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Nod Factors from *Sinorhizobium saheli* and *S. teranga* bv. *sesbaniae* Are Both Arabinosylated and Fucosylated, a Structural Feature Specific to *Sesbania rostrata* Symbionts

Jean Lorquin,[1,2] Gilles Lortet,[1,3] Myriam Ferro,[2] Nathalie Méar,[1] Bernard Dreyfus,[1] Jean-Claude Promé,[2] and Catherine Boivin[1]

[1]Laboratoire de Microbiologie, ORSTOM, BP. 1386, Dakar, Sénégal; [2]Institut de Pharmacologie et de Biologie structurale, CNRS, 205 Route de Narbonne, 31077 Toulouse, France; [3]Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, INRA-CNRS, BP27, 31326 Castanet-Tolosan Cedex, France

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***Sesbania* spp. can establish symbiotic interactions with rhizobia from diverse taxonomical origins, including the newly described *Sinorhizobium saheli* and *S. teranga* bv. *sesbaniae*, in addition to the *Sesbania rostrata*–specific symbiont *Azorhizobium caulinodans*. These different rhizobia exhibit a narrow host range, which is limited mainly to *Sesbania* spp. Nod factors from overproducing strains of *S. saheli* ORS611 were purified and their structures determined. Remarkably, the terminal reducing glucosamine of most compounds was found to bear both an arabinosyl group on C-3 and a fucosyl substitution on C-6. Other structural features are as follows: Nod factors are mainly chitopentameric compounds, *N*-methylated, *O*-carbamoylated and *N*-substituted either by a $C_{18:1}$ or a $C_{16:0}$ acyl chain at their nonreducing end. Nod factors from an overproducing strain of *S. teranga* bv. *sesbaniae* ORS604 were found to be identical to those of *S. saheli* on the basis of high-pressure liquid chromatography separation and liquid secondary ion mass spectrometry analysis. The Nod factor double glycosylation, i.e., arabinosylation and fucosylation, which appears to be unique to *Sesbania* symbionts, probably reflects a high selection pressure from *Sesbania* host plants.**

Additional keywords: nitrogen fixation.

Symbiotic soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Sinorhizobium*, collectively referred to as rhizobia, elicit on their leguminous hosts the formation of specialized organs called nodules, in which atmospheric nitrogen fixation occurs. This symbiosis is specific since each rhizobium has a defined plant host range. Infection, nodulation, and host specificity are largely controlled by signal exchanges between the two symbionts. Legumes secrete phenolic inducing compounds (mainly flavonoids) that regulate the expression of the bacterial nodulation genes (the *nod*, *nol*, and *noe* genes) (Fellay et al. 1995). In turn, these genes are involved in the production of signal molecules, the Nod factors, that trigger the nodule developmental program (reviewed in Fisher and Long 1992; Dénarié and Cullimore 1993).

All Nod factors identified so far are β-1,4-linked *N*-acetyl-D-glucosamine oligomers varying in length from 3 to 6 sugars, mono-*N*-acylated at the nonreducing end, and diversely substituted on the two terminal glucosamine residues. Modifications frequently found include (i) an *N*-methyl group, an *O*-acetyl group, carbamoyl groups, and different fatty acid groups on the terminal nonreducing sugar, and (ii) an acetyl or a sulfate group or several sugars such as arabinose, fucose, or 2-*O*-methyl fucose that can be either acetylated or sulfated on the O-6 position, on the terminal reducing glucosamine. Occasionally, the terminal glucosaminyl residue can be glycosidically linked to glycerol or to a neutral sugar. The particular combination of different substitutions, which depends on species and/or strains, makes the core signaling molecules plant specific (Schultze et al. 1994; Fellay et al. 1995; Dénarié et al. 1996). Indeed, the purified Nod factors induce on host roots the same type of biological responses as do the corresponding rhizobium strains: e.g., root induction and deformation, cortical cell activation, and nodule formation (Mylona et al. 1995; Heidstra and Bisseling 1996). By acting as mediators in plant morphogenesis, these lipo-chitooligosaccharides may represent a new class of plant growth regulators (Dénarié et al. 1996).

The aquatic, tropical legume *Sesbania rostrata*, which exhibits high nitrogen accumulation rates, has become one of the most valuable legumes in lowland rice intercropping (Ndoye and Dreyfus 1988; Ladha et al. 1992). This African legume is known to establish a highly specific interaction with the atypical rhizobium species *Azorhizobium caulinodans* (Dreyfus et al. 1988; de Bruijn 1989; Boivin et al. 1997b), which induces effective nodules on both the root and stem of its homologous host (Tsien et al. 1983; Duhoux 1984). An overproducing

Corresponding author: C. Boivin; E-mail: boivinc@belair.orstom.sn

Present address of C. Boivin and B. Dreyfus: LSTM, Campus de Baillarguet, BP 5035, 34 032 Montpellier Cedex, France.

strain derived from *A. caulinodans* ORS571 was shown to produce Nod factors bearing an unusual substitution, a D-arabinose branch, at the reducing terminal end, which is presumably involved in determining its host specificity (Mergaert et al. 1993). Recently, it was shown that *S. rostrata* and other *Sesbania* spp. can also enter into symbiosis with bacteria belonging to the recently described species *Sinorhizobium saheli* and *S. teranga* bv. *sesbaniae* (de Lajudie et al. 1994; Lortet et al. 1996). *S. teranga* and *S. saheli* belong to the *R. meliloti–R. fredii* branch, and all of these species have recently been proposed to be placed in the genus *Sinorhizobium*, which is phylogenetically distant from *A. caulinodans*, a *Xanthobacter*-related bacterium (Dreyfus et al. 1988). The genetically dissimilar *Sesbania* symbionts *A. caulinodans*, *S. saheli*, and *S. teranga* bv. *sesbaniae* are of special interest in addressing the question of whether Nod factor structure is related to host range or to bacterial taxonomy. This paper describes the purification and the determination of the structure of Nod factors from *S. saheli* ORS611 and *S. teranga* bv. *sesbaniae* ORS604 strains isolated from *Sesbania grandiflora* and *S. aculeata*, respectively. Major compounds were found to exhibit a peculiar structural feature, i.e., the double glycosylation of the reducing end. This result, together with the recent structural determination of Nod factors produced by the *A. caulinodans* wild-type strain (Mergaert et al. 1997), suggests that this unusual feature is specific to *S. rostrata* symbionts.

RESULTS

Host specificity of *S. saheli* ORS611 and *S. teranga* bv. *sesbaniae* ORS604.

S. saheli and *S. teranga* bv. *sesbaniae* share with *A. caulinodans* the ability to nodulate many *Sesbania* spp. (Boivin et al. 1997a). To evaluate and compare the extent of their host range, *S. saheli* ORS611, *S. teranga* bv. *sesbaniae* ORS604, and *A. caulinodans* ORS571 were tested for their ability to nodulate a variety of other tropical legumes. None of the following plants, *Acacia senegal, A. nilotica, A. raddiana, A. albida, Leucaena leucocephala, Neptunia oleacera,* and *Indigofera astragalina,* were nodulated by either ORS611, ORS604, or ORS571 in our laboratory conditions. Delayed induction of pseudonodules by the three strains could be observed on *Macroptilium atropurpureum.* Ineffective or very poorly effective nodules were induced on the widely compatible legume *A. seyal.* Only ORS571 induced a weak and delayed nodulation on *Aeschynomene afraspera* and *Tephrosia purpurea.* From these preliminary results, *S. saheli* ORS611, *S. teranga* bv. *sesbaniae* ORS604, and *A. caulinodans* ORS571 appear to have a similar narrow host range.

Production of Nod factors by *S. saheli* ORS611 and *S. teranga* bv. *sesbaniae* ORS604.

