plasmids carry *nodD* and *nodL* (indicated by the L at the top of the lane).

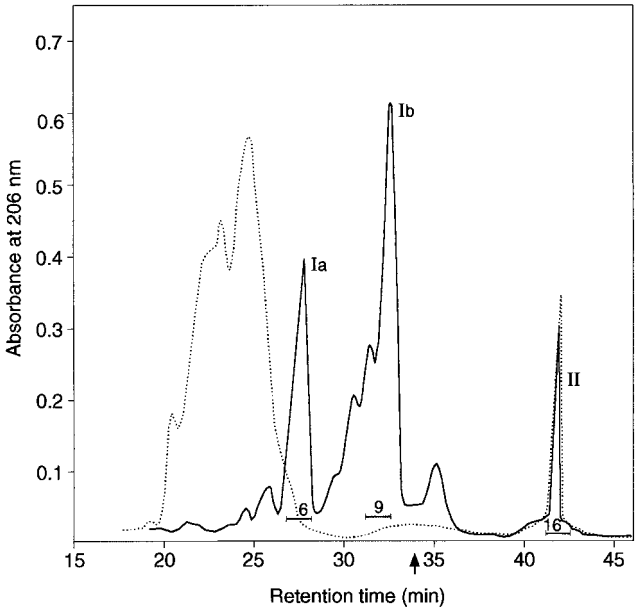

FIG. 3. HPLC chromatogram obtained using UV detection at 206 nm of LCOs from *M. loti* strains. Dotted line, LCOs from *M. loti* E1R(pMP2112); solid line; LCOs from *M. loti* E1R(pMP2109) (expressing *nodL*). The arrow on the *x* axis at 34 min indicates the point at which the solvent changed from 40 to 60% acetonitrile. The fractions collected are indicated by bars labeled with the fraction number.

HPLC purification process than those from the strain bearing *nodD* only, indicating the production of more-hydrophobic structures. Furthermore, instead of one broad peak I, there are two peaks eluting in 40% acetonitrile, Ia and Ib, which were collected mainly in fractions 6 and 9, respectively (Fig. 3).

M. loti is known (15) to produce Mlo-V ($C_{18:1}$, NMe, Carb, AcFuc) (*m/z* 1501) and Mlo-V($C_{18:0}$, NMe, Carb, AcFuc) (*m/z* 1503). The major peak of UV absorbance (fraction 9 [Fig. 3]) isolated from the *nodL*-bearing *M. loti* yielded an MS with a major $[M + H]^+$ pseudomolecular ion at *m/z* 1529, together with a thioglycerol adduct ion at *m/z* 1637, indicating the presence of an LCO bearing an unsaturated fatty acyl chain. To our surprise, these data are not immediately interpretable in terms of the known *M. loti* LCO structure bearing an extra acetyl group (mass increment, 42 amu) but instead indicate the incorporation of a mass increment of only 28 amu. On CID tandem mass spectrometric analysis, when the pseudomolecular ion is fragmented on collision with nitrogen and the fragment ions generated are recorded, B-type ions, formed on glycosidic cleavage, were observed at *m/z* 511, 714, 917, and 1120 (Fig. 4b). These ions indicate that the 28-amu increment is located on the nonreducing terminal $GlcNH_2$ residue and that the reducing terminal residue bears an acetyl-fucose residue. Similarly, the later-eluting peak II collected in fraction 16 yielded a major $[M + H]^+$ pseudomolecular ion at *m/z* 1531 but no thioglycerol adduct, consistent with the presence of an LCO bearing a saturated fatty acyl chain. The CID tandem MS of this species contained fragment ions at *m/z* 513, 716, 919, and 1122 (data not shown), again indicating the presence of a 28-amu increment attached to the nonreducing residue and an acetyl-fucose substituent on the reducing terminal GlcNAc residue. Given the fact that the *nodL* gene is known to encode acetyl transferase activity (3), the most obvious explanation for these observations is that the gene does indeed act to transfer an acetyl group to C-6 of the nonreducing terminal residue and that the net 28-amu mass increment results from the addition of the acetyl group together with the failure to transfer the methyl group to the amide nitrogen of the nonreducing terminal residue (+42 − 14 = +28). Proof for this hypothesis was sought by subjecting the two fractions to mildly basic conditions to remove the ester-bound acetyl groups. The FAB-MS of the products yielded $[M + H]^+$ pseudomolecular ions at *m/z* 1445 (fraction 9) and 1447 (fraction 16), consistent with the removal of two ester-linked acetyl groups from the LCOs. On tandem mass spectrometric analysis, fragment ions were observed at *m/z* 469, 672, 875, and 1078 (fraction 9) (Fig. 4c) and 471, 674, 877, and 1080 (fraction 16) (not shown), consistent with a GlcNAc5 LCO bearing a carbamoyl group and either a $C_{18:1}$ or $C_{18:0}$ fatty acyl group on the reducing terminal residue, as well as a fucosyl susbstituent on the nonreducing terminal residue but, importantly, no methyl group such as that found in the LCOs from wild-type *M. loti* on the nonreducing terminal residue.

