NodZ of Bradyrhizobium extends the nodulation host range of Rhizobium by adding a fucosyl residue to nodulation signals

Lopez-Lara, I.M.; Blok-Tip, L.; Quinto, C.; Garcia, M.L.; Stacey, G.; Bloemberg, G.V.; ... ; Spaink, H.P.

Molecular Microbiology (1996) **21**(2), 397–408

NodZ of *Bradyrhizobium* extends the nodulation host range of *Rhizobium* by adding a fucosyl residue to nodulation signals

Isabel M. López-Lara,[1†] Leonore Blok-Tip,[2] Carmen Quinto,[4] Minviluz L. Garcia,[3] Gary Stacey,[3] Guido V. Bloemberg,[1] Gerda E. M. Lamers,[1] Ben J. J. Lugtenberg,[1] Jane E. Thomas-Oates[2] and Herman P. Spaink[1]*

[1]*Institute of Molecular Plant Sciences, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands.*
[2]*Bijvoet Centre for Biomolecular Research, Department of Mass Spectrometry, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands.*
[3]*Centre for Legume Research, Departments of Microbiology, Ecology and Evolutionary Biology, The University of Tennessee, Knoxville, Tennessee 37996-0845, USA.*
[4]*Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apartado Postal 510-3, Cuernavaca Morelos 62271, Mexico.*

Summary

The nodulation genes of rhizobia are involved in the production of the lipo-chitin oligosaccharides (LCO), which are signal molecules required for nodule formation. A mutation in *nodZ* of *Bradyrhizobium japonicum* results in the synthesis of nodulation signals lacking the wild-type 2-*O*-methylfucose residue at the reducing-terminal *N*-acetylglucosamine. This phenotype is correlated with a defective nodulation of siratro (*Macroptilium atropurpureum*). Here we show that transfer of *nodZ* to *Rhizobium leguminosarum* biovar (bv) *viciae*, which produces LCOs that are not modified at the reducing-terminal *N*-acetylglucosamine, results in production of LCOs with a fucosyl residue on C-6 of the reducing-terminal *N*-acetylglucosamine. This finding, together with *in vitro* enzymatic assays, indicates that the product of *nodZ* functions as a fucosyltransferase. The transconjugant *R. leguminosarum* strain producing fucosylated LCOs acquires the capacity to nodulate *M. atropurpureum*, *Glycine soja*, *Vigna unguiculata* and *Leucaena leucocephala*. Therefore, *nodZ* extends the narrow host range of *R. leguminosarum* bv. *viciae* to include various tropical legumes. However, microscopic analysis of nodules induced on siratro shows that these nodules do not contain bacteroids, showing that transfer of *nodZ* does not allow *R. leguminosarum* to engage in a nitrogen-fixing symbiosis with this plant.

Received 16 February, 1996; revised 15 April, 1996; accepted 24 April, 1996. †Present address: TU Berlin, Institut für Biotechnologie, FG Technische Biochemie, 13353 Berlin, Germany. *For correspondence. Tel. (71) 5275055; Fax (71) 5275088.

Introduction

The symbiotic relationship between legumes and rhizobia (i.e. *Rhizobium*, *Bradyrhizobium* or *Azorhizobium*) can result in the formation of a nitrogen-fixing root organ, the nodule. 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 governing the early steps of this interaction (*nod*, *nol* and *noe* genes). Many of these genes are involved in the synthesis and secretion of lipo-chitin oligosaccharides (LCOs). The backbone of all LCOs (also known as Nod factors) consists of an oligomer of three to six *N*-acetylglucosamine residues, *N*-acylated on the non-reducing-terminal residue. This backbone is synthesized by the co-operative action of NodA, NodB and NodC (Atkinson *et al*., 1994; Geremia *et al*., 1994; Kamst *et al*., 1995; Mergaert *et al*., 1995; Rohrig *et al*., 1994; Spaink *et al*., 1994). Additional gene products provide chemical decorations which, in some cases, have been shown to determine host specificity (for reviews see Carlson *et al*., 1994; Dénarié and Cullimore, 1993; Downie, 1994; Schultze *et al*., 1994; Spaink, 1995). For example, NodFE of *Rhizobium leguminosarum* bv. *viciae* and *Rhizobium leguminosarum* bv. *trifolii* are involved the synthesis of highly unsaturated fatty acyl moieties which play a role in the determination of specificity for *Vicia* and *Trifolium* plants, respectively (Spaink *et al*., 1995b). Purified LCOs are able to elicit various responses on the host-plant root. For instance, they can induce the deformation of root hairs, the formation of pre-infection threads, and the division of root cortical cells. LCOs isolated from *Rhizobium meliloti* trigger the formation of fully developed nodules on *Medicago* (Truchet *et al*., 1991). Co-inoculation of purified LCOs from the broad-host-range strain *Rhizobium* sp. NGR234, together with

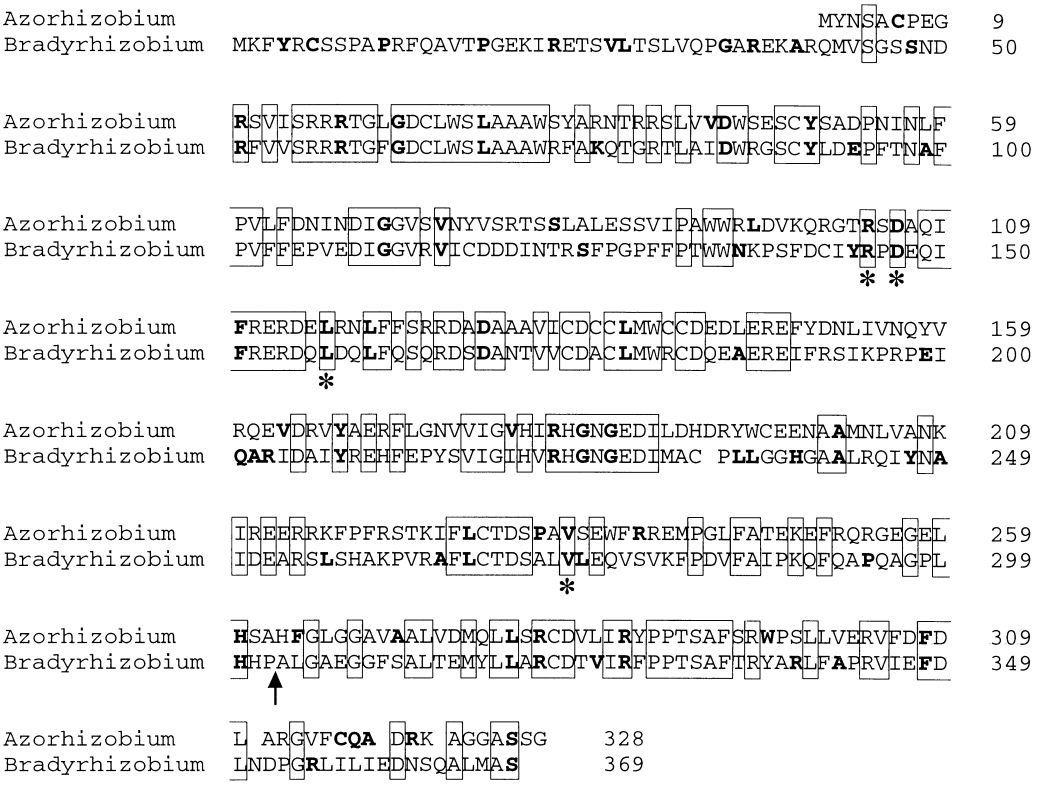

Fig. 1. Alignment of the predicted protein sequence of NodZ of *Azorhizobium,* as reported in the accompanying paper of Mergaert *et al*. (1996), and NodZ of *Bradyrhizobium*. The arrow indicates where the reading frame changes in the corrected sequence. Amino acids identical in both NodZ proteins are boxed. Residues in bold are conserved in the human ELAM-1 ligand fucosyl transferase (Goelz *et al*., 1990), and the asterisks indicate residues conserved in all reported fucosyl transferases (accession numbers: P22083, U11282, M81485 and U40028).

LCO-production-deficient mutants of the same strain, results in nitrogen-fixing nodules, showing that LCOs permit the entry of the mutants into the roots (Relić *et al*., 1993).