As preliminary attempts to detect Nod factors from the wild-type strains ORS604 and ORS611 with the thin-layer chromatography (TLC) system (Spaink et al. 1992) were unsuccessful, overproducing strains were constructed by introducing pMH682, a multicopy plasmid containing *nodD3* and *syrM* regulatory genes from the related *R. meliloti* (Lortet et al. 1996). Cloned *nodD3-syrM* genes have been shown to direct flavonoid-independent *nod* gene transcription in *R. meliloti* (Maillet et al. 1990). ORS611(pMH682) and ORS604(pMH682) exhibited TLC profiles similar to each other (Lortet et al. 1996). An increased Nod factor production was obtained when overproducing strains were grown in the presence of luteolin, as assessed by the TLC system (data not shown). Indeed, luteolin was identified as a good *nod* gene inducer in *S. saheli* and *S. teranga* (Lortet et al. 1996). Furthermore, high-pressure liquid chromatography (HPLC) profiles of butanol extracts of luteolin-induced cultures were found to be identical for both strains in the Nod factor region (data not shown). Hence, *S. saheli* Nod factors were first studied.

Purification of Nod factors from *S. saheli* ORS611(pMH682).

Large-scale, luteolin-induced cultures of ORS611(pMH682) were extracted with the polymeric XAD-4 resin, since this gave higher Nod factor yields than with butanol extraction. The XAD-4 extract was then fractionated by HPLC with four consecutive purifications, three on a reverse phase column and the fourth on a normal phase column (Fig. 1). Several purifications were necessary to isolate six fractions (I to VI) corresponding to pure Nod factors, as indicated by liquid secondary ion mass spectrometry (LSI-MS). Nod factor production was too weak to develop a valuable and precise HPLC method of quantification of these compounds. Production was thus only approximately evaluated by quantifying the total amount of Nod metabolites eluted in the first C_{18}-HPLC step and this was found to be about 0.1 mg per liter of culture. Nod factors I and VI were present in the largest amounts.

Constituent analysis of Nod factors.

After complete acid hydrolysis, *N*-acetylglucosamine, *N*-methylglucosamine, and arabinose were detected in all the fractions. Fucose was found in fractions I, III, IV and VI (data not shown). Gas chromatography (GC) analysis of the peracetylated (–)-2-butylglycosides assigned *N*-acetylglucosamine, *N*-methyl-glucosamine, and arabinose to the D series, whereas fucose was assigned to the L series (data not shown). A single fatty acid was liberated from each fraction by acid hydrolysis. By comparison of the GC retention time of the fatty acid methyl esters with authentic standards and confirmation of the structures by charge remote fragmentation of the carboxylate anions (Promé et al. 1987), palmitic acid ($C_{16:0}$, fractions I and II) and *cis*-vaccenic acid ($C_{18:1}$, fractions III to VI) were identified (data not shown).

Nod factors are mainly di-substituted on their reducing end.

Analysis of Nod factor–containing fractions by LSI-MS ionization in an acidified matrix led to the formation of the corresponding $(M+H)^+$ ions together with a series of fragments (Figs. 2 and 3). Clear fragmentation of the backbone was seen and peaks were separated by 203 mass units, assigning an *N*-acetylglucosamine oligomer core, characteristic of Nod metabolites and confirming carbohydrate determination studies. The fragmentation series ended at *m/z* 483 (fractions III, IV, V, and VI) or *m/z* 457 (fractions I and II), which was attributed to oxonium ions from the first sugar residue at the nonreducing end. These ions all contained substituents attached to the nonreducing end. The odd number values of these masses indicated the presence of a nitrogen-

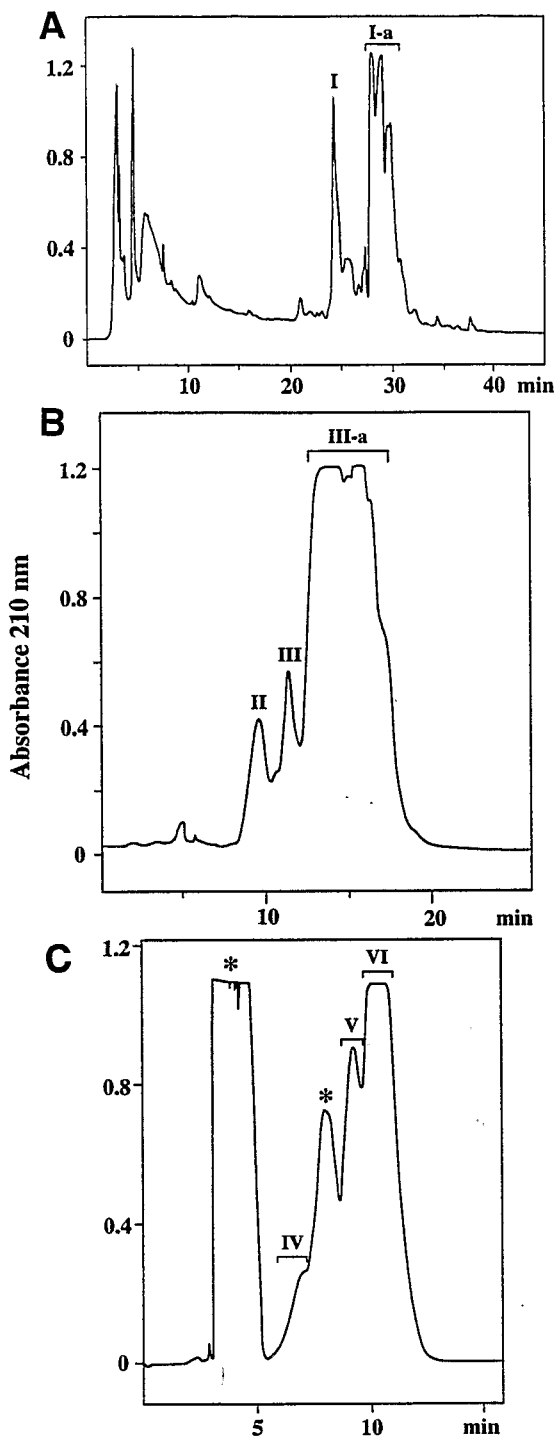


Fig. 1. High-pressure liquid chromatography (HPLC) profiles of XAD-4 extract from the culture supernatant of luteolin-induced *Sinorhizobium saheli* ORS611(pMH682). **A,** Semipreparative C_{18} column eluted with a 30 to 70% aqueous acetonitrile linear gradient for 40 min, at a flow rate of 2 ml/min, after a first separation on a 20 to 100% aqueous acetonitrile gradient (see Materials and Methods). **B,** C_{18}-HPLC profile of fraction I-a (from A) with a 45 to 60% aqueous acetonitrile gradient for 45 min, at a flow rate of 2 ml/min. **C,** Normal phase HPLC separation of fraction III-a (from **B**) with a linear gradient running from 18 to 40% water in acetonitrile for 30 min, on a Carbohydrate OH-100 (4.6 × 250 mm, Alltech, Templeuve, France) column, at a flow rate of 1 ml/min. Peaks I, II, III, IV, V, and VI correspond to Nod factors. * = peaks that do not correspond to a chito-oligomeric sequence, as assayed by liquid secondary ion mass spectrometry.

containing substituent, which is very likely a carbamoyl group, as has been reported for the *Rhizobium* sp. NGR234, *A. caulinodans,* and *S. teranga* bv. *acaciae* Nod metabolites (Price et al. 1992; Mergaert et al. 1993; Lorquin et al. 1997). This hypothesis of a carbamoyl substituent is consistent with the calculated 43-mass-unit difference between these *m/z* values and that of an *N*-methyl-glucosamine residue acylated by each of the two fatty acids identified earlier. Presence of the carbamoyl group was supported by the nuclear magnetic resonance (NMR) spectra of Nod factors I and VI (Fig. 4) showing this residue (δ 6.5) and its corresponding enol form (δ 6.7). Furthermore, examination of the high-mass region of the LSI-MS spectra showed that the most prominent ions were accompanied by a weaker satellite peak 43 mass units less (not shown). Thus, *m/z* 483 and *m/z* 457 indicated a vaccenoyl or palmitoyl substitution, respectively.