The remaining fractions from *M. loti* together with the fractions obtained on HPLC fractionation of the LCOs from the other three species were analyzed using FAB-MS before and after de-O-acetylation and, where amounts permitted, CID-MS-MS. The results are summarized in Table 2. Most of the LCOs produced by *R. etli* CE3(pMP280) are eluted in a broad peak (4). As with the LCOs from *M. loti* containing *nodL,* the LCOs from the *nodL*-bearing strain of *R. etli* were separated

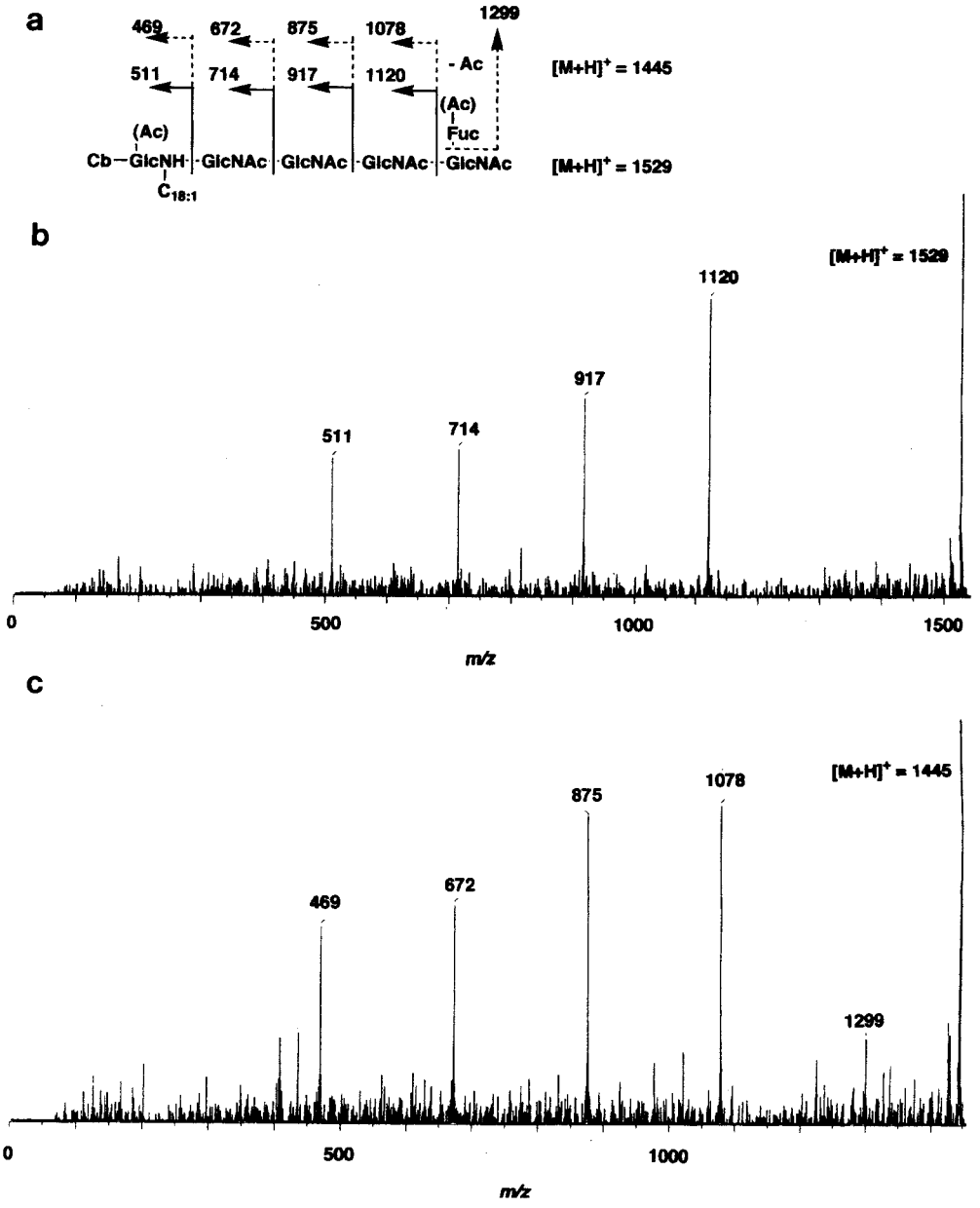

FIG. 4. CID MS obtained from major component in fraction 9 from *M. loti* containing the *nodL*gene. (a) Fragmentation scheme; (b) native (parent ion *m/z* 1529) (solid lines in panel a); (c) after de-O-acetylation (parent ion *m/z* 1445) (broken lines in panel a).