Using a probe comprising the *nod* genes from *Rhizobium* sp. NGR234, Nieuwkoop *et al*. (1987) isolated a siratro (*Macroptilium atropurpureum*)-specific nodulation locus in *B. japonicum* downstream of *nodIJ*. Recently a detailed characterization of this locus and the identification of the *nodZ* gene encoded by this locus have been reported (Stacey *et al*., 1994). In *Bradyrhizobium japonicum,* this gene is unusual, compared to other *nod* genes, because its expression is not controlled by the regulatory NodD protein. In contrast, in *Rhizobium* sp. NGR234 there is a flavonoid-inducible promoter upstream of ORFB that is homologous to *nodZ* of *B. japonicum* (Fellay *et al*., 1995).

Chemical analysis of the LCOs produced by a *nodZ* mutant of *B. japonicum* suggested that NodZ is essential for the fucosylation of the terminal reducing *N*-acetylglucosamine (Stacey *et al*., 1994). In this study we show that transfer of the *B. japonicum nodZ* gene to *R. leguminosarum* (*Rl*) bv. *viciae* leads to the biosynthesis of LCOs that are fucosylated on C-6 of the reducing-terminal *N*-acetylglucosamine (GlcNAc). The presence of *nodZ* in *Rl. bv. viciae* extends its host range to several tropical leg- umes such as *Macroptilium*, *Glycine*, *Vigna* and *Leucaena*.

Results

nodZ *sequence revision and cloning of* nodZ *under the control of a flavonoid-inducible promoter*

Resequencing of *nodZ* from *B. japonicum* USDA110 showed that an error was present in the sequence previously published by Stacey *et al*. (1994). There is a frameshift deletion in the original sequence making it 46 amino acids shorter than the corrected sequence (Fig. 1). At position 1410 there is an additional cytosine. Translation of the corrected DNA sequence would yield a protein of 369 amino acids. In contrast, the previously published sequence predicted a protein of 323 amino acids. The alignment of NodZ from *B. japonicum* with that of the recently identified NodZ from *Azorhizobium caulinodans* (Mergaert *et al*., 1996 – accompanying article) is presented in Fig. 1. The corrected reading frame shows 43% identity and 63% similarity to the *A. caulinodans*

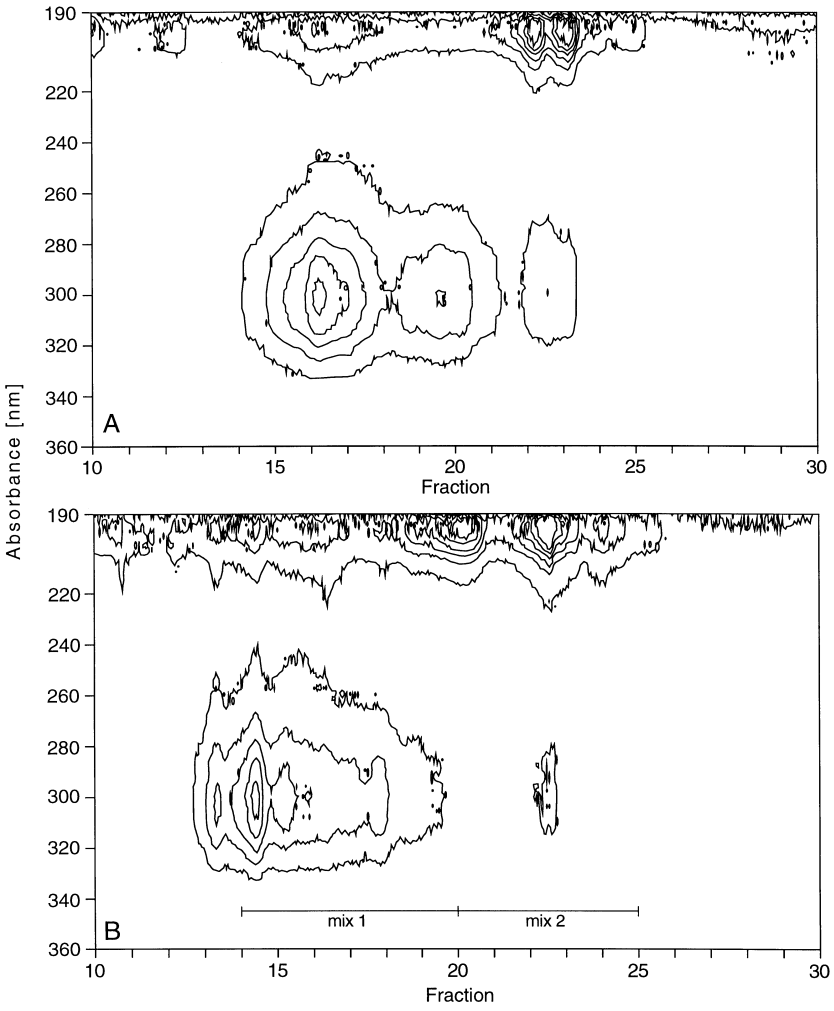

Fig. 2. Reversed-phase HPLC analysis of LCOs using photodiode-array detection. Isogram of LCO-containing *n*-butanol extracts from strain RBL5560 (A) and RBL5560 (pMP2450) (B). 2 min fractions were collected. Pooled fractions, indicated by Mix 1 and Mix 2, were used for mass spectrometric analyses.

NodZ sequence. Comparison of NodZ sequences with the human ELAM-1 ligand fucosyltransferase (Goelz *et al*., 1990) shows that these proteins share only 16% amino acid identity. We have identified four residues that are conserved among all identified fucosyltransferases and the NodZ sequences (Fig. 1).

The expression of *B. japonicum nodZ* is not regulated by NodD (Stacey *et al*., 1994). In order to introduce and express *nodZ* of *B. japonicum* in *Rl*. bv. *viciae* strain RBL5560, we cloned *nodZ* under the control of the *nodA* promoter (pA) of *Rl*. bv. *viciae* in an IncP vector, resulting in plasmid pMP2450 (see the *Experimental procedures*).

Introduction of nodZ *of* B. japonicum *in* R. leguminosarum *bv.* viciae

The product of *nodZ* from *B. japonicum* USDA110 is required for the addition of a 2-*O*-methylfucosyl residue to the reducing terminus of LCOs (Stacey *et al*., 1994). To further analyse the role of *nodZ* in the production of methylfucosylated LCOs, the plasmid pMP2450 was introduced into *Rl*. bv. *viciae* strain RBL5560. As strain RBL5560 produces LCOs that are not substituted at the reducing terminus (Spaink *et al*., 1991), it is a suitable background strain in which to determine a putative (methyl)fucosylating activity of NodZ. The LCOs produced by the transconjugant strain RBL5560 (pMP2450) were compared with those produced by the wild-type strain by high-performance liquid chromatography (HPLC) using photodiode-array detection. The isograms derived from both strains contain a major 303 nm-absorbing peak, but that obtained from strain RBL5560(pMP2450) elutes 2 min earlier than that from the wild type (Fig. 2). The results show that in the presence of *nodZ* there is a shift in the retention times of all LCO species from the transconjugant strain compared with those of the LCOs produced by the wild-type strain.

In order to study the structural modification that is responsible for this shift, the structures of the LCOs produced by strain RBL5560(pMP2450) were analysed following extraction, isolation, pre-purification and separation on reversed-phase HPLC with photodiode-array

detection. Pooled fractions representing early-eluting fractions (Mix 1) and late-eluting fractions (Mix 2) from strain RBL5560(pMP2450) were collected (Fig. 2). The chemical structures of the LCOs in the two resulting samples were studied using mass spectrometry (MS).

