Structurally Diverse Chitolipooligosaccharide Nod Factors Accumulate Primarily in Membranes of Wild Type *Rhizobium leguminosarum* biovar *trifolii*†

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ABSTRACT: The general view on *Rhizobium* chitolipooligosaccharides (CLOS) is that they are made in very low levels as diffusible molecules and are primarily secreted by the bacteria into the extracellular milieu where they interact with the host. However, the structural and predicted physicochemical properties of these amphiphilic molecules led us to postulate that they should normally be targeted to bacterial membranes after synthesis. Thus, we analyzed membrane lipid extracts of *Rhizobium leguminosarum* bv. *trifolii* wild-type strain ANU843 cells and the corresponding culture supernatants for CLOS-type glycolipids. As predicted, fractionation of the membrane extracts from pelleted cells led to the isolation of a diverse family of CLOS in high yield (≥15 mg/L of culture), whereas all attempts to isolate CLOS from the corresponding culture supernatant failed. Structural analyses reveal that the membrane CLOS of ANU843 consist of a complex mixture of O-acetylated or non-O-acetylated chito- tri-, -tetra-, and pentasaccharides bearing an *N*-acyl moiety at the nonreducing glucosamine residue. *cis*-Vaccenic acid was the predominant acyl substituent (>70%), but several other saturated, unsaturated, and 3-hydroxy fatty acids were found in the CLOS glycolipids. Membrane accumulation of CLOS in ANU843 is promoted by the presence of 4′,7-dihydroxyflavone and pSym *nod* genes. Potential host-selective biological activity of the purified membrane CLOS fraction from ANU843 was indicated by its ability to elicit meristems resembling rudimentary nodule primordia in the root cortex of axenic seedlings of the host legume, white clover, but not of the nonhost legumes hairy vetch or alfalfa. These results indicate that, as predicted, a very diverse family of chitolipooligosaccharide Nod factors accumulate primarily in bacterial membranes of wild-type *R. leguminosarum* bv. *trifolii*, and our protocol which readily isolates these biologically active glycolipids in high yield from this source eliminates the need to use recombinant "overproducing" strains to obtain sufficient quantities for structural analyses. These results lead us to predict that these glycolipids may perform important membrane functions for wild-type rhizobia in the host root environment, and that these bacterial factors are likely to operate primarily at short range rather than as freely diffusible extracellular molecules during development of the *Rhizobium*−legume symbiosis.

The development of the nitrogen-fixing *Rhizobium*-legume symbiosis is modulated by the bacterial induction of the plant host symbiotic program. A crucial event in this plant−microbe interaction is the activation by plant flavonoids of several bacterial nodulation (*nod*)[1] genes, located on the symbiotic plasmid (pSym) (Djordjevic & Weinman, 1991; Long, 1992). The *nod* functions are pivotal in the bilateral signaling that eventually enables *Rhizobium* to infect and nodulate its specific legume host (Brewin, 1991; Dénarié et al., 1992; Hirsch, 1992; Verma, 1992).

The action of certain *nod* genes has been linked to the production of "Nod factors" represented by chitolipooligosaccharides (CLOS). The first reported CLOS, called NodRm1, was isolated and characterized by Lerouge et al. (1990) from the culture supernatant of a *nod* overexpressing strain of *Rhizobium meliloti*. NodRm1 consisted of a glucosamine tetrasaccharide substituted at the nonreducing terminus with an amide-linked C16 bisunsaturated fatty acid, bearing an *N*-acetyl group on each of the remaining glucosamines, and a sulfate group at the C-6 position of the reducing *N*-acetylglucosamine (GlcNAc) terminus. Subsequent studies showed that a given strain of *R. meliloti* produces a large and highly complex family of sulfated or unsulfated CLOS molecules, including tri-, tetra-, and pentasaccharides which are N-acylated with either C16:0,

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[1] Abbreviations: CLOS, chitolipooligosaccharides; D_2O, deuterium oxide; DHF, 4′,7-dihydroxyflavone; DMSO, dimethyl sulfoxide; dp, degree of polymerization; EPS, extracellular acidic heteropolysaccharide; FAB-MS, fast atom bombardment mass spectrometry; FID-GC, flame ionization detection gas chromatography; GC-MS, gas chromatography coupled to mass spectrometry; GlcNAc, *N*-acetylglucosamine; HMQC, heteronuclear multiquantum coherence spectroscopy; HPLC, high-pressure liquid chromatography; Kan, kanamycin; NF, nitrogen free medium; MS-MS, mass spectrometry−mass spectrometry; *nod*, nodulation gene; NMR, nuclear magnetic resonance; NodRlt, nodulation factors from *Rhizobium leguminosarum* biovar *trifolii;* pSym, symbiotic plasmid; Tn, transposon.

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C18:0, C16:1, C16:2, C16:3 or a series of (ω-1) hydroxylated C18−C26 fatty acids and are *O*-acetylated at the C-6 position of the nonreducing terminus or unsubstituted at the same location (Lerouge et al., 1990; Roche et al., 1991; Schultze et al., 1992; Demont et al., 1993). A large diversity of CLOS molecules have also been isolated and characterized from culture supernatants of a wild-type strain of *Bradyrhizobium japonicum* (Sanjuan et al., 1992; Carlson et al., 1993), *Rhizobium tropici* (Poupot et al., 1993), and *Rhizobium fredii* (Bec-Ferté et al., 1993), and from *nod*-overexpressing recombinant strains of *Rhizobium leguminosarum* bv. *viciae* (Spaink et al., 1991), *Rhizobium* sp. NGR234 (Price et al., 1992), and *Azorhizobium caulinodans* (Mergaert et al., 1993). The CLOS from these other rhizobia differ in oligosaccharidic chain length (dp 3−5), structure of the *N*-fatty acyl substituent (C16:0, C18:0, C18:1, C18:4), and the presence or absence of other substituents (e.g., sulfate, acetyl, carbamoyl, *N*-methyl, arabinosyl, fucosyl, and 2-*O*-methylfucosyl) on the glucosamine backbone.

The general view on *Rhizobium* CLOS is that they are made in very low levels and secreted as diffusible molecules by the bacteria into the extracellular milieu where they interact with the host. However, on the basis of structural, physicochemical, and physiological arguments, we hypothesized that these amphiphilic glycolipids should normally be targeted to bacterial membranes after synthesis. Several lines of evidence suggest that CLOS might reside primarily in rhizobia bacterial membranes. First, the diversity of fatty acyl moieties in the family of CLOS made by a given strain of rhizobia is typical of what one would expect of bacterial membrane glycolipids. Second, approximately 70% of the butanol-extractable, *nod*-dependent labeled lipid originating from [^{14}C]acetate labeling of *Rhizobium leguminosarum* bv. *trifolii* cultures grown with *nod*-inducing flavones is extracted from pelleted cells (McKay & Djordjevic, 1993). Third, CLOS could be extracted with aqueous methanol from cell pellets of a recombinant strain consisting of a pSym-cured derivative of *R. leguminosarum* bv. *phaseoli* (strain 8401) carrying the entire cloned *nod* gene region from *R. leguminosarum* bv. *viciae* strain TOM on a multicopy plasmid (Firmin et al., 1993). In addition, we recently established that the membrane accumulation of another *R. leguminosarum* bv. *trifolii* glycolipid, namely, a diglycosyldiacylglycerol, was significantly increased in wild-type ANU843 grown with *nod*-activating flavone, significantly diminished in *nodA*::Tn*5* and *nodD*::Tn*5* mutant derivatives, and exhibited a potent mitogenic activity on host roots (Orgambide et al., 1992, 1994b).

