

Sinorhizobium fredii and *Sinorhizobium meliloti* Produce Structurally Conserved Lipopolysaccharides and Strain-Specific K Antigens

BRADLEY L. REUHS,^{1*} DANIEL P. GELLER,¹ JOHN S. KIM,¹ JENNIFER E. FOX,¹
V. S. KUMAR KOLLI,¹ AND STEVEN G. PUEPPKE^{2†}

Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602-4712,¹
and Department of Plant Pathology, University of Missouri, Columbia, Missouri 65211²

Received 3 June 1998/Accepted 6 October 1998

Lipopolysaccharides (LPS) and capsular polysaccharides (K antigens) may influence the interaction of rhizobia with their specific hosts; therefore, we conducted a comparative analysis of *Sinorhizobium fredii* and *Sinorhizobium meliloti*, which are genetically related, yet symbiotically distinct, nitrogen-fixing microsymbionts of legumes. We found that both species typically produce strain-specific K antigens that consist of 3-deoxy-D-manno-2-octulosonic acid (Kdo), or other 1-carboxy-2-keto-3-deoxy sugars (such as sialic acid), and hexoses. The K antigens of each strain are distinguished by glycosyl composition, anomeric configuration, acetylation, and molecular weight distribution. One consistent difference between the K antigens of *S. fredii* and those of *S. meliloti* is the presence of N-acetyl groups in the polysaccharides of the latter. In contrast to the K antigens, the LPS of *Sinorhizobium* spp. are major common antigens. Rough (R) LPS is the predominant form of LPS produced by cultured cells, and some strains release almost no detectable smooth (S) LPS upon extraction. *Sinorhizobium* spp. are delineated into two major RLPS core serogroups, which do not correspond to species (i.e., host range). The O antigens of the SLPS, when present, have similar degrees of polymerization and appear to be structurally conserved throughout the genus. Interestingly, one strain was found to be distinct from all others: *S. fredii* HH303 produces a unique K antigen, which contains galacturonic acid and rhamnose, and the RLPS did not fall into either of the RLPS core serogroups. The results of this study indicate that the conserved S- and RLPS of *Sinorhizobium* spp. lack the structural information necessary to influence host specificity, whereas the variable K antigens may affect strain-cultivar interactions.

The cell surface polysaccharides of rhizobia are believed to be involved in key aspects of symbiosis, such as infection thread initiation and development, nodule invasion, and host specificity; however, unlike those of the Nod factor (20, 34), the specific functions of the polysaccharides have not been determined. The uncertainty about their functions is due in part to a lack of structural data on the cell surface components of related rhizobia. In order to develop a general model, we examined the lipopolysaccharides (LPS) and capsular polysaccharides (K antigens) from representative strains of *Sinorhizobium fredii* and *Sinorhizobium meliloti*, which are genetically related, yet symbiotically distinct, nitrogen-fixing microsymbionts of legumes (13).

Cultured cells of *Sinorhizobium* spp. typically produce two forms of LPS: rough (R) LPS, which consists of the lipid A membrane anchor and core oligosaccharide, and smooth (S) LPS, which includes an O antigen (3, 5). The K antigens, in contrast, lack a lipid anchor and are structurally distinct from the LPS (28). The two types of cell surface polysaccharides can be identified on polyacrylamide gel electrophoresis (PAGE) gels by differential staining (29), and they can be separated from one another by preparative PAGE, based on the presence of the hydrophobic lipid A moiety on the LPS (16).

In this report, we show that the K antigens of *S. fredii* and *S. meliloti* typically comprise small repeating units of hexoses and 3-deoxy-D-manno-2-octulosonic acid (Kdo) or other 1-carboxy-

2-keto-3-deoxy sugars. The polysaccharides of each strain are distinguished by size range and structural features, such as glycosyl residue composition and substituents. These polysaccharides, therefore, are strain-specific antigens. In contrast, the RLPS are common antigens that are delineated into two major serogroups, which do not correspond to species. Little or no SLPS is released from these rhizobia upon extraction and, when present, the O antigens show similar degrees of polymerization and lack structural variation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Cells were stored at -70°C in 7.5% (vol/vol) glycerol and cultured in yeast extract-mannitol (*S. fredii*) or tryptone-yeast extract broth (*Sinorhizobium* sp. strain NGR234 and *S. meliloti*) at 28°C , as previously described (29, 30).

Analysis of crude polysaccharide preparations. For PAGE analysis, the cell-associated polysaccharides were extracted from cell pellets (3-ml cultures) by a mini-phenol-water extraction technique adapted by B. L. Reuhs and J. S. Kim: prior to extraction, the cells are resuspended in H_2O and pelleted to remove exopolysaccharides (EPS). Past studies have shown that no trace of EPS is found in the subsequent extract (27). The cells were pelleted (5 min; $10,000 \times g$) in a microcentrifuge tube (Dot Scientific, Burton, Mich.), and the supernatant was removed. The pellet was resuspended in 0.5 ml of solution A (0.05 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 M EDTA, 0.05% NaN_3 , pH 7) and vortexed thoroughly; 0.5 ml of solution B (90% phenol) was added, and the suspension was vortexed. The tubes were placed in a 65°C heating block for 15 min, with thorough vortexing every 5 min, and then cooled in crushed ice for 5 min. After centrifugation (5 min; $10,000 \times g$), the water phase was removed, dialyzed (6,000- to 8,000-molecular-weight cutoff) against deionized H_2O (dH_2O), and freeze-dried. This fraction typically contains both the LPS and the K antigens. The phenol phase may also be dialyzed and analyzed, if necessary. The lyophilized material was then dissolved in 10 to 20 μl of PAGE sample buffer (see below).

