Structures of Nodulation Factors from the Nitrogen-Fixing Soybean Symbiont *Rhizobium fredii* USDA257†

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ABSTRACT: We have isolated and characterized the extracellular Nod factors of *Rhizobium fredii* USDA257, a nitrogen-fixing symbiont of soybean [*Glycine max* (L.)Merr.] and several other legume species. These signals are produced upon exposure to the isoflavone genistein and consist of a series of substituted, β1,4-linked tri-, tetra-, and pentamers of *N*-acetylglucosamine. *N*-Vaccenic acid replaces acetate on the nonreducing residue, and the reducing residue contains α-linked 2-*O*-methylfucose on carbon 6. Small amounts of a fucose-containing tetramer also were present. The Nod factors elicit root-hair deformations on soybean and two other plants at concentrations ranging from 10^{-6} to 10^{-12} M.

Rhizobium and the allied genera *Bradyrhizobium* and *Azorhizobium* are soil dwelling bacteria with the unique ability to invade and fix nitrogen in association with roots of leguminous plants. The nodulation process is characterized by varying degrees of biological specificity (Young & Johnston, 1989). Some rhizobia, including *Rhizobium meliloti, Azorhizobium caulinodans*, and the biovars of *Rhizobium leguminosarum,* nodulate just a few legume species, while organisms such as *Rhizobium* sp. NGR234 have wide host ranges. Conversely, legumes such as alfalfa (*Medicago sativa* L.) are specific in their *Rhizobium* requirements, but others accept diverse types of rhizobia. Thus the garden bean, *Phaseolus vulgaris* L., is symbiotically compatible with *Rhizobium etli* and *Rhizobium tropici* (Martínez-Romero *et al.*, 1991; Segovia *et al.*, 1993), and the soybean, *Glycine max* (L.)Merr., is nodulated by three distinct types of rhizobia: *Rhizobium fredii, Bradyrhizobium japonicum,* and *Bradyrhizobium elkanii* (Keyser *et al.*, 1982; Scholla & Elkan, 1984; Kuykendall *et al.*, 1992). Boundaries sometimes are transgressed, as is the case for strain Or191, which can nodulate both *Medicago sativa* and *P. vulgaris* (Eardly *et al.*, 1992), and *R. fredii,* which can also form nitrogen-fixing nodules on a variety of species, including *P. vulgaris* (Sadowsky *et al.*, 1988).

Although the molecular basis for these complex and overlapping symbiotic interactions is not well understood, nodule initiation depends at least in part on the exchange of molecular signals between the participating symbionts (Dénarié *et al.*, 1992; Fisher & Long, 1992). The expression of a series of *Rhizobium* nodulation genes (*nod* genes) is triggered by flavonoid signals from the plant host (Phillips, 1992) and leads to the biosynthesis of molecules termed Nod factors (Lerouge *et al.*, 1990; Roche *et al.*, 1991a,b; Spaink *et al.*, 1991). These compounds alter the growth of root hairs, the target sites for *Rhizobium* infection, and they may induce nodulin gene expression and the formation of nodule-like meristematic foci (Truchet *et al.*, 1991; vanBrussel *et al.*, 1992; Carlson *et al.*, 1993; Horvath *et al.*, 1993; Relic *et al.*, 1993, 1994; Stokkermans & Peters, 1994). All known Nod factors are modified oligomers of *N*-acetylglucosamine with a long-chain *N*-acyl derivative on the nonreducing glucosamine and one or more *O*-substitutions, including sulfate, acetyl, carbamoyl, and simple or modified monosaccharides. The degree of polymerization, the substitution pattern, and the total number of structurally different molecules that are produced all vary among *Rhizobium* species, and they are believed to help define host specificity of nodulation (Dénarié *et al.*, 1992).

We have become intrigued with *R. fredii*, a recently discovered root-nodule organism from China. This bacterium was first isolated from soybean [*G. max* (L.)Merr.] and its wild progenitor species, *Glycine soja* Sieb. & Zucc. (Keyser *et al.*, 1982), and some strains, typified by USDA257, exhibit a curious and unique blend of symbiotic characteristics. USDA257 has a broad host range for legumes, forming nitrogen-fixing nodules on nearly 20% of 324 species that have been tested (Pueppke and Broughton, unpublished data), but it can only nodulate certain cultivars of its original host, soybean (Keyser *et al.*, 1982; Heron & Pueppke, 1984). This unusual combination of symbiotic competence with diverse legume species yet restricted compatibility with soybean raises thought-provoking questions about the basis for symbiotic specificity and the involvement of Nod factors in the process. The availability of additional types of soybean-nodulating bradyrhizobia adds to the attractiveness of the soybean system as a model.

We show here that flavonoids stimulate *R. fredii* USDA257 to synthesize a family of oligochitin Nod factors that are *N*-substituted with a monounsaturated $C_{18:1}$ acyl chain on the nonreducing glucosamine and containing L-fucose or 2-*O*-methyl-L-fucose on the reducing *N*-acetylglucosamine. Two of these lipooligosaccharide compounds are structurally identical to Nod factors of *B. japonicum.* The compounds

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vary in their abilities to elicit root-hair deformation on three legume species.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. Wild-type *R. fredii* USDA257 (Keyser *et al.*, 1982) and 257B3 (Heron *et al.*, 1989; Krishnan & Pueppke, 1991b) were maintained on yeast extract–mannitol agar (Vincent, 1970). Cells were cycled through two successive precultures at 30 °C prior to initiation of cultures for chemical analysis. Cells for Nod factor production were grown for 20 h at 30 °C; the medium contained the following: mannitol (10 g/L), sodium glutamate (1.1 g/L), K_2HPO_4 (0.22 g/L), NaCl (0.1 g/L), $MgSO_4$ (0.1 g/L), $FeCl_3$ (0.02 g/L), and biotin (0.5 mg/L). Cultures were induced with 15 μM genistein (Krishnan & Pueppke, 1991b) and, when appropriate, labeled with sodium (^{14}C)-acetate or sodium (^{35}S)sulfate at 2.5 μCi/mL.