On the basis of the above points, we investigated the occurrence of CLOS in membrane lipid extracts of pelleted cells of wild-type *R. leguminosarum* bv. *trifolii* ANU843 and developed a protocol which showed that this family of membrane glycolipids can be obtained from this source in quantities over 1000-fold greater than those obtained from the corresponding culture supernatant. The structures of several different membrane CLOS from wild-type *R. leguminosarum* bv. *trifolii* ANU843, their pSym *nod* dependence, and tests for host specificity of their mitogenic activity are also reported. Portions of this work were presented at the 7th International Symposium on Molecular Plant-Microbe Interactions (Orgambide et al., 1994a).

MATERIALS AND METHODS

Bacterial Cultures and Cell Extraction. Strains of *R. leguminosarum* bv. *trifolii* used in this study were wild type ANU843 (Rolfe et al., 1982), a pSym-cured derivative ANU845 (Rolfe et al., 1982), and a *nodC*::Tn*5* transposon insertion mutant derivative ANU277 (Djordjevic et al., 1985). Each strain was grown at 29 °C in 4 L shaken flasks containing 2 L of BIII broth (Dazzo, 1982) supplemented with 0.45 mM $CaCl_2$ (McKay & Djordjevic, 1993), with or without 4 μM 4′,7-dihydroxyflavone (DHF) as a *nod* gene inducer (Redmond et al., 1986). BIII medium was supplemented with 30 μg/mL Kan for growth of the Tn*5* mutant ANU277. Cells were grown to a density of 9×10^8 cells/mL, harvested by centrifugation at 10000*g* for 45 min at 4 °C, washed by resuspension in 200 mL distilled water, and recentrifuged. The cell pellet was extracted with 200 mL of 1:2:2:3 chloroform/propanol/methanol/water by vigorous stirring at 37 °C for 24 h under nitrogen atmosphere. The extraction mixture was centrifuged in Corex glass tubes at 30000*g* for 45 min at 7 °C. The supernatant of membrane lipid extract was collected and dried by flash evaporation at 40 °C.

Purification of the Membrane Chitolipooligosaccharides from ANU843. The membrane lipid extract from ANU843 grown with DHF (843+DHF) was redissolved in 100 mL of 20% acetonitrile and loaded onto a LiChroprep RP-18 column (EM Separations, Gibbstown, NJ; 50 mL gel bed). Three successive elutions (3 × 50 mL each) were made with 20% acetonitrile, 50% acetonitrile, and 100% acetonitrile, respectively. The three fractions were dried by flash evaporation, weighed, and analyzed for sugar and fatty acid content by GC-MS of the methyl glycoside peracetate and methyl ester derivatives, respectively. The material eluted with 50% acetonitrile was further fractionated by chromatography of 5 mg aliquots on Sep-Pak C18 (Waters-Millipore Corp. Milford, MA) with stepwise elutions of increasing concentrations of acetonitrile in water (5% increments). Fractions eluting between 35% and 50% acetonitrile were further processed by gel permeation on Sephacryl S-100 (solvent 1: 1:4 propanol/water), followed by anion exchange chromatography on QMA Sep-Pak (solvent 2: 2:1:3 propanol/acetonitrile/water), and cation exchange chromatography on Dowex AG50W-X12 (H^+ form, solvent 2). The final purified fraction consisting of the family of membrane chitolipooligosaccharides from ANU843 was thereafter referred to as the "NodRlt factors".

Membrane lipid extracts obtained from ANU843 cells grown without DHF (843−DHF), ANU845 grown with DHF (845+DHF), and ANU277 grown with DHF (277+DHF) were processed through the step of LiChroprep RP-18 flash chromatography as described above. This generated fractions chromatographically equivalent to those obtained at that primary stage of purification from the ANU843+DHF membrane lipid extract. C18 reversed-phase HPLC of the CLOS fraction from 843+DHF, or chromatographically equivalent fractions from 843−DHF, 845+DHF, and 277+DHF, was achieved using a 250 × 10 cm Selectosil 5 μm C18 column (Phenomenex, Torrance, CA). Elution was performed using a linear gradient from 30% to 85% acetonitrile in water, at a flow rate of 1.2 mL/min for over 30 min, with continuous monitoring for ultraviolet absorbance at 206 nm.

Chemical Analyses of the Membrane Chitolipooligosaccharides. One milligram of the NodRlt factors was methanolyzed with either 0.7 M or 4 M HCl in methanol for 20 h at 80 °C. Fatty acid methyl esters were extracted in chloroform, and the methyl glycosides were peracetylated (1:1 acetic anhydride/pyridine, 15 h, 25 °C). Fatty acid and carbohydrate derivatives were then identified by GC-MS analysis (retention times and mass spectra matched with those of authentic standards when available) and quantified by FID-GC. The GC-MS analyses of fatty acid methyl esters were carried out on a Hewlett-Packard 5890 gas chromatograph equipped with a Supelco DB-1 column and coupled to a JEOL AX-50511 mass spectrometer. GC-MS of the methylglycosides was done on a Hewlett-Packard 5995C instrument fitted with a Supelco DB225 column. FID-GC analyses were performed on a Varian 3740 gas chromatograph fitted with a Supelco SP-2330 column for the peracetylated methyl glycosides and a DB-1 column for the fatty acid methyl esters. Ozonolysis of the CLOS-derived fatty acid methyl esters (from 0.5 mg of NodRlt factors) was achieved by bubbling a gentle stream of ozone into a 0.5 mL dichloromethane solution of the derivatives for 7 min, followed by addition of 5 mg of triphenylphosphine. Glycosidic linkage determination by methylation analysis of the NodRlt factors was performed as described elsewhere (Waeghe et al., 1983).

Five milligrams of the NodRlt factors was subjected to mild methanolysis (1 M HCl in methanol, 1 h, 80 °C) to release the *N*-acylated glucosamine residue at the nonreducing end of the oligosaccharides. The methanolysate was neutralized with 1 M NaOH and loaded onto a C18 Sep-Pak. The GlcNAc monosaccharides were eluted from the cartridge with 20% acetonitrile whereas the *N*-acylated glucosamine residues were recovered in the 100% acetonitrile eluate. One milligram of the CLOS sample was de-*O*-acetylated by treatment with 0.1 M NaOH for 5 min at 25 °C. The reaction mixture was neutralized by addition of 1 M HCl. The de-*O*-acetylated glycolipid was desalted by chromatography on a C18 Sep-Pak.

