Common Links in the Structure and Cellular Localization of *Rhizobium* Chitolipooligosaccharides and General *Rhizobium* Membrane Phospholipid and Glycolipid Components†

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ABSTRACT: Several common links between the structural chemistry of the chitolipooligosaccharides of *Rhizobium* and the general rhizobial membrane lipid and lipopolysaccharide chemistry of these bacteria have been uncovered. Aspects of common chemistry include sulfation, methylation, and the position and extent of fatty acyl chain unsaturation. We find that bacteria which are known to synthesize sulfated chitolipooligosaccharides (such as *Rhizobium meliloti* strains and the broad-host-range *Rhizobium* species strain NGR234) also have sulfated lipopolysaccharides. Their common origins of sulfation have been demonstrated by using mutants which are known to be impaired in sulfating their chitolipooligosaccharides. In such cases, there is a corresponding diminution or complete lack of sulfation of the lipopolysaccharides. The structural diversity of the fatty acids observed in the chitolipooligosaccharides is also observed in the other membrane lipids. For instance, the doubly unsaturated fatty acids which are known to be predominant components of *R. meliloti* chitolipooligosaccharides were also found in the usual phospholipids and glycolipids. Also, the known functionalization of the chitolipooligosaccharides of *R.* sp. NGR234 by O- and N-methylation was also reflected in the lipopolysaccharide of this organism. The common structural features of chitolipooligosaccharides and membrane components are consistent with a substantial degree of biosynthetic overlap and a large degree of cellular, spatial overlap between these molecules. The latter aspect is clearly demonstrated here since we show that the chitolipooligosaccharides are, in fact, normal membrane components of *Rhizobium*. This increases the importance of understanding the role of the bacterial cell surface chemistry in the *Rhizobium*/legume symbiosis and developing a comprehensive understanding of the highly integrated membrane lipid and glycolipid chemistry of *Rhizobium*.

The lipopolysaccharides (LPS)[1] of *Rhizobium* have been implicated in several stages of the symbiotic nitrogen-fixing process between these bacteria and legumes. These stages include infection, bacterial release from infection threads, and bacteroid transformation and maturation (Puvanesarajah et al., 1987; Cava et al., 1989; deMaag et al., 1989; Priefer, 1989; Williams et al., 1990; Dazzo et al., 1991; Zhang et al., 1992). A definitive involvement of capsular polysaccharide biosynthesis and structure is also well established. Specific mutations of bacterial genes involved in the various stages of capsular polysaccharide biosynthesis show a high correlation with symbiotic deficiencies in nodulation (Leigh & Walker, 1994). The cell surface components of *Rhizobium*, then, have always been considered as essential in the bacteria's very specific interaction with legumes. This interaction is known to be controlled from the bacteria's side by the host specific *nod* genes. These genes, along with the common *nod* genes which are essential for nodulation in all *Rhizobium* species, have been shown to be involved in the synthesis of specific bacterial signal molecules which regulate plant responses to *Rhizobium*. These signal molecules are (chito)lipooligosaccharides (Figure 1) containing β-1,4-linked *N*-acetylglucosamine residues which, in the case of *Rhizobium meliloti* and *Rhizobium* sp. NGR234, are sulfated (Lerouge et al., 1990; Roche et al., 1991a; Price et al., 1992). In *R. meliloti*, two of the more important host specific genes, *nod*PQ and *nod*H, are known to be involved in the sulfation

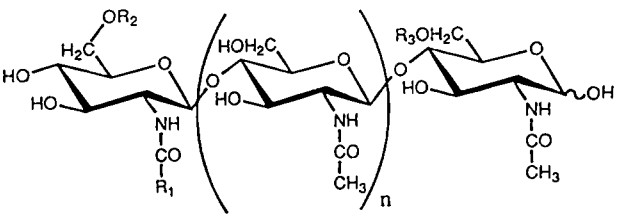

FIGURE 1: General structure of chitolipooligosaccharides found in *Rhizobium*. R_1 is an alkyl chain containing various degrees of unsaturation and hydroxylation. R_2 is H or acetate depending on species or biovar. R_3 can be H, sulfate, or another substituted glycosyl component. $n = 1-3$.

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[1] Abbreviations: LPS, lipopolysaccharide; NMR, nuclear magnetic resonance; GC/MS, gas chromatography/mass spectrometry; FAB-MS, fast-atom bombardment mass spectrometry; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; KDO, 2-keto-3-deoxyoctulosonic acid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PMME, phosphatidylmonomethylethanolamine; HMQC, heteronuclear multiple-quantum coherence spectroscopy; FAME, fatty acid methyl ester.

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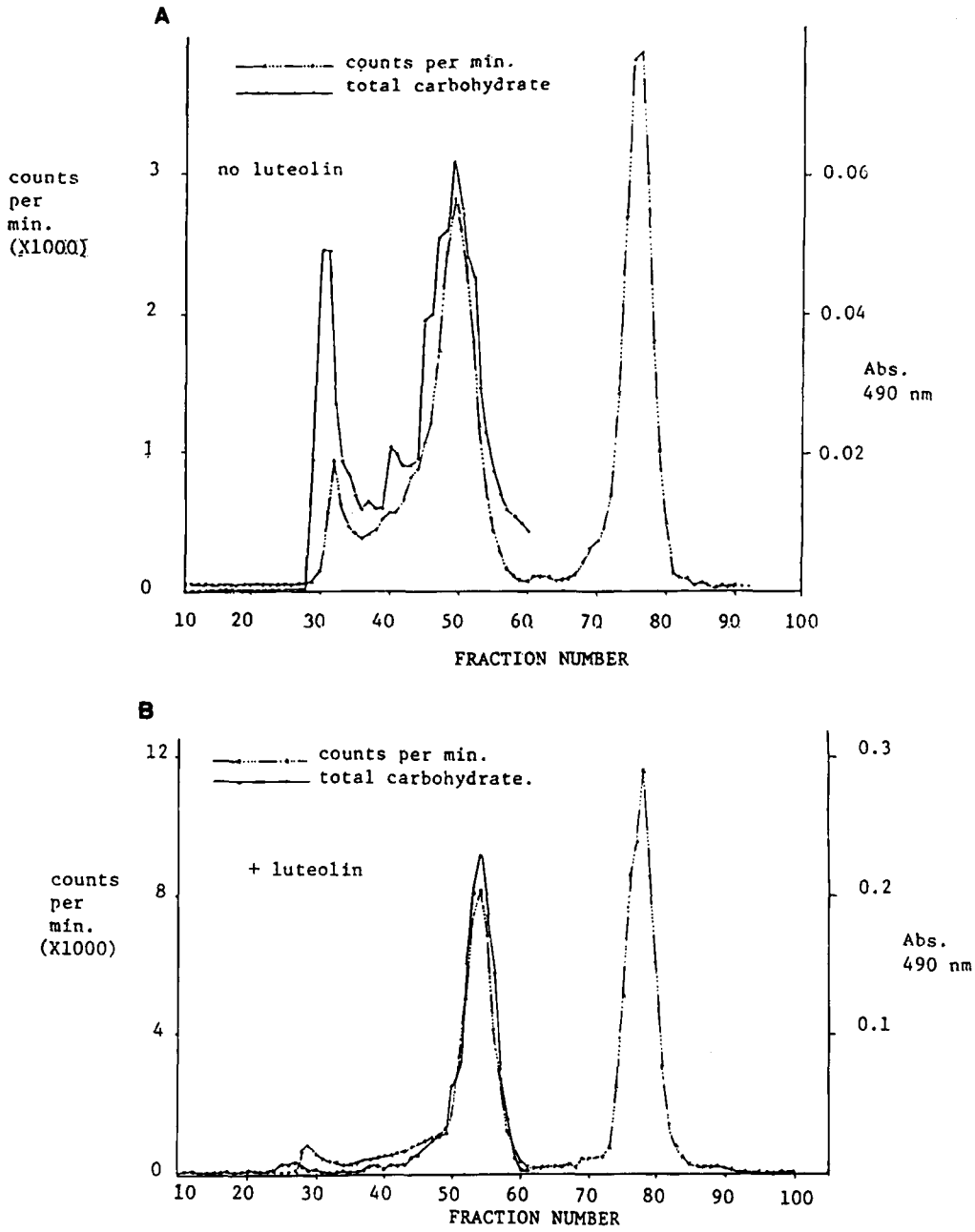