Structural determination of LCOs

Positive-ion-mode fast atom bombardment (FAB) mass spectra were obtained from Mix 1 and Mix 2. The mass spectrum of Mix 1 contained low abundance $[M+H]^+$ pseudomolecular ions at *m/z* 1438 and 1444 (with their corresponding thioglycerol adducts at *m/z* 1546 and 1552) corresponding to an LCO-V (C18:4, Ac, Deoxyhex) species (for nomenclature see the *Experimental procedures*), and its C18:1 fatty acyl-containing analogue, respectively. Ion intensities were too low to permit further analysis with tandem mass spectrometry. In contrast, Mix 2 yielded an intense spectrum with $[M+H]^+$ pseudomolecular ions at *m/z* 1444 and 1241 (thioglycerol adduct ions at *m/z* 1552 and 1349) for the $GlcNAc_5$ and $GlcNAc_4$ LCO analogues that bear a C18:1 fatty acyl chain, an acetate, and a deoxyhexose residue. Low intensity ions at *m/z* 1402 and 1199 represent either mass spectrometric or chemical loss of the acetate group from the two species, while weak ions at *m/z* 1298 and 1095 represent a small amount of species lacking the deoxyhexose residue, either as a result of mass spectrometric β-cleavage or incomplete biosynthesis. Collision-induced dissociation (CID) MS–MS analysis of the abundant ion at *m/z* 1241 yielded oxonium ions at *m/z* 468, 671, and 874, with a further ion at *m/z* 1095 generated on β-cleavage of the deoxyhexose residue (Fig. 3). These ions allow the structure of the major species in Mix 2 to be assigned as having a linear $GlcNAc_4$ backbone, which is acetylated and fatty acylated (with a C18:1 chain) on the non-reducing-terminal residue and bears a branching deoxyhexose residue on the reducing-terminal residue. A similar experiment carried out on the less-abundant $GlcNAc_5$ species yielded oxonium ions at *m/z* 468, 671, 874, and 1077, and a β-cleavage ion at *m/z* 1298, again defining the site of deoxyhexose substitution as the reducing-terminal residue. These data demonstrate that, in the presence of the *nodZ* gene, a deoxyhexose residue is transferred as a branch to the reducing-terminal GlcNAc residue of the LCOs normally made by *Rl.* bv. *viciae.*

In order to identify the deoxyhexose residue, Mix1 and Mix2, together with authentic fucose and rhamnose standards, were subjected to methanolysis and trimethylsilylation, and the resulting monosaccharide derivatives were separated and analysed using capillary gas chromatography (GC)–MS. Both samples yielded four abundant peaks corresponding to tetramethylsilane (TMS) methylglycosides, which co-elute with the four peaks typically obtained from standard fucose. Peaks with retention times corresponding to standard rhamnose were completely absent. These results allow us to assign as fucose the deoxyhexose residue transferred in the presence of the *nodZ* gene.

The linkage of the fucose to the reducing-terminal residue was determined following preparation of partially methylated alditol acetates (PMAAs) from the two fractions, and identification of these derivatives following capillary GC–MS. Both fractions yielded three peaks; these were identified as being derived from non-reducing *N*-acetylhexosamine (HexNAc), 4-substituted HexNAc, and 4,6-branched HexNAc, consistent with the fucose residue being transferred to C-6 of the reducing-terminal GlcNAc residue.

We can thus conclude that NodZ mediates the transfer of a fucose residue to C-6 of the reducing-terminal GlcNAc residue of LCOs produced by strain *Rl*. bv. *viciae* RBL5560.

In vitro *activity of NodZ*

In order to further analyse the function of the *nodZ* gene, we performed *in vitro* enzymatic assays for transfucosylation activity. The results show that crude cell-free extracts of RBL5560 transformed with pMP2450 (RBL5560 (pMP2450)) are able to transfer fucosyl residues from ^{14}C-labelled GDP-fucose to acceptor molecules such as chitin trisaccharides, and pentasaccharides. The resulting radiolabelled oligosaccharide derivatives run slightly slower than the corresponding chitin pentamer or trimer (Fig. 4) and could be enzymatically digested by chitinase (data not shown). No fucosyltransferase activity could be detected in extracts from strain RBL5560 lacking the *nodZ* gene. These data suggest that NodZ is able to fucosylate chitin oligomers *in vitro*.

Symbiotic phenotype

Since mutations in *nodZ* result in defective nodulation of siratro, *nodZ* was characterized as a siratro-specific nodulation locus of *B. japonicum* (Nieuwkoop *et al*., 1987; Stacey *et al*., 1994). We tested whether *nodZ* could confer on strain RBL5560 the ability to nodulate siratro plants. Twenty-five days after inoculation, 57% of siratro plants inoculated with strain RBL5560(pMP2450) form nodules, while no nodules at all are observed after inoculation with the wild-type strain RBL5560 (Table 1). The transconjugant strain starts to nodulate siratro 4 d later than homologous rhizobia (*B. japonicum* or *Rhizobium* sp. NGR234).

Siratro is not a natural host for *Rl*. bv. *viciae* and therefore it is possible that the *nod* genes of this strain are not optimally induced by siratro-root exudates. The introduction of the mutant *nodD604* into rhizobia strains results in flavonoid independent transcription activation (FITA)

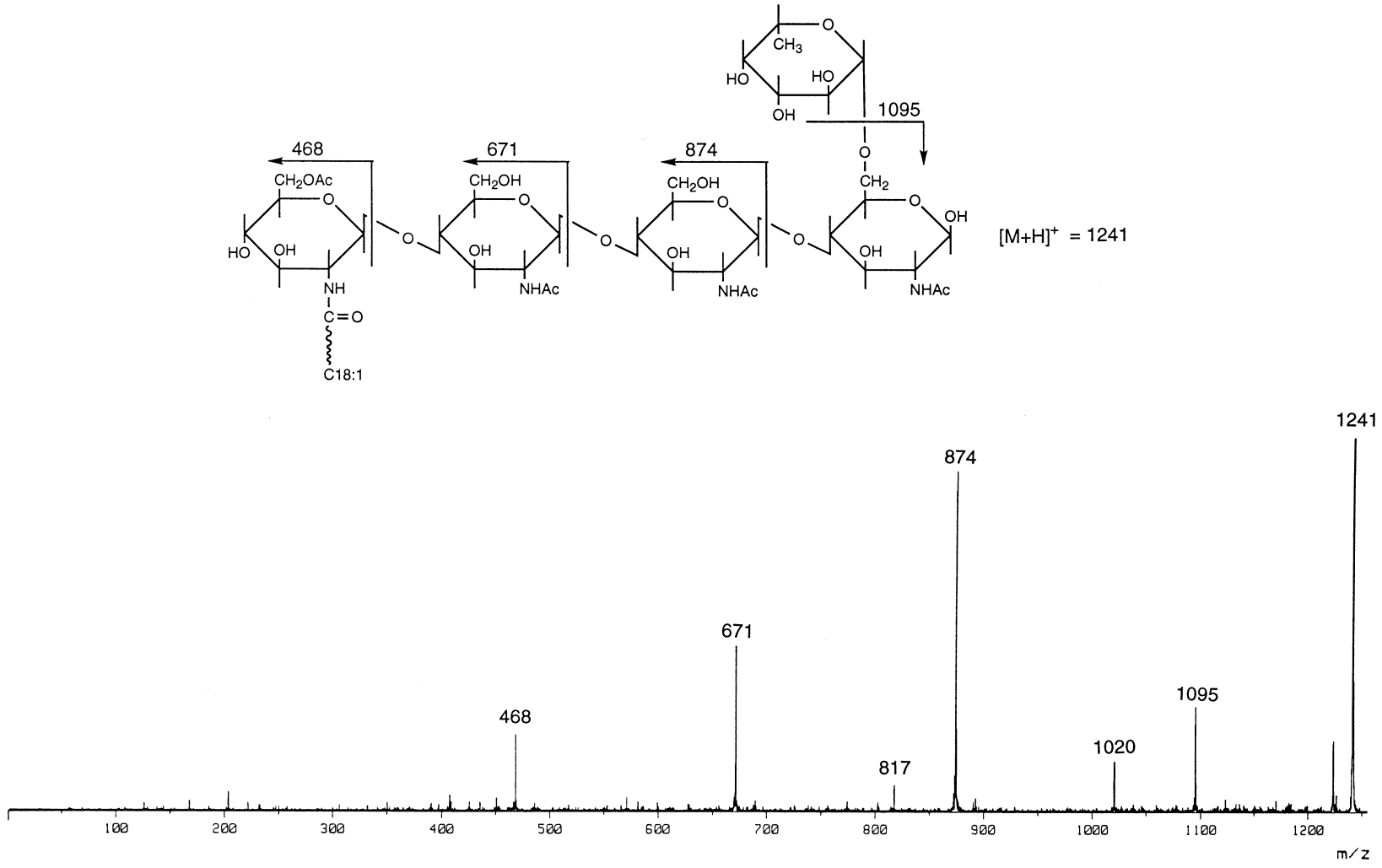

Fig. 3. FAB CID tandem spectrum of the pseudomolecular ion of the $GlcNAc_4$-containing LCO from Mix 2. The ions at *m/z* 817 and 1020 correspond to oxonium ions formed either on rearrangement (Cardenas *et al.*, 1995; M. Ferro, N. Demont, D. Promé, J.-C. Promé, C. Boivin and D. Dreyfus, personal communication) or by internal-residue loss (Kovacik *et al.*, 1995).