NMR spectra were recorded on a Varian VXR500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C) at 25 °C in perdeuterated dimethyl sulfoxide. The chemical shifts are expressed in ppm downfield from an external tetramethylsilane standard. Positive mode FAB-MS was conducted with a JEOL HX-110 instrument with a 1:1 glycerol / nitrobenzyl alcohol matrix, at an accelerating voltage of 10 kV.

Search for Chitolipooligosaccharides in the Extracellular Fraction of ANU843. A broth culture of ANU843 was grown in 8 L of BIII+DHF at 29 °C to a density of 9×10^8 cells/mL and centrifuged to pellet the cells, and the supernatant fluid was extracted with 4 L of *n*-butanol. This butanol extract was washed twice with 2 L of distilled water and dried by flash evaporation, and aliquots were analyzed by ^{1}H NMR and GC-MS of the methylglycoside peracetate derivatives. Another 8 L of culture supernatant was passed through a 2.6 × 40 cm column of polystyrene adsorbent (Bio-Beads SM-2, Bio-Rad, Richmond, CA). The column was then flushed with distilled water, and the retained material was eluted with methanol. In other experiments, the extracellular acidic heteropolysaccharide (EPS) was removed from the culture supernatant prior to butanol extraction. This was done by concentration to 30% of its initial volume by flash evaporation followed by addition of 2 volumes of ethanol at 4 °C. The precipitated EPS was collected by centrifugation, lyophilized, redissolved (5 mg in 2 mL) in a buffer containing 2 M guanidinium chloride and 0.5 M sodium acetate (pH 6.8) and fractionated by dissociative gel permeation chromatography on Sephacryl S-300 (1.6 × 100 cm; Pharmacia, Piscataway, NJ) using the same solvent as eluent.

Axenic Seedling Bioassays. A lyophilized sample of the purified CLOS NodRlt factors (nominal molecular weight of 1000) from ANU843 was resuspended in nitrogen-free (NF) plant growth medium (Fåhraeus, 1957) with the aid of an ultrasonic bath and heated at 100 °C for 20 min. This procedure sterilized the sample, as verified by plating aliquots on BIII medium and tryptic soy agar (Difco, Detroit MI). Assays for mitogenic activity leading to nodule-like cortical meristems were performed on axenic seedlings of the host legume, white clover (*Trifolium repens* L. cv. Dutch), and of the nonhost legumes alfalfa (*Medicago sativa* cv. Gemini) and hairy vetch (*Vicia hirsuta*) under microbiologically controlled conditions as described earlier (Orgambide et al., 1994b). Sterile NF medium was used as the diluent and untreated control throughout.

RESULTS

Isolation of Membrane Chitolipooligosaccharides from ANU843. The major portion (dry weight) of the membrane lipid extract from ANU843 grown with DHF was eluted from the LiChroprep reverse phase column with 20% acetonitrile, while subsequent elutions with 50% acetonitrile and 100% acetonitrile released less abundant fractions. GC-MS of the methyl glycoside peracetate derivatives indicated that glucosamine was only a minor sugar component in the materials recovered in the 20% or 100% acetonitrile eluates, whereas this aminoglycoside represented over 90% of the total sugars detected in the fraction eluted with 50% acetonitrile. Upon further fractionation of the latter sample on reversed-phase C18 Sep-Pak, fractions eluting between 35% and 50% acetonitrile contained glucosamine as the sole sugar plus various fatty acids. In subsequent anion and cation exchange chromatographies, this glucosamine-containing material was not retained, indicating that it was not ionic. A typical yield of 15−20 mg of the purified membrane chitolipooligosaccharides was routinely obtained from pelleted cells (ca. 9×10^{11}) per liter of 843+DHF culture.

Several attempts were made to obtain this class of glycolipids from 8 L of the corresponding culture supernatant, including a direct *n*-butanol extraction or hydrophobic adsorption chromatography on Bio-Beads SM-2 polystyrene adsorbent. GC-MS analyses did not give any indication of glucosamine in the butanol extract nor in the methanol-eluted material preadsorbed onto polystyrene beads. To test the possibility that the abundant bacterial EPS might somehow interfere with efficient extraction of extracellular CLOS, we also removed the EPS from the culture supernatant by ethanol precipitation prior to butanol extraction. Again, no glucosamine was found in such supernatant extracts depleted of EPS. Finally, we evaluated whether putative CLOS might have coprecipitated with the EPS. The ethanol-precipitated EPS was redissolved in a buffer containing a high concentration of a chaotropic agent (2 M guanidinium chloride) to promote the dissociation of potential CLOS noncovalently bound to EPS, and the solution was chromatographed through Sephacryl S-300 using the same dissociative solvent as