over a larger number of fractions than those from the strain lacking *nodL*. Fractions 1 to 5 correlate with the previously described peak I (reference 4 and data not shown). The HPLC profiles of the LCOs isolated from *Rhizobium* sp. strain GRH2(pMP2107) and *R. tropici* CIAT899(pMP2107) are similar to those obtained from the LCOs from the strains lacking the *nodL* gene (10, 16), though again the retention times of the peaks from the strain bearing *nodL* increased. In the case of *Rhizobium* sp. strain GRH2(pMP2107), the major peak, peak 10, observed from the wild-type LCOs was split into two major peaks, 10.1 and 10.2, in the chromatogram obtained from the strain bearing *nodL* (data not shown).

From these results, it is clear that in *M. loti* acetylation due to *nodL* almost totally prevents the transfer of the endogenous methyl group mediated by *nodS* (11) and that a similar although not exclusive effect is observed in *R. etli* and *R. tropici,* while interestingly, in *Rhizobium* sp. strain GRH2, *nodL*-mediated acetylation appears to have little or no effect on the activity of *nodS*-mediated N methylation.

[*methyl*-^{14}C]methionine labeling of LCOs produced by *nodL*-bearing strains. In order to examine this effect in more detail, labeling experiments were set up for each of the four strains, using [*methyl*-^{14}C]methionine as the in vivo methyl donor. The resulting LCOs were extracted into butanol and separated on silica TLC. Radioactive spots were visualized by phosphorimaging (Fig. 5). The results of the labeling experi-

TABLE 2. FAB-MS analysis of LCO fractions of strains containing plasmid pMP2109 (*M. loti*) or pMP2107 (other strains).

Bacterium	Fraction no.	LCO structure	MS/MS
M. loti	6	V(18:1, Cb, Ac, Fuc)	Yes
		V(18:1, Me, Cb, Ac, Fuc)min	No
	9	V(18:1, Cb, Ac, AcFuc)	Yes
	16	V(18:0, Cb, Ac, AcFuc)	Yes
		V(18:0, Me, Cb, Ac, AcFuc)min	
R. tropici	2	V(16:1, Ac, S)maj	
		V(16:1, S)	
		V(16:0, S)	
		V(14:0, Ac, S)	
		V(18:1-OH, Ac, S)	
		V(18:0-OH, Ac, S)	
		V(20:0-OH, Ac, S)	
	3	V(16:0, Ac, S) maj	
		V(18:1, S)	
		V(16:0, S)	
	4	V(18:1, Ac, S)	
	5	V(18:1, Ac, S)	
		V(18:0, Ac, S)	
		IV(18:1, Ac, S) min	
	6	V(18:1, Ac)	
		IV(20:1, Me, Ac)	
R. etli	1	V(16:0, Ac, AcFuc)	Yes
		V(16:0, Cb, Ac, Fuc)	
		V(16:0, Cb, Ac, AcFuc)	Yes
		IV(16:0, Cb, Ac, Fuc)	
		V(18:1, Cb, Ac, Fuc)	
		V(18:1, Ac, Fuc)	
		V(16:0, Me, Ac, AcFuc) min	
		V(16:0, Me, Cb, Ac, Fuc)	
		V(16:0, Me, Cb, Ac, AcFuc)	
	2	V(18:1, Ac, AcFuc)	
		V(18:1, Cb, Ac, AcFuc)	
	3	V(18:1, Ac, AcFuc)	
		V(18:1, Cb, Ac, Fuc)	
		V(18:1, Me, Ac, AcFuc)	
		V(18:1, Me, Cb, Ac, Fuc)	
		V(18:1, Cb, Ac, AcFuc)	
		V(18:1, Me, Cb, Ac, AcFuc)	
	4	V(18:1, Me, Cb, Ac, AcFuc)	
	5	V(18:1, Ac, AcFuc)	
		V(18:1, Me, Cb, Ac, AcFuc)	
Rhizobium sp. strain GRH2	2	V(18:1, Ac, S)	
	3	V(18:1, Me, Ac, S)	
	10.1	V(18:1, Ac) maj	
		IV(20:1, Me, Ac)	
	10.2	V(18:1, Me, Ac) maj	
		IV(22:1, Ac)	
	11	V(18:0, Me, Ac)	

ment (Fig. 5) are consistent with the results of the structural analyses—*M. loti* is unable to incorporate detectable levels of radioactivity from the ^{14}C-labeled methyl donor into its LCOs when carrying the *nodL* gene (Fig. 5, lane 1), although incorporation is normal in strains without this gene (Fig. 5, lane 2). *R. tropici* and *R. etli* strains carrying the *nodL* gene can incorporate only very minor amounts of the ^{14}C-labeled methyl donor into their LCOs (Fig. 5, lanes 6 and 8) in comparison to the strains lacking the *nodL* gene (Fig. 5, lanes 5 and 7). In contrast, *Rhizobium* sp. strain GRH2 shows no reduction in the level of radioactive incorporation from the labeled methyl donor into spots corresponding to LCOs in the presence (Fig. 5, lane 4) or absence (Fig. 5, lane 3) of the *nodL* gene.