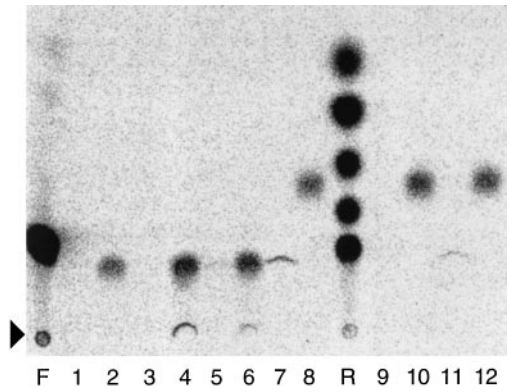

Fig. 4. Analysis of *nodZ*-dependent activity *in vitro* using silica TLC. After incubation, reaction mixtures containing crude cell-free extracts from strain RBL5560 (odd numbers) or RBL5560 (pMP2450) (even numbers), ^{14}C-GDP-fucose and chitin pentamers (lanes 1–6) or trimers (lanes 7–12) were incubated with Dowex resin to eliminate unincorporated GDP-fucose, and analysed using silica TLC. Lanes 1, 2, 7 and 8: 10 min incubation; lanes 3, 4, 9 and 10: 30 min incubation; lanes 5,6, 11 and 12: 60 min incubation. Lane F: ^{14}C-GDP-fucose. Lane R: ^{14}C-labelled standard oligomers of *N*-acetylglucosamine; from top to bottom, monomer to pentamer, respectively (Kamst *et al*., 1995). The arrowhead indicates the origin of the TLC.

and therefore renders production of LCOs independent of the presence of inducer (Spaink *et al*., 1989a). The *NodD* FITA gene was cloned in an IncW vector (resulting in plasmid pMP1604) in order to introduce it into the strain already carrying the IncP vector pMP2450. As shown by radioactive labelling of LCOs and subsequent thin-layer chromatography (TLC) analysis, strain RBL5560 (pMP2450, pMP1604) produces, in the absence of inducer, the same pattern of LCOs as strain RBL5560(pMP2450) induced with naringenin (data not shown). The introduction of *nodD* FITA improves nodulation on siratro by strain RBL5560 carrying *nodZ* (Table 1). In this case, 100% of the plants are nodulated 23 d after inoculation, and the delay in the start of nodulation is reduced by 2 d. Homologous rhizobia nodulated all plants after 17 d.

In order to analyse which other *nod* genes of *Rl*. bv. *viciae* are necessary for nodulation of siratro, we introduced the *nodZ* gene into various *nod* mutants of *Rl*. bv. *viciae* strain RBL5560 and tested the derivatives for nodulation. Transfer of *nodZ* (plasmid pMP2450) to strain RBL5900 (in which the *nodFELMNO* genes are deleted) does not confer the ability to nodulate siratro. However, strain RBL5900 carrying the *nodD* FITA gene in addition to *nodZ* is able to nodulate a small proportion of the plants (Table 1). We introduced plasmid pMP2105, which carries *nodL*, into strain RBL5900(pMP2450). The resulting strain, RBL5900(pMP2450, pMP2105), nodulates siratro with a similar frequency to strain RBL5560(pMP2450). Therefore, in our test system, besides the common *nod* genes, only *nodL* is required for extension of nodulation to siratro by *nodZ* in an *R. leguminosarum* background. We have also introduced the plasmid pMP2450 into a series of derivatives of strain RBL5560 carrying a single Tn*5* insertion (i.e. Tn*5* insertions in *nodE*, *F*, *L*, *M*, *T*, *O*, *I* and *J*). As is the case with strain RBL5560, none of the strains carrying a Tn*5* insertion were able to nodulate siratro. With the exception of the *nodL* mutant strain, all of the mutants carrying *nodZ* in plasmid pMP2450 nodulated siratro with a similar frequency to strain RBL5560(pMP2450). These data support the previous conclusion that *nodL* and *nodZ* are required for the ability of *Rl*. bv. *viciae* to nodulate siratro.

We have also studied the response of other tropical leguminous plants to inoculation with RBL5560(pMP2450, pMP1604). The results presented in Table 2 show that *nodZ* also confers on *Rl*. bv. *viciae* the ability to nodulate *Glycine soja*, *Vigna unguiculata* and *Leucaena leucocephala*. On *Lotus corniculatus* plants, we observed the formation of nodule primordia but the primordia did not develop into complete nodules.

The external morphology of the nodules induced on siratro by strain RBL5560(pMP2450, pMP1604) was similar to that of the nodules induced by the homologous strains. However, in contrast to plants inoculated with the homologous strains, plants nodulated by the transconjugant strain became yellow within four weeks, indicating that they had not fixed nitrogen. Microscopic analysis showed that nodules induced by the transconjugant strain are devoid of bacteroids (Fig. 5). Although the general structure resembles that of rhizobia-induced nodules, they do not develop a vascular bundle surrounding the nodule and instead they present vascular tissue in the centre (Fig. 5). It is also remarkable that the cells of the nodule induced by the transconjugant strain are significantly larger than in the *Bradyrhizobium*-induced nodules. Gram staining of nodule

Table 1. Nodulation in siratro.[a]

Strain	Percentage of nodulated plants[b]	Total no. of plants (no. of assays)
RBL5560	0	60 (6)
RBL5560 (pMP2450)	57	69 (6)
RBL5560 (pMP1604)	0	30 (3)
RBL5560 (pMP2450, pMP1604)	100 (23)	30 (3)
USDA110	100 (17)	55 (6)
NGR234	100 (17)	50 (6)
RBL5900	0	20 (2)
RBL5900 (pMP2450)	0	20 (2)
RBL5900 (pMP1604)	0	20 (2)
RBL5900 (pMP2450, pMP1604)	16	19 (2)
RBL5900 (pMP2105)	0	20 (2)
RBL5900 (pMP2450, pMP2105)	54	30 (2)

a. Nodulation scored 25 d after inoculation.
b. The first day that 100% nodulation was observed is indicated in parentheses.

Table 2. Nodulation capacity on different hosts.[a]

Plant species	Percentage of Nodulated Plants with Strain		
	RBL5560	RBL5560 (pMP1604)	RBL5560(pMP2450, pMP1604)
M. atropurpureum	0	0	88
G. soja	0	0	45
V. unguiculata	0	0	70
L. leucocephala	0	0	36
L. corniculatus	0	0	0[b]

a. 28 d after inoculation.
b. Nodule primordia are formed. The other plant species tested, *Dolichos bifloris* and *Lupinus nanus*, did not respond to inoculation.

sections shows that in the nodules induced by the transconjugant strain, many of the cortical cells contain amyloplasts filled with starch grains (data not shown). In general, the nodule structure we observed to be induced by *Rl*. bv. *viciae* carrying *nodZ*, is very similar to that reported for nodules induced on siratro by purified LCOs from strain NGR234 (Relić *et al*., 1993).