Aliquots of 1 or 2 μl were analyzed by deoxycholic acid-PAGE, using 18% acrylamide gels. PAGE analysis was performed with a Bio-Rad Mini-Protein II cell, and the following protocol is specific for this equipment. The resolving gel (18%) contained 6 ml of A (30% [wt/vol] acrylamide, 0.8% [wt/vol] bisacrylamide), 2 ml of B (22.71 g of Tris base/75 ml of dH_2O [pH 8.8], brought to 100 ml

* Corresponding author. Mailing address: Complex Carbohydrate Research Center, University of Georgia, 220 Riverbend Rd., Athens, GA 30602-4712. Phone: (706) 542-1216. Fax: (706) 542-4412. E-mail: breuhs@ccrc.uga.edu.

† Present address: College of ACES, University of Illinois, Urbana, IL 61801.

TABLE 1. Bacterial strains used in this study

Strain	Characteristics	Source
<i>S. fredii</i>		
USDA191	From soil, Shanghai Province, China; Fix ⁺ with <i>Glycine max</i> cv. Peking and McCall	Keyser et al.
USDA192	From <i>Glycine soja</i> ; Fix ⁺ with <i>G. max</i> cv. Peking; Nod ⁻ with <i>G. max</i> cv. McCall	Keyser et al.
USDA201	From <i>G. max</i> ; Fix ⁺ with cv. Peking; Nod ⁻ with cv. McCall	Keyser et al.
USDA205	From <i>G. max</i> ; Fix ⁺ with cv. Peking; Nod ⁻ with cv. McCall	Keyser et al.
USDA208	From <i>G. max</i> ; Fix ⁺ with cv. Peking; Nod ⁻ with cv. McCall	Keyser et al.
USDA257	From <i>G. max</i> ; Fix ⁺ with cv. Peking; Nod ⁻ with cv. McCall	Keyser et al.
USDA196	From rice field soil, Hubei Province, China; nodulates <i>G. max</i>	Dowdle & Bohlool
USDA197	From rice field soil, Hubei Province, China; nodulates <i>G. max</i>	Dowdle & Bohlool
HH103	From rice field soil, Hubei Province, China; nodulates <i>G. max</i>	Dowdle & Bohlool
HH303	From rice field soil, Hubei Province, China; nodulates <i>G. max</i>	Dowdle & Bohlool
<i>Sinorhizobium</i> sp. strain NGR234	From <i>Lablab purpureus</i> ; broad host range	Trinick
<i>S. meliloti</i>		
AK631	<i>exoB</i> mutant of wild-type strain Rm41	Bánfalvi et al.
Rm1021	Streptomycin-resistant derivative of wild-type strain RCR2011	Walker et al.
NRG23	Wild-type from <i>Medicago sativa</i> L.	Olsen et al.
NRG53	Wild-type from <i>M. sativa</i> L.	Olsen et al.
NRG133	Wild-type from <i>M. sativa</i> L.	Olsen et al.
NRG185	Wild-type from <i>M. sativa</i> L.	Olsen et al.
NRG247	Wild-type from <i>M. sativa</i> L.	Olsen et al.
NRG286	Wild-type from <i>M. sativa</i> L.	Olsen et al.
<i>B. japonicum</i> USDA110	Wild-type strain from <i>G. max</i>	Stacey et al.
<i>R. etli</i> CE3	Streptomycin-resistant derivative of wild-type strain CFN42	Noel et al.

with dH₂O), 2 ml of dH₂O, 17.5 µl of 10% ammonium persulfate, and 8.75 µl of *N,N,N',N'*-tetramethylethylenediamine (TEMED). The stacking gel (4%) contained 0.33 ml of A, 0.5 ml of C (7.69 g of Tris base/75 ml of dH₂O [pH 6.8], brought to 100 ml with dH₂O), 1.67 ml of dH₂O, 12.5 µl of 10% ammonium persulfate, and 6.25 µl of TEMED. The running buffer consisted of 21.7 g of glycine, 4.5 g of Tris base, and 2.5 g of deoxycholic acid in 1 liter of dH₂O. The sample buffer contained 4 ml of C, 5 mg of bromophenol blue, and 2 ml of glycerol, brought to 20 ml with dH₂O. The gels were prerun for 10 min (15.0 mA/gel) prior to the loading of samples and were run for 40 to 60 min (15.0 mA/gel) until the buffer front, not the dye front, reached the bottom of the gel. The gels were either silver stained for LPS (35) or alcian blue-silver stained for the K antigens (6, 29).

For the alcian blue-silver stain, the gel was immediately immersed in 100 ml of alcian blue solution (0.005% [wt/vol] alcian blue in ethanol-acidic acid wash [40% ethanol, 5% acetic acid in dH₂O]) and gently rocked for 30 min. This was followed by a change to 100 ml of fresh solution and overnight rocking. The gel was rinsed for 1 min in dH₂O, oxidized in 100 ml of 0.7% sodium metaperiodate (in dH₂O) for 10 min, and washed five times in 100 ml of dH₂O for 5 min (minimum) each time. One milliliter of the final wash was added to 1 ml of the silver solution (see below); if a precipitate (ppt) formed, the gel was washed two more times. The gel was then stained in 100 ml of silver solution (10% Bio-Rad silver concentrate) for 10 min and rinsed in dH₂O for 1 min. The gel was rinsed again in 50 ml of Bio-Rad developer (3.0 g/200 ml of dH₂O) with agitation until dark ppt formed (about 20 s) and immediately drained to remove all ppt. The color was developed with the remaining developer (2 to 5 min), and the development was stopped in 50 ml of 5% acetic acid (30 s) followed by a 2-min rinse in dH₂O. The developed gels were stored in dH₂O.