Major Nod factors (I and VI) exhibited similar mass fragmentations, differing by 26 mass units, which corresponds to the difference between a $C_{16:0}$ and a $C_{18:1}$ residue situated on the nonreducing end of the molecules (Fig. 3A,B). Both fractions contained chito-pentamers. On the reducing end, protonated molecules of compounds from fraction I appeared at *m/z* 1565, 1433, and 1419 (Fig. 3A). From fraction VI, the most abundant Nod metabolite, $(M+H)^+$ ions are seen at *m/z* 1591 and 1459, with a minor one at *m/z* 1445 (Fig. 3B). It should be noted that the different $(M+H)^+$ ions within fractions I and VI differ from that with the highest mass by either 132 or 146 mass units, values corresponding to the mass of the arabinose or fucose residues, respectively.

Different and less abundant Nod factors were also found. In both fractions II and V, Nod factors were present as almost pure compounds (Fig. 3C,F), whereas fractions III and IV corresponded to a mixture of compounds (Fig. 3D,E). Most of these minor components were easily identified as chitotetramers or pentamers substituted or not either by an arabinose or by both an arabinose and a fucose on their reducing end (see Figure 5). The $(M+H)^+$ ion at *m/z* 1515 (fraction IV, Fig. 3E), however, could not be clearly identified. Constant B/E-linked scan spectra of the parent ion at *m/z* 1515 show a characteristic fragmentation of *N*-acetyl-glucosamine at 1092, 889, 686, and 483, but no clear $(M+H)^+$ ions were observed between 1515 and 1092 (data not shown). Not enough material was available to determine exactly the terminal reducing *N*-acetylglucosamine substitution. Such a mass of *m/z* 1515 could correspond to a Nod factor substituted by an acetylated 2-*O*-methylfucose residue (Price et al. 1992). Surprisingly, we found a $(M+H)^+$ ion of *m/z* 1388 in both fractions III and IV, and an $(M+H)^+$ ion of 1445 in both fractions III and V (Fig. 3D,E,F). These compounds present in two different fractions could differ by the location of arabinose and fucose residues. The very low quantity of minor compounds as well as the difficulty of performing a more efficient purification did not enable us to check this hypothesis.

Location of arabinose, fucose, and carbamoyl residues.

Permethylation of Nod factors I and VI was performed in order to confirm the oligochitin core and to locate L-fucose and D-arabinose residues. $NaBD_4$ reduction followed by permethylation of fraction VI afforded a single compound with a molecular mass of 1848 as determined by LSI-MS (data not shown). This result indicated that (i) the carbamoyl group had

been lost during the chemical process, probably after addition of sodium hydroxide before methylation by iodomethane, and (ii) the hydroxyl and amide groups had been methylated. Acid hydrolysis of $NaBD_4$-reduced and permethylated fractions I and VI, followed by alditol acetate formation, afforded five compounds that were identified by GC-MS as follows: (i) 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-*N*-acetyl-*N*-methylglucosaminitol derived from the glucosamine residue at the nonreducing end of the molecule; (ii) 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-*N*-acetyl-*N*-methylglucosaminitol arising from the three internal residues; (iii) 1-deuterio-3,4,6-tri-*O*-acetyl-1,5-di-*O*-methyl-*N*-acetyl-*N*-methylglucosaminitol derived from a glucosamine residue at the reducing end of the molecule and bearing substituents on the 3 and 6 positions; (iv) 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinositol (furanose form) derived from the D-arabinose residue; and (v) 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucositol (pyranose form) provided by the L-fucose residue (Fig. 6). Therefore, this study confirmed the (1→4) linkage between the five *N*-acetyl-D-glucosamine residues and indicated (1→6) and (1→3) glycosidic linkages between the L-fucose and D-arabinose residues and the reducing *N*-acetyl-D-glucosamine, respectively.

To differentiate the 3 or 6 positions for D-arabinose and L-fucose, enzymatic hydrolysis of Nod factors I and VI with commercial fucosidases was performed. Fucosidase α1-3R had no effect on the main compounds (I and VI). In contrast, fucosidase α1-6 allowed us to recover the corresponding arabinosylated (defucosylated) molecule, with a yield of 100%. Mass spectra obtained after the enzymatic conversions were quite similar to Figure 3C and F for the defucosylated Nod factors I and VI, respectively. These results indicate that the fucose residue was located on O-6, and consequently arabinose was on O-3. Furthermore, they also indicated an α configuration for the anomeric proton of fucose (see Figure 5A).

To locate the carbamoyl residue on the nonreducing end, permethylation was performed by adding NaOH and iodomethane simultaneously in order to retain the carbamoyl group (Lopez-Lara et al. 1995a). No new di-*O*-methylated glucosamine derivative was detected and it is tempting to propose that the carbamoyl group was on O-4. However, the same methylation procedure performed on model compounds that are *O*-carbamoylated on O-6 (Mergaert et al. 1993) led to the partial removal of this substituent and the yield of the expected 3,4-di-*O*-methyl derivative was very low. Thus, another method must be used to reliably locate carbamoyl groups on the sugar backbone.

Proton NMR analysis.

Proton NMR spectra of Nod factors I and VI were recorded to determine the anomeric configurations and to confirm the results obtained by mass spectrometry and chemical analysis. The proton NMR spectrum of Nod factor VI, NodSs-V($C_{18:1}$, N-Me, Carb, Ara, Fuc), is shown in Figure 4. The resonance at δ 4.82 ($J_{H1,2}$ = 2.9 Hz) is consistent with that reported for the anomeric proton of the reducing α-*N*-acetylglucosamine of other Nod metabolites (Lerouge et al. 1990; Spaink et al. 1991; Roche et al. 1991; Bec-Ferté et al. 1994). The resonances between δ 4.35 and 4.40 ($J_{H1,2}$ = 8.2 Hz) are due to the anomeric protons of the β-linked *N*-acetylglucosaminosyl residues as reported for other Nod metabolites (Lerouge et al. 1990; Spaink et al. 1991; Roche et al. 1991; Bec-Ferté et al. 1994). The resonance at δ 4.88 (as a broad singlet) is consistent with an α-linked fucose residue (Altona and Haasnoot 1980) and is identical to that reported for the Nod metabolite from *Bradyrhizobium japonicum* (Sanjuan et al. 1992; Carlson et al. 1993). The anomeric proton of D-arabinofuranose was seen as a broad singlet at δ 5.33 ($J_{H1,2}$ = 0 Hz) and corresponded to an α configuration. Indeed, in aldofuranose derivatives in which H1 and H2 are *trans* and in quasi-equatorial positions (see Figure 5A), the dihedral angle close to 90° induced a very small coupling constant ($J_{H1,2}$ = 0 to 2 Hz) (Rudrum and Shaw 1965; Angyal and Pickles 1972). The sharp singlet at δ 5.23 ($J_{H1,2}$ ≈ 0) was attributed to the vinal protons, since their chemical shift is generally found around this value and their response must be twice that of the arabi-