Discussion

The LCOs produced by strain *B. japonicum* USDA110 contain a 2-*O*-methylfucosyl residue linked to C-6 of the reducing *N*-acetylglucosamine residue (Sanjuan *et al*.,

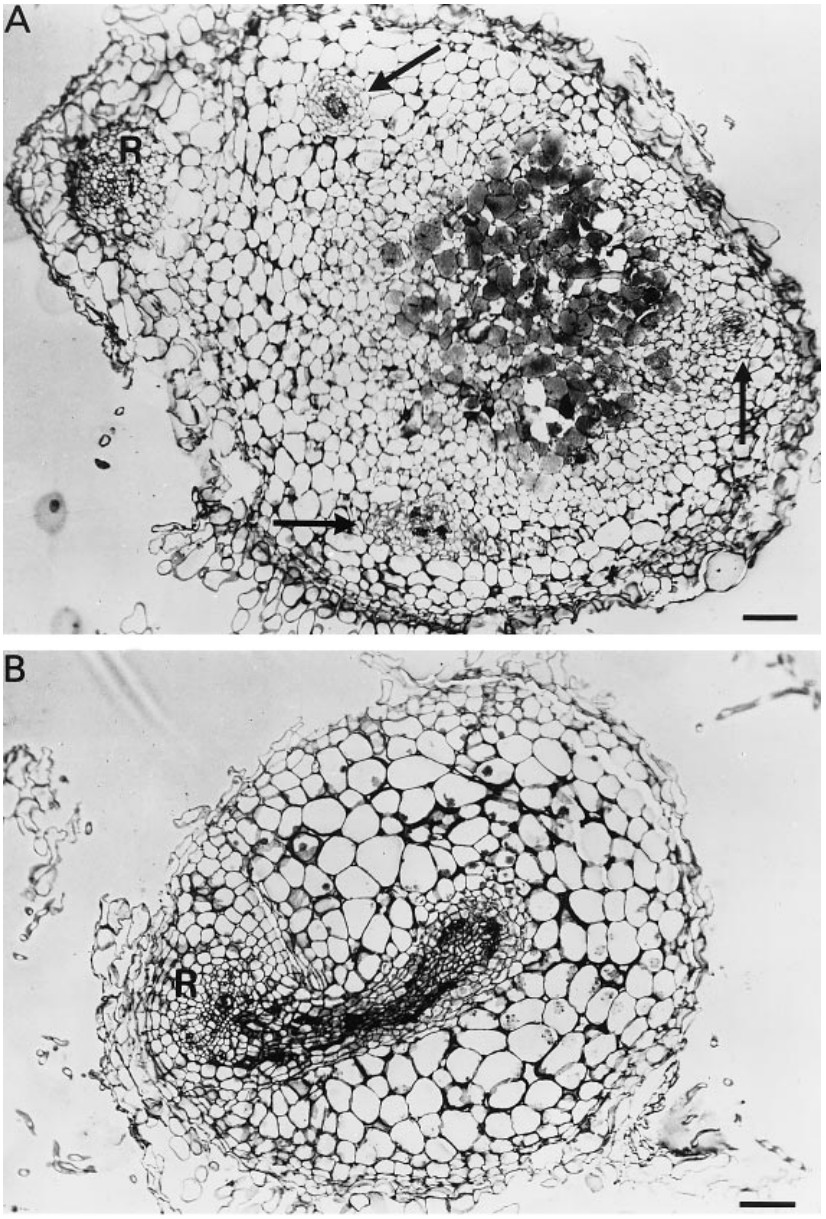

Fig. 5. Light microscopy of transverse sections of siratro nodules elicited by wild-type *B. japonicum* USDA110 (A) and transconjugant RBL5560(pMP2450, pMP1604) (B). Gram staining of serial sections of (A) and (B) showed starch deposition in the cortical cells of (B). R: root; arrows point to vascular bundles. Bar = 100 μm.

1992). The 2-*O*-methylfucosyl residue is absent in the LCOs produced by a *nodZ* mutant, suggesting that NodZ is involved in fucosylation of the core LCO structure (Stacey *et al*., 1994). Our initial TLC analysis of the ^{14}C-labelled LCOs produced by *Rl*. bv. *viciae* carrying *nodZ* from *B. japonicum* suggested that such factors are structurally different from those produced by the wild-type strain. The purification and structural determination of the LCOs produced by the transconjugant strain RBL5560-(pMP2450) showed that, in the presence of *nodZ,* a fucosyl residue is transferred to C-6 of the reducing-terminal GlcNAc of LCOs produced by strain RBL5560. These data strongly suggest that NodZ functions *in vivo* as a fucosyltransferase. We also present *in vitro* data showing that NodZ is involved in producing a fucosylated oligosaccharide when incubated with chitin oligosaccharide acceptors and GDP-fucose as substrates. The fucosyltransferase activity owing to the NodZ protein is currently being further investigated.

Most of the LCO species isolated from *B. japonicum* wild-type strains bear a methyl group linked to the position 2 of the fucosyl residue (Sanjuan *et al*., 1992; Carlson *et al*., 1993). Because NodZ *in vivo* is involved in the transfer of a fucosyl residue (and not a methylfucosyl residue), this indicates that a second, as yet unidentified, gene in *B. japonicum* involved in the methylation of the fucosyl residue does not occur in *R. leguminosarum*.

The fact that *nodZ* extends the host range of *Rl*. bv. *viciae* to include siratro and several other tropical leguminous plants, further supports the role of *nodZ* as a major host-specific determinant for nodulation. Therefore, the fucosyl residue can be considered to be a modification of the core chitin oligomers that confers on LCOs their plant specificity. Two other modifications to the reducing-terminal residue of LCOs that have been shown to be host-specific are sulphation and *O*-acetylation. Sulphation is required for nodulation of alfalfa (Roche *et al*., 1991), while *O*-acetylation of the reducing-terminal residue is required for nodulation of Afghan pea (Firmin *et al*., 1993). Alfalfa and Afghan pea can be nodulated by only a very limited number of rhizobium strains, while siratro is a plant with a broad capacity to be nodulated by different rhizobia (Lewin *et al*., 1987). Among wild-type rhizobia with the ability to nodulate siratro are *B. japonicum*, *Rhizobium* sp. NGR234 and *Rhizobium fredii*, which all produce LCOs having a fucosyl modification of the reducing-terminal residue (Sanjuan *et al*., 1992; Carlson *et al*., 1993; Price *et al*., 1992; Bec-Ferté *et al*., 1994). The broad-host-range strains *R. tropici* and *Rhizobium* sp. GRH2 also include siratro among their hosts (Martinez-Romero *et al*., 1991; López-Lara *et al*., 1995a). However, in these two cases, no fucosyl group is present in the LCOs, but, instead, a fraction of the LCOs is modified with a sulphate group (Poupot *et al*., 1993; López-Lara *et al*., 1995c). Therefore, it seems that siratro can recognize LCOs with either a fucose or a sulphate group modifying the reducing-terminal residue. The responsiveness of siratro to sulphated LCOs is also clear from the fact that constitutive expression of the *nod* genes of *R. meliloti* results in host-range extension to siratro (Spaink *et al*., 1989a; Kondorosi *et al*., 1991). This result indicates that the inability of *R. meliloti* to nodulate siratro originates at the level of *nod* gene activation. In summary, siratro allows quite some variability with respect to LCO structure and these data do not seem to support a simple 'lock and key' model for LCO signal-receptor interaction. An alternative function proposed for the LCO modifications is the protection of the LCOs against inactivation by hydrolytic enzymes produced by the plant (Staehelin *et al*., 1994).

Rl. bv. *viciae* is a narrow-host-range strain that only nodulates the temperate legumes *Vicia*, *Pisum*, *Lathyrus* and *Lens,* which all form indeterminate-type nodules. By introducing one gene, *nodZ* of *B. japonicum*, into this strain, the host range broadens to include tropical legumes, most of which form determinate-type nodules. These data clearly illustrate the role of *nodZ* as a major positive host-range determinant of nodulation. Although the presence of a fucosyl residue on the LCOs produced by a *Rl*. bv. *viciae* is sufficient to induce nodulation on siratro, it does not allow the bacteria to engage in a nitrogen-fixing symbiosis with this plant. A possible explanation is that some other component of the rhizobium strain is missing or it is not properly recognized. Candidates for such components are the exopolysaccharides, lipopolysaccharides, capsular polysaccharides, β(1-2) cyclic glucans or K-antigens, which have been indicated to be involved in efficient infection or formation of the bacteroid (Hirsch, 1992; Kannenberg and Brewin, 1994; Reuhs *et al*., 1993). For example, a strain of NGR234 deficient in the production of exopolysaccharides induces empty nodules on siratro (Djordjevic *et al*., 1987). Using the test strains developed in this study, a gain-of-function approach could be further exploited to identify other factors involved in establishing a successful symbiotic interaction with tropical leguminous plants.