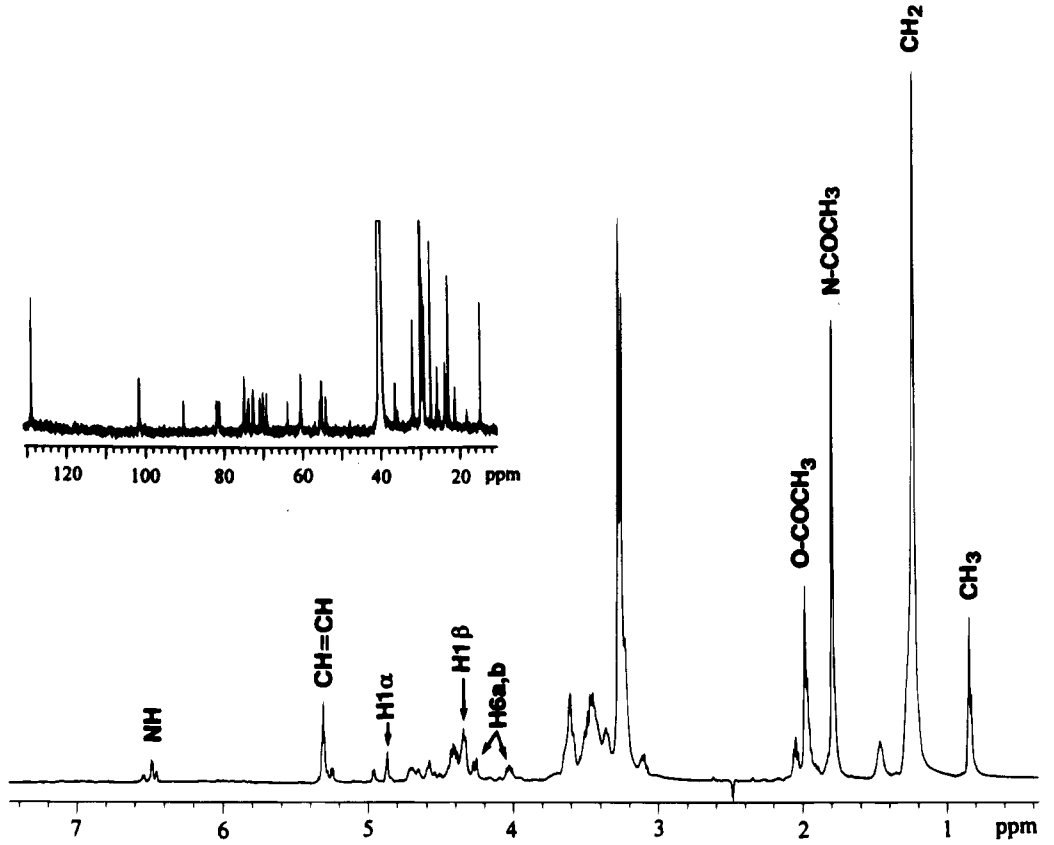


FIGURE 1: ^1H NMR and ^{13}C NMR (inset) spectra of the purified fraction of membrane chitolipooligosaccharides from wild-type *R. leguminosarum* bv. *trifolii* ANU843. The unlabeled resonances at 1.45, 2.05, and between 3.0 and 3.8 ppm are due to the 3- and 2-protons of the attached fatty acid and the glucosamine ring protons, respectively. The signal for the *O*-acetyl moiety coincides with those for the protons adjacent to the vinyl group.

eluent. The polysaccharide [having the ^1H NMR spectrum and glycosyl composition of the acidic heteropolysaccharide of this strain (Hollingsworth et al., 1988)] eluted in the void volume of the column, and a low molecular weight fraction (representing less than 5% by weight of the initial sample) was also obtained. ^1H NMR and GC-MS analyses indicated that the latter fraction contained mannitol and glutamic acid (the major organic components of the BIII culture medium) but no evidence of glucosamine.

Structural Analyses of the Membrane Chitolipooligosaccharides from ANU843. The ^1H NMR spectrum of CLOS (Figure 1) purified from the membrane lipid extract of ANU843 cells grown with 4 μM DHF exhibited a combination of sharp aliphatic (0.8−2.1 ppm) and glycosidic (3.0−5.0 ppm) resonances, consistent with a low molecular weight glycolipid sample. GC-MS analyses of the peracetylated methyl glycosides and fatty acid methyl ester derivatives obtained by extensive methanolysis of the CLOS revealed GlcNAc as the sole glycosyl component, and several classes of fatty acids. GC-MS analysis of the partially methylated alditol acetates prepared from the CLOS sample revealed a mixture of 2-acetamido-2-*N*-methyl-3,4,6-tri-*O*-methylglucosamine diacetate, derived from the nonreducing glucosamine terminus of the oligosaccharides, and 2-acetamido-2-*N*-methyl-3,6-di-*O*-methylglucosamine triacetate indicating the glycosylation at the C4 position of the other GlcNAc residues in the oligosaccharides. Although an *N*-methyl-*N*-acylglucosamine residue has recently been found at the nonreducing terminus of CLOS from *A. caulinodans* (Mergaert et al., 1993), *B. japonicum* (Carlson et al., 1993), and *R. tropici* (Poupot et al., 1993), the ^1H NMR spectrum of the CLOS sample from *R. leguminosarum* bv. *trifolii* (Figure 1) did not show any evidence of *N*-methyl groups on the glucosamine moieties. This methyl group was therefore incorporated during the methylation step. The ^{13}C NMR spectrum of the ANU843 CLOS sample (Figure 1, inset) showed predominant anomeric resonances at 101.8 ppm and a minor anomeric signal at 90.3 ppm correlated in the $^1H/^{13}C$ HMQC NMR spectrum to proton resonances at 4.38 and 4.90 ppm, respectively. The downfield ^{13}C NMR chemical shift of the predominant anomeric resonances (101.8 ppm) was consistent with a β-anomeric configuration of the glycosidic residues in the oligosaccharides (Lipkind et al., 1988; Lerouge et al., 1990). The minor α-anomeric signal at 90.3 ppm arose from the mutarotation equilibrium between the α- and β-anomeric configurations for the reducing glucosamine residue of the oligosaccharides. The series of small doublets at 6.4−6.6 ppm, as well as most of the minor signals in the region of 4.4−4.8 ppm of the proton spectrum, disappeared when 10% D_2O was added to the perdeuterated DMSO solvent prior to NMR analysis. These were assigned to amide and hydroxylic exchangeable protons, respectively (Gasa et al., 1983).

Treatment of the CLOS sample with 1 M HCl in methanol for 1 h at 80 °C did not release the fatty acyl moieties and instead yielded a mixture of methyl glycosides of free *N*-acetylglucosamine and of a family of (*N*-)acylglucosamine monomers as determined by GC-MS. The latter compounds corresponded to the acylated glucosamine residues at the nonreducing end of the oligosaccharides, with the same series of fatty acid substituents as found in the native oligomers. This result indicating a stability of the acyl linkage under