FIGURE 2: (A) Sepharose 4B gel filtration profile of the crude phenol extract of ^{35}S-labeled *R. meliloti* 2011 cells grown in the absence of luteolin. Fractions were assayed for radioactivity by scintillation counting and for total carbohydrate. The second eluting peak (fractions 35−60) is due to LPS; note the presence of radiolabel. The first eluting peak (fraction 28−34) is due largely to a high molecular weight capsular polysaccharide. There is, however, a small amount of a coincident very high molecular weight LPS form. Note the presence of radioactivity in these components. The late eluting peak (fractions 68−82) contains free sulfate and a β-1,2-glucan as well as low molecular weight sulfur-containing metabolites. (B) Similar profile for *R. meliloti* 2011 cells grown in the presence of luteolin. Note that the LPS is still radiolabeled and that the level of radiolabeling (counts/absorbance) is essentially the same as that observed in the absence of luteolin. Note that the presence of luteolin also has an effect on the extent of encapsulation as evidenced by the decrease in intensity of the first eluting peak.

of the lipooligosaccharides (Schwedock & Long, 1990; Roche et al., 1991b). The function of *nod*PQ in the biosynthesis of the activated sulfate molecule has been clearly demonstrated (Schwedock & Long, 1990, 1992).

One of the confounding aspects of *Rhizobium* chitolipooligosaccharide structure is its tremendous variability, even in the same strain. The first studies on *R. meliloti* chitolipooligosaccharides promoted the idea that each species of *Rhizobium* synthesized, almost exclusively, only one molecule with no variation in carbohydrate or fatty acid chain length, degree of sulfation or acetylation, or position or extent of alkyl chain unsaturation (Lerouge et al., 1990). Subsequent studies, however, have demonstrated that any given bacterial species, including *R. meliloti*, can produce chitolipooligosaccharides containing a variety of different fatty acids. Such lipooligosaccharides may also contain between zero and four double bonds in their acyl chains, differing degrees of sulfation, varying numbers of glucosaminyl residues, and various substituents ranging from methyl and carbamoyl to entire glycosyl groups (Denarie et al., 1993; Poupot et al., 1993; Vijn et al., 1993). In one case, 18 different chitolipooligosaccharides were identified or characterized in one bacterial strain (Price et al., 1992). The structural diversity of chitolipooligosaccharides is such that

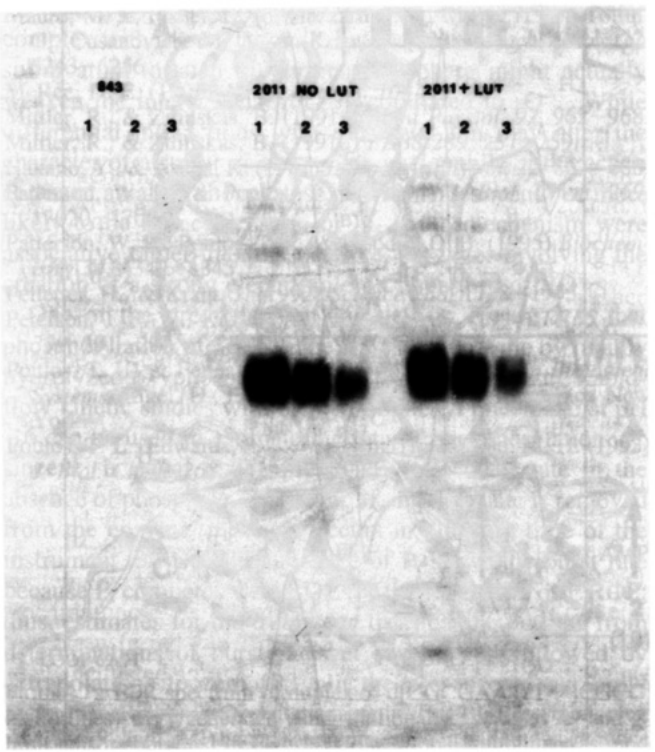

FIGURE 3: Autoradiograph of a sodium dodecyl sulfate gel showing the separation of ^{35}S-labeled LPS isolated from *R. trifolii* ANU 843 and *R. meliloti* 2011 in the presence and absence of flavone inducers. The samples for each condition were applied in triplicate using decreasing amounts (3:2:1) for each lane. Note that the *R. meliloti* LPS is radiolabeled independently of flavone (luteolin). Note also that there is no incorporation of label in the *R. trifolii* LPS. Only a slight shift of one of the minor bands is observed for the *R. meliloti* LPS isolated from cells grown in the presence of flavone compared to the result obtained for cells grown in the absence of flavone.

the relatively sparse number of *nod* genes cannot account for it. The only location in the cell, known to contain the vast repository of enzymatic activities capable of producing such a myriad of structures, is the membrane. In order to use this machinery during their biosynthesis, chitolipooligosaccharides would have to be membrane localized. If this were true, the structural modifications one finds in chitolipooligosaccharides should also be found in the other membrane components of that strain. If chitolipooligosaccharides were membrane localized, the more complete picture that emerges should have the bacterial cell surface and membrane chemistry as the critical factors in determining host range and the outcome of a potential symbiotic event between *Rhizobium* and legumes.

MATERIALS AND METHODS

Strains and Bacterial Cultures. R. meliloti 1021 mutants were obtained from Dr. Sharon Long (Stanford University, CA). All bacterial strains were grown in liquid cultures using modified Bergensen's media as previously described (Dazzo, 1982) except that ^{35}S-labeled sulfate in the form of sulfuric acid (New England Nuclear) at a level of 100 μCi/L of culture was added before adjusting the pH of the medium to neutrality. This medium contains sulfate as the only form of sulfur.

Nuclear Magnetic Resonance (NMR) Spectroscopy and Mass Spectrometry. NMR spectra were recorded on a Varian VXR-500 spectrometer operating at 500 MHz for protons. Spectra were acquired in deuterium oxide containing 10% deuterated methanol with chemical shifts being referenced relative to external TMS. Gas chromatography/mass spectrometry (GC/MS) was performed on a JEOL 505 mass spectrometer interfaced to a Hewlett Packard 5890 gas chromatograph. Spectra were recorded in the positive ion mode using electron impact with an ionization voltage of 70 eV. For the analyses of carbohydrate derivatives, a J and W DB225 30 m capillary column was used with a temperature program of 180−230 °C at 3 °C/min and a 30 min hold at the upper temperature. Fast atom bombardment-mass spectra (FAB-MS) were recorded on a JEOL 110-HX-HF instrument in both the negative and positive ion modes. The matrix used for chitolipooligosaccharide analysis (performed in the negative ion mode) was 1:1 glycerol/4-nitrobenzyl alcohol. For phospholipid analyses, the matrices were 4-nitrobenzyl alcohol/15-crown-5/camphorsulfonic acid for positive ion mode and 4-nitrobenzyl alcohol/15-crown-5/*tert*-butyl ammonium hydroxide for negative ion mode. Electrospray mass spectrometry was performed on a Fisons Platform I instrument operating in the negative ion mode. The sample was infused into the instrument in 1:1 acetonitrile/water.