Experimental procedures

Strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 3. Broad-host-range plasmids were mobilized from *Escherichia coli* to rhizobia using pRK2013 as a helper plasmid (Ditta *et al*., 1980). *Rhizobium* spp. were grown in B$^-$ medium (Spaink *et al*., 1989b). Antibiotics were added, when required, at the following final concentration (μg ml^{-1}): tetracycline, 2; spectinomycin, 200. For induction, naringenin was added to the medium to a final concentration of 1.5 μM.

The source of *nodZ* gene was plasmid pSL32. pSL32 was constructed by replacing a 0.5 kb *Ssp*I fragment of pSUP202 (Simon *et al*., 1983) with the 3.4 kb fragment

Table 3. Bacterial strains and plasmids used in this study.

Strain/ Plasmid	Relevant characteristic	Source/Reference
Strain		
R. leguminosarum bv. *viciae*		
RBL5560	pSym pRL1JI	Wijffelman *et al.* (1985)
RBL5900	Strain LPR5045 containing the Sym plasmid of strain A69	Ritsema *et al.* (1994)
A69	pSym pRL1JI containing the deletion *nod*Δ99	Downie and Surin (1990)
B. japonicum		
USDA110	Wild type	Kuykendall and Elkan (1976)
Rhizobium sp.		
NGR234(Rif^R)	Rif^R wild-type NGR234	Lewin et al. (1990)
Plasmid		
pRK2013	IncColE1 Tra$^+$ Km^R	Ditta *et al.* (1980)
pSUP202	Mob Tc^R Amp^R Cm^R	Simon *et al.* (1983)
pRP14	*nodZ* Amp^R	Stacey *et al.* (1994)
pSL32	*nodZ* Tc^R Amp^R	This work
pMP3510	IncP, cloning vector, pA Tc^R	Spaink *et al.* (1995a)
pMP2450	IncP *nodZ* pA Tc^R	This work
pMP2105	IncW *nodL* pA Spc^R Sm^R	Spaink *et al.* (1995a)
pMP604	IncP *nodD* FITA	Spaink *et al.* (1989a)
pRI40	IncW Spc^R Sm^R	Innes *et al.* (1988)
pMP1604	IncW *nodD* FITA	This work

Rif^R, Tc^R, Amp^R, Cm^R, Spc^R, Sm^R, and Km^R: rifampicin, tetracycline, ampicillin (carbenicillin), chloramphenicol, spectinomycin, streptomycin and kanamycin resistance, respectively. pA, promoter of *nodA*; Mob, plasmid mobilizing region; Inc, plasmid incompatibility group.

of pRP14 (Stacey *et al*., 1994) encompassing the *nodZ* gene. Plasmid pMP2450 was derived by cloning the 2.5 kb *Pst*I fragment from pSL32 (containing *nodZ*) into pMP3510 (Spaink *et al*., 1995a), orientated such that the *nodA* promoter is upstream of *nodZ*. pMP1604 was constructed by inserting the *Hin*dIII fragment from pMP604 containing the *nodD* FITA gene into plasmid pRI40 (Innes *et al*., 1988).

Detection and purification of LCOs

LCOs were radioisotope-labelled using [1-^{14}C]-D-glucosamine (Amersham; specific activity 54 mCi mmol^{-1}) as precursor. One ml of the appropriate cells (OD at 620 nm = 0.1) were grown overnight at 28°C in the presence of naringenin, when required. Then 0.1 μCi of [1-^{14}C]-D-glucosamine was added and the cultures were grown for 5 h. LCOs were isolated from the cultures using *n*-butanol extraction. Samples were concentrated by evaporation and chromatographed on reversed-phase C18-coated silica plates (Sigma) using a mobile phase of 1:1 (v:v) acetonitrile:water. Radioisotope-labelled components were detected using a Molecular Dynamics PhosphoImager and the Image Quant™ software.

LCOs were purified from 2 l cultures as previously described (López-Lara *et al*., 1995b). The HPLC elution was performed at a flow rate of 0.7 ml min^{-1}, and the UV absorption of the eluent was analysed with a photodiode-array detector (Pharmacia, LKB). Fractions (1.4 ml) were collected, pooled and dried.

LCO nomenclature

LCO nomenclature is according to Spaink *et al*. (1991). Briefly, the chain length of the oligosaccharide is indicated by a latin numeral (e.g. V) followed by terms in parentheses to indictate particular substitutions (e.g. C18:4 for the presence of a fatty acyl moiety containing four unsaturations, or deoxyhex for the presence of a deoxyhexose).

FAB–MS and CID tandem MS

Positive-ion FAB mass spectra 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. 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 HP9000 data system running JEOL COMPLEMENT software.

CID–MS were recorded using the same machine, with air 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.

Samples were dissolved in 30 μl of dimethylsulphoxide (DMSO), and 1.5 μl aliquots of sample solution were loaded into a matrix of mono-thioglycerol.

Gas chromatography–MS

LCOs were converted to their TMS methylglycosides on methanolysis and trimethylsilylation, as described (López-Lara *et al*., 1995b), and were separated using GC–MS, or were converted to their PMAAs on permethylation, hydrolysis, reduction, and acetylation (López-Lara *et al*., 1995b) and the resulting derivatives were analysed using GC–MS. GC–MS analyses were performed using a Fisons MD800 mass spectrometer fitted with a Carlo Erba GC8060 gas chromatograph, an on-column injector and using helium as the carrier gas. Monosaccharide derivatives were separated on a DB-5MS column (0.25 mm × 30 m; J + W Scientific). TMS methyl glycosides were injected directly from solution in the TMS reagents (1 μl injected) and separated using the following temperature programme: 110°C for 2 min, then ramping at 30°C min^{-1} to 140°C, holding for 2 min, then ramping at 4°C min^{-1} to 180°C, holding for 30 min, then finally ramping at 30°C min^{-1} to 250°C and holding for 10 min. Partially methylated alditol acetates were injected in solution in dichloromethane (1 μl injected) and separated using the following temperature programme: 50°C for 2 min, then ramping at 40°C min^{-1} to 130°C, holding for 2 min, then ramping at 4°C min^{-1} to 230°C, and holding for 15 min. Mass spectra were recorded under conditions of electron impact in the positive-ion mode with an electron energy of 70 eV, and were recorded using linear scanning from m/z 50–350 over 1 s.

In vitro *activity of NodZ*

For the assay of the *in vitro* activity of NodZ, crude cell-free extracts were obtained using a French pressure cell as described previously (Bloemberg *et al*., 1994). Samples equivalent to 500 μl of cell cultures were incubated in the presence of 50 nCi of [1-^{14}C]-GDP-fucose (Amersham) and 100 μg of the chitin oligomer at 28°C. After the reaction, free GDP-fucose was removed by incubation with Dowex1 ion-exchange resin (Sigma). Samples were concentrated by evaporation and chromatographed on silica plates (Merck) using as mobile phase 5:3:1 (v:v:v) *n*-butanol:ethanol:water. Radioisotope-labelled components were detected using a Molecular Dynamics PhosphoImager and the Image Quant™ software. Chitinase from *Streptomyces griseus* (Sigma; 001 units per assay) was used for digestion of chitin oligosaccharides.

Plant assays

Siratro seeds were germinated as previously described (van Brussel *et al*., 1986). For all nodulation assays with siratro, except those in Table 2, plants were grown in agar-slant tubes (van Brussel *et al*., 1982). Each seedling was inoculated with 100 μl of a suspension of the rhizobial strain in plant growth medium ($OD_{620} = 0.1$).

For the nodulation experiment described in Table 2, seeds of *M. atropurpureum*, *G. soja* (PI #1468397), *V. unguiculata* cv. Caloona, *L. leucocephala* cv. Cunningham, *L. corniculatus*, *Dolichos biflorus* and *Lupinus nanus* were surface-sterilized as described by Nieuwkoop *et al*. (1987). After germination, seedlings were transferred into sterile Leonard jars containing vermiculite and half-strength plant-nutrient solution (Wacek and Brill, 1976). Two days after planting, each seedling was inoculated with 5×10^8 cells of the desired strain. Plants were grown in a greenhouse under 16 h light per day. Nodulation was examined 28 d after inoculation.