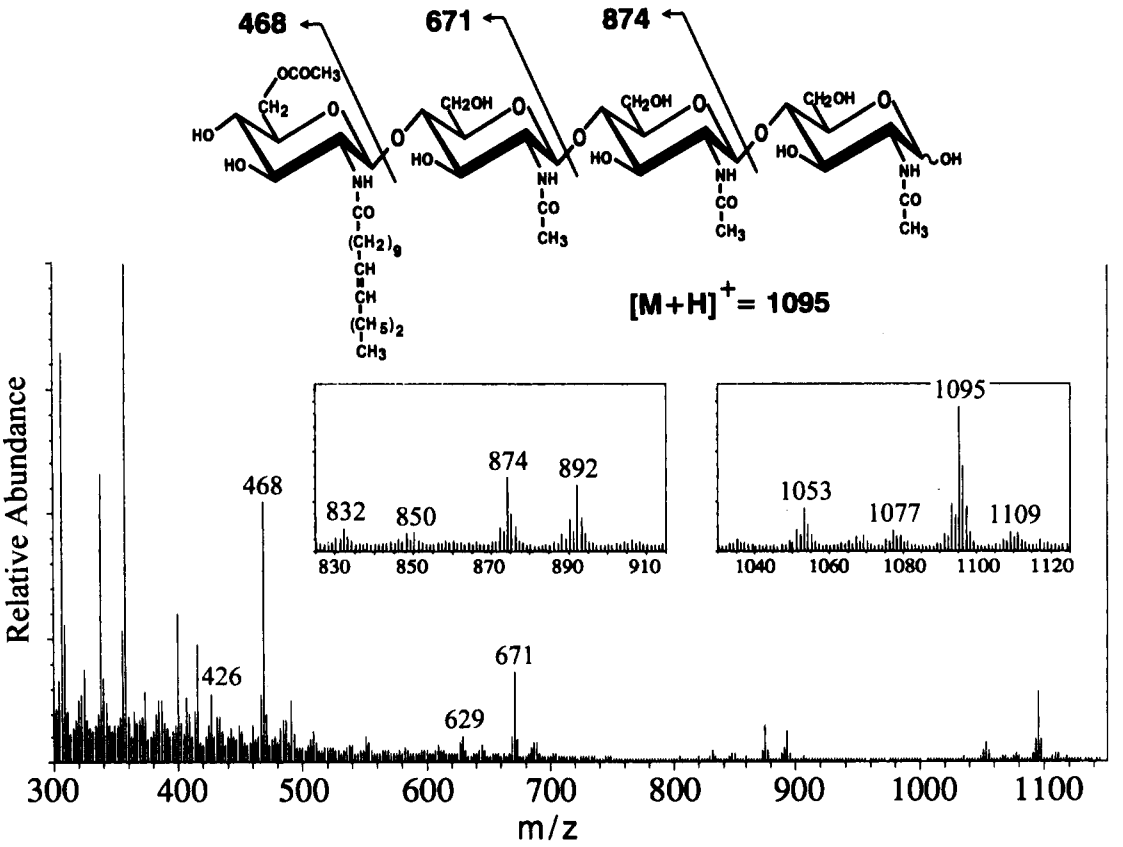


FIGURE 2: Positive mode FAB-MS of the family of membrane chitolipooligosaccharide NodRlt factors from wild-type *R. leguminosarum* bv. *trifolii* ANU843. The fragmentation sequence of the predominant membrane CLOS [NodRltIV(Ac, C18:1] is illustrated at the top. Portions of the FAB-MS spectrum between *m/z* 825−915 u and 1030−1125 u are enlarged within boxed inserts.

the above acidic conditions was consistent with the fatty acids being amide-linked rather than ester-linked to the nonreducing glucosamine terminus.

The complete release of the fatty acyl substituents from the CLOS sample was achieved by a more stringent methanolysis using 4 M HCl in methanol for 18 h at 80 °C. GC-MS and FID-GC of the methyl ester derivatives revealed a large predominance of octadecenoic acid (18:1, >70% of the total fatty acids), smaller proportions of a variety of other alkyl chains, including 16:0 (3.5%), 16:1 (1.5%), 18:0 (14.6%), 18:2 (1%), and also several hydroxylated fatty acids (3OH-14:0, 3OH-16:0, 3OH-18:0) that together accounted for 5−7% of the total fatty acids in the purified NodRlt CLOS sample.

The location of the double bond on the CLOS-derived octadecenoic (C18:1) acid was established as Δ11 by ozonolysis treatment followed by GC-MS analysis. This treatment produced an oxidative cleavage of the double bond and complete conversion of the 18:1 fatty acid methyl ester into a truncated C-11 aldehyde derivative that had the same mass spectrum and retention time in GC-MS as the ozonolysis product of an authentic vaccenic acid ($18{:}1^{\Delta 11}$) methyl ester standard. In addition, the CLOS-derived native octadecenoic acid coeluted with *cis*-vaccenic acid ($18{:}1^{\Delta cis11}$) upon GC analysis of the methyl ester derivatives under chromatographic conditions that separated *cis*- and *trans*-vaccenic acid standards.

A second fatty acid analysis of the CLOS sample was performed using a lower concentration of HCl in methanol (0.7 M, 18 h, 80 °C) in order to favor the recovery of potential acid-labile fatty acids, but no additional fatty acyl components were found under those conditions. In addition, only a partial release of the fatty acids was achieved under the latter conditions of solvolysis, as indicated by the detection of residual, unhydrolyzed *N*-acylglucosamine monomers in the GC-MS analysis.

The number of glycosyl residues in different species of CLOS NodRlt factors was determined by positive mode FAB-MS (Figure 2). This spectrum was dominated by the molecular ions and ion fragments from four CLOS species having pseudomolecular ions $[M + H]^+$ in the high mass range at *m/z* 1095, 1053, 892, and 850 u. These were assigned to mono-*O*-acetylated tetramer (1095), non-*O*-acetylated tetramer (1053), mono-*O*-acetylated trimer (892), and non-*O*-acetylated trimer (850) of *N*-acetylglucosamine bearing a single N-vaccenyl substituent.

The proportion of non-*O*-acetylated trisaccharide CLOS species (*m/z*: 848 and 850 u) in the total family of NodRlt factors was low (Figure 2). To verify their presence, further reversed-phase HPLC was performed. This led to a fraction containing predominantly trisaccharide CLOS species for which the ions at *m/z* 848 and 850 u in the corresponding FAB-MS spectrum were now predominant (figure not shown).

All of the above described pseudomolecular ions were examined by link scan MS-MS analysis (see for example Figure 3) and displayed similar patterns of fragmentation, notably a loss of 220 u followed by one loss (from ions at *m/z*: 892 and 850) or two consecutive losses (from ions at *m/z* 1095 and 1053) of 203 u. These ion sequences were as follows: *m/z* 1095/874/671/468, 1053/832/629/426, 892/671/468, and 850/629/426. This pattern of fragmentation was