Lipopolysaccharide Isolation. Lipopolysaccharide (LPS) was isolated and purified by gel filtration chromatography on Sepharose 4B columns as previously described (Williams et al., 1990; Zhang et al., 1992). Column effluents were monitored for radioactivity by liquid scintillation. The purity of LPS preparations was established by compositional analysis for fatty acids and carbohydrates by GC and GC/MS (see below). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis was performed using 16% acrylamide gels. LPS on gels was detected both by silver staining and by exposure of the dried, 2,5-diphenyloxazole-impregnated gels to KODAK X-omat X-ray plates at −75 °C for 4 days.

Chitolipooligosaccharide Isolation and Purification. Two liters of broth culture were typically grown for chitolipooligosaccharide isolation. Cells were harvested in late stationary phase by centrifugation, and lipids in the cell pellet were extracted by stirring either with a mixture of chloroform, *n*-butanol, methanol, and water (2:1:1:4) or chloroform, methanol, and water (4:1:5). The organic layers were removed, and the aqueous layers were concentrated to dryness and then redissolved in 5 mL of water and adsorbed onto a short column of C-8 or C-18 reverse phase packing (5 cm × 2.5 cm). The column was eluted sequentially with 30 mL each of water, 4:1 water/methanol, 1:1 methanol/water, 4:1 methanol/water, and pure methanol. Fractions were analyzed by proton NMR spectroscopy; those containing the characteristic *N*-acetyl methyl signals at about 2 ppm were subjected to further purification by reverse-phase high-performance liquid chromatography on a C-8 reverse-phase column using ultraviolet (UV) detection and monitoring at 220 nm. A linear solvent gradient from 10% acetonitrile in water to 80% acetonitrile in water was used for the separation. The chitolipooligosaccharides could also be purified by gel filtration on Bio-Gel P6 using acetonitrile/water (1:1) as the solvent. Peaks were analyzed for glucosamine content by gas chromatography while radiolabeled samples were also analyzed by scintillation counting. The typical yield of product from 2 L of cells was 8−12 mg and was not significantly affected by the addition of flavone (luteolin). Products were analyzed by NMR spectroscopy,

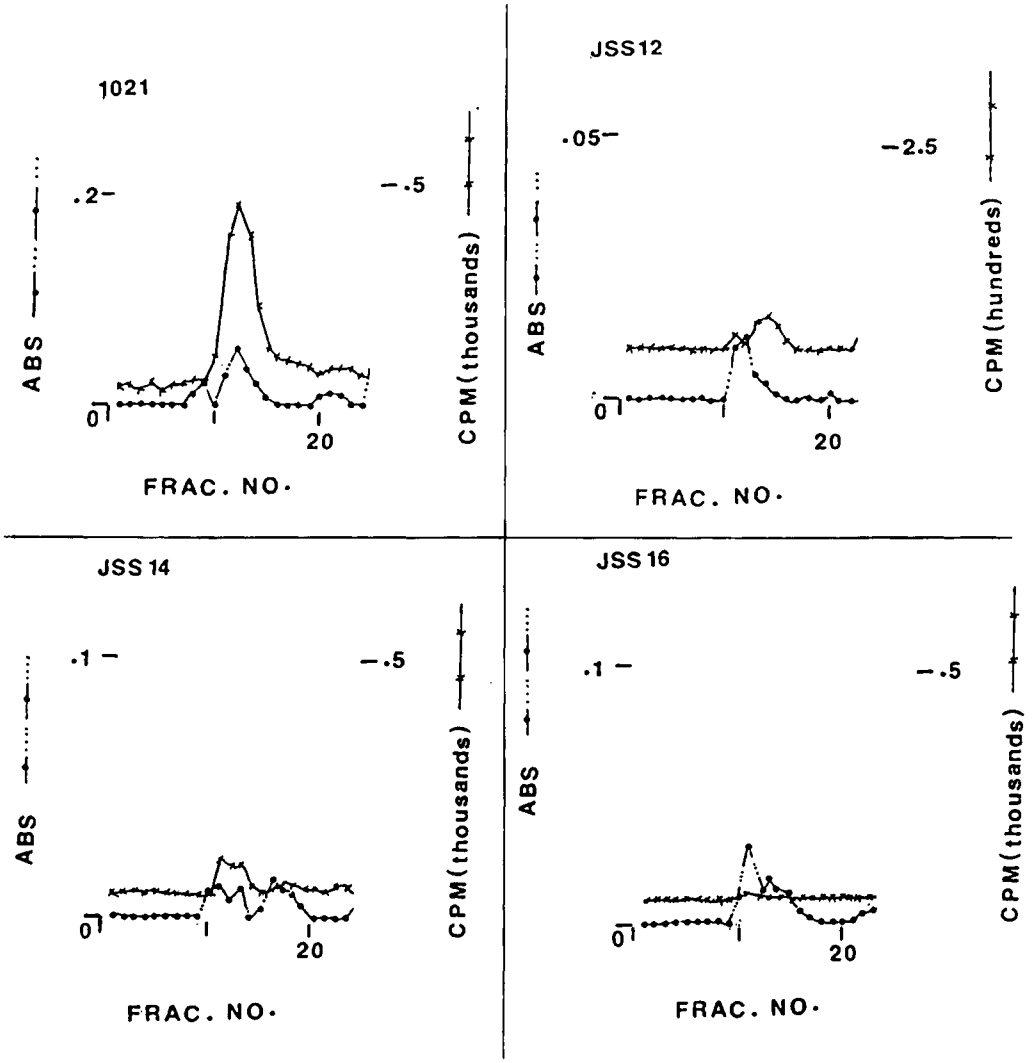

FIGURE 4: Sepharose 4B gel filtration profiles of LPS isolated from ^{35}S-labeled *R. meliloti* 1021 cells and three Tn5 mutants assaying for total carbohydrate and radioactivity. The small, earlier eluting peak lacking radioactivity (fractions 7−10 in the first panel) has been attributed to a capsular polysaccharide related to the *E. coli* K antigen (Reuhs et al., 1993). Note that there is significant reduction of the level of sulfation in the *nod*Q_1 and *nod*Q_2 mutants (JSS12 and JSS14, respectively) in the region of LPS elution (fraction 10−15). Note also that sulfation of the LPS is totally abolished in the case of the double mutant (JSS16). Loss of sulfate groups on the LPS of all three mutants results in increased levels of synthesis of the non-sulfated protective capsular polysaccharide. (Note: A late eluting peak of totally included free radiolabeled sulfate and β-1,2-glucan has been omitted for clarity.)

fast atom bombardment mass spectrometry, and electrospray mass spectrometry.