For serotype analysis, unstained PAGE gels of the cell extracts were immediately blotted to Nytran⁺ (Schleicher and Schuell, Keene, N.H.) with a Trans-Blot SD apparatus (Bio-Rad). The membranes were separately incubated with polyclonal rabbit antiserum raised against *S. meliloti* Rm41 or *S. fredii* USDA205 and then with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma, St. Louis, Mo.). The blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) for 5 to 15 min. Anti-Rm41 was provided by Dale Noel (Marquette University, Milwaukee, Wis.), and anti-Rf205 was produced by B. L. Reuhs as a graduate student in the Botany Department, Eastern Illinois University. Antiserum development and immunoblotting protocols have been described previously (25).

Polysaccharide purification and analysis. For large-scale preparations, the polysaccharides were extracted from cell pellets (5-liter cultures) with hot phenol-water and the water phase material was dialyzed (against H₂O) and freeze-dried. The RLPS, SLPS, and K antigens were isolated by size exclusion chromatography (Sephadex G-150 superfine; Pharmacia, Uppsala, Sweden) of the water phase material, as previously described (9, 29, 30, 32). The eluted fractions were assayed colorimetrically for 1-carboxy-2-keto-3-deoxy sugars by the thiobarbituric acid assay (36) and for hexose with phenol-sulfuric acid (38); LPS-containing

fractions were identified by PAGE analysis. In some cases, there was minor contamination of the K-antigen preparation by SLPS; when necessary, the intact K-antigen fractions were subjected to polymyxin-agarose affinity chromatography (Detoxi-Gel; Pierce Chemical Co.). After addition of the sample solution (in H₂O), the column was washed with H₂O (flowthrough fraction), which eluted the K antigens, and the LPS was then eluted with 1% deoxycholic acid in H₂O.

Composition analysis of the K antigens and RLPS was performed on the intact preparations (from G-150 chromatography) by gas chromatography-mass spectrometry (GC-MS) of the trimethylsilyl (TMS) methyl glycoside derivatives (38) with a 30-m DB1 fused silica column (J&W Scientific, Folsom, Calif.) on a model 5890A gas chromatograph-mass selective detector (Hewlett-Packard, Palo Alto, Calif.). Inositol was used as an internal standard, and retention times were compared to those of authentic monosaccharide standards. The relative ratios of the glycosyl residues from the RLPS were established compared to the β-OH-fatty acids of the lipid A, which are considered constant within the genus. This showed that the glucose (Glc) content was also a constant; therefore, the molar ratios were normalized to Glc.

Proton nuclear resonance (¹H NMR) spectroscopy was performed with a Bruker AM 250 spectrometer. The G-150-purified K antigens were dissolved in ²H₂O, and the spectra were obtained at 296 or 316 K. Chemical shifts (reported as parts per million) were established relative to acetone. Solvent suppression in the analysis of the *Sinorhizobium* sp. strain NGR234 K antigen was achieved by low-energy presaturation of the ²H₂O signal.

Fast-atom bombardment (FAB)-MS was performed with a JEOL (Tokyo, Japan) SX/SX 102A tandem four-sector instrument, in the negative mode, using a xenon FAB gun (6 kV) and an accelerating voltage of 10 kV. The scan range was 300 to 3,000 *m/z*. The K antigens were partially hydrolyzed with dilute acid (1% acetic acid at 100°C for 10 min), which hydrolyzed the acid-labile ketosidic linkages of Kdo and sialic acid and generated oligosaccharides of 1 to 4 disaccharide repeat units. The oligosaccharides were then freeze-dried to remove the acetic acid. A 1-µl aliquot of a saturated sample solution (in H₂O) was added to a matrix of thioglycerol (2 µl) and applied to the FAB probe.

High-performance anion-exchange chromatography (HPAEC) of the LPS core oligosaccharides was performed with a metal-free BioLC (Dionex Corp., Sunnyvale, Calif.) with a CarboPac PA1 anion-exchange column (4 by 250 mm) and a pulsed amperometric detector (PAD), as previously described (29, 30). The core oligosaccharides were prepared for HPAEC by mild acid hydrolysis (1% acetic acid at 100°C for 3 to 5 h) of the RLPS, which cleaves the Kdo linkages, followed by centrifugation to remove the insoluble lipid A.

RESULTS

K antigens. (i) PAGE analysis. The polysaccharide extracts from cultured cells of 16 strains of *Sinorhizobium* spp. were analyzed by PAGE-silver staining (Fig. 1), using an alcian blue