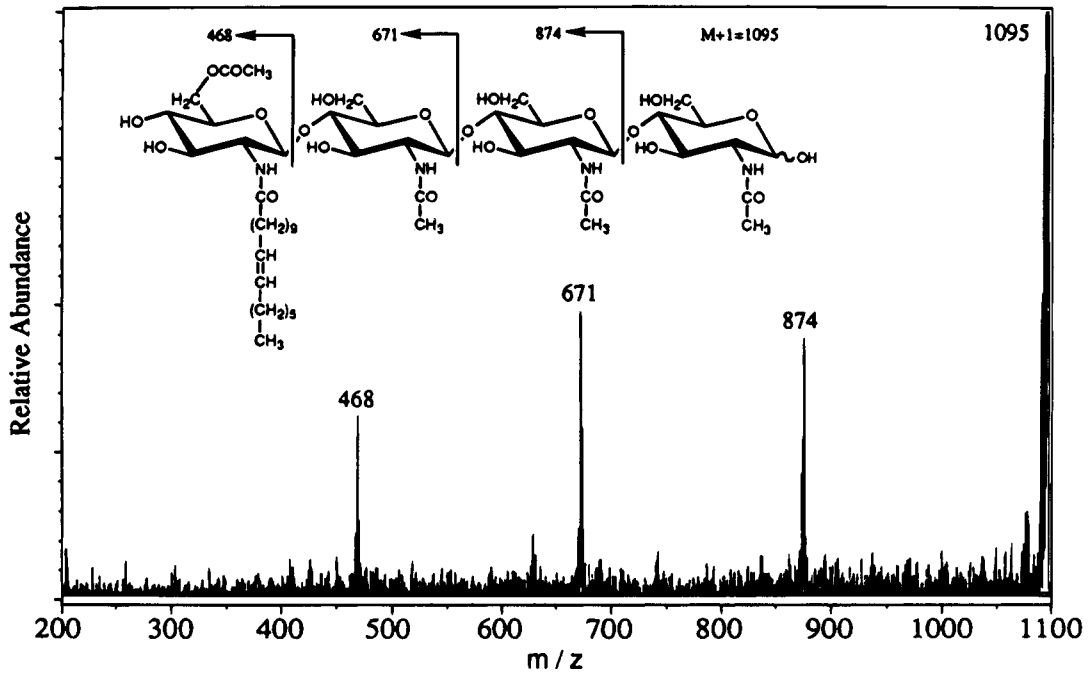


FIGURE 3: Constant B/E link scan of the parent ion (*m/z* 1095 u) from the FAB mass spectrum of the predominant membrane chitolipooligosaccharide [NodRltIV(Ac, C18:1] of wild-type *R. leguminosarum* bv. *trifolii* ANU843. Also shown are the molecular structure and fragmentation pattern dominated by scission between the glycosyl units of the oligosaccharide chain.

due to interglycosidic cleavages resulting in the elimination of the reducing GlcNAc terminus from the actual molecular ion (to generate the fragment $[M-220]^+$) and subsequent eliminations of GlcNAc residues (to generate the fragments $[M-220-203]^+$ and $[M-220-2 \times 203]^+$), as illustrated in Figure 3. In each of the above cases, these losses corresponded to non-*O*-acetylated, non-*N*-acylated GlcNAc residues. Thus, both the *O*-acetyl and *N*-fatty acyl substituents were located on the same nonreducing glucosamine terminus of the above four major chitolipooligosaccharides. The low molecular mass ions at *m/z* 468 and 426 u (Figure 2) were consistent with the masses of mono-*O*-acetylated and non-*O*-acetylated *N*-18:1-glucosamine ion fragments.

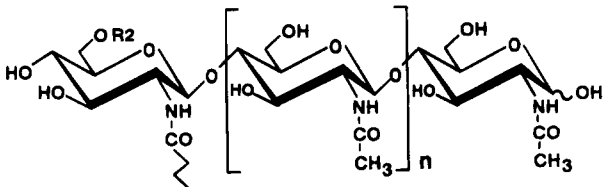
The FAB-MS spectrum of the CLOS NodRlt factors (Figure 2) also contained several minor high mass ions which gave a fragmentation sequence similar to that described above for the major CLOS species. These additional molecular masses were assigned to minor CLOS species having a diversity of fatty acids consistent with that described above. The family of CLOS NodRlt factors was again further fractionated by C18 reverse-phase HPLC in order to produce subfractions enriched in these minor CLOS species and enhance their detection by FAB-MS. One of the subfractions gave a FAB-MS spectrum containing weak high mass ions at *m/z* 1298 and 1256 u, consistent with chitopentaose oligomers *N*-acylated with an 18:1 fatty acid and bearing one or no *O*-acetyl substituent, respectively. In a different fraction, low intensity molecular ions were tentatively assigned to chitotriose or chitotetraose moieties *N*-acylated with 3OH-14:0, 3OH-16:0, or 3OH-18:0 fatty acids.

The exact location of the *O*-acetyl group in the membrane CLOS NodRlt factors was determined by NMR spectroscopy. ^{1}H NMR analysis of the CLOS sample exhibited intense singlets at 1.79 and 1.98 ppm (Figure 1), consistent with the resonances of the methyl group from the *N*-acetyl moieties of the *N*-acetyl glucosamine residues, and from the *O*-acetyl substituent, respectively. Mild alkaline hydrolysis selectively removed the *O*-acetyl group, resulting in the disappearance of the methyl resonance of the *O*-acetyl moiety and also of the two well-resolved resonances at δH 4.30 and 4.28 ppm. A two-dimensional HMQC analysis of the native CLOS sample showed that these two deshielded proton resonances correlated with the same carbon at δC 63.9 ppm, indicating attachment was to a site bearing two protons, i.e., the C-6 position. The resonance of the primary alcohol carbon (C-6) of a non-glycosylated *N*-acyl-GlcN in the β-anomeric configuration is typically at δC 62.1 ppm (Lipkind et al., 1988). The *O*-acetylation of this carbon normally induces a slight downfield shift (+1−2 ppm) of its resonance (Gagnaire et al., 1978), resulting in a chemical shift fully consistent with that of the carbon identified above as the site of *O*-acetylation. These NMR data are consistent with the FAB-MS results which indicate that an *O*-acetyl substituent is located at the C-6 position of the *N*-acylglucosamine forming the nonreducing termini of some of the ANU843 membrane CLOS species.

Figure 4 summarizes the definitively and tentatively identified species of CLOS NodRlt factors isolated from membrane lipid extracts of ANU843 cells. Although 19 distinct species of CLOS have been identified so far, HPLC and other analyses indicate the presence of several additional minor CLOS species which add to the diversity of this family of membrane glycolipids in ANU843 and remain to be identified.

Influence of nod Expression on Membrane Accumulation of the CLOS NodRlt Factors. The first step in fractionation (C18 reversed-phase chromatography) of the membrane lipid extract from 843+DHF cells yielded a fraction with glucosamine representing over 90% of the total sugars detected by GC-MS and diagnostic peaks characteristic of CLOS in its ^{1}H NMR spectrum (Figure 5A). GC-MS, ^{1}H NMR, and analytical C18 reversed-phase HPLC analyses of material purified to this stage were used as semiquantitative measures

Membrane Chitolipooligosaccharides of Wild Type R. trifolii ANU843



Assignments of 19 Individual Species of Chitolipooligosaccharides

M_r	Assignment	M_r	Assignment
848	NodRlt-III(C18:2)	1055	NodRlt-IV(C18:0)
850	NodRlt-III(C18:1)	1067	NodRlt-IV(Ac, C16:1)
854	NodRlt-III(Ac, 3OH-C14:0)*	1069	NodRlt-IV(Ac, C16:0)
890	NodRlt-III(Ac, C18:2)	1077	NodRlt-IV(C20:3)*
892	NodRlt-III(Ac, C18:1)	1093	NodRlt-IV(Ac, C18:2)
894	NodRlt-III(Ac, C18:0)	1095	NodRlt-IV(Ac, C18:1)
1043	NodRlt-IV(3OH-C16:0)*	1097	NodRlt-IV(Ac, C18:0)
1051	NodRlt-IV(C18:2)	1113	NodRlt-IV(Ac, 3OH-C18:0)*
1053	NodRlt-IV(C18:1)	1256	NodRlt-V(C18:1)
		1298	NodRlt-V(Ac, C18:1)*

*Tentative assigments of minor species

FIGURE 4: Summary of structures found within the family of membrane chitolipooligosaccharides isolated from pelleted cells of wild-type *R. leguminosarum* bv. *trifolii* ANU843.