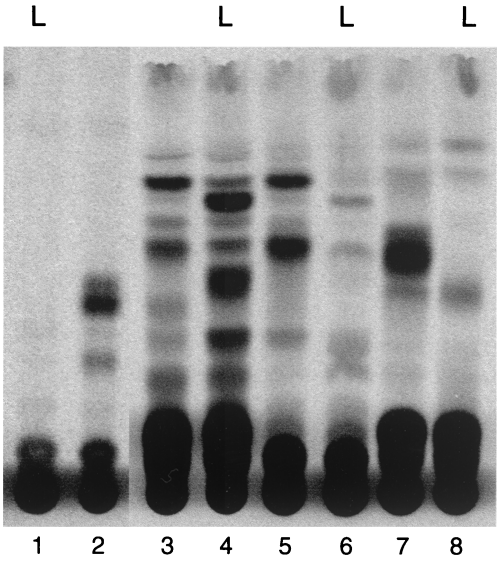

FIG. 5. Silica TLC separation of *methyl*-^{14}C-labeled LCOs from different strains with and without *nodL*. Lanes: 1, *M. loti* E1R(pMP2109); 2, *M. loti* E1R(pMP2112); 3, *Rhizobium* sp. strain GRH2(pMP280); 4, *Rhizobium* sp. strain GRH2(pMP2107); 5, *R. tropici* CIAT899(pMP280); 6, *R. tropici* CIAT899(pMP2107); 7, *R. etli* CE3(pMP280); 8, *R. etli* CE3(pMP2107) (all induced with naringenin) In lanes 2, 3, 5, and 7, the plasmids carry only *nodD,* while in lanes 1, 4, 6, and 8, the plasmids carry *nodD* and *nodL* (indicated by the L at the top of the lane).

Cloning of *M. loti nodS* and overexpression of its product. Since the apparent interference of *nodL* with *nodS* is most pronounced in *M. loti,* we chose to use this strain to carry out further studies. We began by identifying and cloning *nodS* from *M. loti*. In order to identify the *nodS* gene in PCR products, two primers, S1 and S2, based on conserved regions of known NodS proteins, were designed. Using these primers in a PCR with *M. loti* E1R DNA, a PCR product of the expected size was obtained. In order to obtain the complete ORF for the gene, primers flanking the gene were required. In most rhizobia, *nodS* is found in an operon where it is followed by *nodU,* and in some cases preceded by *nodC,* or is found as the first gene in the operon. Consequently, U3, a primer based on a conserved region of the *nodU* genes, was designed, together with C1, based on the sequence of *nodC* of *M. loti* NZP2037 (6), which could be replaced by a *nod* box-based primer (NB) for those cases when *nodS* is the first gene in the operon.

Using the primers NB and U3 in a PCR produced a product which, in a nested PCR with primers S1 and S2, yielded a smaller product of the expected size, confirming that the PCR product obtained using the first set of primers harbors the *M. loti nodS* gene. We thus conclude that in *M. loti* E1R *nodS* is followed by *nodU* but is not preceded by *nodC*. The amino acid sequence of the *M. loti nodS* gene was deduced from the nucleotide sequence of the gene and compared with those known for other NodS proteins. The similarities in the sequences of the NodS proteins from *M. loti, R. tropici,* and *R. etli* are particularly interesting, considering that these are the species that, when carrying the *nodL* gene, fail to incorporate radiolabel efficiently from [*methyl*-^{14}C]methionine, suggesting