Compositional and Linkage Analysis of Chitolipooligosaccharides. The carbohydrate compositions of chitolipooligosaccharides were determined by hydrolysis of approximately 1 mg of sample with 2 M trifluoroacetic acid (1 mL) at 120 °C for 2 h followed by extraction of the free fatty acids from the monosaccharides with chloroform. The aqueous layer was then concentrated to dryness under a stream of nitrogen and the product redissolved in water and reduced to alditols using sodium borohydride (1 mg). The excess borohydride was decomposed by treatment with 30 μL of acetic acid and the resulting solution concentrated to dryness. Methanol (2 mL) containing 2% acetic acid was then added and the solution concentrated to dryness again. This process of adding acetic acid in methanol and concentrating was repeated five times to remove all traces of boric acid as the volatile methyl ester. The residue was finally peracetylated by treatment with pyridine (0.2 mL) and acetic anhydride (0.2 mL) at room temperature for 24 h. The reagents were removed under a stream of nitrogen, and the residue was analyzed by GC/MS. Alternatively, both the fatty acid and the carbohydrate compositions were determined simultaneously by methanolysis of a sample of lipooligosaccharide (0.5 mg) with 3% HCl in methanol at 70 °C for 24 h, peracetylation of the mixture with acetic anhydride and pyridine, and performing GC and GC/MS analyses directly on the mixture using the same column conditions as described above. Methylation analysis was performed using silver oxide as the base and methyl iodide as the methylating agent in dimethylformamide (DMF) as the solvent. A sample of lipooligosaccharide was dissolved in dry DMF (1 mL), and methyl iodide (0.2 mL) and silver oxide (50 mg) were added. The mixture was stirred at room temperature for 36 h and then diluted with chloroform (2 mL) and filtered. The filtrate was concentrated to dryness, and then the residue was hydrolyzed, reduced with sodium borohydride, peracetylated, and analyzed by GC and GC/MS as described above.

Phospholipid Isolation and Characterization. Phospholipids were separated and isolated by thin layer chromatography (TLC) on silica gel using a solvent system composed

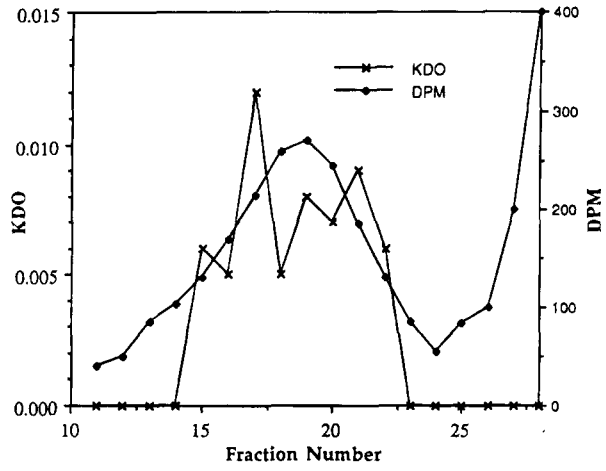

FIGURE 5: Sepharose 4B gel filtration profile of extract from ^{35}S-labeled *R.* sp NGR234 cells showing the correspondence of radioactivity to the LPS marker component KDO. The late eluting peak of radioactivity is due to free sulfate and other small sulfur-containing substituents.

of chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1 by volume). Bands were detected by removing the edges of the plates and spraying them with 5% phosphomolybdic acid followed by heating at 120 °C for 5 min. The bands were removed from the layers by scraping and eluting with chloroform/methanol (2:1). The components thus isolated were analyzed by fast atom bombardment mass spectrometry in both the positive and negative ion modes. The fatty acids were analyzed by GC and GC/MS after methanolysis.

RESULTS AND DISCUSSION

The Sepharose 4B gel filtration chromatogram of the LPS of *R. meliloti* 2011 showed it to be labeled by ^{35}S (Figure 2) in a flavone-independent manner. The ratio of ^{35}S counts to carbohydrate absorbance units for the LPS peak (fractions 45−60) in induced and noninduced cultures is approximately the same in each case. The addition of luteolin does have some (albeit slight) effects on the size distribution of the lipopolysaccharide as well as the extent of membrane lipid synthesis and cell encapsulation. This latter point is reflected in the reduction of carbohydrate content in the fractions preceding those of the LPS. These components have been described (Reuhs et al., 1993) as KDO-rich capsular antigens related to the *Escherichia coli* K antigen and are structurally distinct from LPS. The reduction in amount observed when the cells are grown in the presence of luteolin is not surprising since luteolin is, in fact, bacteriocidal at higher concentrations and should provoke stress responses from the bacteria even in moderate concentrations. In order to reinforce these results and verify that the label was attached to the LPS and not free, a sample of the LPS was subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) in parallel with a similar preparation from *Rhizobium trifolii* ANU 843. The gels were examined by silver staining and by autoradiography. These analyses also indicated that the LPS of *R. meliloti* was radiolabeled and that the level of incorporation of label was independent of flavone (Figure 3). In contrast, the *R. trifolii* LPS was found to contain no sulfur. These results also reflected the aforementioned changes in molecular weight distribution due to the presence of the flavone. Silver-stained gels (not shown) reflected exactly what is portrayed in the autoradiograms. In SDS−PAGE, the LPS of wild-type rhizobial strains generally appears as only one major band with a smaller amount of a faster moving component which is thought to be rough LPS. The form of the sulfur containing species was definitively identified as sulfate by liberating it from a Sepharose 4B purified LPS sample by mild acid hydrolysis and precipitating the free sulfate as barium sulfate. No radioactivity was detected in the solution containing the carbohydrate components. Based on the level of radioactivity and the amount of LPS isolated (assuming a molecular mass of about 30 000 Da for a smooth LPS), a conservative estimate of 1.3 sulfate groups per LPS molecule was obtained. It should be noted that, because of the extreme lability of sulfate groups, this estimate is likely to be lower

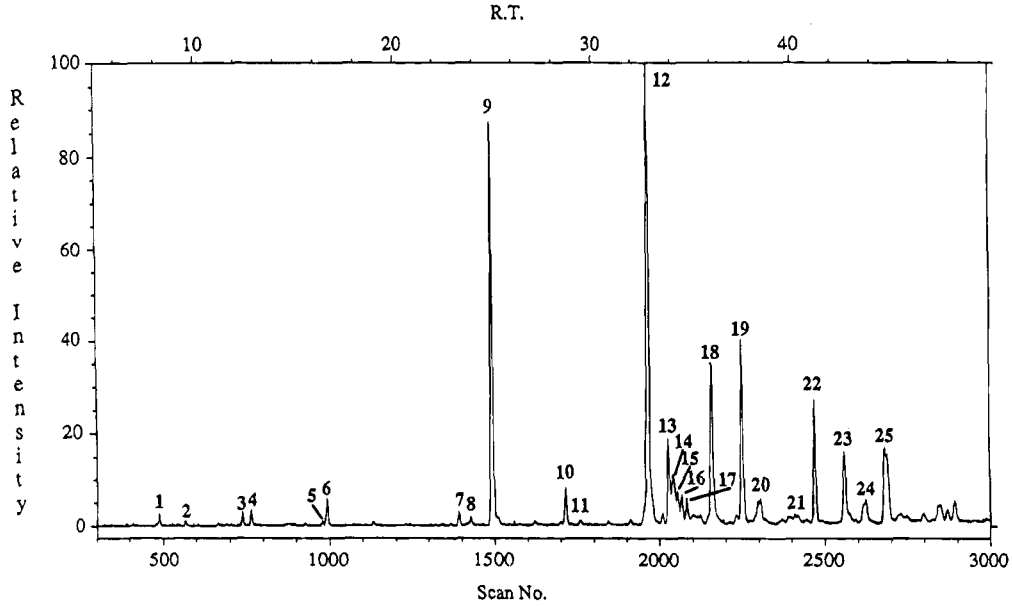

FIGURE 6: Total ion chromatogram showing the typical fatty acid species in membrane lipids from *R. meliloti* 2011 derivatized as methyl esters. Note the high occurrence of diunsaturated fatty acids. The large number of isomers could arise from isomerization during the derivatization. Note also the frequency of methoxylated methyl esters. These are expected to arise from addition of methanol to unsaturations and scission of cyclopropyl rings. See Table 1 for assignments.