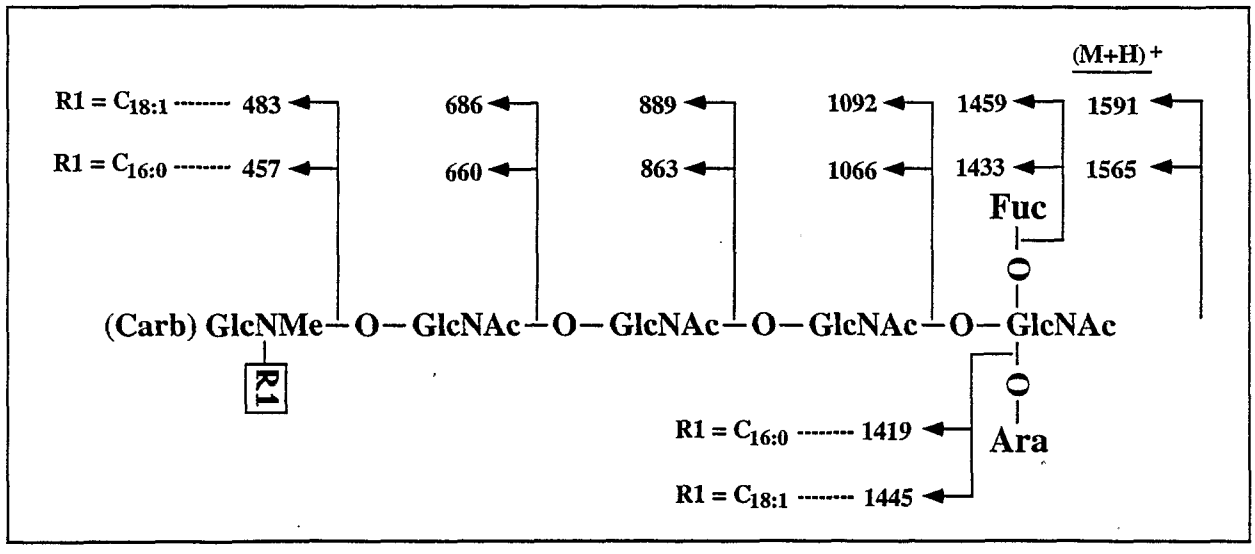


Fig. 2. Fragmentation patterns corresponding to the main ions observed in positive liquid secondary ion mass spectrometry (see Figure 3). Fragmentation pattern of minor Nod metabolites is similar except that for tetramers, a 203 mass unit corresponding to one *N*-acetylglucosamine residue is to be substracted from parent ions mass.

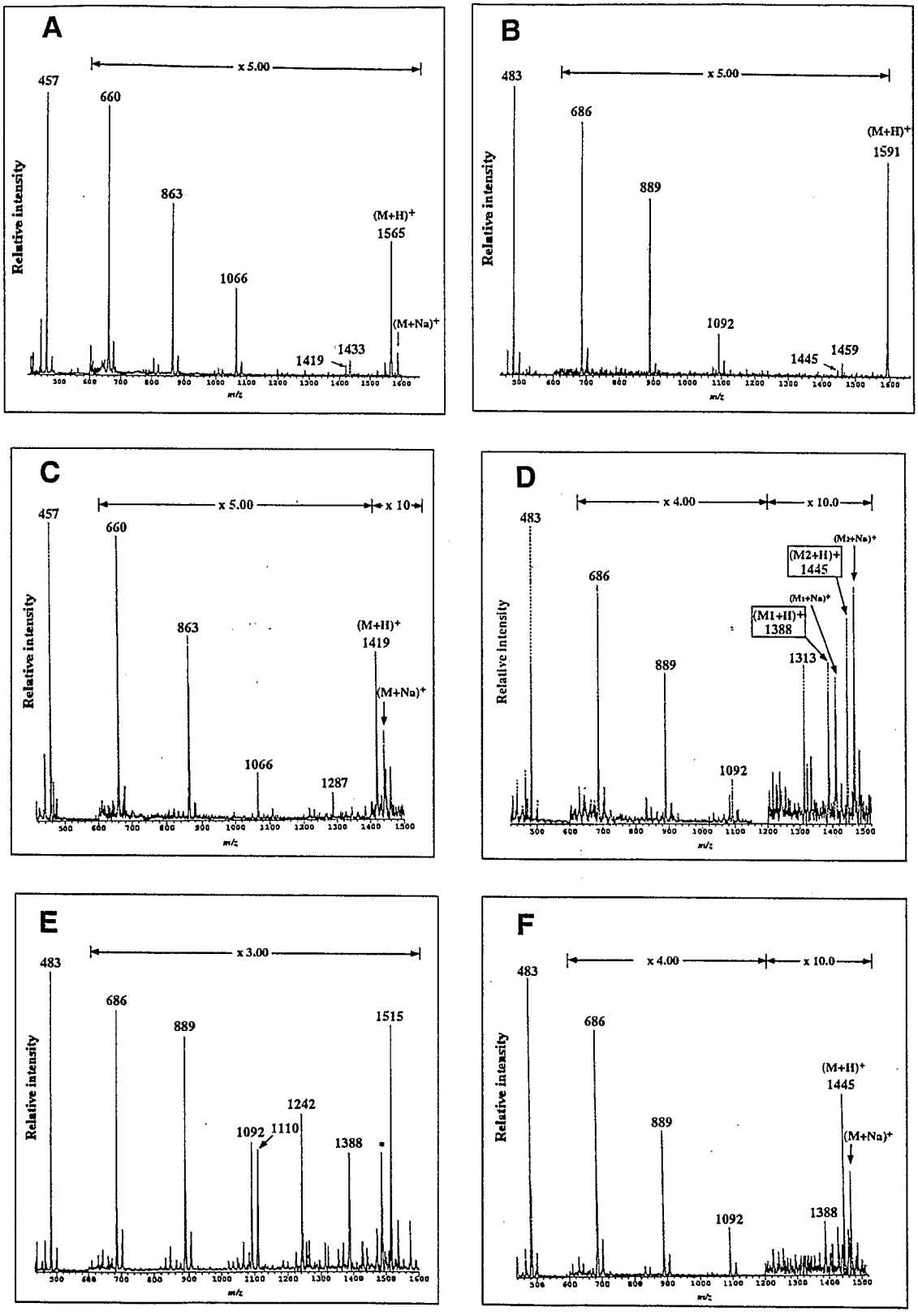


Fig. 3. Positive ion liquid secondary ion mass spectra of the high-pressure liquid chromatography fractions from *Sinorhizobium saheli* ORS611(pMH682). Matrix was a 1:1 mixture of glycerol and *m*-nitrobenzyl alcohol spiked with 1% trichloroacetic acid. For peak assignments see Figure 5. **A,** Nod factor I. **B,** Nod factor VI. **C,** Fraction II. **D,** Fraction III. **E,** fraction IV. * = peak due to an unidentified contaminant. **F,** fraction V. Presence of a weak compound ([[M+H]$^+$ ion at *m/z* 1388) was observed in this fraction.

nose anomeric protons. The coupling constant of these vinal protons is small, indicating a *cis* configuration as has already been observed in the same solvent conditions (Carlson et al. 1993; Sanjuan et al. 1992). Other signals, classically described for the NMR spectra of nodulation factors, were also identified (Fig. 4), particularly the characteristic *N*-methyl protons (δ 2.7), and the *N*-acetyl protons (δ 1.81).

***S. teranga bv. sesbaniae* ORS604(pMH682) and *S. saheli* ORS611(pMH682) produce identical Nod factors.**

For comparison, Nod metabolites produced by ORS604 (pMH682) and ORS611(pMH682) were partially characterized after butanol extraction and HPLC separation (see Materials and Methods). These strains exhibited similar HPLC profiles. The six fractions obtained for each strain were analyzed by LSI-MS and each fraction was found to correspond to the same compound for each strain (data not shown). As with ORS611(pMH682), the most abundant Nod metabolites from ORS604(pMH682) were found to be NodSt-V($C_{16:0}$, N-Me, Carb, Ara, Fuc) and NodSt-V($C_{18:1}$, N-Me, Carb, Ara, Fuc). Other Nod factors were also present as minor compounds in butanol extracts and were found to be identical to those summarized in Figure 5B. In addition, we found a Nod metabolite in both strains that exhibited an $(M+H)^+$ at *m/z* 1110 (data not shown) and that corresponds to a nonsubstituted tetramer with the following nomenclature: NodSs (or St)-IV($C_{18:1}$, N-Me, Carb). More of this compound was produced at 30 than at 37°C. An increase of the culture temperature may activate arabinosyl and fucosyl transferases and thus be responsible for such a difference in Nod factor production. These results indicate that strains ORS611(pMH682) and ORS604(pMH682) synthesize identical Nod factors, thus confirming the previous TLC profile studies (Lortet et al. 1996).

DISCUSSION

As the wild-type strains *Sinorhizobium saheli* ORS611 and *S. teranga* bv. *sesbaniae* ORS604 both produced very low amounts of Nod factors, we used overproducing strains to enable structural studies. These strains were constructed by introducing the regulatory genes *nodD3* and *syrM* from the related *R. meliloti*, cloned on the multicopy plasmid pMH682 (Lortet et al. 1996). Despite this, Nod factor secretion by overproducing strains remained low and production yield was about 0.1 mg/liter.