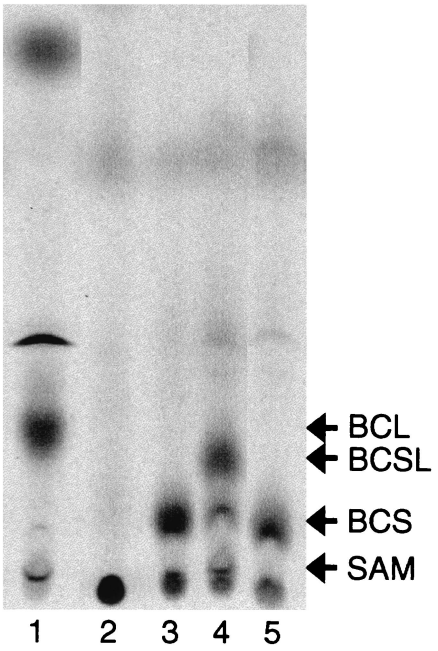

FIG. 6. Silica TLC separation of ^{14}C-labeled oligosaccharides produced on incubation of $GlcNH_2$-$GlcNAc_3$ in the presence of a radioactively labeled precursor. Lanes: 1, ^{14}C-labeled acetyl CoA plus NodL protein; 2, [^{14}C]SAM and cell extract of BL21 (DE3) (no *nodS*); 3, [^{14}C]SAM and cell extract of BL21(pMP4600) (i.e., *nodS*); 4, first [^{14}C]SAM and cell extract of BL21(pMP4600), and then cold acetyl CoA and NodL protein 5, first cold acetyl CoA, and NodL protein, and then [^{14}C]SAM and cell extract of BL21(pMP4600).

that their *nodS* genes (or their products) are all similarly affected by the presence of the *nodL* gene (or its product).

The ORF encoding NodS from *M. loti* was cloned, as described in Materials and Methods, in pET16b, resulting in plasmid pMP4601. After induction with IPTG of BL21(DE3) carrying pMP4601, the majority of the His tag-NodS fusion protein was found in inclusion bodies. It proved impossible to isolate an active NodS protein using a Ni column (data not shown). Subsequently, the *nodS* gene was cloned from pMP4601 in pET3a, resulting in pMP4600. The strain BL21(DE3) carrying pMP4600 was used to obtain a cell extract containing soluble NodS protein that was used in further experiments.

Functional interference between NodS and NodL activities in vitro. In order to determine whether the apparent interference of *nodL* with *nodS* observed in vivo occurs at the level of the genes or their products, the cell extract from BL21(DE3)(pMP4600) (the *E. coli* strain overproducing NodS from *M. loti*) was tested in a variety of in vitro enzyme assays with NodL. The so-called NodBC tetrasaccharide ($GlcNH_2$-GlcNAc-GlcNAc-GlcNAc) was used as the substrate, and *S*-[*methyl*-^{14}C]adenosyl methionine was used as the methyl donor. The products were analyzed by silica TLC, with radioactive compounds detected by phosphorimaging (Fig. 6).

In the absence of NodS and labeled SAM, and instead using ^{14}C-labeled acetyl CoA and the NodL protein (Fig. 6, lane 1), a spot of radioactivity is observed, which we assign as the product of NodL-mediated acetylation of the NodBC metabolite (NodBCL metabolite) (3). Incubation of the NodBC metabolite with [^{14}C]SAM and a cell extract of *E. coli* BL21(DE3) carrying only the pET3a vector (i.e., no *nodS* gene and therefore in the absence of NodS) results in a single radioactive spot that fails to migrate and is thus observed at the origin (Fig. 6, lane 2) and that corresponds to the unused radioactive donor [^{14}C]SAM. Inclusion of the cell extract of *E. coli* BL21(DE3) carrying pMP4600 (i.e., *nodS* of *M. loti* cloned in pET3a) results in the appearance of a second radioactive spot (Fig. 6, lane 3) that migrates slightly from the origin and corresponds to a NodBCS metabolite formed on incorporation of a radiolabeled methyl group into the NodBC metabolite. Subsequent incubation of the NodBCS metabolite thus generated using the cell extract overexpressing *M. loti* NodS with cold acetyl CoA and NodL yields three radioactive spots (Fig. 6, lane 4). The first remains at the origin and corresponds to unincorporated [^{14}C]SAM, and the second has an R_f value consistent with its representing a NodBCS metabolite, while the third, fastest-migrating radioactive spot is assigned as corresponding to a NodBCSL metabolite formed on NodL-mediated transfer of an acetyl group to the NodBCS metabolite. Very interestingly, however, when the analogous experiment was carried out in the reverse order, i.e., incubating the NodBC metabolite first with cold acetyl CoA and NodL and subsequently with [^{14}C]SAM and cell extract overproducing NodS from *M. loti,* the third radioactive spot was not formed (Fig. 6, lane 5) while the spots corresponding to unincorporated label and to the NodBCS metabolite (presumably formed from the small residual amounts of unacetylated NodBC metabolite remaining after NodL treatment) were. These results demonstrate that the NodBCL metabolite is not a substrate for the NodS protein from *M. loti* while the NodL protein can accept both the NodBC and the NodBCS metabolites as substrates and that the functional interference of the two genes is at the level of their gene products, the proteins.