Detection of Nod Factors. Supernatant solutions from labeled cultures were extracted on a C_{18} reversed-phase Sep-Pak cartridge (Waters Associates). After washing with water, Nod factors were eluted with 5 mL of methanol and concentrated to ca. 100 μL. The methanol extracts were deposited on octadecyl silica reversed-phase TLC[1] plates, which were developed with a 1:1 (v/v) mixture of H_2O and CH_3CN. Radioisotope-labeled compounds were detected by autoradiography on Kodak X-Omat film.

Alternatively, the concentrated methanol extracts were loaded onto a HPLC system fitted with a C_{18} reversed-phase column (Colochrom, 7.5 × 250 mm, Spherisorb, ODS2–5 μm column) and eluted at 2.0 mL/min. The following gradient was used: 5 min with an isocratic solvent system of 20% HPLC-grade CH_3CN in water (solvent A), followed by a linear gradient up to 30% aqueous CH_3CN (solvent B) for 5 min, then 5 min isocratic with solvent B, and finally a linear gradient up to 100% CH_3CN in 10 min. The column was subsequently washed with 100% CH_3CN and reequilibrated with solvent A. The separations were monitored at 206 nm. HPLC fractions were collected every 2 min for radioactivity measurement.

Purification of Nod Factors. Six liters of cultured cells, which had been induced with 15 μM genistein, were filtered through a tangential filter (0.45 μm). The cell-free culture medium was passed twice through an Amberlite XAD-4 column (Fluka), and the retained products were eluted with methanol. The XAD-4 extracted compounds were further purified on an open glass column filled with C_{18} reversed-phase adsorbent (140 × 8 mm; Lichroprep RP-18, particle size 25–40 μm, Merck). After washing with 20 mL of a 4:1 (v/v) mixture of H_2O and CH_3CN, the column was eluted with 20 mL of 1:1 (v/v) H_2O and CH_3CN. This fraction was dried, redissolved in a small volume of 1:1 H_2O and CH_3CN, and then purified by C_{18} reversed-phase HPLC with a linear gradient from 20% to 100% aqueous CH_3CN in 30 min. The flow rate was 2 mL/min, and detection was at 206 nm. Fractions corresponding to the elution volume of Nod factors were pooled and purified by a second C_{18} reversed-phase HPLC run. The gradient (2 mL/min; 40 min total) was from 35% to 70% aqueous CH_3CN.

Carbohydrate Determination and Fatty Acid Analysis. Nod factors were hydrolyzed in 3 N HCl for 3 h at 80 °C. Fatty acids were extracted by dichloromethane, methylated with diazomethane, and analyzed either by capillary GC or by GC/MS. Sugars from the aqueous phase were derivatized as alditol acetates and identified by capillary GC. 2-Butyl glycosides were prepared by reaction with 1 N anhydrous HCl in (±)- or (–)-2-butanol (3 h; 60 °C). These derivatives were further acetylated [1:1 (v/v) acetic anhydride and pyridine, 3 h at 60 °C] and analyzed by capillary GC (Gerwig *et al.*, 1979). $NaBH_4$-reduced Nod factors were permethylated (Ciucanu & Kerek, 1984) for linkage analysis. The permethylated oligosaccharides were hydrolyzed with 4 N trifluoroacetic acid (4 h at 110 °C). Partially methylated monosaccharides were reduced with $NaBH_4$ and acetylated with acetic anhydride/pyridine as described above. Partially methylated alditol acetates were analyzed by GC/MS.

Analytical Methods. HPLC was performed on a Gilson system (Gilson France S.A.). GC was on a Girdel Series 30 instrument equipped with an OV1 bound capillary column (0.32 mm × 30 m, Spiral, France), a Ross injector, and a flame ionization detector. Helium was the carrier gas. The temperature gradient was 3 °C/min from 100 to 280 °C. Mass spectra were recorded on an Autospec instrument (FISONS, VG Analytical, U.K.). The acceleration voltage was 8 kV. A 25-keV cesium ion bombardment was used for LSIMS ionization. The matrix was a 1:1 (v/v) mixture of *m*-nitrobenzyl alcohol and glycerol, spiked with either 1% trichloroacetic acid in water or a solution of sodium iodide in water (1 mg/mL). ^1H-NMR spectra were collected on a Brüker AHX-500 spectrometer (Karlsruhe, Germany) with 1 mg of sample dissolved in 0.5 mL of dimethyl sulfoxide-d_6 (99.9% from Serva); 80 scans were accumulated at 318 K.

Tests for Biological Activity. Seeds of cowpea [*Vigna unguiculata* (L.)Walp. cv. Pink Eye Purple Hull] were from Hastings Seed Co., Atlanta, GA. Seeds of soybean [*G. max* (L.)Merr. cv. Peking and McCall] were from the Department of Agronomy, University of Missouri, and from Eric Pueppke, Erie, ND, respectively. R. L. Weaver of Texas A & M University, College Station, supplied seeds of siratro [*Macroptilium atropurpureum* (DC)Urb.]. Seeds were surface-sterilized and germinated aseptically on 1.5% agar plates at 30 °C for 3 days (Pueppke, 1983). For each experiment, five seedlings were transferred individually to sterile 8 × 1.2 cm plastic test tubes, the lower portions of which had been painted black. Each tube contained 5 mL of Nod factor (10^{-6}–10^{-2} M) in Jensen's N-free medium (Vincent, 1970). The mouths of the tubes were covered loosely with aluminum foil, and the tubes were placed in a growth chamber at 26 °C, with a 12-h light cycle. After incubation for 3 d, roots were examined under a microscope for root-hair curling and deformation. The experiments were repeated three times.

RESULTS

R. fredii USDA257 Synthesizes Nod Factors. R. fredii USDA257 was cultured in medium supplemented with genistein as *nod* gene inducer and either (^{14}C)acetate or (^{35}S)-sulfate as label. The mutant strain 257B3, with deleted common *nodABC* genes, was cultured under the same conditions as a control. Culture media were extracted on C_{18} Sep-Pak cartridges that were further eluted with methanol and analyzed by TLC.