The *S. saheli* derivative strain ORS611(pMH682) synthesizes a population of Nod factor molecules that are mainly pentameric chito-oligomers, substituted on their nonreducing

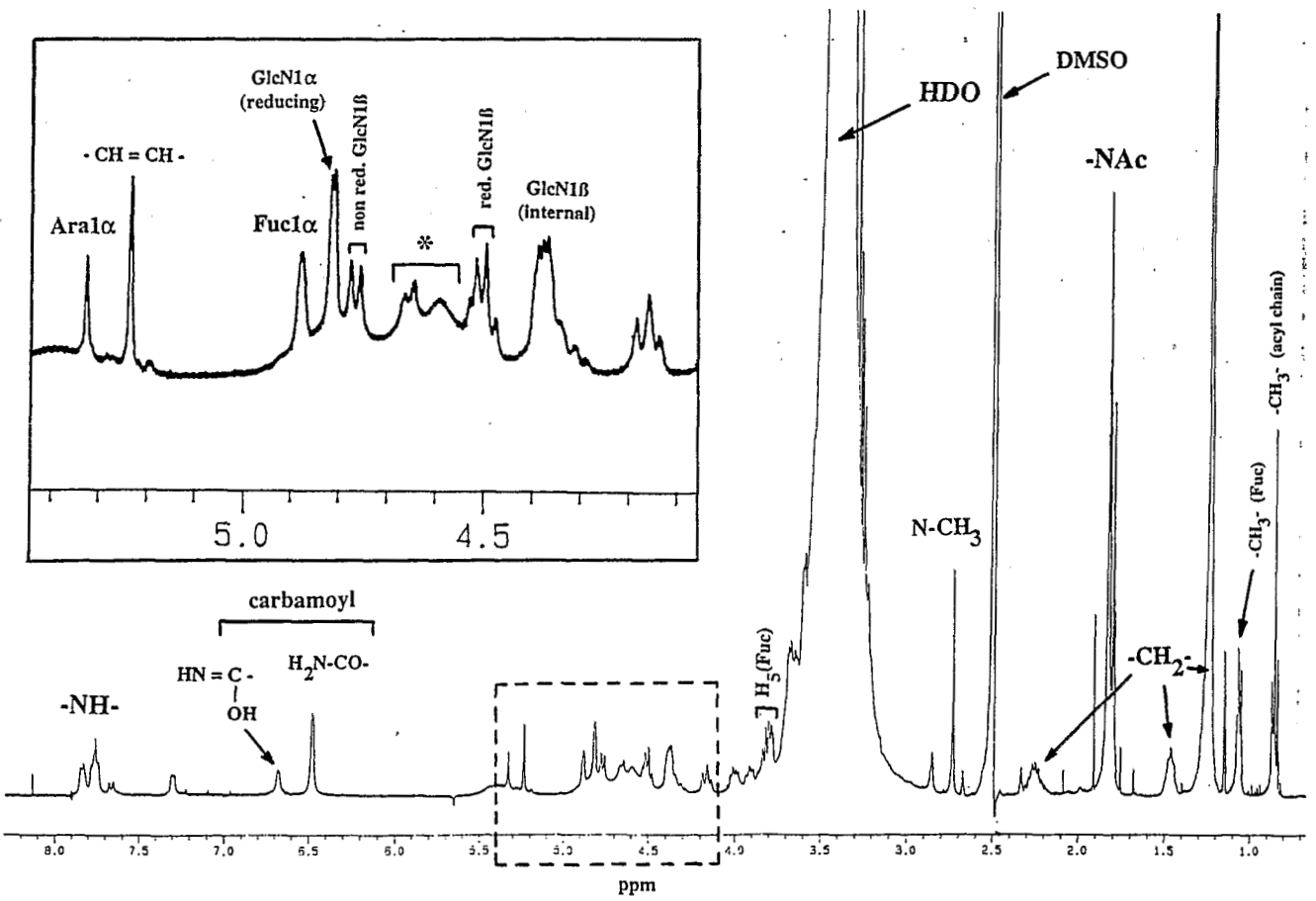
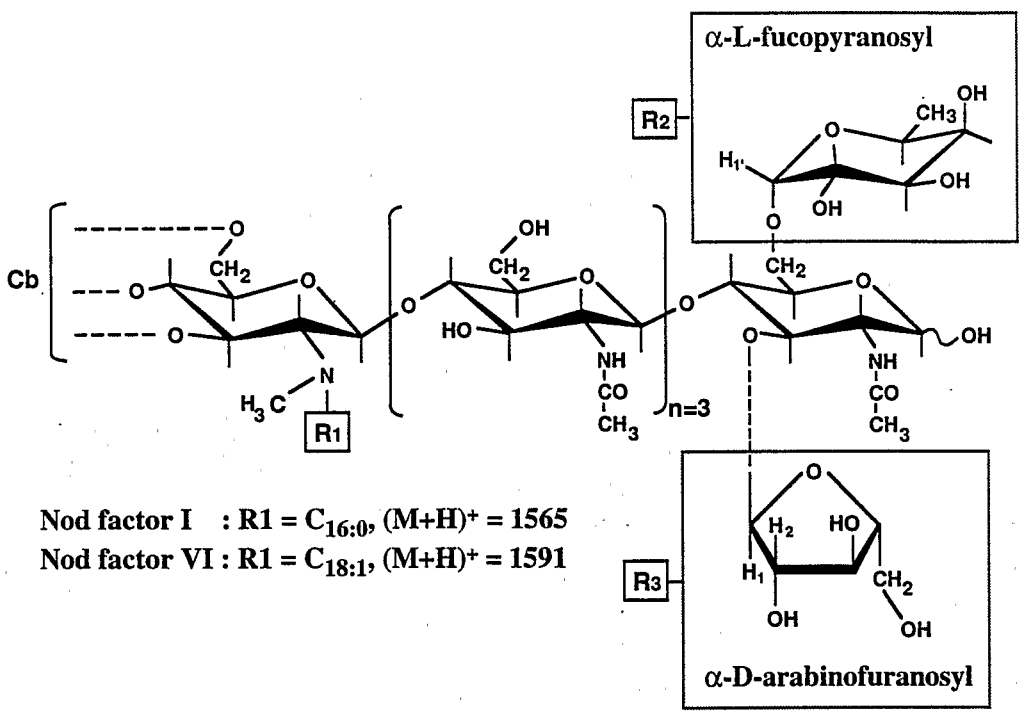


Fig. 4. Proton nuclear magnetic resonance spectrum of Nod factor VI (NodSs-V($C_{18:1}$, NMe, Carb, Ara, Fuc) purified from strain ORS611(pMH682) Insert shows the anomeric proton (4.3 to 5.4 ppm). Resonances due to the methylene (-CH_2-) and methyl groups (CH_3) of the fatty acyl residue, *N*-acety groups are as indicated. Response of the vinal protons (δ 5.23) is twice that of the arabinofuranose (δ 5.33) or fucopyranose (δ 4.88) anomeric protons Fuc, fucose; Ara, arabinose; GlcN, *N*-acetylglucosamine; Ac, acetyl; DMSO, dimethylsulfoxyde. * = peaks due to unidentified contaminants.

end by *N*-methyl and *O*-carbamoyl groups, and *N*-acylated by either vaccenic ($C_{18:1}$) or palmitic ($C_{16:0}$) acids. The reducing *N*-acetyl-glucosaminyl end is glycosylated either by both D-arabinosyl and L-fucosyl residues or only by a D-arabinosyl residue. Respective positions of the two sugar substitutions have been determined for Nod factors I (NodSs-V($C_{16:0}$, N-Me, Carb, Ara, Fuc)) and VI (NodSs-V($C_{18:1}$, N-Me, Carb, Ara, Fuc)). With fucosidase α1-6R, it was demonstrated that the fucosyl residue is on O-6 and therefore arabinose is on O-3 in the double branched molecule (Fig. 5A). Because of the scarcity of the corresponding molecules, no attempts to locate the arabinosyl residue in mono-branched molecules were made. The structures of Nod factors produced by *S. teranga* bv. *sesbaniae* ORS604 appear to be identical to those of *S. saheli* ORS611, as evaluated by HPLC separation and LSI-MS analysis.