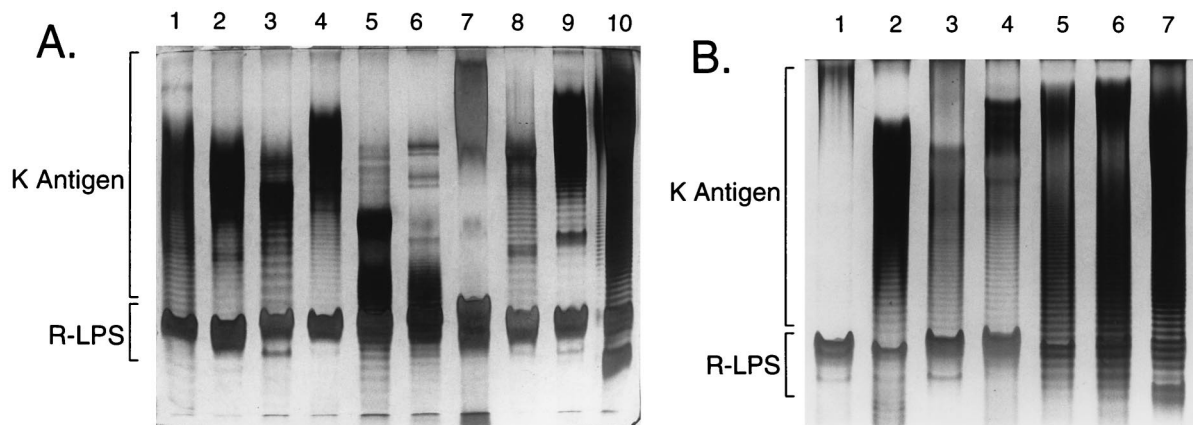


FIG. 1. PAGE analysis of the K antigens. The gels were prestained with alcian blue, a cationic dye that binds the acidic polysaccharides, prior to silver staining. (A) Lanes: 1, *S. fredii* USDA205; 2, *S. fredii* USDA257; 3, *S. fredii* USDA201; 4, *S. fredii* USDA208; 5, *Sinorhizobium* sp. strain NGR234; 6, *S. fredii* HH103; 7, *S. fredii* HH303; 8, *S. fredii* USDA192; 9, *S. fredii* USDA191; 10, *S. meliloti* AK631. (B) Lanes: 1, *S. meliloti* NRG133; 2, *S. meliloti* NRG23; 3, *S. meliloti* NRG185; 4, *S. meliloti* NRG247; 5, *S. meliloti* NRG286; 6, *S. meliloti* NRG53; 7, *S. meliloti* AK631.

prestain to visualize the acidic K antigens (compare Fig. 3). These polysaccharides commonly migrate in a ladder pattern, which is due to a sequential degree of polymerization of structurally constant repeating units. The K antigens of *S. fredii* USDA205 and *S. fredii* USDA257 have been shown to consist of disaccharide repeats (9, 29), and the polysaccharides from 9 of the other 14 strains appear to be similar. Although the size range of each is strain specific, the ladder patterns had comparable band separations. In contrast, the polysaccharides from *S. fredii* USDA201, USDA192, and USDA191 (Fig. 1A, lanes 3, 8, and 9, respectively) migrated with a relatively wide band separation, indicating that they consist of larger repeating units, and the K antigens of *S. fredii* HH303 and *S. meliloti* NRG133 (Fig. 1A, lane 7, and Fig. 1B, lane 1, respectively) migrated as "smears," with no obvious ladder patterns. Importantly, the PAGE analysis demonstrated that each of the 16 strains produce acidic polysaccharides that are unrelated to the LPS. The distinction of the K antigens from the LPS was also shown by nondetergent PAGE (not shown): the K antigens migrate into the gels, whereas the LPS do not (16).

(ii) **Structural analyses.** The K antigens from six *Sinorhizobium* strains were selected for further analysis: these strains included *S. fredii* USDA201 and *S. fredii* HH303, which yielded atypical PAGE migration patterns; *S. meliloti* NRG247 and *S. meliloti* NRG185, which had been used in interesting immunochemical studies by P. Olsen et al. (23, 24); *Sinorhizobium* sp. strain NGR234, a well-studied, broad-host-range organism; and *S. fredii* USDA208, which was randomly chosen. The K antigens were separated from the LPS by size exclusion chromatography of the phenol-water extracts (water phase), as described in past reports (9, 29); the intact polysaccharides were analyzed for glycosyl residue composition (by GC-MS) and by ^1H NMR spectroscopy, and the repeat oligosaccharides, generated by mild acid hydrolysis, were analyzed by FAB-MS.

***S. meliloti* NRG247.** The K antigen from *S. meliloti* NRG247 was found to contain equimolar amounts of *N*-acetylneuraminic acid (NeuNAc; commonly termed sialic acid) and Glc, and a repeating unit mass ion of m/z 470 ($M-1$ in negative-mode FAB-MS) confirmed that it consisted of Glc-NeuNAc disaccharides. The NeuNAc was determined to be in the α configuration by ^1H NMR analysis (Fig. 2A), with methylene proton resonances at 1.7 ($\text{H}_{3,\text{a}}$) and 2.8 ($\text{H}_{3,\text{c}}$) ppm; the Glc is also α linked, with an anomeric proton resonance at 4.96 ppm

and a small $J_{1,2}$ coupling. The *N*-acetyl methyl resonances are found at 1.98 ppm.

***S. meliloti* NRG185.** *S. meliloti* NRG185 produces a K antigen consisting of disaccharide repeats of *N*-acetylglucosamine (GlcNAc) and Kdo, as determined by equimolar amounts of the glycosyl residues and a repeating unit mass ion of m/z 440 ($M-1$ for GlcNAc-Kdo). ^1H NMR analysis (Fig. 2B) showed that the Kdo is β linked, with characteristic resonances at 1.8 ppm ($\text{H}_{3,\text{a}}$) and 2.35 ppm ($\text{H}_{3,\text{c}}$), and that the GlcNAc is also β linked. The chemical shift of the anomeric proton is 4.55 ppm, and the large $J_{1,2}$ coupling is also indicative of β -linked aldoses. The polysaccharide is *N* acetylated (2.0 ppm) on the GlcNAc and *O* acetylated (2.1 ppm) at unknown locations.