Figure 1A is an autoradiogram of a reversed–phase TLC plate. Three ^{14}C-labeled spots were resolvable from the extract of wild-type USDA257 cells that had been induced with genistein. These spots were absent in induced cultures of mutant strain 257B3. No spots at these R_f values were detected

[1] Abbreviations: GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-pressure liquid chromatography; MIKE, mass-analyzed ion kinetic energy; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

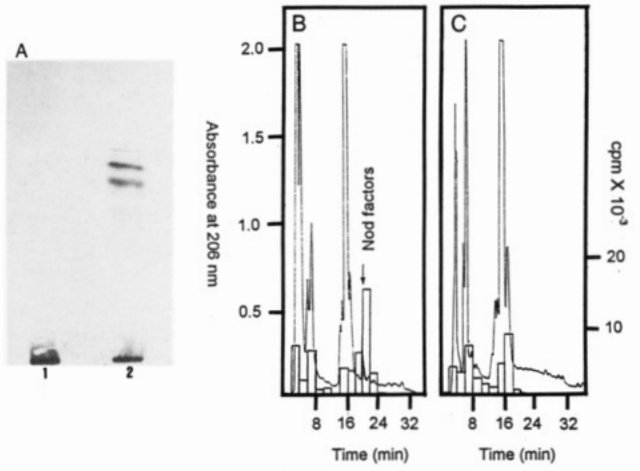

FIGURE 1: (A) TLC analysis of ^{14}C-labeled Nod factors produced by wild-type *R. fredii* USDA257 (lane 2) and from *nodABC* mutant 257B3 (lane 1). Cultures were induced with 15 μM genistein. HPLC and radioactivity profiles of extracts from the induced, ^{14}C-labeled cultures of USDA257 and mutant 257B3 are in panels B and C, respectively.

in ^{35}S-labeled extracts from either strain under any conditions, indicating that the Nod factors lack sulfate groups (data not shown). The ^{14}C-labeled extracts were analyzed by C_{18} reversed-phase HPLC. The two HPLC profiles arising from the wild-type strain and the *nodABC* mutant are given in panels B and C, respectively, of Figure 1. The radioactive peak eluting at about 20 min was specifically detected from the wild-type strain and attributed to Nod factors.

Large-scale genistein-induced cultures of USDA257 were used for preparative purification of Nod factors. The culture medium was passed through an Amberlite XAD-4 column and purified once on an open C_{18}-bonded silica glass column with monitoring of separation by reversed-phase TLC. The crude Nod factor mixture was purified again on a C_{18} reversed-phase HPLC column with a gradient of CH_3CN in water. Fractions containing Nod factors were pooled and analyzed by MS. Alternatively, this material was fractionated again on a C_{18} reversed-phase column, which facilitated the separation of the individual compounds (Figure 2).

The Nod Factors Are Substituted Oligomers of N-Acetylglucosamine. The molecular weights of the Nod factors were deduced from LSIMS mass spectra after sodium ion cationization, a procedure that strongly reduces fragmentation reactions (data not shown). Three peaks separated by 203 mass units were observed at *m/z* 1032, 1235 (major), and 1438. Another small peak was observed 14 mass units below *m/z* 1235. The 203 mass unit intervals are characteristic of compounds with varying numbers of *N*-acetylglucosamine residues in the lipooligosaccharide backbone. Identification of the saccharide components was facilitated by acid hydrolysis, followed by $NaBH_4$ reduction and peracetylation. Two different sugars were detected: *N*-acetylglucosamine and 2-*O*-methylfucose. The latter was characterized by two independent methods. One was by combined GC and GC/MS analysis of the peracetylated alditols. The other involved preparation of the (–)-2-butyl glycoside peracetyl derivative and confirmation that this compound coelutes with the authentic (–)-2-butyl glycosyl derivative of 2-*O*-methylfucose by capillary GC. A small amount of fucose also was detected. *N*-Acetylglucosamine was assigned to the D-series and fucose derivatives to the L-series by capillary GC of their (–)-2-butyl glycosides. Acid hydrolysis also released a fatty acid that was identified as *cis*-vaccenic acid [11(*Z*)-octadecenoic acid] by capillary GC.

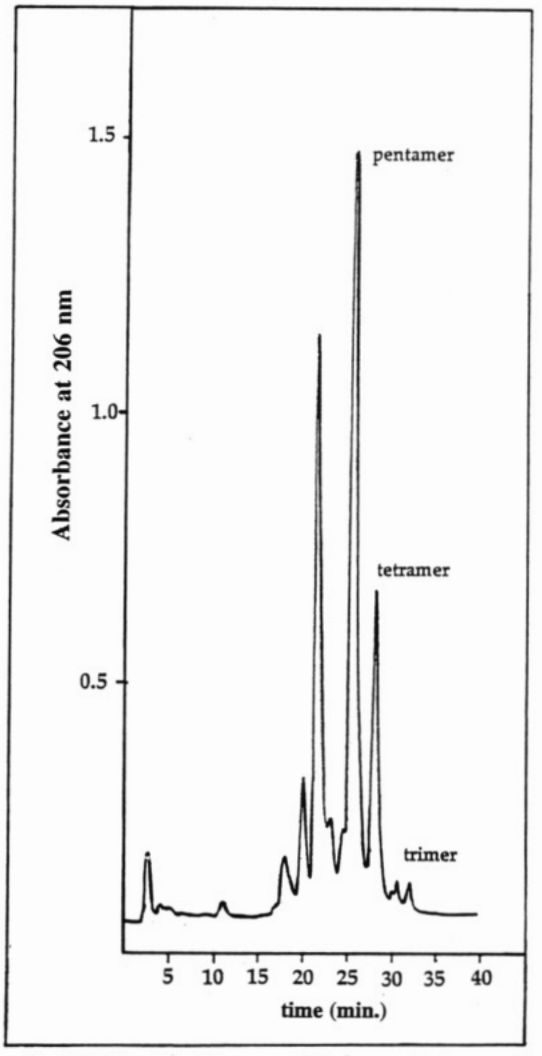

FIGURE 2: HPLC profile of the second C_{18} reversed-phase purification of the Nod factors from USDA257. Peaks corresponding to NodRf factors are pentamers, tetramers, and trimers.