A



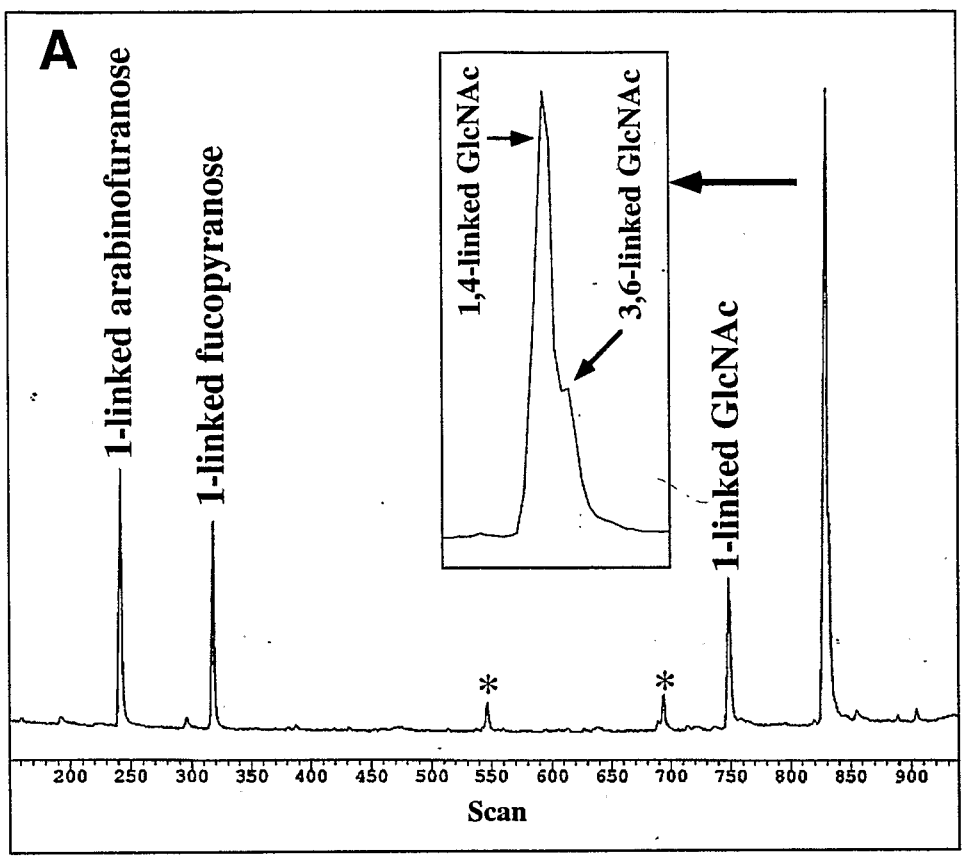
Nod factor I : R1 = $C_{16:0}$, $(M+H)^+$ = 1565
Nod factor VI : R1 = $C_{18:1}$, $(M+H)^+$ = 1591

B

Nod Metabolite	HPLC fraction	n	R1	R2	R3	$(M+H)^+$
NodSs-V($C_{16:0}$, N-Me, Carb, Ara)	II	3	$C_{16:0}$	Ara/H [a]	H/Ara [a]	1419
NodSs-V($C_{18:1}$, N-Me)		3	$C_{18:1}$	H	H	1313
NodSs-IV($C_{18:1}$, N-Me, Carb, Ara, Fuc)	III	2	$C_{18:1}$	Ara/Fuc [a]	Fuc/Ara [a]	1388
NodSs-V($C_{18:1}$, N-Me, Carb, Ara)		3	$C_{18:1}$	Ara/H [a]	H/Ara [a]	1445
NodSs-V($C_{18:1}$, N-Me)		3	$C_{18:1}$	H	H	1110
NodSs-IV($C_{18:1}$, N-Me, Carb, Ara)	IV [b]	2	$C_{18:1}$	Ara/H [a]	H/Ara [a]	1242
NodSs-IV($C_{18:1}$, N-Me, Carb, Ara, Fuc)		2	$C_{18:1}$	Ara/Fuc [a]	Fuc/Ara [a]	1388
NodSs-V($C_{18:1}$, N-Me, Carb, Ara)	V	3	$C_{18:1}$	Ara/H [a]	H/Ara [a]	1445

Fig. 5. Chemical structures of Nod metabolites from *Sinorhizobium saheli* ORS611. Location of carbamoyl group in the metabolites is not determined and could be at C-3, C-4 or C-6. **A,** Two major Nod factors. **B,** Minor Nod factors. Nomenclature is as previously described (Roche et al. 1991). [a] Position of arabinose and fucose was not determined because of the too low quantity of compounds. [b]In this fraction, we found a Nod metabolite with a $(M+H)^+$ ion at *m/z* 1515 that has not clearly been identified (see text). Ss, *Sinorhizobium saheli*; Carb, carbamoyl; Fuc, fucose; Ara, arabinose.

The Nod factors from *S. saheli* and *S. teranga Sesbania*-nodulating strains exhibit two particular features that make them very unusual: (i) the terminal reducing glucosamine of major compounds is glycosylated with two different sugars, L-fucose and D-arabinose, the latter one being an unusual substitution only encountered in *A. caulinodans* (Mergaert et al. 1993); and (ii) a substitution is present on atom C-3 of the reducing glucosaminyl residue. These characteristics have only been described in *A. caulinodans*. Indeed, Nod factors from the wild-type strain *A. caulinodans* ORS571 were character-



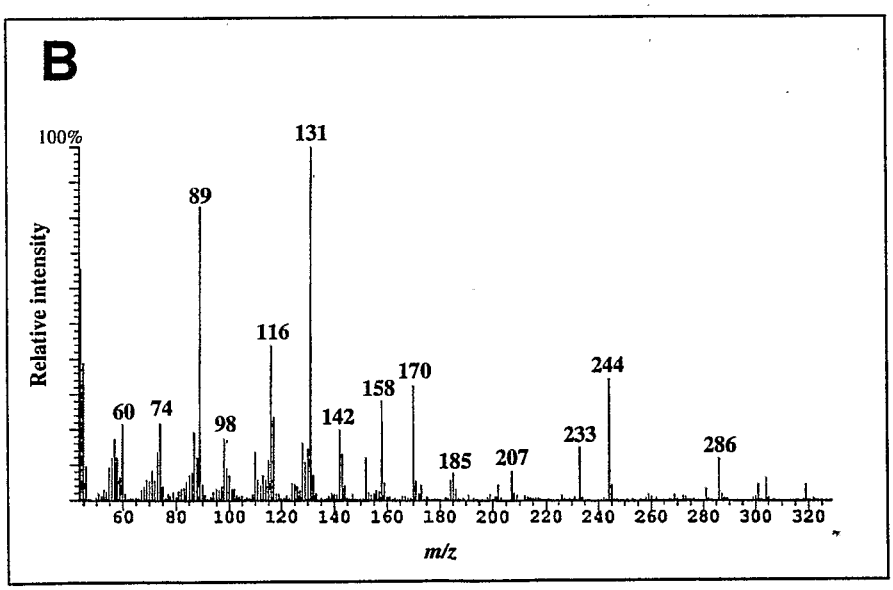


Fig. 6. Analysis of partially methylated alditol acetates arising from Nod factor VI. **A,** Total ion current-gas chromatogram (electron impact mode) Linkages of different monosaccharides within lipochitooligomers are labeled. * = two peaks that are due to contaminants. Insert represents a larger view of the major peak that shows the presence of a 3,4,6-tri-*O*-acetyl-1,5-di-*O*-methyl compound (O-3 and O-6 substitution). **B,** Mass spectrum of this compound and its characteristic fragmentation.

ized in a recent study and the major compounds were also found to bear both an arabinosyl group on C-3 and a fucosyl substitution on C-6 at their reducing end (Mergaert et al. 1997). This structure is different from that of previously identified Nod factors of *A. caulinodans*, which were first reported to only harbor a D-arabinose branch in position C-6 of the reducing glucosaminyl residue (Mergaert et al. 1993). The *A. caulinodans* strain originally used for chemical analysis was a genetically modified strain ORS571(pRG70), containing some multicopy structural *nod* genes. Differences in Nod factor structures observed between ORS571 and ORS571(pRG70) are presumably caused by gene dosage effects, since the *nolK* locus, involved in azorhizobial Nod factor fucosylation (Mergaert et al. 1996), was absent in pRG70. However, Nod factors from ORS604(pMH682) and ORS611(pMH682) should be close in structure to those produced by wild-type strains, as plasmid pMH682 contains two regulatory nodulation genes, *nodD3* and *syrM,* but no structural *nod* genes. Multicopy *nodD3* and *syrM* genes were demonstrated to be involved in the biosynthesis of particular Nod factors *N*-acylated by (ω–1)-hydroxylated fatty acids in *R. meliloti* (Demont et al. 1994). Such acyl moieties were not detected in this study.