***S. fredii* USDA208.** The K antigen of *S. fredii* USDA208 contained Gal and Kdo in a 1:1 molar ratio and was determined to consist of Gal-Kdo disaccharides by FAB-MS, with a repeating unit mass ion of m/z 399. The ^1H NMR spectrum (Fig. 2C) contained resonances associated with β -linked Kdo (1.8 and 2.4 ppm) and α -linked Gal (5.05 ppm). The off-scale resonance at 4.0 ppm is due to contamination.

***S. fredii* USDA201.** The K antigen isolated from *S. fredii* USDA201 contained Kdo, Gal, and an unidentified 2-*O*-methylhexose (2-*O*-MeHex) in a 1:1 molar ratio of Kdo to the neutral sugars. Partial acid hydrolysis released the expected disaccharides of Gal-Kdo and 2-*O*-MeHex-Kdo (m/z 399 and 413, respectively), but unlike the K antigens of *S. fredii* USDA205 and *S. fredii* USDA257 (9, 29), a mass ion of m/z 795 showed that both the Gal-Kdo and the 2-*O*-MeHex-Kdo disaccharides were present in the same polysaccharide. Thus, the unusual PAGE banding pattern of the *S. fredii* USDA201 K antigen (Fig. 1) is due to a tetrasaccharide repeating unit, although it has not been determined how the two disaccharides are linked in the tetrasaccharide. The hexoses were determined to be α linked by ^1H NMR analysis (Fig. 2D), but the complexity of the anomeric region (5.0 to 5.2 ppm) suggests that there may be a nonlinear arrangement of the tetrasaccharides (possibly in branches). The resonances associated with the β -linked Kdo were also complex (1.7 to 2.4 ppm).

***Sinorhizobium* sp. strain NGR234.** Only Glc was detected in the GC-MS analysis of the *Sinorhizobium* sp. strain NGR234 K antigen; however, the ^1H NMR spectrum (Fig. 2E) contained the methylene proton resonances, at 1.65 ($\text{H}_{3,\text{a}}$) and 2.6 ($\text{H}_{3,\text{c}}$) ppm, of a 1-carboxy-2-keto-3-deoxy sugar. Two-dimensional

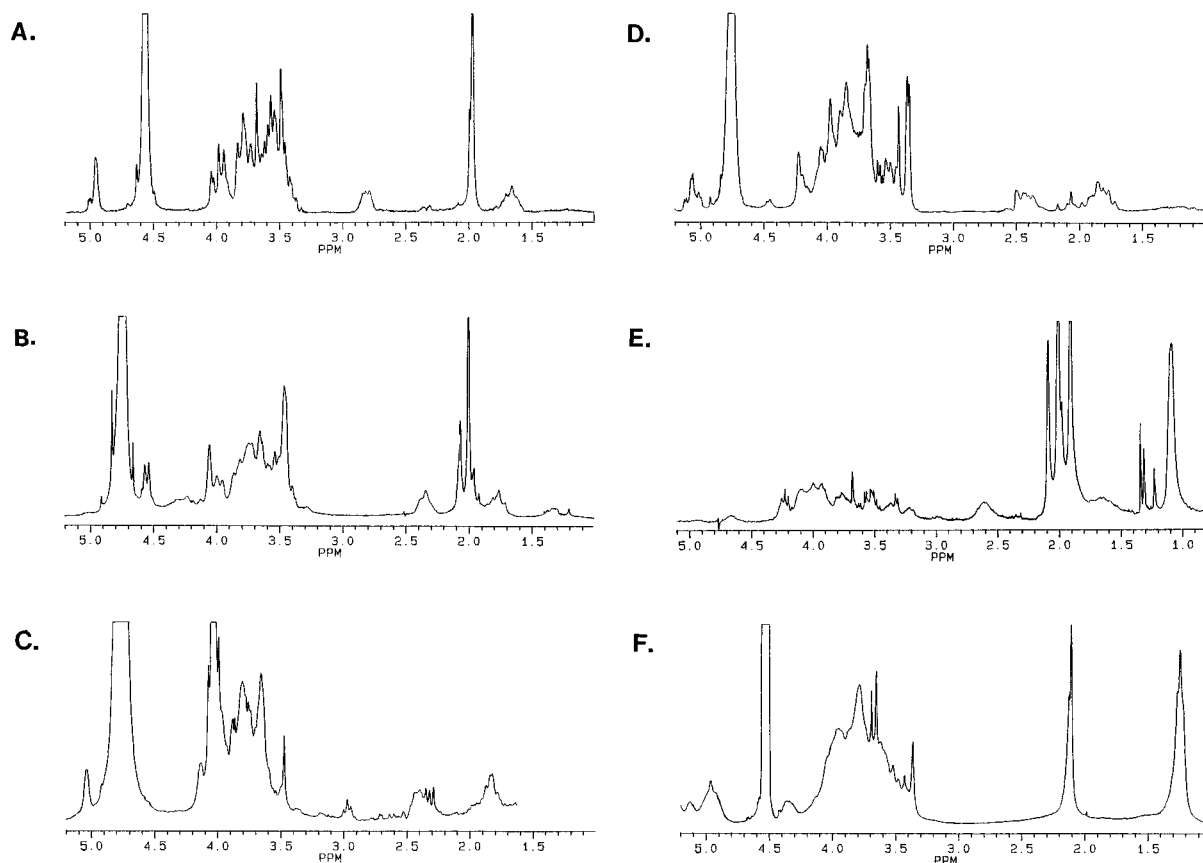


FIG. 2. ^1H NMR spectra of the K antigens from *S. meliloti* NRG247 (A), *S. meliloti* NRG185 (B), *S. fredii* USDA208 (C), *S. fredii* USDA201 (D), *Sinorhizobium* sp. strain NGR234 (E), and *S. fredii* HH303 (F). The chemical shift of the ^2HOH resonance (4.55 or 4.75 ppm) is temperature dependent. See the text for detailed descriptions.