Permethylation of the $NaBH_4$-reduced Nod factors followed by acid hydrolysis, peracetylation, and GC/MS analysis of the partially methylated alditol acetates confirmed that all glucosamines were 1,4-linked. Moreover, the terminal reducing residue was additionally substituted on O-6. ^{1}H-NMR spectra confirmed β-linkage between the glucosamines ($J_{H1,2}$ = 8.5 Hz) and α-linkage of 2-*O*-methylfucose ($J_{H1,2}$ = 3.75 Hz), as is the case for previously described Nod factors (Price *et al.*, 1992; Sanjuan *et al.*, 1992). The complete ^{1}H-NMR spectrum of the pentameric compound is given in Figure 3.

The final structures of the Nod factors were deduced from liquid secondary ion mass spectra, obtained from an acidified matrix (Figure 4). Protonated molecular ions and several ion fragments were produced by this method. Fragmentation of each individual $(M + H)^+$ species was studied by MIKE spectrometry (Figure 5). Most cleavages occurred at the interglycosidic bonds with charge retention on the nonreducing end (B-ions). A loss of 381 mass units was detected from each predominant $(M + H)^+$ ion, corresponding to the elimination of the terminal 2-*O*-methylfucosylated *N*-acetylglucosamine (Figure 5a). This mode of decomposition, together with data from methylation analysis, indicates that the 2-*O*-methylfucose substituent is located on O-6 of the reducing glucosamine. Below the $(MH - 381)^+$ ion, the ion series appearing at 203 mass unit intervals was characteristic of the chitin oligomeric backbone. This series ended at *m/z*

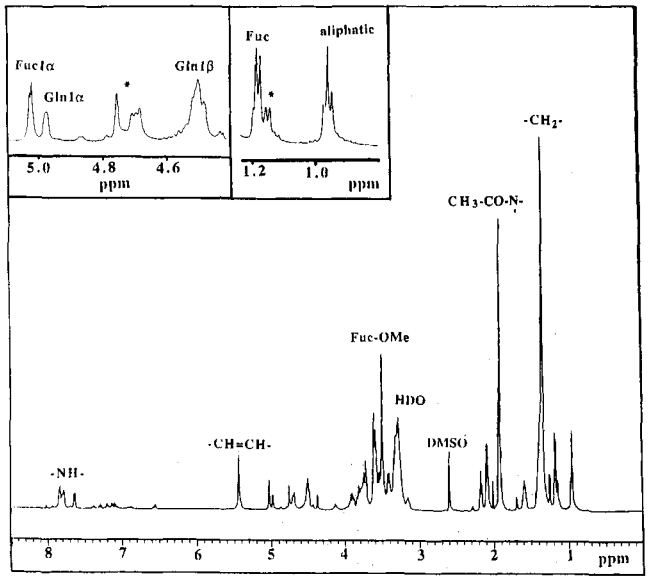

FIGURE 3: ^{1}H-NMR spectrum of the pentameric Nod factor. The inserts show the anomeric proton (4.4–5.1 ppm) and the methyl regions (0–1.2 ppm). The resonances labeled Fuc and Gln are due to 2-*O*-methylfucosyl and the *N*-acetylglucosamine residues, respectively. DMSO = dimethylsulfoxide. Peaks due to unidentified contaminants are indicated with asterisks.

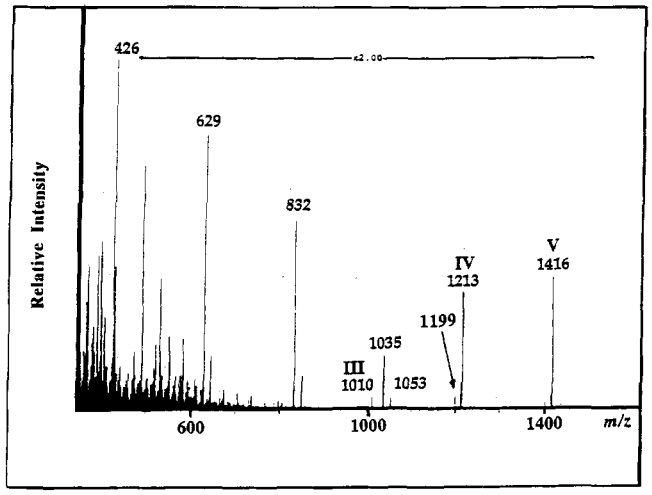

FIGURE 4: Positive ion liquid secondary ionization mass spectra of the Nod fraction in a 1:1 *m*-nitrobenzyl alcohol/glycerol matrix spiked with 1% trichloroacetic acid. Peaks labeled V, IV, and III, respectively, correspond to $(M + H)^+$ ions of pentameric, tetrameric, and trimeric chitin oligomers, each bearing one *N*-vaccenoyl and one 2-*O*-methylfucosyl group.

426 for all compounds. This ion corresponds to the oxenium ion of a *N*-vaccenoyl glucosamine, thus locating the acyl substituent at the nonreducing end.

The loss from the minor peak 14 mass units below the $(M + H)^+$ ion of the main species was 367 mass units instead of 381 units (Figure 5b). This indicates a fucosylated *N*-acetylglucosamine at the reducing end. Another series starting with the loss of 221 mass units (*N*-acetylglucosamine moiety) from both protonated ions, giving *m/z* 992 *m/z* 978, respectively, and followed by two successive losses of 203 mass units, suggested the presence of isomeric compounds with a fucosyl derivative linked to the nonreducing end or to interval residues of glucosamine. However, this series is not seen in the spectrum from the source (Figure 4), and other additional data (NMR and methylation analysis) do not provide evidence for the presence of such isomeric compounds. Thus, it is likely that a partial gas phase isomerization reaction precedes fragmentation reactions of long-lived ions, as analyzed by mass spectrometry. Such gas phase reactions are now under study.