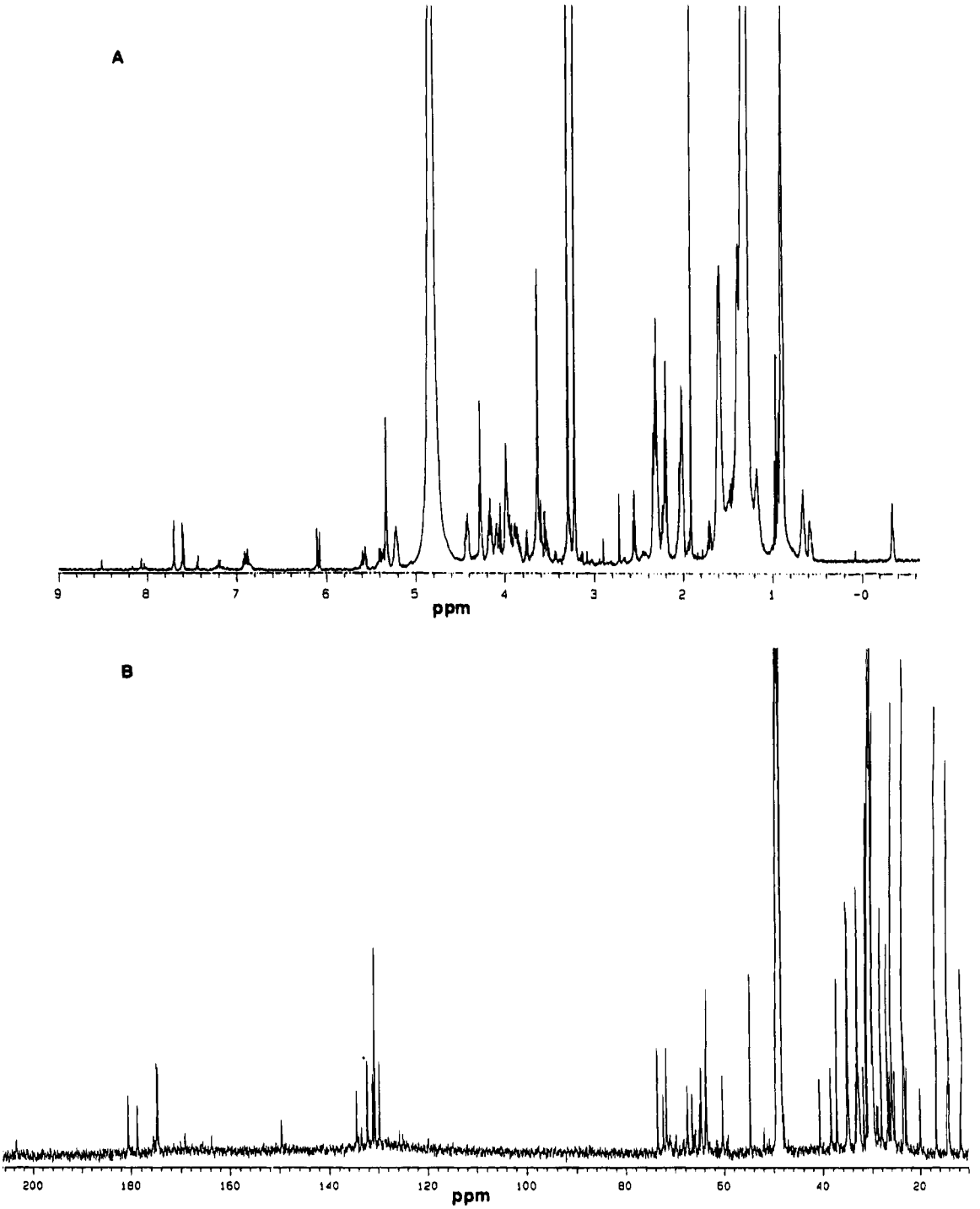

FIGURE 7: (A) Proton NMR spectrum of the total membrane extract from *R. meliloti* 2011. Note the presence of a sharp singlet at 3.22 ppm due to methyl protons on the choline headgroup. The methylene signals appear at approximately 3.61 and 4.25 ppm. (B) ^{13}C NMR spectrum of the total membrane extract from *R. meliloti* 2011.

than the actual value since desulfation is likely to have occurred during the isolation and purification processes. Both UV and SDS−PAGE analyses indicated that there was no protein contamination in the LPS preparations. In addition, free sulfate was not present since it would, of course, be separated from the polysaccharide during the chromatography and would also run with the ion front in the SDS gel. We have reported the carbohydrate and fatty acid compositions of the LPS of *R. meliloti* 2011 before (Williams et al., 1990; Bhat et al., 1991).

The effect(s) of impairment of the *nod*Q_1 and *nod*Q_2 genes on the sulfation of the lipopolysaccharide was next examined. The *nod*Q_1 and *nod*Q_2 mutants used in the study nodulate more slowly than the parent strain *R. meliloti* 1021 while the *nod*Q_1Q_2 double mutant is ineffective in nodulation (Schwedock & Long, 1992). These mutant strains were all derived from *R. meliloti* 1021 by Tn5 insertion. Assaying fractions of the Sepharose 4B gel filtration column of the parent strain clearly showed that the LPS was sulfated (Figure 4, fractions 10−15). A similar analysis on the *nod*Q_1 mutant (JSS12) showed a decrease in the level of sulfation of the LPS in comparable fractions. The same result was observed with the LPS of the *nod*Q_2 mutant (JSS14). However, this same LPS region in the profile of the Q_1Q_2 double mutant