Unlike other tropical rhizobia, *S. saheli* ORS611, *S. teranga* bv. *sesbaniae* ORS604, and *A. caulinodans* ORS571 have a narrow host range (this study) and share the ability to nodulate a variety of *Sesbania* spp. (Boivin et al. 1997a), which is an unusual property among tropical rhizobia (Lewin et al. 1987). Moreover, these three rhizobial species can effectively stem-nodulate *S. rostrata* (Tomekpe et al. 1996; Boivin et al. 1997a). This strongly suggests that both Nod factor arabinosylation and fucosylation, specifically found in *Sesbania* symbionts, are structural requirements for the nodulation of these tropical legumes. As *A. caulinodans* was recently shown to be highly specific to the aquatic legume *S. rostrata*, while *S. saheli* and *S. teranga* are effective symbionts of all *Sesbania* spp. (Boivin et al. 1997a), these particular Nod factor structures may be especially involved in *S. rostrata* nodulation. Such a hypothesis is supported by the fact that glycosylated Nod factors are biologically more active on *S. rostrata* than unglycosylated molecules, as assessed by a root hair formation assay (Mergaert et al. 1997). Quantitative biological activity tests, performed on *S. rostrata* and various *Sesbania* spp. with the different purified molecules produced by the *Sesbania* symbionts, together with nodulation behavior analysis of *nod* mutants, should determine the exact structural requirements for the nodulation of these tropical legumes.

In addition to *S. teranga* bv. *sesbaniae,* the species *S. teranga* consists of another biovar, *S. teranga* bv. *acaciae,* which contains *Acacia* isolates unable to nodulate *Sesbania* spp. (Lortet et al. 1996). The recent determination of the chemical structure of the Nod factors produced by *S. teranga* bv. *acaciae* (Lorquin et al. 1997) shows that they are chitopentameric compounds, *N*-methylated, *O*-carbamoylated, and *N*-substituted either by a $C_{18:0}$, $C_{18:1}$, or $C_{16:0}$ acyl chain at their nonreducing end and partially sulfated on their reducing glucosamine. The Nod factors of *S. teranga* bv. *sesbaniae* thus mainly differ from those of *S. teranga* bv. *acaciae* in the specific decoration of the reducing end. Structural variations within a rhizobial species have already been described in *R. leguminosarum. R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* Nod factors differ in the length and unsaturation of the fatty acyl groups at the nonreducing end (Spaink et al. 1995). *R. leguminosarum* bv. *viciae* produces Nod factors that contain common fatty acyl groups or a highly unsaturated group ($C_{16:4}$), while *R. leguminosarum* bv. *trifolii* produces molecules that contain a complex mixture of other unsaturated acyl groups ($C_{18:3}$, $C_{20:3}$, $C_{20:4}$) instead of the $C_{18:4}$ fatty acyl (Spaink et al. 1991, 1995). The host specificity of these two biovars was shown to be determined by the hydrophobicity of the highly unsaturated fatty acyl moieties of the Nod factors (Spaink et al. 1995). Moreover, in *R. leguminosarum* bv. *viciae* strain TOM, the specific acetylation of the Nod factor terminal reducing glucosamine has been correlated with the ability to nodulate the pea cultivar Afghanistan (Firmin et al. 1993).

Similarities in Nod factors of taxonomically different symbionts of the same legume have also been observed in the cases of *Acacia* trees and soybean. The *Acacia* nodulating strains S. *teranga* bv. *acaciae, Rhizobium* sp. ORS1001, *Rhizobium* sp. GRH2, and *R. tropici,* originating from Africa or America, all produce Nod factors that are partially sulfated at their reducing end, and substituted by a common fatty acyl group and *N*-methylated at their nonreducing end (Lopez-Lara et al. 1995b; Lorquin et al. 1997). The presence or absence of an *O*-carbamoyl group on the nonreducing glucosamine represents the major structural variation within *Acacia* symbionts. In the same way, the soybean symbionts *B. japonicum, B. elkanii,* and *R. fredii* all produce Nod factors substituted by a methyl fucose or a fucose at the reducing end (Sanjuan et al. 1992; Carlson et al. 1993; Bec-Ferté et al. 1994). Additional molecules bearing a combination of diverse substitutions, i.e., *O*-carbamoyl, *N*-methyl, or *O*-acetyl, can also be found, especially in the case of the broad host range *B. elkanii* (Carlson et al. 1993; Stokkermans et al. 1996). It is interesting to note that the very low structural Nod factor variability within *S. rostrata* symbionts, compared with that in *Acacia* or soybean strains, correlates with their high host specificity. The structural heterogeneity inside the species *S. teranga*, together with the similarities of Nod factors produced by the different *Sesbania* nodulating species, thus represents a new and very clear illustration of the close relationship between Nod factor structure and host specificity, regardless of the taxonomy of the bacterial symbiont.

Given the specificity and the range of active concentrations of Nod factors, high affinity receptors of specific Nod factors are hypothesized to be involved in the recognition mechanism between rhizobia and legumes (Dénarié and Cullimore 1993). It was suggested that, in most cases, the acyl moiety permits diffusion and insertion of signaling molecules in the host membrane while other substitutions may be important in receptor activation (Dénarié et al. 1996). The fact that *S. saheli, S. teranga* bv. *acaciae,* and *A. caulinodans,* which are all isolated from *Sesbania* nodules and are able to both stem and root nodulate *S. rostrata,* harbor the same unusual substitutions on the reducing glucosamine, reveals the importance of the Nod factor reducing end for their recognition in the *S. rostrata*–rhizobia tropical symbioses.

Diverse genetic mechanisms may lead to the correct combination of *nod* genes allowing the synthesis of the Nod factors required for the nodulation of a defined legume. Rhizobia in the soil undergo various genetic events such as duplications, mutations, and deletions. Moreover, horizontal gene transfer

can occur in the field, as was demonstrated in the case of *Lotus* rhizobia (Sullivan et al. 1995). Acquisition of *nod* genes by horizontal transfer in the *Xanthobacter*-related *A. caulinodans* is supported by the fact that *nod* regions are flanked by repetitive elements homologous to insertion sequences (Goethals et al. 1992; Geelen et al. 1995), and are characterized by a guanine-cytosine content different from the overall guanine-cytosine content of the chromosome (Dreyfus et al. 1988). Since *Sesbania* sinorhizobia and azorhizobia originate from the same tropical area in which *S. rostrata* is endemic, it is tempting to speculate that *nod* gene horizontal transfer occurred between these two genera. Surprisingly, *S. saheli* ORS611 and *S. teranga* ORS604 lack the azorhizobial *nodZ* sequence, as determined by Southern hybridization (Lortet et al. 1996), indicating a great divergence in the genes responsible for Nod factor fucosylation in *A. caulinodans* and *Sesbania* sinorhizobia. Plant nodulation probably proceeds through the selection of Nod factors derived from various genetic events in rhizobia, thus explaining the Nod factor structural convergence of taxonomically different symbionts of the same legume.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media.