NMR indicated that the acidic sugar is α -linked *N*-acetyl-5,7-diamino-3,5,7,9-tetradeoxyonulosonic acid (pseudaminic acid; Pse5NAc7NAc), which is labile to the (TMS methyl glycoside) derivitization process and mild acid hydrolysis (27). The resonance at 1.1 ppm in the ^1H NMR spectrum is due to the C-9 (methyl) protons of the Pse5NAc7NAc. Due to numerous *N*-acetyl groups (1.9 and 2.0 ppm) and *O*-acetyl groups (2.1 ppm), the polysaccharide was much less soluble than the others, and this resulted in poor resolution; therefore, solvent suppression was required to resolve the anomeric resonance of the β -linked Glc, at 4.65 ppm, yielding a diminished signal shown in Figure 2E. Integration showed that the Glc and Pse5NAc7NAc residues were present in a 1:1 molar ratio (27). Thus, the polysaccharide consists of disaccharide repeats of Glc-Pse5NAc7NAc.

***S. fredii* HH303.** The K antigen preparation from *S. fredii* HH303 was found to contain rhamnose (Rha) and galacturonic acid (GalA) in a 1:1 molar ratio. ^1H NMR analysis (Fig. 2F) confirmed that no labile 1-carboxy-2-keto-3-deoxy sugars, such as Pse5NAc7NAc, were present. The resonance at 1.3 ppm is due to the C-6 (methyl) protons of the Rha component, and the polysaccharide was shown to be *O* acetylated (2.1 ppm). Strain HH303 is the only strain of *S. fredii* that has been found to produce an acetylated K antigen (references 9 and 29 and this report). The polysaccharide could not be degraded into subunits by dilute acid hydrolysis, so MS was not applied.

LPS. (i) PAGE and immunoblot analyses. The same polysaccharide extracts used in the PAGE analysis of the K antigens (Fig. 1) were subjected to PAGE-silver staining, omitting

the alcian blue pre-stain (Fig. 3), for the analysis of the LPS. This revealed a common pattern of LPS migration for *Sinorhizobium* spp., including both the high-mobility RLPS and the low-mobility SLPS. When present, the SLPS of most strains commonly migrates in two distinct banding regions (*S. meliloti* Rm41 *exoB* [AK631] differs because the *exoB* mutation affects LPS production).

In contrast to other gram-negative bacteria, including *Rhizobium elii* and *Rhizobium leguminosarum* (3), the RLPS is the major form of LPS produced by laboratory-cultured cells of *Sinorhizobium* spp. Often, little SLPS appeared on the gels, and in no case was there an extensive ladder pattern, as with *Salmonella* LPS (37). Thus, the LPS O antigens of *Sinorhizobium* spp. do not appear to be highly variable, strain-specific antigens.

As the RLPS of *Sinorhizobium* spp. appeared to be important surface antigens, PAGE and immunoblot analyses of the polysaccharide extracts were performed to assess structural variation in the RLPS and to establish LPS core serotypes for each strain. The blots were probed with polyclonal antisera raised against whole cells of *S. fredii* USDA205 (anti-Rf205) and *S. meliloti* Rm41 (anti-Rm41), which contain anti-RLPS antibodies against the core oligosaccharides, not the membrane-bound lipid A moiety. PAGE acted as a purification step in these analyses, and the identity of the various products (i.e., RLPS, SLPS, and K antigens) on PAGE gels had already been established (29, 30, 32); therefore, this yielded a direct comparison of the affinities of the antisera for specific bacterial products. Examples of the results obtained for 10 strains are