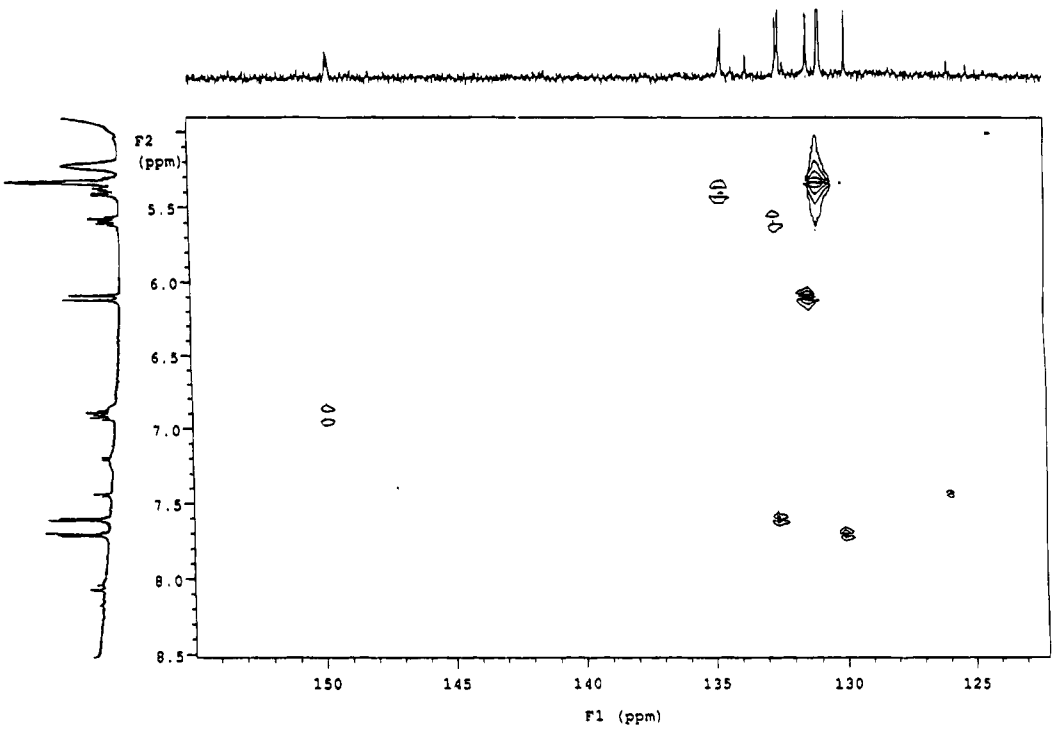

FIGURE 8: $^{1}H-^{13}C$ multiple quantum coherence spectrum (HMQC) of the total membrane extract from *R. meliloti* 2011. Connectivities confirm assignments for unsaturations and are explained in the text.

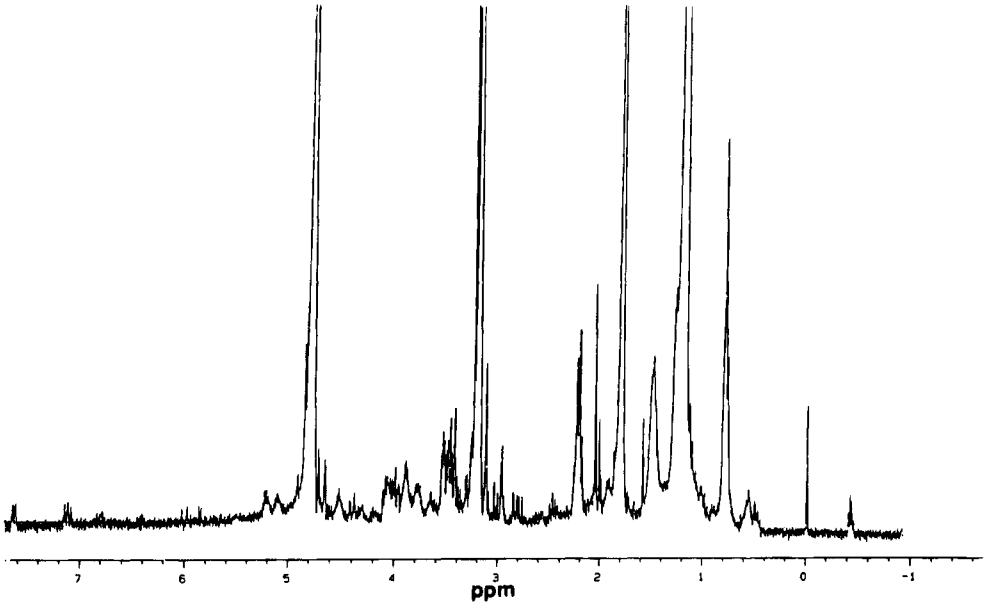

FIGURE 9: Proton NMR spectrum of sulfoquinovosyl diacylglycerol from mutant JSS16. Note the very high level of fatty acid unsaturation giving rise to the signals downfield of 6.0 ppm. This is very unlike the situation with the parent strain again underlining the high degree of coupling and interdependence of membrane lipid chemistry.

(JSS16) contained no sulfate. These results indicate that the current models explaining the molecular basis of host specificity should be revised in order to factor out the role(s) of LPS from those of the glucosamine-containing lipo-oligosaccharides. This work shows unequivocally that the lipopolysaccharide is a substrate for enzymes encoded for by *nod* genes. The fact that the sulfation of LPS is flavone-independent but the *nod*Q genes are (supposedly) flavone-regulated requires some explanation. One possible explanation is that only one of the *nod*Q genes is dependent on flavone for its expression.

In order to investigate further the relationship between chitolipooligosaccharide and typical membrane lipid and glycolipid synthesis, we also studied the promiscuous *Rhizobium* species strain NGR234. This strain nodulates at least 70 different genera of legumes and has been shown to synthesize chitolipooligosaccharides bearing a wide assortment of functionalities (Price et al., 1992). The most notable features of the NGR234 chitolipooligosaccharides is the presence of a methylated 6-deoxyhexosyl residue on the reducing end (that also may or may not contain a sulfate moiety) and the methylation of nitrogen on glucosamine (Price et al., 1992). *R.* sp. NGR234 was grown in the presence of ^{35}S-labeled sulfate and the LPS isolated and purified. Assays of the Sepharose 4B column eluate showed radioactivity coinciding with the marker LPS component

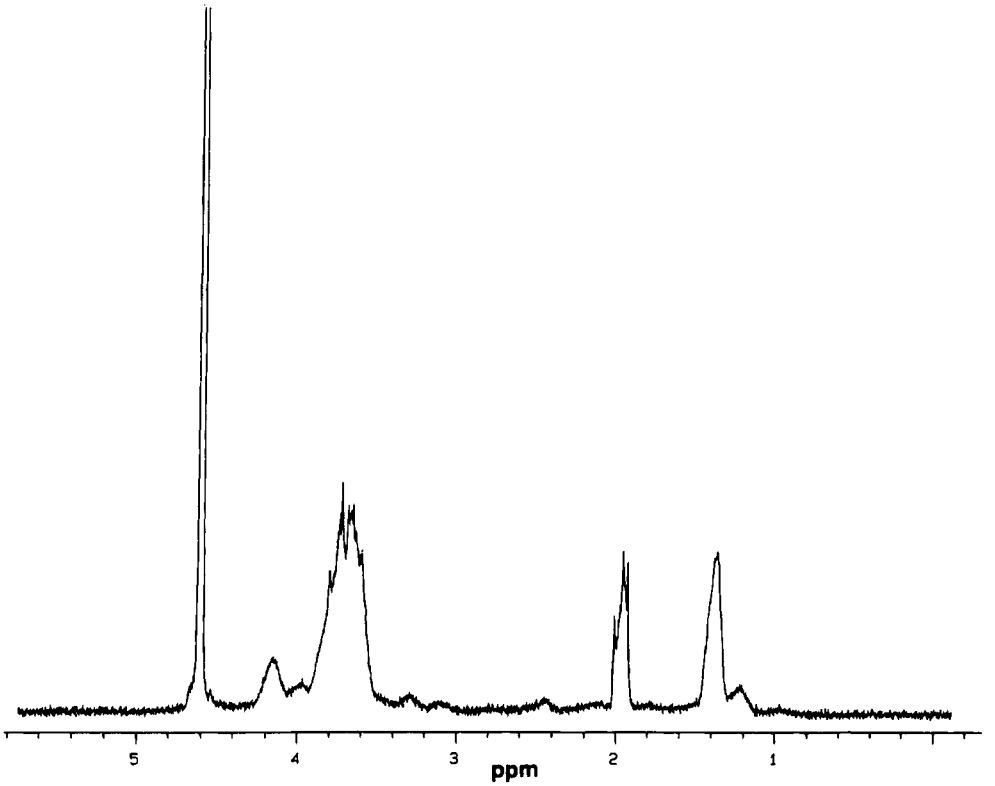

FIGURE 10: Proton NMR spectrum of chitolipooligosaccaride fraction from *R. meliloti* 2011 membranes of cells grown in the absence of flavone. The spectrum was measured in D_2O at 50 °C. The anomeric protons can be seen at the edge of the residual water line at 4.60 ppm. Note the prominent acetyl group signals at ~2 ppm. The fatty acid signals appear between 1.1 and 1.6 ppm.