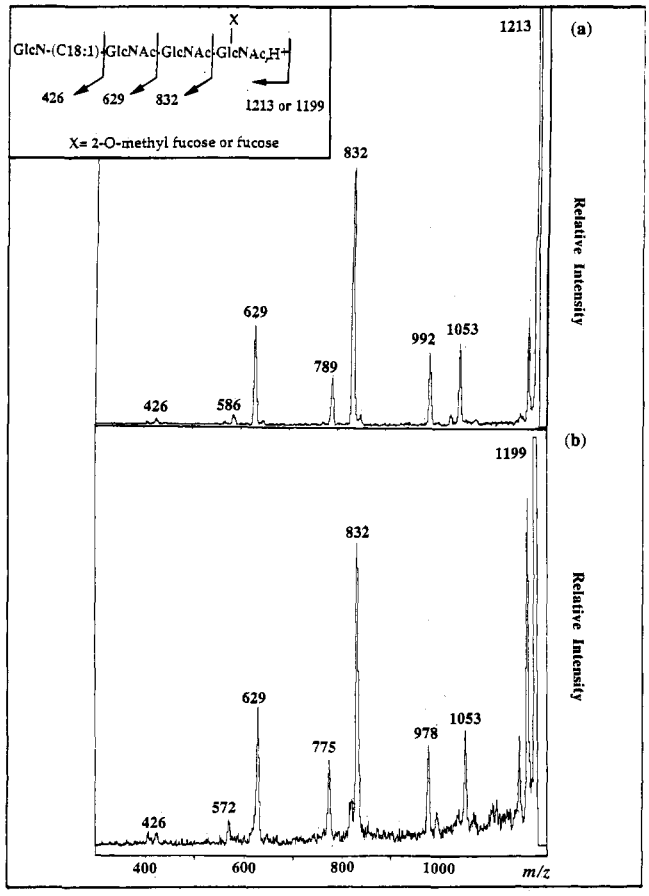

FIGURE 5: Mass ionized ion kinetic spectra of the protonated tetramers bearing either a 2-*O*-methylfucosyl group (*m/z* 1213) (a) or a fucosyl group (*m/z* 1199) (b).

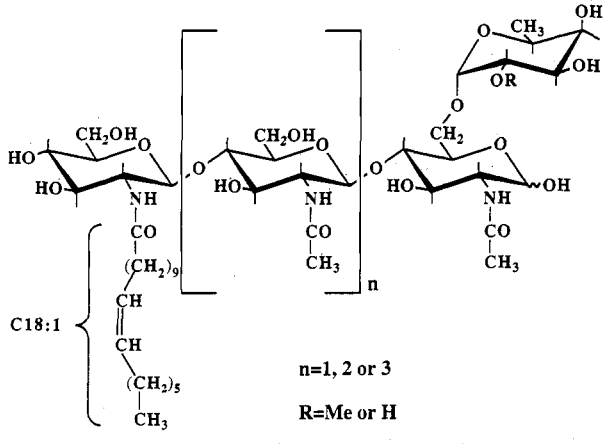

FIGURE 6: Complete chemical structures of the Nod factors from *R. fredii* USDA257.

Figure 6 summarizes the structures of Nod factors isolated from *R. fredii* USDA257. They are mixtures of β(1,4)-glucosamine pentamers, tetramers, and trimers that bear a *N*-vaccenoyl group on the nonreducing residue and *N*-acyl groups on the other residues. The reducing end is substituted on O-6 with 2-*O*-methylfucose. In the unique case of the glucosamine tetramer, a fucosyl moiety may replace the 2-*O*-methylfucose residue, giving a minor additional species. In accord with the nomenclature of Roche *et al.* (1991b), we designate the main 2-*O*-fucosylated pentamer, tetramer, and trimer as NodRf-V($C_{18:1}$,MeFuc), NodRf-IV($C_{18:1}$,MeFuc),

Table 1: Root Hair Deformation Activity of Nod Factors from *R. fredii* USDA257

Nod factor and legume	Nod factor concentration (M)						
	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
NodRf-III($C_{18:1}$,MeFuc)							
G max (McCall soybean)	nd[a]	+	+	+	–	–	–
G. max (Peking soybean)	nd	nd	+	+	nd	nd	nd
M. atropurpureum (siratro)	nd	+	+	+	+	–	–
V. unguiculata (cowpea)	nd	+	+	+	–	–	–
NodRf-IV($C_{18:1}$,MeFuc)							
G. max (McCall soybean)	nd	–	–	+	+	+	+
G max (Peking soybean)	nd	nd	nd	nd	+	+	nd
M. atropurpureum (siratro)	nd	–	–	+	+	+	+
V. unguiculata (cowpea)	nd	–	–	+	+	+	+
NodRf-V($C_{18:1}$,MeFuc)							
G. max (McCall soybean)	+	–	–	nd	nd	nd	nd
G. max (Peking soybean)	+	–	–	nd	nd	nd	nd

[a] nd, not determined.

and NodRf-III($C_{18:1}$,MeFuc), respectively. The minor fucosylated compound is NodRf-IV($C_{18:1}$,Fuc).

The Nod Factors Are Biologically Active. Table 1 summarizes the biological activities of the three 2-*O*-methylfucosylated Nod factors in a root-hair deformation assay. All three compounds triggered various sorts of reactions by root hairs of all three legume species, but at distinctly different threshold concentrations. Thus the tetramer generally was more active than the trimer, which in turn was more active than the pentamer. The responses of Peking soybean, which forms nitrogen-fixing nodules with USDA257, and McCall soybean, which does not, were indistinguishable.