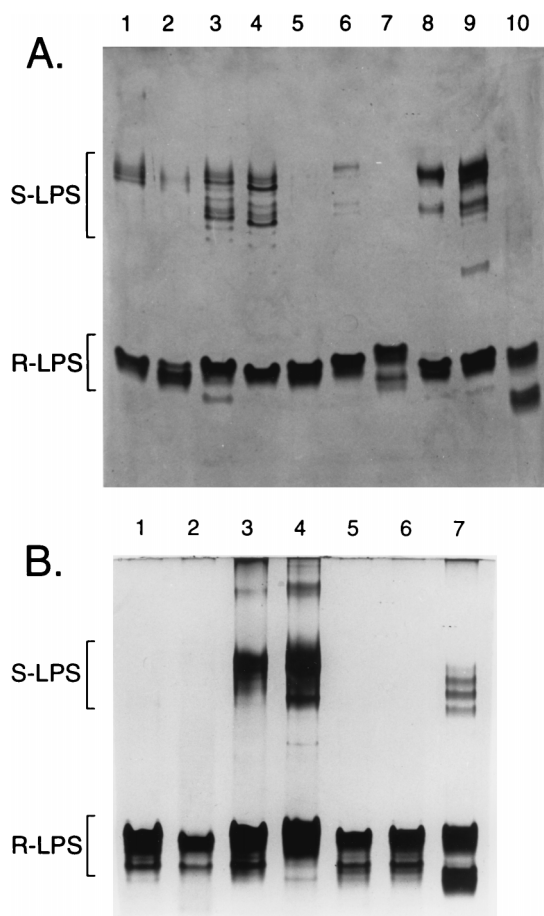


FIG. 3. PAGE analysis of the LPS. The gels were silver stained; the omission of the alcian blue pre-stain precludes the staining of the K antigens (compare Fig. 1) and allows the LPS to be clearly visualized. (A) Lanes: 1, *S. fredii* USDA205; 2, *S. fredii* USDA257; 3, *S. fredii* USDA201; 4, *S. fredii* USDA208; 5, *Sinorhizobium* sp. strain NGR234; 6, *S. fredii* HH103; 7, *S. fredii* HH303; 8, *S. fredii* USDA192; 9, *S. fredii* USDA191; 10, *S. meliloti* AK631. (B) Lanes: 1, *S. meliloti* NRG133; 2, *S. meliloti* NRG23; 3, *S. meliloti* NRG185; 4, *S. meliloti* NRG247; 5, *S. meliloti* NRG286; 6, *S. meliloti* NRG53; 7, *S. meliloti* AK631 (included on both gels for comparison).

presented in Fig. 4, and the results of all serotype analyses (19 *Sinorhizobium* strains and two controls) are summarized in Table 2. The negative responses with *R. etli* and *Bradyrhizobium japonicum* excluded the possibility that the binding of the antiserum to the RLPS was nonspecific, and the lack of uniform reaction to all *Sinorhizobium* strains indicates that there is a significant degree of specificity in these analyses.

The anti-Rm41 bound the RLPS of all *S. meliloti* strains examined and several *S. fredii* strains (total, 14), whereas the anti-Rf205 recognized the RLPS of *Sinorhizobium* sp. strain NGR234 and three *S. fredii* strains. Surprisingly, the serogroups were essentially mutually exclusive (some minor cross-reaction occurs if the gels are greatly overloaded or the blots are allowed to develop for several days). These results demonstrated that two major RLPS serogroups exist within the genus *Sinorhizobium* and that they are not correlated with species (i.e., host range). The reactions of the *S. meliloti* NRG23 and *S. meliloti* NRG53 RLPS with anti-Rm41 were very weak, and the RLPS of *S. fredii* HH303 did not bind either antiserum, indicating that there are at least four serogroups.

The immunoblot analyses also indicated that a conserved production of the O antigen is common within the genus *Sino-*

rhizobium (Fig. 4A) and confirmed that the SLPS is much less abundant than the RLPS. As in the PAGE analysis, the immunoblot showed that the degrees of polymerization of the O antigens of all strains are similar; when present, the SLPS consistently migrates as two distinct banding regions. The cross-reaction of anti-Rm41 antibodies with the SLPS (O antigens) of *Sinorhizobium* sp. strain NGR234 and *S. fredii* USDA205, USDA208, and USDA191 (the anti-Rf205 serogroup) is of particular interest, as the RLPS of these strains did not bind the anti-Rm41. Previous studies have established that the LPS cores in both the R- and SLPS of *Sinorhizobium* spp. are similar (30); therefore, antibody recognition of the SLPS of these strains is specific for the O antigens.

(ii) **Composition analyses.** In order to determine the basis for serogroup identity, further analyses were performed on the RLPS of selected strains from each of the major serogroups. The RLPS were isolated by size exclusion chromatography of the phenol-water extracts (references 9, 29, and 32 and this report). From glycosyl residue composition analysis, the RLPS were found to consist primarily of Glc, GalA, and Kdo, with lesser amounts of Gal, Man, and glucuronic acid (GlcA) (Table 3). There was some difference in the relative abundances of Gal and Man in the RLPS from the different strains, but it did not delineate the serogroups. However, there was a striking difference between the relative amounts of GalA in the RLPS from the Rf205 serogroup (*S. fredii* USDA205 and *Sinorhizobium* sp. strain NGR234) and those in the RLPS from the

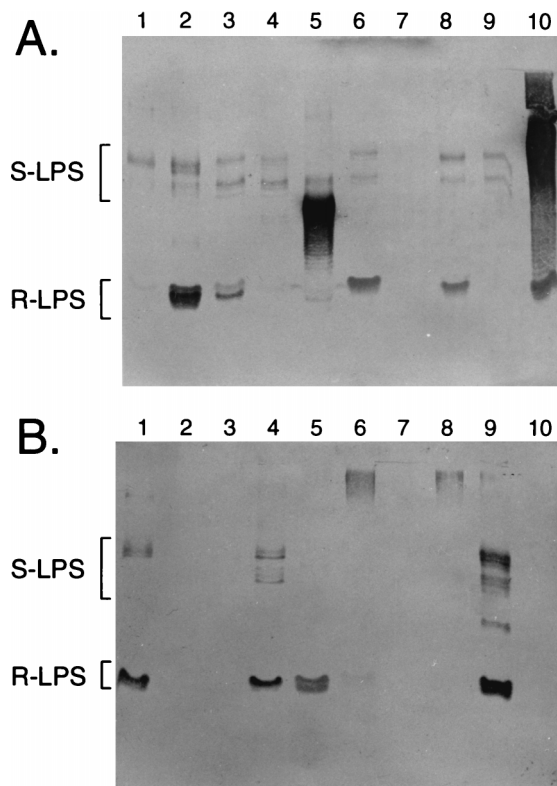


FIG. 4. Serotype analysis of the RLPS. The immunoblots were probed with polyclonal antisera raised against whole cells of *S. meliloti* Rm41 (anti-Rm41) (A) and *S. fredii* USDA205 (anti-Rf205) (B). Lanes: 1, *S. fredii* USDA205; 2, *S. fredii* USDA257; 3, *S. fredii* USDA201; 4, *S. fredii* USDA208; 5, *Sinorhizobium* sp. strain NGR234; 6, *S. fredii* HH103; 7, *S. fredii* HH303; 8, *S. fredii* USDA192; 9, *S. fredii* USDA191; 10, *S. meliloti* AK631. The dark areas in panel A, lanes 5 and 10, show cross-reaction of the anti-Rm41 with the *Sinorhizobium* sp. strain NGR234 and *S. meliloti* AK631 K antigens, both of which contain Pse (Table 4).