2-keto-3-deoxyoctulosonic acid (KDO) (Figure 5). This fraction was pooled and rechromatographed on Bio-Gel P6 on which it eluted as one radioactive fraction which tested positive for LPS by the KDO assay. The peak was analyzed by GC and GC/MS to determine its carbohydrate content. Like the chitolipooligosaccharide isolated from this strain, the lipopolysaccharide was also sulfated and both O- and N-methylated. The GC/MS analyses indicated that, like the chitolipooligosaccharide, a 2-*O*-methyl-6-deoxyhexose was a component of the LPS. In addition, methylation of the nitrogen of a 2-amino-2,6-dideoxyhexose component was also observed. The predominant glycosyl component was glucose.

The fatty acid components of chitolipooligosaccharides of *Rhizobium* are often cited as being the primary deteminants of their ability to induce host specific responses in legume plants. In the case of *R. meliloti*, for instance, much importance has been placed on the presence of a 2,9-doubly unsaturated C-16 fatty acid in which one of the double bonds is conjugated to the carbonyl group and has a trans configuration (Lerouge et al., 1990; Roche et al., 1991a). These latter unsaturated fatty acids are still thought to be critical even though it was also demonstrated that there were several other fatty acid components, including α, β, γ, and δ chains and saturated chains of varying lengths, in chitolipooligosaccharides from *R. meliloti* (Schultze et al.,1992). The fatty acid composition of the membrane lipids and the number and stereochemistry of the unsaturated linkages therefore required special attention. The modifications of the membrane lipids were generally similar to those reported for the chitolipooligosaccharides. The phospholipids could be identified from their molecular ions in positive and negative mode FAB-MS. Further information was obtained from additional diagnostic ions corresponding to the loss of phosphate esters in positive ion mode and the acyl fragment ion in negative ion mode. Based on the information on the molecular ions, the losses of the headgroups, and the carboxylate anions, the mass spectra of the TLC bands of the total lipid extracts from *R. meliloti* 2011 revealed the presence of homologous series of lysophosphatidylcholines (LPC), phosphatidylcholines (PC), phosphatidylglycerols (PG), and phosphatidylmonomethylethanolamines (PMME). The major carboxylate anions ($RCOO^-$) appeared at the following *m/z* values: 255 (16:0), 267 (17:cy), 281 (18:1), 283 (18:0), and 295 (19:cy). Carboxylate ions corresponding to doubly unsaturated fatty acids were also observed but at lower intensity. The presence of C18:2 fatty acids could be discerned by GC and GC/MS analyses. A total ion chromatogram from a representative membrane lipid fatty acid methyl ester analysis of *R. meliloti* 2011 is shown in Figure 6, and a list of the components is given in Table 1. The high incidence of methoxy fatty acids can be attributed to artifacts due to the addition of methanol to unsaturations and to methanolysis of cyclopropane groups. It should be pointed out that this list contains both outer and inner membrane lipid fatty acids and chitolipooligosaccharides would, more than likely, be restricted to the outer membranes since they share sulfation with lipopolysaccharides.

The NMR spectra of total membrane lipids gave definitive information on the types of fatty acids which were present in the total lipid extracts of the bacteria especially with regard to the number and types of double bonds. Hence in the case of *R. meliloti* 2011, the proton spectra (Figure 7A) and the ^{13}C NMR spectra (Figure 7B) both indicated that unsaturated fatty acids were major components of the phospholipids. Unsaturation was indicated in the proton spectrum by the presence of signals downfield of 6 ppm and in the ^{13}C spectrum by signals between 130 and 150 ppm. One

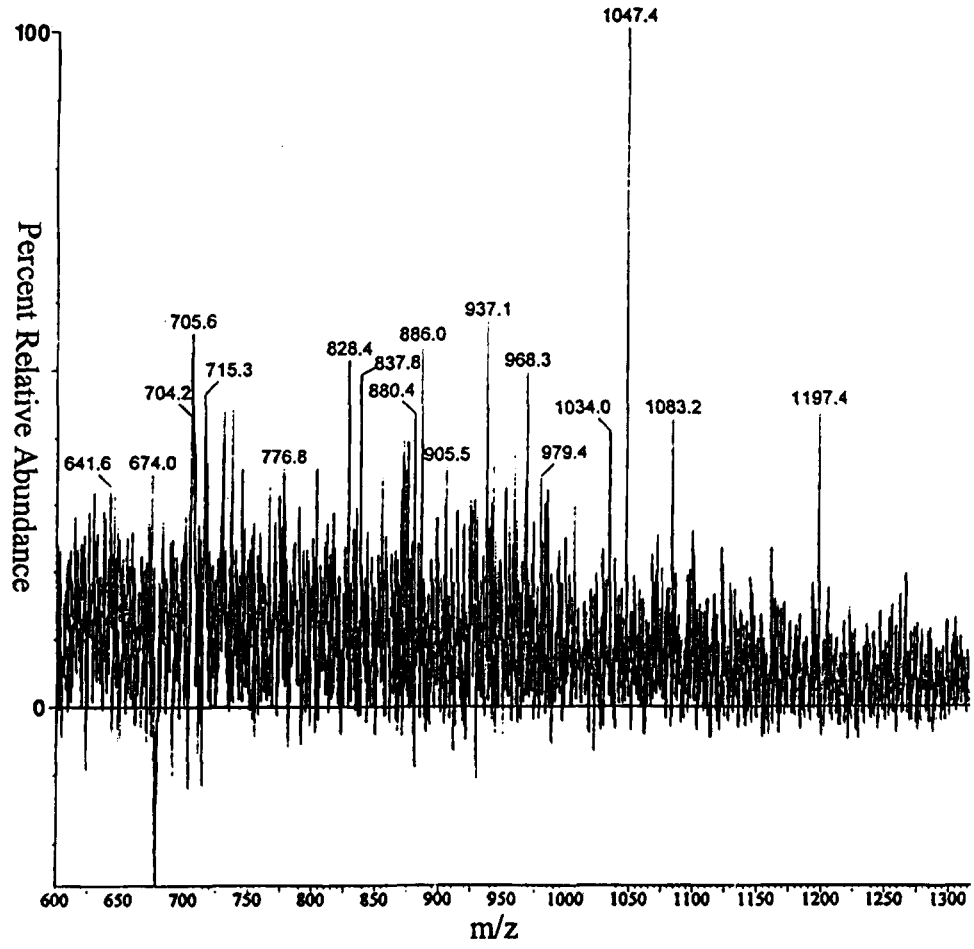

FIGURE 11: Electrospray (negative ion) mass spectrum of a chitolipooligosaccharide preparation from *R. meliloti* 2011. The complexity of peaks indicates a high level of heterogeneity due to differences in substitution. The ion at *m/z* 1047 corresponds to the M−H ion of a chitolipooligosaccharide (trisaccharide) bearing one sulfate and one *O*-acetate group and a hexadecanoyl residue. The less intense ion at *m/z* 1197 corresponds to an unacetylated tetrasaccharide species. The linkages were comfirmed by methylation analysis. Lower masses correspond to species with various levels of desulfation, de-*O*-acylation, and fatty acid variability.