Plant responses to the Nod factors are illustrated in Figure 7. Untreated root hairs were elongated and perpendicular to the axis of the root (Figure 7A). Treatment with Nod factors induced tip bending and waviness, as well as tip adhesions (Figure 7B, D–F, H). Treated root hairs also sometimes grew like corkscrews (Figure 7C) or wrapped around one another to form regular braided structures (Figure 7G). Root-hair tips assumed neither the markedly curled morphology as described for alfalfa (Truchet *et al.*, 1985) nor the short, pivoted morphology as described for cowpea, soybean, and siratro (Pueppke, 1983; Turgeon & Bauer, 1985; Ridge & Rolfe, 1986).

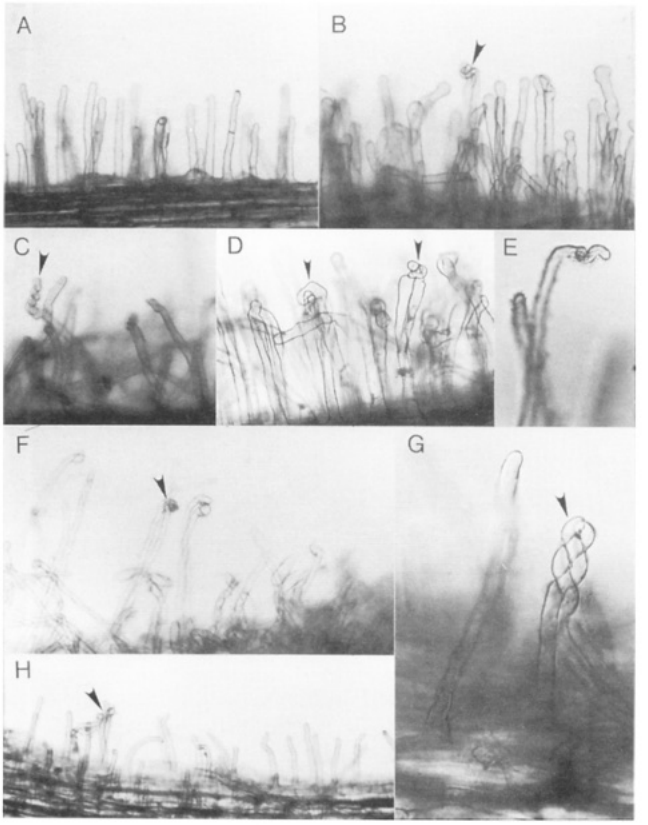

FIGURE 7: Responses of root hairs to purified Nod factors. Seedlings were germinated aseptically and exposed to Nod factors for 3 d prior to microscopic inspection of root hairs. (A) Untreated siratro root hairs. (B and D) Siratro treated with 10^{-8} M NodRf-III($C_{18:1}$,MeFuc). The arrowheads mark tip adhesions between root hairs. (C and E) Siratro treated with 10^{-10} M NodRf-IV($C_{18:1}$,MeFuc). The arrowhead in (C) marks a root hair that has coiled like a corkscrew. (F and G) McCall soybean treated with 10^{-11} M NodRf-IV($C_{18:1}$,MeFuc). The arrowhead in (F) marks a tip adhesion, and that in (G) shows two root hairs that have braided together. (H) Cowpea treated with 10^{-11} M NodRf-IV($C_{18:1}$,MeFuc). The arrowhead marks an area of tip adhesion.

DISCUSSION

The involvement of Nod factors in the legume–*Rhizobium* symbiosis is supported by two main lines of evidence, one genetic and the other physiological. The first stems from the discovery that the biosynthesis of Nod factor molecules is regulated by bacterial genes known to influence nodule initiation (Roche *et al.*, 1991a,b; Spaink *et al.*, 1991; Price *et al.*, 1992; Sanjuan *et al.*, 1992; Schultze *et al.*, 1992; Carlson *et al.*, 1993; Demont *et al.*, 1993; Firmin *et al.*, 1993; John *et al.*, 1993; Mergaert *et al.*, 1993; Poupot *et al.*, 1993; Stacey *et al.*, 1994). And second, purified Nod factors have been shown to substitute for *Rhizobium* cells in triggering plant responses, which include various sorts of root-hair deformation, dedifferentiation of cortical cells, and initiation of nodule meristems (Truchet *et al.*, 1991; vanBrussel *et al.*, 1992; Relic *et al.*, 1993, 1994; Heidstra *et al.*, 1994; Stokkermans & Peters, 1994). These observations have stimulated intense interest in the biological roles of Nod factors and have led to the proposal that these compounds are hormone-like signals with key functions in nodulation (Dénarié *et al.*, 1992).

We have shown here that genistein, an isoflavone elaborated by seedlings of soybean and other legumes (Cho & Harper, 1991; Graham, 1991; Phillips, 1992), causes *R. fredii* USDA257 to synthesize one minor and three major Nod factors. These compounds induce various sorts of root-hair deformations in legume species that are hosts of USDA257, and these deformations resemble the responses of other plants to Nod factors from their *Rhizobium* symbionts (Roche *et al.*, 1991a; Sanjuan *et al.*, 1992; Carlson *et al.*, 1993; Heidstra *et al.*, 1994). It is important to point out, though, that the deformed root hairs shown in Figure 7 are not similar to the short, curled root hairs of the type that contain infection threads in cowpea, soybean, and siratro (Pueppke, 1983; Turgeon & Bauer, 1985; Ridge & Rolfe, 1986).

Structurally, the *R. fredii* Nod factors are substituted chitin oligomers that contain vaccenic acid on the nonreducing glucosamine and differ from one another only in the length of the glucosamine backbone, except in the case of the minor compound, which lacks 2-*O*-methylation of the fucose residue (Figure 6). In these respects, the *R. fredii* compounds are similar to lipooligosaccharide Nod factors that have been isolated from other root-nodule organisms (Dénarié *et al.*, 1992). The Nod factors from *R. fredii* are not sulfated and thus differ from those of *R. meliloti* (Lerouge *et al.*, 1990), *R. tropici* (Poupot *et al.*, 1993), and *Rhizobium* sp. NGR234 (Price *et al.*, 1992), and they also lack the *O*-acetyl and *N*-methyl groups that are characteristic of Nod factors from some of these organisms.