of the CLOS content in equivalent wild-type and mutant membrane lipid extracts. Only very low levels of glucosamine (<2% of the total sugar content) were detected in the GC-MS analysis of the methyl glycoside peracetate derivatives of these CLOS-equivalent fractions from 843-DHF, 845+DHF and 277+DHF cells. Although the ^{1}H NMR spectra of these latter CLOS-equivalent fractions indicated the presence of aliphatic moieties (strong alkyl proton resonances at δH 0.7−2.4 ppm), they were devoid of diagnostic resonances for CLOS, as illustrated by the absence of intense resonances of methyl groups of the *N*-acetyl (δH 1.79 ppm) and *O*-acetyl (δH 1.98 ppm) moieties (Figure 5A). Whereas the C18 reversed-phase HPLC profile of the 843+DHF fraction was dominated by a cluster of intense CLOS peaks (eluting with retention times between 22−39 min), only background level peaks were present in the same chromatographic region in the profiles of the 843−DHF, 845+DHF, and 277+DHF CLOS-equivalent fractions (Figure 5B). These three lines of evidence indicate that DHF and pSym *nod* genes promote the membrane accumulation of chitolipooligosaccharides in wild-type ANU843.

Biological Activity of Membrane CLOS NodRlt Factors from ANU843. The membrane CLOS NodRlt factors from ANU843 exhibited a very potent biological activity capable of inducing rudimentary nodule-like meristems in the root cortex of axenic seedlings of Dutch white clover (a host plant for *R. leguminosarum* bv. *trifolii*) at a minimum threshold concentration of 10^{-11} M. This mitogenic activity exhibited potential host selectivity since the ANU843 CLOS NodRlt factors at 10^{-7}, 10^{-9}, or 10^{-11} M did not induce nodule-like meristems on the non-host legumes hairy vetch or alfalfa.

DISCUSSION

The general view on *Rhizobium* chitolipooligosaccharides is that they are made in very low levels as freely diffusible

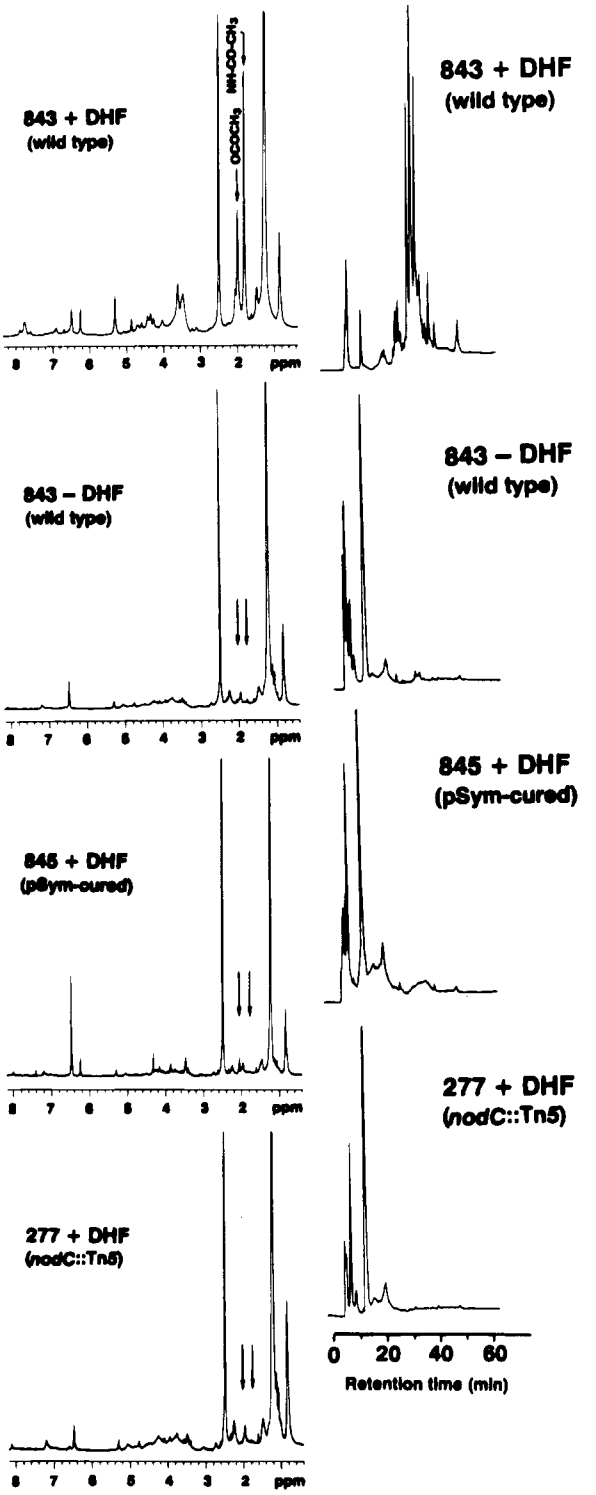


FIGURE 5: Influence of flavone and pSym *nod* genes on accumulation of membrane chitolipooligosaccharides in wild-type *R. leguminosarum* bv. *trifolii* ANU843. (A, left) ^{1}H NMR spectra of the partially purified CLOS fraction (20−50% acetonitrile eluate from C-18 reversed phase) of the membrane lipid extract of ANU843 cells grown with DHF (843+DHF) and the equivalent fractions from ANU843 grown without flavone (843-DHF), the pSym-cured derivative grown with DHF (845+DHF), and the *nodC*::Tn*5* derivative grown with DHF (277+DHF). Arrows indicate the position of the diagnostic methyl resonances of *N*-acetyl and *O*-acetyl substitutions of CLOS from the 843+DHF culture (top). (B, right) C18 reversed-phase HPLC profiles of the same fractions. Peaks eluting with retention times between 22 and 39 min correspond to the various CLOS species. Methods to isolate these fractions are described in the text.

molecules and are primarily secreted by the bacteria into the extracellular milieu where they interact with the host. This view is based in part on the minute quantities of CLOS which typically accumulate in the extracellular fraction of *Rhizobium* cultures grown to express their *nod* genes. Indeed, the isolation of CLOS in sufficient quantities for structural analysis from the culture supernatant of many wild-type strains of *Rhizobium* has proved to be extremely difficult. In several cases, genetically engineered *Rhizobium* strains that overexpress *nod* genes had to be constructed for successful isolation of CLOS molecules from this extracellular source. Even with such recombinant strains, the highest reported yield of CLOS was lower than 4 mg/L of culture.