common site of unsaturation was α,β- to the ester carbonyl group. This was indicated by the presence of a doublet (J = 12 Hz) at 6.10 ppm, attributable to the α proton and a doublet of triplets (J = 12 + 7 Hz) at 6.88 Hz attributable to the β proton. The proton−^{13}C multiquantum coherence spectrum (HMQC) confirmed the assignments for the vinylic protons and carbons (Figure 8). Hence the signal assigned to the β proton of the α,β-unsaturated system showed a connectivity to a ^{13}C resonance at the characteristic position of 149.6 ppm. The signal at 6.10 ppm in the proton spectrum (assigned to the α proton) displayed a connectivity to a signal at 131.5 ppm in the ^{13}C spectrum. The HMQC spectrum also showed a connectivity between a signal at 5.32 ppm in the proton dimension and 130.8 ppm in the ^{13}C dimension. This was assigned to the presence of an isolated double bond. The NMR spectra confirmed the TLC and mass spectrometric results indicating that the predominant lipid component was phosphatidylcholine as evidenced by the characteristic signals for its headgroup (Figure 7A). The information obtained from NMR spectroscopy on the extent, position, and type of unsaturation was especially important since it cannot be obtained by mass spectrometric analysis of fatty acid methyl esters. One complication in the latter type of analyses is that α,β-unsaturated esters easily add nucleophiles such as alcohols and hydroxide ions under acid or base catalysis and a large proportion of such fatty acids is lost during their liberation.

Since lipopolysacccharides, chitolipooligosaccharides, and other glycolipids are likely to share similar cellular localizations (spatial overlap), we examined the possibility that other rhizobial glycolipids might have similar types of fatty acids as were reported for the chitolipooligosaccharides. One such glycolipid is sulfoquinovosyldiacylglycerol (Cedergren & Hollingsworth, 1994). Hence the proton NMR spectrum of sulfoquinovosyldiacylglycerol from *nod*PQ mutants contained signals between 6 and 8 ppm indicating that α,β,γ,δ-unsaturated fatty acids were present (Figure 9). This was readily confirmed by a proton two-dimensional, double quantum filtered, J-correlated NMR spectrum (not shown).

Finally, our last goal was to obtain more direct evidence that chitolipooligosaccharides were actually membrane components as the above studies indicate, and not really excreted metabolites. If this were true, we supposed, it would then be possible to obtain substantial quantities from cells after disrupting the membrane structure. This was accomplished by extracting cells with an organic solvent mixture to remove phospholipids and disrupt the membrane thus releasing the more polar lipids (such as chitolipooligosaccharides) which, along with the lipopolysaccharides, should partition into the more aqueous layer. The components of the aqueous layer were absorbed onto a C-18 reverse-phase cartridge. The cartridge was then eluted with an increasingly nonpolar mixture of water and methanol. Using radioactivity counts (from ^{35}S) and glucosamine content as an index, further

Table 1: List of Fatty Acids Normally Present in the Membranes of *R. meliloti* Lipids

peak no. (Figure 6)	structure	molecular weight
1	Unidentified	216
2	$CH_3OOC(CH_2)_7COOCH_3$	214
3	$OHC(CH_2)_9COOCH_3$	230
4	$CH_3OOC(CH_2)_8COOCH_3$	242
5	$CH_3(CH_2)_{12}COOCH_3$, 14:0	244
6	$CH_3OOC(CH_2)_9COOCH_3$	
7	Unidentified	268
8	$CH_3(CH_2)_xCH{=}CH(CH_2)_yCOOCH_3$ (x+y=12), 16:1	270
9	$CH_3(CH_2)_{14}COOCH_3$, 16:0	282
10	$CH_3(CH_2)_xCH(CH_2)CH(CH_2)_yCOOCH_3$ (x+y=12), 17:0Δ	284
11	$CH_3(CH_2)_{15}COOCH_3$, 17:0	296
12	$CH_3(CH_2)_xCH{=}CH(CH_2)_yCOOCH_3$ (x+y=14), 18:1	298
13	$CH_3(CH_2)_{16}COOCH_3$, 18:0	310
14	Unidentified	294
15	$CH_3(CH_2)_xCH{=}CH(CH_2)_yCH{=}CH(CH_2)_zCOOCH_3$ (x+y+z=12), 18:2	294
16	Same as above	294
17	Same as above	294
18	Same as above	310
19	$CH_3(CH_2)_xCH(CH_2)CH(CH_2)_yCOOCH_3$ (x+y=14), 19:0Δ	
20	Methoxylated FAME	
21	Methoxylated FAME	
22	Methoxylated FAME	310
23	Unsaturated keto FAME	
24	Methoxylated FAME	
25	Unidentified	

purification yielded material which contained glucosamine as the only carbohydrate component. Methylation analysis under the mild silver oxide conditions indicated a 1,4-linkage between the glucosamine residues. There was a minor component with a different methylation pattern which was attributed to the nonreducing terminal. Under these conditions, the reducing terminal is expected be methylated at the 1-position and suffer some degree of desulfation to give the same methylation pattern as the internal residues. The proton NMR spectra of the chitolipooligosaccharides obtained from *R. meliloti* strains 2011 grown in the absence of flavone is shown in Figure 10. The level of unsaturation of the fatty acids in chitolipooligosaccharides from bacteria grown in the absence of flavone was generally lower than when the bacteria were grown in the presence of flavone. There was also a significant difference between the levels of unsaturation of the fatty acids of the 2011 and 1021 lipooligosaccharides. The latter tended to have a lower degree of unsaturation. The electrospray mass spectrum of the chitolipooligosaccharide from strain 2011 grown in the absence of luteolin is shown in Figure 11. Trisaccharide species seem to predominate. The presence of a sulfate group was confirmed by radiolabeling with ^{35}S-labeled sulfate. Typically, several milligrams of chitolipooligosaccharides were obtained per liter of culture. Significant (though lower) quantities were obtained in the absence of flavone.

The results of this study clearly demonstrate biochemical and spatial links between the chitolipooligosaccharides of *Rhizobium* and the other membrane lipid and glycolipid components of these organisms. There are important parallels between normal lipid structure and chitolipooligosaccharide structure especially with respect to unsaturation. The important feature of chitolipooligosaccharide sulfation is that it is coupled to lipopolysaccharide sulfation. Chitolipooligosaccharides themselves are membrane components. In a recent publication it was suggested that the unsaturated fatty acids, which eventually become part of chitolipooligosaccharide structure, are first put on phospholipids and then transferred to the chitolipooligosaccharide (Geiger et al., 1994). Whereas this would have been quite remarkable if chitolipooligosaccharides were synthesized and excreted as was first thought, this overlap in fatty acid structure is hardly surprising in light of the evidence presented here that these molecules are actually membrane components. The shuffling and transfer of fatty acids between membrane lipid components is a well documented phenomenon (Hazel et al., 1990; Shibuya, 1992) as are the common modifications, such as hydroxylation, desaturation, and methylation, of fatty acids from different lipid species in the same membrane. *Nod* genes, therefore, encode functions that clearly affect and determine bacterial membrane and general surface chemistry. In light of this finding, it is reasonable to conclude that bacterial membrane and surface chemistry are critical determinants of the outcome of the symbiotic relationship between *Rhizobium* and legumes.

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