Wild-type strains of *S. teranga* bv. *sesbaniae* ORS604, *S. saheli* ORS611, and *A. caulinodans* ORS571 were originally isolated in Senegal from *S. grandiflora* root nodules, *S. aculeata* root nodules (de Lajudie et al. 1994), and *S. rostrata* stem nodules (Dreyfus et al. 1988) respectively. Strains used for Nod factor purifications were derived from streptomycin-resistant mutants of strains ORS604 and ORS611, respectively (Lortet et al. 1996) by introducing pMH682, a tetracycline-resistant IncP vector containing *nodD3* and *syrM* genes from *R. meliloti* SU47 (Honma et al. 1990). The complete media for the growth of *Sinorhizobium* and *Azorhizobium* strains were yeast extract–mannitol medium (de Lajudie et al. 1994) and lactate medium (Dreyfus et al. 1983), respectively. The minimal medium for Nod factor production (V medium) was as described by Roche et al. (1991). Tetracycline (5 μg/ml) was added when required (strains harboring pMH682).

TLC detection.

An overnight culture was diluted in fresh V medium to an A_{600} of 0.02, in 2.5 ml of final volume. 10 μCi of [^{14}C]acetate (56 mCi/mmol, Amersham, les Ulis, France) was added. Cells were simultaneously noninduced or induced with 10 μM luteolin and incubated at 37°C for 24 h. Supernatants were extracted with 1-butanol and applied on C_{18} coated-silica TLC plates (Sigma, St. Louis, MO) with acetonitrile/water (1:1) as solvent and revealed by autoradiography as described by Spaink et al. (1992).

Purification of Nod factors.

Cells were cycled through two successive precultures at 30°C in V medium, prior to initiation of cultures in the same medium. Luteolin (10 μM) was added and cultures were grown at 37°C. A total of 60 liters of culture from each strain was used. When cultures reached an A_{600} of 1.0 to 1.2, cells were pelleted by centrifugation (8,000 × *g*, 45 min). Supernatants were extracted with XAD-4 resin (Sigma) as previously described (Bec-Ferté et al. 1994). Products in water were further washed with ethyl acetate (Roche et al. 1991) and concentrated by evaporation, and the solution was clarified through 0.22-μm filters. For HPLC separations, UV absorbance was monitored at 210 nm, and LSI-MS was used to detect the presence of Nod factors in the collected fractions. These separations were first carried out on a semipreparative C_{18} reverse phase column (7.5 × 250 mm, Spherisorb ODS2, 5 μm, Alltech, Templeuve, France), with an isocratic step of 15 min with water/acetonitrile 80:20 (vol/vol), followed by a linear gradient running from this solvent to pure acetonitrile for 50 min, at a 2 ml/min flow rate. A broad region corresponding to the Nod factors (≈55% acetonitrile) was collected and further re-injected twice on the same semipreparative C_{18} column (Fig. 1A,B) and finally on an analytical normal phase column (Fig. 1C). Fractions I to VI were obtained, evaporated, and vacuum dried. To compare structures of Nod factors produced by strains ORS604(pMH682) and ORS611(pMH682), cultures of 7.5 liters were grown at 30°C. Nod factors were extracted with 1-butanol (Roche et al. 1991) and fractionated by a one-step C_{18} HPLC (see first HPLC step above for conditions).

Analytical methods.

HPLC was carried out with a Waters 600E pump, a diode array detector (model 1000S, Applied Biosystems, Foster City, CA), and a Rheodyne injector (model 7125) fitted with a 500-μl loop. GC analyses were performed on a Girdel 30 gas chromatograph and LSI mass spectra were recorded in the positive ion mode on an AutoSpec instrument (Fisons, VG Analytical, Manchester, U.K.), as already described (Poupot et al. 1993; Bec-Ferté et al. 1994). B/E-linked scans were performed to study metastable ion decay in the first field-free region without the use of a collision gas. Glycosyl composition, glycosyl linkages, and residue positions were determined by GC-MS analysis of partially methylated alditol acetates (Bec-Ferté et al. 1994). Proton NMR spectra were collected on a Brüker ARX400 MHz spectrometer (Karlsruhe, Germany). Sample was dissolved in dimethylsulfoxyde-d_6 (99.9% from Serva) as the solvent at a concentration of 1 mg per 0.3 ml.

Constituent determination and chemical modifications on purified Nod factors.

Nod factors were hydrolyzed in 3 N HCl (3 h, 80°C). After extraction with dichloromethane, sugars from the aqueous phase were derivatized as alditol acetates and identified by capillary GC. Fatty acids from the dichloromethane phase were analyzed as methyl esters as already described (Bec-Ferté et al. 1994). Alternatively, 2-butyl glycosides of individual sugars were also prepared and analyzed by capillary GC (Gerwig et al. 1979). For reduction, 500 μl of 10 mg/ml $NaBD_4$ (Aldrich Chimie, St. Quentin Fallavier, France) in ethanol/water (1:1) was added to the purified Nod factor (about 20 to 50 μg) and this mixture was left at room temperature for 2 h. The reaction was quenched with a few drops of acetic acid, then coevaporated three times with 10% acetic acid in methanol and again four times with pure methanol. The residue was then dissolved in water and extracted with 1-butanol. The butanol layer was evaporated and dried under P_2O_5. Acetylation was performed as previously described (Poupot et al. 1993; Bec-Ferté et al. 1994). Permethylation of Nod factors was done according to the method of Ciucanu and

Kerek (1984) with some modifications. About 50 µg of dried $NaBD_4$–reduced sample was redissolved in 1.5 ml of anhydrous dimethylsulfoxyde (Fluka, Buchs, Switzerland). After a few minutes of agitation under argon, a few ground NaOH (previously dried at 100°C) pellets were added. After 30 min (or immediately, if the carbamoyl group was to be retained), 300 µl of iodomethane (Sigma) was added and the mixture was stirred gently. After 10 min, 500 µl more was added. After 10 min of stirring, 1 ml was again added and the solution was left in agitation for 30 min. The reaction was stopped with 1 ml of a sodium thiosulfate solution (100 mg/ml), immediately followed by the addition of 1 ml of chloroform. After shaking, the aqueous layer was removed and the chloroform washed about 10 times with water until the organic layer became clear and evaporated to dryness. Hydrolysis was achieved with a 2 N trifluoroacetic acid solution (2 h, 110°C), and acid was removed by coevaporation with methanol. Partially methylated sugars were then reduced with $NaBH_4$ and acetylated, and the corresponding alditol acetates analyzed by GC-MS for determination of glycosyl composition and glycosyl linkages.

Enzymatic conversions.

To determine the respective position of D-arabinose and L-fucose on the reducing end, purified Nod factors were treated with two commercial fucosidases: fucosidase α1-3,4R from almonds and fucosidase α1-6R from bovine epididymis (Oxford GlycoSystems, Abingdon, U.K.). Digestions were carried out for 20 h at 37°C, with 0.2 U of enzyme per ml at a substrate concentration of about 40 µM in a 50 mM sodium acetate buffer, pH 5.0 containing bovine serum albumin 0.18 µg/ml for fucosidase α1-3,4R and in a 20 mM citrate-phosphate buffer, pH 6.0 for fucosidase α1-6R. Products were directly extracted with 1-butanol. The butanol layer was then evaporated to dryness and the residue analyzed by LSI-MS in order to control products of the enzymatic reaction.

Plant assays.

Seeds were surface sterilized by immersion in concentrated sulfuric acid for 30 to 150 min, washed, and soaked in water for 24 h. Surface-sterilized seeds were germinated at 30°C for 24 to 48 h and then transferred to Gibson tubes containing Jensen slant agar and liquid medium (Vincent 1970). Plants were grown under continuous light (20 W/m^2) at 28°C. Eight plants were tested for each strain. Plants were observed for nodule formation during 6 to 8 weeks, and effectiveness was estimated by visual observation of plant vigor and foliage color.

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