We analyzed membrane lipid extracts of wild-type *R. leguminosarum* bv. *trifolii* ANU843 cells [carrying only one copy of pSym *nod* genes (Djordjevic & Weinman, 1991)] and the corresponding culture supernatants for CLOS-type glycolipids. As predicted, fractionation of the membrane lipid extracts from pelleted cells led to the isolation of a family of CLOS in high yield, whereas all attempts to isolate CLOS-type glycolipids from the corresponding culture supernatant failed to yield detectable amounts of this glycolipid. In preliminary studies, we found that *n*-butanol did not extract CLOS from cell pellets of ANU843. This indicates that CLOS glycolipids in ANU843 behave primarily as integral cell components, most likely residing within membranes, rather than as "loosely associated" cell components. A successful extraction of CLOS therefore necessitated the formulation of a solvent system capable of completely disrupting membrane structures and solubilizing their lipid components. Assuming a conservative lower limit of 15 μg/L needed to successfully isolate and purify CLOS from the culture supernatant, one would conclude that at least 1000-fold higher levels of these CLOS NodRlt factors accumulate in ANU843 as cell-bound glycolipids than are secreted from an equivalent number of cells into the extracellular milieu. Knowing that the cell density at harvest was ca. 9×10^{11} cells per liter of culture, this calculates to a minimum average yield of approximately 10^7 CLOS molecules per bacterial cell. Structural analyses indicate that these cellular CLOS have the diversity of fatty acyl moieties that one would typically expect of bacterial membrane glycolipids. This family of membrane CLOS NodRlt factors from ANU843 at a concentration as low as 10^{-11} M can induce cortical meristems resembling rudimentary nodule primordia on axenic seedling roots of the white clover host. All of these results indicate that these symbiotically important CLOS glycolipids accumulate primarily in cell membranes of wild-type *R. leguminosarum* bv. *trifolii*, and our protocol which readily isolates these NodRlt factors in high yield from this source eliminates the need to use recombinant "overproducing" strains to obtain sufficient quantities for structural analyses. The accumulation of CLOS primarily as membrane components is not unique to wild-type *R. leguminosarum* bv. *trifolii*, since diverse families of CLOS also accumulate primarily in cell membranes of several other wild-type rhizobia used extensively in studies of the root nodule symbiosis, including *R. meliloti* 2011, *R. leguminosarum* bv. *viciae* 300, and *R. sp.* (siratro) NGR234 (R. Hollingsworth and R. Cedergren, unpublished data).

The above result is not unusual for amphiphilic molecules which exhibit potent biological activities, e.g., bacterial endotoxin or the phosphodiglyceride platelet activation factor (Morgan, 1937; Hanahan, 1986). Both of these bioactive molecules were first observed as "extracellular factors" in quantities which defied isolation and characterization, until they were found to be largely membrane localized and available in large quantities from that source. The extremely low "extracellular" concentrations of authentic chitolipooligosaccharides in *nod*-expressing broth cultures of wild-type rhizobia could be explained by organic extraction of residual cells in the centrifuged culture supernatant and/or normal shedding of membrane vesicles from the bacteria during cell division. Related to the latter phenomenon is the finding that lipopolysaccharide, a major amphiphilic glycolipid in the outer membrane of *Rhizobium*, is released from the bacteria into the external root environment where it interacts with host root hairs and modulates infection thread development in the *R. leguminosarum* bv. *trifolii*-white clover symbiosis (Dazzo et al., 1991).

Structural analyses indicate that the family of membrane CLOS synthesized by wild-type ANU843 is comprised of a large diversity of glycolipids which vary in glucosamine oligomerization (dp 3, 4, or 5), presence or absence of an *O*-acetyl substituent, and type of amide-linked fatty acyl moiety. *cis*-Vaccenic acid ($18{:}1^{\Delta cis11}$) is a predominant *N*-acyl substituent in membrane CLOS of ANU843. In addition, GC-MS and FAB-MS analyses also indicate other saturated (16:0, 18:0), unsaturated (18:2, 20:3), and 3-hydroxy fatty acids (3OH-14:0; 3OH-16:0; 3OH-18:0) as *N*-acyl moieties of these CLOS glycolipids. A diversity of amide-linked fatty acids in membrane CLOS was found in several batch cultures of ANU843 grown under the same conditions. However, as for all bacterial membrane lipids, it is likely that the composition and relative proportion of fatty acyl moieties in the membrane CLOS of ANU843 will vary somewhat depending on its physiological status and condition of growth. In this study, considerable care was made to control this variable by harvesting the cultures at a specific stage of growth in BIII medium in which the inoculum of *R. leguminosarum* bv. *trifolii* is most interactive with white clover (Dazzo et al., 1979; Hrabak et al., 1981).

In general, the biosynthesis of CLOS has been linked to the expression and function of *nod* genes in rhizobia. The pSym *nod*-encoding region of wild-type *R. leguminosarum* bv. *trifolii* ANU843 contains a single copy of *nod* genes encoding common and host specific functions and whose expression is activated by DHF, a flavone secreted from roots of the white clover host (Redmond et al., 1986; Djordjevic & Weinman, 1991). Our data indicate that the presence of pSym *nod* genes and this *nod*-activating flavone promotes the accumulation of various membrane CLOS made by wild-type ANU843. On the other hand, the fatty acids identified so far in membrane CLOS from ANU843 have also been found previously in membrane phospholipids, lipid A, or whole cell lipid extracts from this same wild-type strain grown without flavone or in the pSym-cured derivative ANU845 (Orgambide et al., 1993; Hollingsworth & Lill-Ellighan, 1991).

Our finding that chitolipooligosaccharide Nod factors accumulate primarily in bacterial membranes of wild type *R. leguminosarum* bv. *trifolii* not only simplifies the logistics of isolating sufficient quantities of these glycolipids for structural elucidations but also influences the current thinking on the biological fate and action of CLOS signal molecules for the bacteria themselves and in symbiosis with the legume

host. For instance, these results suggest that, in addition to activating segments of the host symbiotic program, CLOS may also perform important physiological functions within membranes of wild-type rhizobia themselves. Another important implication of the primary accumulation of CLOS in rhizobial membranes is the likelihood that their amphiphilic nature limits the spatial distribution of their action during the normal infection process. CLOS released from rhizobia during growth in the partially hydrated environment of the rhizosphere would immediately form extracellular micelles that would be more restricted in dispersal than would freely diffusible molecules. These extracellular micelles would tend to fuse with and spontaneously incorporate into neighboring membranes that they encounter. Such action will be favored by close proximity of bacterial and host membranes as would occur when the bacteria are sequestered within the shepherd's crook morphology of the root hair tip. We propose that these short range effects describe how intact membrane chitolipooligosaccharides behave during normal development of the *Rhizobium*–legume symbiosis.

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SUPPLEMENTARY MATERIAL AVAILABLE

The FAB-MS spectrum of a reversed-phase HPLC subfraction enriched in NodRlt-III(C18:1), NodRlt-III(C18:2), NodRlt-III(Ac,C18:1), and NodRlt-III(Ac,C18:2) CLOS species (1 page). Ordering information is given on any current masthead page.

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