DISCUSSION

Our results show that the rhizobial *nodS*-dependent *N*-methyl transferase and the *nodL O*-acetyl transferase activities functionally interfere and that this interference occurs at the level of the activities of the primary gene products. The NodBCL metabolite, the product of NodL acetyl transferase activity on the NodBC metabolite, is not used by the NodS protein as a substrate, while the NodBCS metabolite produced by the action of NodS is recognized and used as a substrate by NodL. Clearly, the presence of a substituent on O-6 of the nonreducing terminal residue blocks the action of NodS. Based on our observations, we predict that in the biosynthesis of LCOs that bear both an *N*-methyl group and a substituent, such as a carbamoyl group, on the non-reducing O-6, as is the case in the LCOs from *Azorhizobium caulinodans* and *Rhizobium* sp. strain NGR234, NodS acts on the NodBC product (11, 18), while we predict that the carbamoyl transferase NodU or its analogue acts on a more mature acylated chitin oligosaccharide derivative.

It is known that NodL acts preferentially on the NodBC metabolite rather than on a more mature acyl-bearing species (2). From our observations, we suggest that NodS-mediated N methylation of such NodBCL metabolites may well be impossible and that N-methylated, nonreducing-terminally O-acety-

lated LCOs would therefore be very unlikely to be biosynthesized. We are unaware of any reports of such LCO structures. In *Bradyrhizobium elkani* USDA 61, about 20 different LCOs have been identified, among them LCO species carrying acetate on C-6 of the nonreducing terminal residue as well as other LCO species carrying an N-methyl group. Both the O-acetylated and the N-methylated species can carry additional carbamoyl groups on the nonreducing terminal residue, but interestingly, species carrying both the *O*-acetyl and the *N*-methyl groups were not observed (5, 29). Examples of functional interference of *nod* gene products are not abundant, although the description of LCO structures from the type strain of *M. loti,* NZP2213, in which C-3 of the subterminal GlcNAc residue is fucosylated (21), noted that those fucosylated LCO structures appear not to be methylated. This could be an example analogous to that reported here and should therefore allow us to predict that the $\alpha(1 \rightarrow 3)$ transferase must act earlier in LCO biosynthesis than the *N*-methyl transferase activity.

Our results and those of others (11, 18) show that only a de-N-acetylated chitooligosaccharide can be used as a substrate by NodS. Fully N-acetylated chitooligosaccharides or unmethylated LCOs are not methylated in vitro by NodS (11). In constrast, the NodL protein is able to acetylate, in addition to terminally de-N-acetylated chitooligosaccharides, various other substrates, such as LCOs, chitin oligosaccharides, chitosan oligosaccharides, *N*-acetylglucosamine, and cellopentaose (2, 3). From all these data it is clear that NodS is highly substrate specific while NodL has a broad substrate specificity. The differences in the degrees of specificity may also account for the effect observed in vivo with the strains carrying *nodL*. While NodS can act only at a very precise step in LCO biosynthesis (i.e., after NodB deacetylation and before NodA-mediated acylation), NodL acts preferentially at this point but can also act directly on the product of NodC or on a completely mature LCO (i.e., at any point in LCO biosynthesis). Mergaert et al. (18) have shown that an *E. coli* strain expressing *nodCS* produces unmethylated chitin oligosaccharides. Based on our in vitro data, we predict that an *E. coli* strain expressing *nodCL* should be able to produce acetylated chitin oligosaccharides.

In spite of the functional interference of the *nodS* and *nodL* gene products in vivo, we have developed an in vitro system for synthesizing NodBCSL metabolites that in most cases would not be possible to synthesize using an in vivo system. It remains to be seen whether this system can be used to generate mature LCOs that are both N methylated and O acetylated in order to carry out bioactivity experiments.

Having determined the sequence of the *nodS* gene from *M. loti,* we have deduced its amino acid sequence and demonstrated its similarity to the analogous proteins from *R. etli* and *R. tropici*. This similarity is consistent with our observation that the NodS proteins from all three strains are interfered with by the NodL protein from *R. leguminosarum* bv. viciae. Having demonstrated in vivo that the *nodS* gene from *Rhizobium* sp. strain GRH2 is apparently not interfered with by the presence of the *nodL* gene, it will be interesting to see how different the GRH2 NodS protein appears to be from the other three proteins when its sequence becomes known.

The transconjugant strains carrying the *nodL* gene are all able to nodulate the same natural host plants as their wild-type counterparts. Since a *nodS* mutant of *R. tropici* CIAT899 is unable to nodulate *Phaseolus* (32), we propose that in the *nodL* transconjugant strain the lack of the *N*-methyl group is compensated for by the presence of the *O*-acetyl substitution. Apart from a role in host specificity, it is proposed that the substituents on LCOs make them more resistant to degradation by enzymes produced by the plant or present in the rhizosphere. It is possible that the function of *O*-acetyl or *N*-methyl substituents on the nonreducing terminus is thus to render LCOs more resistant to degradation. This proposal is supported by the recent finding that LCOs produced by *Rhizobium fredii* (a strain that produces non-N-methylated LCOs) are de-N-acylated by fatty acyl amidase II secreted by *Dictyostelium discoideum* but, interestingly, N-methylated LCOs from a transgenic *R. fredii* strain carrying *nodS* are protected against degradation by this enzyme (31).