Microscopy and photography

Root nodules of siratro were excised four weeks after inoculation. They were fixed in glutaraldehyde buffer and dehydrated through an ethanol series to 96% ethanol. Infiltration and embedding in Historesin (Jung) was carried out according to the manufacturer's specifications. Sections of 4 μm were stained with toluidine blue and examined by light microscopy. Gram staining was performed as described (Friedly, 1985). Pictures were taken with a Leitz Laborlux using Delta (Ilford) film.

Acknowledgements

We are very grateful to Marco van der Laan for his help in the initial stages of this research, and to Peter Hock for his assistance with the preparation of the figures. This work was supported by contracts from: the European Union, Grant BIO2-CT92-5112 (to I.M.L.-L.) and Grant BIO2-CT93-0400 (DG12 SSMA); the Netherlands Foundation for Chemical Research (SON) (to J.T.O. and G.V.B.); the Netherlands Organization for the Advancement of Pure Research (NWO-PIONIER) (to H.P.S.); and the US Department of Energy Grant 92ER20072 (to G.S.). C.Q. was supported by a Marie Curie fellowship from the European Union for a research project at Leiden University.

References

Atkinson, E.M., Palcic, M.M., Hindsgaul, O., and Long, S.R. (1994) Biosynthesis of *Rhizobium meliloti* lipooligosaccharide Nod factors: NodA is required for an *N*-acyltransferase. *Proc Natl Acad Sci USA* **91:** 8418–8422.

Bec-Ferté, M.P., Krishnan, H.B., Promé, D., Savagnac, A., Pueppke, S.G., and Promé, J.C. (1994) Structures of nodulation factors from the nitrogen-fixing soybean symbiont *Rhizobium fredii* USDA257. *Biochemistry* **33:** 11782–11788.

Bloemberg, G.V., Thomas-Oates, J.E., Lugtenberg, B.J.J., and Spaink, H.P. (1994) Nodulation protein NodL of *Rhizobium leguminosarum O*-acetylates lipo-oligosaccharides, chitin fragments and *N*-acetylglucosamine *in vitro*. *Mol Microbiol* **11:** 793–804.

van Brussel, A.A.N., Tak, T., Wetselaar, A., Pees, E., and Wijffelman, C.A. (1982) Small leguminosae as test plants for nodulation of *Rhizobium leguminosarum* and other *Rhizobia* and *Agrobacteria* harbouring a leguminosarum plasmid. *Plant Sci Lett* **27:** 317–325.

van Brussel, A.A.N., Zaat, S.A.J., Canter Cremers, H.C.J., Wijffelman, C.A., Pees, E., Tak, T., and Lugtenberg, B.J.J. (1986) Role of plant root exudate and sym plasmid-localized nodulation genes in the synthesis by *Rhizobium leguminosarum* of Tsr factor which causes thick and short roots on common vetch. *J Bacteriol* **165:** 517–522.

Cardenas, L., Dominquez, J., Quinto, C., López-Lara, I.M., Lugtenberg, B.J.J., Spaink, H.P., Rademaker, G.J., Haverkamp, J., and Thomas-Oates, J.E. (1995) Isolation, chemical structure and biological activity of the lipo-chitin oligosaccharide nodulation signals from *Rhizobium etli. Plant Mol Biol* **29:** 453–464.

Carlson, R.W., Sanjuan, J., Bhat, R., Glushka, J., Spaink, H.P., Wijfjes, A.H.M., van Brussel, A.A.N., Stokkermans, T.J.W., Peters, K., and Stacey, G. (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.

Carlson, R.W., Price, N.P.J., and Stacey, G. (1994) The of rhizobial lipo-oligosaccharide nodulation signal molecules. *Mol Plant–Microbe Interact* **7:** 684–695.

Dénarié, J., and Cullimore, J. (1993) Lipo-oligosaccharide nodulation factors: A new class of signaling molecules mediating recognition and morphogenesis. *Cell* **74:** 951–954.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (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.

Djordjevic, S.P., Chen, H., Batley, M., Redmond, J.W., and Rolfe, B.G. (1987) Nitrogen fixation ability of exopolysaccharide synthesis mutants of *Rhizobium* sp. strain NGR234 and *Rhizobium trifolii* is restored by the addition of homologous exopolysaccharides. *J Bacteriol* **169:** 53–60.

Downie, J.A. (1994) Signalling strategies for nodulation of legumes by rhizobia. *Trends Microbiol* **2:** 318–324.

Downie, J.A., and Surin, B.P. (1990) Either of two *nod* gene loci can complement the nodulation defect of a *nod* deletion mutant of *Rhizobium leguminosarum* bv. *viciae*. *Mol Gen.Genet* **222:** 81–86.

Fellay, R., Perret, X., Viprey, V., Broughton, W.J., and Brenner, S. (1995) Organization of host-inducible transcripts on the symbiotic plasmid of *Rhizobium* sp. NGR234. *Mol Microbiol* **16:** 657–667.

Firmin, J.L., Wilson, K.E., Carlson, R.W., Davies, A.E., and Downie, J.A. (1993) Resistance to nodulation of cv Afghanistan peas is overcome by *nodX* which mediates an *O*-acetylation of the *Rhizobium leguminosarum* lipo-oligosaccharide nodulation factor. *Mol Microbiol* **10:** 351–360.

Friedly, G. (1985) Importance of bacterial stains in the diagnosis of infectious diseases. *J Med Tech* **1:** 823–833.

Geremia, R.A., Mergaert, P., Geelen, D., Van Montagu, M., and Holsters, M. (1994) The NodC protein of *Azorhizobium caulinodans* is an *N*-acetylglucosaminyltransferase. *Proc Natl Acad Sci USA* **91:** 2669–2673.

Goelz, S.E., Hession, C., Goff, D., Griffiths, B., Tizard, R., Newman, B., and Chi-Rosso, G. (1990) ELFT: a gene that directs the expression of an ELAM-1 ligand. *Cell* **63:** 1349–1356.

Hirsch, A.M. (1992) Developmental biology of legume nodulation. *New Phytol* **122:** 211–237.

Innes, R.W., Hirose, M.A., and Kuempel, P.L. (1988) Induction of nitrogen-fixing nodules on clover requires only 32 kilobase pairs of DNA from the *Rhizobium trifolii* symbiosis plasmid. *J Bacteriol* **170:** 3793–3802.

Kamst, E., van der Drift, K.M.G.M., Thomas-Oates, J.E., Lugtenberg, B.J.J., and Spaink, H.P. (1995) Mass spectrometric analysis of chitin oligosaccharides produced by *Rhizobium* NodC protein in *Escherichia coli*. *J Bacteriol* **177:** 6282–6285.

Kannenberg, E.L., and Brewin, N.J. (1994) Host-plant invasion by *Rhizobium*: the role of cell-surface components. *Trends Microbiol* **2:** 277–283.

Kondorosi, E., Buire, M., Cren, M., Iyer, N., Hoffmann, B., and Kondorosi, A. (1991) Involment of the *syrM* and *nodD3* genes of *Rhizobium meliloti* in *nod* gene activation and in optimal nodulation of the plant host. *Mol Microbiol* **5:** 3035–3048.

Kovacik, V., Hirsch, J., Kovac, P., Heerma, W., Thomas-Oates, J., and Haverkamp, J. (1995) Oligosaccharide characterization using collision-induced dissociation fast atom bombardment mass spectrometry: evidence for internal monosaccharide residue loss. *J Mass Spectrom* **30:** 949–958.

Kuykendall, L.D., and Elkan, G.H. (1976) *Rhizobium japonicum* derivatives differing in nitrogen-fixing efficiency and carbohydrate utilization. *Appl Env Microbiol* **32:** 511–519.

Lewin, A., Rosenberg, C., Meyer, H., Wong, C.H., Nelson, L., Manen, J.-F., Stanly, J., Dowling, D.N., Dénarié, J., and Broughton, W.J. (1987) Multiple host-specificity loci of the broad host-range *Rhizobium* sp. NGR234 selected using the widely compatible legume *Vigna unguiculata*. *Plant Mol Biol* **8:** 447–459.

Lewin, A., Cervantes, E., Wong, C.-H., and Broughton, W.J. (1990) *nodSU*, two new *nod* genes of the broad host range *Rhizobium* strain NGR234 encode host-specific nodulation of the tropical tree *Leucaena leucocephala*. *Mol Plant–Microbe Interact* **3:** 317–326.