Four kinds of bacteria, *B. japonicum*, *B. elkanii*, *Rhizobium* sp. strain NGR234, and *R. fredii*, form nodules on roots of soybean. With the completion of this work, the Nod factors from all four are known, and thus the relationships among these compounds can be established for the first time. NodRf-V($C_{18:1}$,MeFuc) of *R. fredii* USDA257 is in fact identical to NodBj-V($C_{18:1}$,MeFuc), the sole Nod factor of *B. japonicum* USDA110, and one of an array of compounds elaborated by *B. japonicum* USDA135 and *B. elkanii* USDA61. Moreover, NodRf-IV($C_{18:1}$,MeFuc) is identical to NodBj-IV($C_{18:1}$,-MeFuc) of *B. elkanii* USDA61 (Carlson *et al.*, 1993; Sanjuan *et al.*, 1992). All of these Nod factors from *Bradyrhizobium* are inducible by genistein, as the case with their counterparts from *R. fredii*, and thus it seems likely that they share a common role in nodulation of a common host, soybean. NGR234, in contrast, elaborates a complex mixture of Nod factors (Price *et al.*, 1992). These Nod factors all contain 2-*O*-methylfucose, but they differ from the *Bradyrhizobium* and *R. fredii* compounds in other respects, e.g., the presence of carbamoyl substituents on the nonreducing glucosamine and, in some cases, sulfation or acetylation of the fucose residue. Although it is not known to what extent, if any, these substituents influence symbiosis, nodules produced on soybean by NGR234 often fail to fix nitrogen or are minimally effective (Morrison *et al.*, 1986; Nayudu & Rolfe, 1987; Relic *et al.*, 1993; Balatti, Krishnan, and Pueppke, unpublished data).

The cultivar specificity of USDA257 on soybean represents an additional order of complexity for symbioses involving *R. fredii*. This strain forms nitrogen-fixing nodules on soybean cultivar Peking, but not on cultivar McCall. Although two groups of genes are known to influence this process (Krishnan & Pueppke, 1991a; Meinhardt *et al.*, 1993), the physiological basis for cultivar specificity remains obscure. One likely possibility, that cultivar specificity reflects differential sensitivity of Peking and McCall to the Nod factors of USDA257, seems unlikely in light of our inability to detect a differential, cultivar-specific response to any of the three major Nod factors. This observation stands in contrast to data from peas, where *O*-acetylation of a Nod factor from *R. leguminosarum* bv. *viciae* appears to be of key significance for nodulation of the pea cultivar Afghanistan (Firmin *et al.*, 1993).

A final issue concerns the relationship between Nod factors and broad host range, e.g., the ability of a *Rhizobium* strain to nodulate many different legume species. NGR234 is perhaps the best known such strain, and it has been proposed that this strain nodulates many species because it can synthesize an array of Nod factors with various sorts of substitution that can impart an adjustable signaling capacity (Price *et al.*, 1992). Recent evidence that sulfated Nod factors from NGR234 can extend the host range of USDA257 to *Calopogonium caeruleum* is in accord with this theory (Relic *et al.*, 1994). Although the host range of *R. fredii* is not so broad as that of NGR234 (Krishnan & Pueppke, 1994), USDA257 nevertheless can form nitrogen-fixing nodules on nearly 20% of 324 tested species, versus 33% of these species for NGR234 (Pueppke and Broughton, unpublished data). The structural variability of Nod factors from *R. fredii* USDA257 seems modest in comparison to this ability to nodulate so many legumes, and so USDA257 may at least partially rely on alternative mechanisms to establish its broad host range. Comparative analysis of these two strains, which is already underway (Krishnan *et al.*, 1992; Relic *et al.*, 1993, 1994), should prove valuable for sorting out these distinctions.

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REFERENCES

Carlson, R. W., Sanjuan, J., Bhat, U. R., Glushka, J., Spaink, H. P., Wijfjes, A. H. M., vanBrussel, A. A. N., Stokkermans, T. J. W., & Stacey, G. (1993) *J. Biol. Chem. 268*, 18372–18381.

Cho, M.-J., & Harper, J. E. (1991) *Plant Physiol. 95*, 435–442.

Ciucanu, I., & Kerek, F. (1984) *Carbohydr. Res. 131*, 209–217.

Demont, N., Debellé, F., Aurelle, H., Dénarié, J., & Promé, J.-C. (1993) *J. Biol. Chem. 268*, 20134–20142.

Dénarié, J., Debellé, F., & Rosenberg, C. (1992) *Annu. Rev. Microbiol. 46*, 497–531.

Eardly, B. D., Young, J. P. W., & Selander, R. K. (1992) *Appl. Environ. Microbiol. 58*, 1809–1815.

Firmin, J. L., Wilson, K. E., Carlson, R. W., Davies, A. E., & Downie, J. A. (1993) *Mol. Microbiol. 10*, 351–360.

Fisher, R. W., & Long, S. R. (1992) *Nature 357*, 655–660.

Geremia, R. A., Mergaert, P., Geelen, D., vanMontagu, M., & Holsters, M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2669–2673.

Gerwig, G. J., Kamerlin, J. P., & Vliegenthart, J. F. G. (1979) *Carbohydr. Res. 77*, 1–7.

Graham, T. L. (1991) *Plant Physiol. 95*, 594–603.

Heidstra, R., Geurts, R., Franssen, H., Spaink, H. P., van-Kammen, A., & Bisseling, T. (1994) *Plant Physiol. 105*, 787–797.

Heron, D. S., & Pueppke, S. G. (1984) *J. Bacteriol. 160*, 1061–1066.

Heron, D. S., Érsek, T., Krishnan, H. B., & Pueppke, S. G. (1989) *Mol. Plant-Microbe Interact. 2*, 4–10.