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REFERENCES

1. **Bladergroen, M. R., and H. P. Spaink.** 1998. Genes and signal molecules involved in the rhizobia-Leguminosae symbiosis. Curr. Opin. Plant Biol. **1:**353–359.
2. **Bloemberg, G. V., R. M. Lagas, S. V. Leeuwen, G. A. V. D. Marel, J. H. V. Boom, B. J. J. Lugtenberg, and H. P. Spaink.** 1995. Substrate specificity and kinetic studies of nodulation protein NodL of *Rhizobium leguminosarum*. Biochemistry **34:**12712–12720.
3. **Bloemberg, G. V., J. E. Thomas-Oates, B. J. J. Lugtenberg, and H. P. Spaink.** 1994. Nodulation protein NodL of *Rhizobium leguminosarum O*-acetylates lipo-oligosaccharides, chitin fragments and *N*-acetylglucosamine in vitro. Mol. Microbiol. **11:**793–804.
4. **Cárdenas, L., J. Domínguez, C. Quinto, I. M. López-Lara, B. J. J. Lugtenberg, H. P. Spaink, G. J. Rademaker, J. Haverkamp, and J. E. Thomas-Oates.** 1995. Isolation, chemical structure and biological activity of the lipo-chitin oligosaccharide nodulation signals from *Rhizobium etli*. Plant Mol. Biol. **29:**453–464.
5. **Carlson, R. W., J. Sanjuan, U. R. Bhat, J. Glushka, H. P. Spaink, A. H. M. Wijfjes, A. A. N. V. Brussel, T. J. W. Stockermans, K. Peters, and G. Stacey.** 1993. The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and type II strains of *Bradyrhizobium japonicum*. J. Biol. Chem. **268:**18372–18381.
6. **Collins-Emerson, J. M., E. A. Terzaghi, and D. B. Scott.** 1990. Nucleotide sequence of *Rhizobium loti nodC*. Nucleic Acids Res. **18:**6690.
7. **Dénarié, J., F. Debellé, and J.-C. Promé.** 1996. Rhizobium lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. **65:**503–535.
8. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
9. **Downie, J. A.** 1998. Functions of rhizobial nodulation genes, p. 387–402. In H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The *Rhizobiaceae* Kluwer, Dordrecht, The Netherlands.
10. **Folch-Mallol, J. L., S. Marroquí, C. Sousa, H. Manyani, I. M. López-Lara, K. M. G. M. V. D. Drift, J. Haverkamp, C. Quinto, A. Gil-Serrano, J. E. Thomas-Oates, H. P. Spaink, and M. Megías.** 1996. Characterization of *Rhizobium tropici* CIAT899 nodulation factors: the role of *nodH* and *nodPQ* genes in their sulfation. Mol. Plant-Microbe Interact. **9:**151–163.
11. **Geelen, D., B. Leyman, P. Mergaert, K. Klarskov, M. V. Montagu, R. Geremia, and M. Holsters.** 1995. NodS is an S-adenosyl-L-methionine-dependent methyltransferase that methylates chitooligosaccharides deacetylated at the non-reducing end. Mol. Microbiol. **17:**387–397.
12. **Geelen, D., P. Megaert, R. A. Geremia, S. Goormachtig, M. V. Montagu, and M. Holsters.** 1993. Identification of *nodSUIJ* genes in Nod locus 1 of *Azorhizobium caulinodans*: evidence that *nodS* encodes a methyltransferase involved in Nod factor modification. Mol. Microbiol. **9:**145–154.
13. **Jabbouri, S., R. Fellay, F. Talmont, P. Kamalaprija, U. Burger, B. Relic, J.-C. Prome, and W. J. Broughton.** 1995. Involvement of *nodS* in *N*-methylation and *nodU* in 6-*O*-carbamoylationof *Rhizobium* sp. NGR234 Nod factors. J. Biol. Chem. **270:**22968–22973.
14. **Kamst, E., H. P. Spaink, and D. Kafetzopoulos.** 1998. Biosynthesis and secretion of rhizobial lipochitin-oligosaccharide signal molecules, p. 29–71.

In B. B. Biswas and H. K. Das (ed.), Plant-Microbe Interactions. Plenum Press, New York, N.Y.