López-Lara, I.M., Orgambide, G., Dazzo, F.B., Olivares, J., and Toro, N. (1995a) Surface polysaccharide mutants of *Rhizobium* sp. (*Acacia*) strain GRH2: Major requirement of lipopolysaccharide for successful invasion of *Acacia* nodules and host range determination. *Microbiology* **141:** 573–581.

López-Lara, I.M., van den Berg, J.D.J., Thomas-Oates, J.E., Glushka, J., Lugtenberg, B.J.J., and Spaink, H.P. (1995b) Structural identification of the lipo-chitin oligosaccharide nodulation signals of *Rhizobium loti*. *Mol Microbiol* **15:** 627–638.

López-Lara, I.M., van der Drift, K.M.G.M., van Brussel, A.A.N., Haverkamp, J., Lugtenberg, B.J.J., Thomas-Oates, J.E., and Spaink, H.P. (1995c) 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.

Martinez-Romero, E., Segovia, L., Martins Mercante, F., and Franco, A.A. (1991) *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int J Syst Bacteriol* **41:** 417–426.

Mergaert, P., D'Haeze, W., Geelen, D., Promé, D., Van Montagu, M., Promé, J.-C., and Holsters, M. (1995) Biosynthesis of *Azorhizobium caulinodans* Nod factors. *J Biol Chem* **270:** 28217–29223.

Mergaert, P., D'Haeze, W., Fernandez-Lopez, M., Geelen, D., Goethals, K., Promé, J.-C., Van Montagu, M., and Holsters, M. (1996) Fucosylation and arabinosylation of Nod factors in *Azorhizobium caulinodans*: involment of *nolK*, *nodZ*, and *noeC* and/or downstream genes. *Mol Microbiol* **21:** 409–419.

Nieuwkoop, A.J., Banfalvi, Z., Deshmane, N., Gerhold, D., Schell, M.G., Sirotkin, K.M., and Stacey, G. (1987) A locus encoding host range is linked to the common nodulation genes of *Bradyrhizobium japonicum*. *J Bacteriol* **169:** 2631–2638.

Poupot, R., Martinez-Romero, E., and Promé, J.-C. (1993) Nodulation factors from *Rhizobium tropici* are sulphated or non-sulphated chitopentasaccharides containing an *N*-methyl-*N*-acylglucosaminyl terminus. *Biochemistry* **32:** 10430–10435.

Price, N.P.J., Relić, B., Taimont, F., Lewin, A., Promé, D., Pueppke, S.G., Maillet, F., Dénarié, J., Promé, J.-C., and Broughton, W.J. (1992) Broad-host-range *Rhizobium* species strain NGR234 secretes a family of carbamoylated, and fucosylated, nodulation signals that are *O*-acetylated or sulphated. *Mol Microbiol* **6:** 3575–3584.

Relić, B., Talmont, F., Kopcinska, J., Golinowski, W., Promé, J.-C., and Broughton, W.J. (1993) Biological activity of *Rhizobium* sp. NGR234 Nod-factors on *Macroptilium atropurpureum*. *Mol Plant–Microbe Interact* **6:** 764–774.

Reuhs, B.L., Carlson, R.W., and Kim, J.S. (1993) *Rhizobium fredii* and *Rhizobium meliloti* produce 3-deoxy-d-manno-2-octulosonic acid-containing polysaccharides that are structurally analogous to group II K antigens (capsular polysaccharides) found in *Escherichia coli*. *J Bacteriol* **175:** 3570–3580.

Ritsema, T., Geiger, O., van Dillewijn, P., Lugtenberg, B.J.J., and Spaink, H.P. (1994) Serine residue 45 of nodulation protein NodF from *Rhizobium leguminosarum* bv. *viciae* is essential for its biological function. *J Bacteriol* **176:** 7740–7743.

Roche, P., Debellé, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Dénarié, J., and Promé, J.C. (1991) Molecular basis of symbiotic host specificity in *Rhizobium meliloti*: *nodH* and *nodPQ* genes encode the sulfation of lipooligosaccharides signals. *Cell* **67:** 1131–1143.

Rohrig, H., Schmidt, J., Wieneke, U., Kondorosi, E., Barlier, I., Schell, J., and John, M. (1994) Biosynthesis of lipooligosaccharide nodulation factors: *Rhizobium* NodA protein is involved in *N*-acylation of the chitooligosaccharide backbone. *Proc Natl Acad Sci USA* **91:** 3122–3126.

Sanjuan, J., Carlson, R.W., Spaink, H.P., Bhat, U.R., Barbour, W.M., Glushka, J., and Stacey, G. (1992) A 2-*O*-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. *Proc Natl Acad Sci USA* **89:** 8789–8793.

Schultze, M., Kondorosi, E., Ratet, P., Buire, M., and Kondorosi, A. (1994) Cell and molecular biology of *Rhizobium*–Plant interactions. *Int Rev Cytol* **156:** 1–75.

Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engeneering: transposon mutagenesis in gram negative bacteria. *Biotechnology* **1:** 784–791.

Spaink, H.P. (1995) The molecular basis of infection and nodulation by rhizobia: the in and outs of sympathology. *Annu Rev Phytopatol* **33:** 345–368.

Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Tak, T., Goosen-deRoo, L., Pees, E., van Brussel, A.A.N., and Lugtenberg, B.J.J. (1989a) Symbiotic properties of rhizobia containing a flavonoid-independent hybrid *nodD* product. *J Bacteriol* **171:** 4045–4053.

Spaink, H.P., Wijffelman, C.A., Okker, R.J.H., and Lugtenberg, B.J.J. (1989b) Localization of functional regions of the *Rhizobium nodD* product using hybrid *nodD* genes. *Plant Mol Biol* **12:** 59–73.

Spaink, H.P., Sheeley, D.M., van Brussel, A.A.N., Glushka, J., York, W.S., Tak, T., Geiger, O., Kennedy, E.P., Reinhold, V.N., and Lugtenberg, B.J.J. (1991) A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. *Nature* **354:** 125–131.

Spaink, H.P., Wijfjes, A.H.M., van der Drift, K.M.G.M., Haverkamp, J., Thomas-Oates, J.E., and Lugtenberg, B.J.J. (1994) Structural identification of metabolites produced by the NodB and NodC proteins of *Rhizobium leguminosarum*. *Mol Microbiol* **13:** 821–831.

Spaink, H.P., Wijfjes, A.H.M., and Lugtenberg, B.J.J. (1995a) *Rhizobium* NodI and NodJ proteins determine the efficiency of secretion of lipo-chitin oligosaccharides. *J Bacteriol* **177:** 6276–6281.

Spaink, H.P., Bloemberg, G.V., van Brussel, A.A.N., Lugtenberg, B.J.J., van der Drift, K.M.G.M., Haverkamp, J., and Thomas-Oates, J.E. (1995b) Host specificity of *Rhizobium leguminosarum* is determined by the hydrophobicity of highly unsaturated fatty acyl moieties of the nodulation factors. *Mol Plant–Microbe Interact* **8:** 155–164.

Stacey, G., Luka, S., Sanjuan, J., Banfalvi, Z., Nieuwkoop, A.J., Chun, J.Y., Forsberg, L.S., and Carlson, R. (1994) *nodZ*, a unique host-specific nodulation gene, is involved in the fucosylation of the lipooligosaccharide nodulation signal of *Bradyrhizobium japonicum*. *J Bacteriol* **176:** 620–633.

Staehelin, C., Schultze, M., Kondorosi, E., Mellor, R.B., Boller, T., and Kondorosi, A. (1994) Structural modifications in *Rhizobium meliloti* Nod factors influence their stability against hydrolysis by root chitinases. *Plant J* **5:** 319–330.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Promé, J.-C., and Dénarié, J. (1991) Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. *Nature* **351:** 670–673.

Wacek, T.J., and Brill, W.J. (1976) Simple, rapid assay for screening nitrogen-fixing ability in soybean. *Crop Sci* **15:** 519–523.

Wijffelman, C.A., Pees, E., van Brussel, A.A.N., Okker, R.J.H., and Lugtenberg, B.J.J. (1985) Genetic and functional analysis of the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. *Arch Microbiol* **143:** 225–232.