Horvath, B., Heidstra, R., Lados, M., Moerman, M., Spaink, H. P., Promé, J.-C., vanKammen, A., & Bisseling, T. (1993) *Plant J. 4*, 727–733.

John, M., Röhrig, H., Schmidt, J., Wieneke, U., & Schell, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 625–629.

Keyser, H. H., Bohlool, B. B., Hu, T. S., & Weber, D. F. (1982) *Science 215*, 1631–1632.

Krishnan, H. B., & Pueppke, S. G. (1991a) *Mol. Microbiol. 5*, 737–745.

Krishnan, H. B., & Pueppke, S. G. (1991b) *Mol. Plant-Microbe Interact. 4*, 512–520.

Krishnan, H. B., & Pueppke, S. G. (1994) *Plant Soil 161*, 21–29.

Krishnan, H. B., Lewin, A., Fellay, R., Broughton, W. J., & Pueppke, S. G. (1992) *Mol. Microbiol. 6*, 3321–3330.

Kuykendall, L. D., Saxena, B., Devine, T. E., & Udell, S. E. (1992) *Can. J. Microbiol. 38*, 501–505.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.-C., & Dénarié, J. (1990) *Nature 344*, 781–784.

Martinez-Romero, E., Segovia, L., Mercante, F. M., Franco, A., Graham, P., & Pardo, M. A. (1991) *Int. J. Syst. Bacteriol. 41*, 417–426.

Meinhardt, L. W., Krishnan, H. B., Balatti, P. A., & Pueppke, S. G. (1993) *Mol. Microbiol. 9*, 17–29.

Mergaert, P., vanMontagu, M., Promé, J.-C., & Holsters, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1551–1555.

Morrison, N. A., Trinick, M. J., & Rolfe, B. G. (1986) *Plant Soil 92*, 313–331.

Nayudu, M., & Rolfe, B. G. (1987) *Mol. Gen. Genet. 206*, 326–337.

Phillips, D. A. (1992) in *Phenolic Metabolism in Plants* (Stafford, H. A., & Ibrahim, R. K., Eds.) pp 201–231, Plenum Press, New York.

Poupot, R., Martinez-Romero, E., & Promé, J.-C. (1993) *Biochemistry 32*, 10430–10435.

Price, N. P. J., Relic, B., Talmont, F., Lewin, A., Promé, D., Pueppke, S. G., Maillet, F., Dénarié, J., Promé, J.-C., & Broughton, W. J. (1992) *Mol. Microbiol. 6*, 3575–3584.

Pueppke, S. G. (1983) *Can. J. Microbiol. 29,* 69–76.
Ranga Rao, V., & Keister, D. L. (1978) *Protoplasma 97,* 311–316.
Relic, B., Talmont, F., Kopcinska, J., Golinowski, W., Promé, J.-C., & Broughton, W. J. (1993) *Mol. Plant-Microbe Interact. 6,* 764–774.
Relic, B., Perret, X., Estrada-Garcia, M. T., Kopcinska, J., Golinowski, W., Krishnan, H. B., Pueppke, S. G., & Broughton, W. J. (1994) *Mol. Microbiol. 13,* 171–178.
Ridge, W. W., & Rolfe, B. G. (1986) *J. Plant Physiol. 122,* 121–137.
Roche, P., Debellé, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Dénarié, J., & Promé, J.-C. (1991a) *Cell 67,* 1131–1143.
Roche, P., Lerouge, P., Ponthus, C., & Promé, J.-C. (1991b) *J. Biol. Chem. 266,* 10933–10940.
Röhrig, H., Schmidt, J., Wieneke, U., Kondorosi, E., Barlier, I., Schell, J., & John, M. (1994). *Proc. Natl. Acad. Sci. U.S.A. 91,* 3122–3126.
Sadowsky, M. J., Cregan, P. B., & Keyser, H. H. (1988) *Appl. Environ. Microbiol. 54,* 1907–1910.
Sanjuan, J., Carlson, R. W., Spaink, H. P., Bhat, U. R., Barbour, W. M., Glushka, J., & Stacey, G. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 8789–8793.
Scholla, M. H., & Elkan, G. H. (1984) *Int. J. Syst. Bacteriol. 34,* 484–486.
Schultze, M., Quiclet-Sire, B., Kondorosi, E., Virelizier, H., Glushka, J. N., Endre, G., Géro, S., & Kondorosi, A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 192–196.
Segovia, L., Young, J. P. W., & Martinez-Romero, E. (1993) *Int. J. Syst. Bacteriol. 43,* 374–377.
Spaink, H. P., Sheeley, D. M., vanBrussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., & Lugtenberg, B. J. J. (1991) *Nature 354,* 125–130.
Stacey, G., Luka, S., Sanjuan, J., Banfalvi, Z., Nieuwkoop, A. J., Chun, J. Y., Forsberg, L. S., & Carlson, R. (1994) *J. Bacteriol. 176,* 620–633.
Stokkermans, T. J. W., & Peters, N. K. (1994) *Planta 193,* 413–420.
Truchet, G., Debellé, F., Vasse, J., Terzaghi, B., Garnerone, A.-M., Rosenberg, C., Batut, J., Maillet, F., & Dénarié, J. (1985) *J. Bacteriol. 164,* 1200–1210.
Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., deBilly, F., Promé, J.-C., & Dénarié, J. (1991) *Nature 351,* 670–673.
Turgeon, B. G., & Bauer, W. D. (1985) *Planta 163,* 328–349.
vanBrussel, A. A. N., Bakhuizen, R., vanSpronsen, P. C., Spaink, H. P., Tak, T., Lugtenberg, B. J. J., & Kijne, J. W. (1992) *Science 257,* 70–72.
Vincent, J. M. (1970) *A Manual for the Practical Study of Root-Nodule Bacteria,* Blackwell Scientific Publications, Oxford.
Young, J. P. W., & Johnston, A. W. B. (1989) *Trends Ecol. Evol. 4,* 341–349.