15. **López-Lara, I. M., J. D. J. V. D. Berg, J. E. Thomas-Oates, J. Glushka, B. J. J. Lugtenberg, and H. P. Spaink.** 1995. Structural identification of the lipo-chitin oligosaccharide nodulation signals of *Rhizobium loti*. Mol. Microbiol. **15:**627–638.
16. **López-Lara, I. M., K. M. G. M. V. D. Drift, A. A. N. V. Brussel, J. Haverkamp, B. J. J. Lugtenberg, J. E. Thomas-Oates, and H. P. Spaink.** 1995. Induction of nodule primordia on *Phaseolus* and *Acacia* by lipo-chitin oligosaccharide nodulation signals from broad-host-range *Rhizobium* strain GRH2. Plant Mol. Biol. **29:**465–477.
17. **Martínez, E., M. A. Pardo, R. Palacios, and M. A. Cevallos.** 1985. Reiteration of nitrogen fixation gene sequences and specificity of *Rhizobium* in nodulation and nitrogen fixation in *Phaseolus vulgaris*. J. Gen. Microbiol. **131:**1779–1786.
18. **Mergaert, P., W. D'Haeze, D. Geelen, D. Promé, M. V. Montagu, R. Geremia, J.-C. Promé, and M. Holsters.** 1995. Biosynthesis of *Azorhizobium caulinodans* Nod factors. J. Biol. Chem. **270:**29217–29223.
19. **Mergaert, P., M. V. Montagu, and M. Holsters.** 1997. Molecular mechanisms of Nod factor diversity. Mol. Microbiol. **25:**811–817.
20. **Noel, K. D., A. Sánchez, L. Fernández, J. Leemans, and M. A. Cevallos.** 1984. *Rhizobium phaseoli* symbiotic mutants with Tn5 insertions. J. Bacteriol. **158:** 148–155.
21. **Olsthoorn, M. M. A., I. M. López-Lara, B. O. Petersen, K. Bock, J. Haverkamp, H. P. Spaink, and J. E. Thomas-Oates.** 1998. Novel branched Nod factor structure results from α-(1–3) fucosyl transferase activity: the major lipo-chitin oligosaccharides from *Mesorhizobium loti* strain NZP2213 bear an α-(1–3) fucosyl substituent on a nonterminal backbone residue. Biochemistry **37:**9024–9032.
22. **Ritsema, T., O. Geiger, P. V. Dillewijn, B. J. J. Lugtenberg, and H. P. Spaink.** 1994. Serine residue 45 of nodulation protein NodF from *Rhizobium leguminosarum* bv. *viciae* is essential for its biological function. J. Bacteriol. **176:**7740–7743.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
25. **Schultze, M., E. Kondorosi, P. Ratet, M. Buire, and A. Kondorosi.** 1994. Cell and molecular biology of *Rhizobium*-plant interactions. Int. Rev. Cytol. **156:** 1–75.
26. **Spaink, H. P.** 1996. Regulation of plant morphogenesis by lipo-chitin oligosaccharides. Crit. Rev. Plant Sci. **15:**559–582.
27. **Spaink, H. P., C. A. Wijffelman, R. J. H. Okker, and B. J. J. Lugtenberg.** 1989. Localization of functional regions of the *Rhizobium nodD* product using hybrid *nodD* genes. Plant Mol. Biol. **12:**59–73.
28. **Spaink, H. P., C. A. Wijffelman, E. Pees, R. J. H. Okker, and B. J. J. Lugtenberg.** 1987. *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. Nature (London) **328:**337–340.
29. **Stokkermans, T. J. W., R. Orlando, V. S. K. Kolli, R. W. Carlson, and N. K. Peters.** 1996. Biological activities and structures of *Bradyrhizobium elkanii* low abundance lipochitin-oligosaccharides. Mol. Plant-Microbe Interact. **9:**298–304.
30. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
31. **Sutherland, P. J., A. E. Tobin, C. L. Rutherford, and N. P. J. Price.** 1998. *Dictyostelium discoideum* fatty-acyl amidase II has deacilase activity on *Rhizobium* nodulation factors. J. Biol. Chem. **273:**4459–4464.
32. **Waelkens, F., T. Voets, K. Vlassak, J. Vanderleyden, and P. V. Rhijn.** 1995. The *nodS* gene of *Rhizobium tropici* strain CIAT899 is necessary for nodulation on *Phaseolus vulgaris* and on *Leucaena leucocephala*. Mol. Plant-Microbe Interact. **8:**147–154.
33. **Yang, G.-P., F. Debellé, A. Savagnac, M. Ferro, O. Schiltz, F. Maillet, D. Promé, M. Treilhou, C. Vialas, K. Lindstrom, J. Dénarié, and J.-C. Promé.** 1999. Structure of the *Mesorhizobium huakii* and *Rhizobium galegae* Nod factors: a cluster of phylogenetically related legumes are nodulated by rhizobia producing Nod factors with α,β-unsaturated *N*-acyl substitutions. Mol. Microbiol. **34:**227–237.