TABLE 2. Serotype analysis of the RLPS

RLPS source	Antiserum affinity ^a	
	Anti-Rf205	Anti-Rm41
<i>S. fredii</i> USDA191	+++	-
<i>S. fredii</i> USDA205	+++	-
<i>Sinorhizobium</i> sp. strain NGR234	+++	-
<i>S. fredii</i> USDA208	++	-
<i>S. meliloti</i> AK631	-	+++
<i>S. meliloti</i> Rm1021	-	+++
<i>S. meliloti</i> NRG133	-	+++
<i>S. fredii</i> USDA192	-	+++
<i>S. fredii</i> USDA196	-	+++
<i>S. fredii</i> USDA197	-	+++
<i>S. fredii</i> USDA257	-	+++
<i>S. meliloti</i> NRG185	-	++
<i>S. meliloti</i> NRG247	-	++
<i>S. meliloti</i> NRG286	-	++
<i>S. fredii</i> USDA201	-	++
<i>S. fredii</i> HH103	-	++
<i>S. meliloti</i> NRG23	-	+/-
<i>S. meliloti</i> NRG53	-	+/-
<i>S. fredii</i> HH303	-	-
<i>R. etli</i> CE3	-	-
<i>B. japonicum</i> USDA110	-	-

^a Polyclonal antisera included Anti-Rf205, raised against *S. fredii* USDA205, and anti-Rm41, raised against *S. meliloti* Rm41. Affinities were determined by immunoblot analysis (Fig. 4). +/-, weakly positive; ++, positive; +++, strongly positive; -, negative. *R. etli* CE3 and *B. japonicum* USDA110 were included as negative controls.

Rm41 serogroup (*S. fredii* USDA257 and *S. meliloti* Rm1021): the former contain approximately one less GalA residue per core (based on a comparison to the fatty acid content [see Materials and Methods]). The excess of Gal in the RLPS preparation of *S. fredii* USDA205 is probably due to minor contamination from the Gal-containing K antigen produced by that strain (29).

The core oligosaccharides from the RLPS of *S. meliloti* Rm1021 (Rm41 serogroup) and *S. fredii* USDA205 (Rf205 serogroup) were released from the lipid A moiety by mild acid hydrolysis and analyzed by HPAEC-PAD (Fig. 5). The complexity of the HPAEC chromatograms results from heterogeneity in composition and the acid-catalyzed conversion of some reducing-end Kdo residues of each oligosaccharide to various anhydrous forms during hydrolysis (4). For example, the broad peak at 35.5 min was the anhydro-Kdo derivative (FAB-MS showed a mass ion of m/z 1,640) of the oligosaccharide which eluted at 35 min (m/z 1,658); the difference of 18 represents the loss of water (i.e., anhydro). The broad-peak shape and relative retention times are characteristic of these derivatives (4).

Both strains yielded three common core oligosaccharides

TABLE 3. Relative glycosyl residue compositions of RLPS

Strain	Monosaccharides ^a					
	Gal	Glc	Man	Kdo	GalA	GlcA
<i>S. fredii</i> USDA205	1.25	5.00	0.75	2.50	1.25	Tr
<i>S. fredii</i> USDA257	0.25	5.00	0.75	3.00	2.50	Tr
<i>S. meliloti</i> Rm1021	0.50	5.00	0.25	3.00	2.50	Tr
<i>Sinorhizobium</i> sp. strain NGR234	0.50	5.00	0.25	3.00	1.50	Tr

^a The glycosyl residue compositions were determined by GC analysis of the TMS methyl glycosides. The retention times and response factors of component sugars were compared to those of authentic standards that had been treated in an identical manner. Molar ratios were normalized to glucose (see Methods) and rounded to the nearest 0.25. Tr, trace.

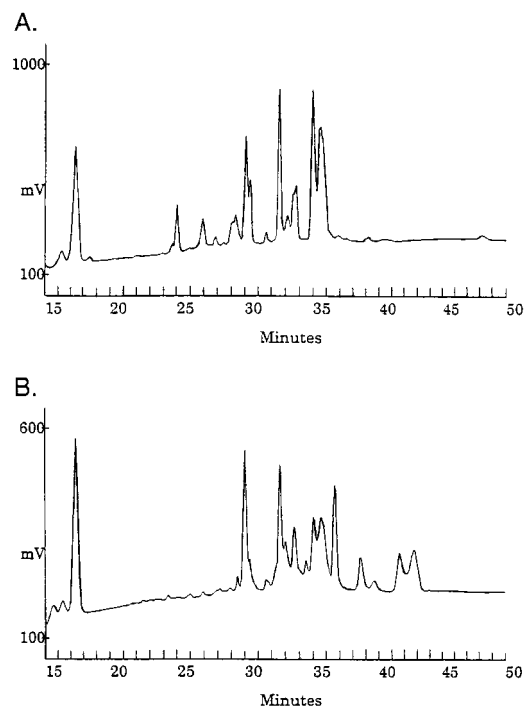


FIG. 5. HPAEC-PAD analysis of the LPS core oligosaccharides released from RLPS of *S. meliloti* Rm1021 (anti-Rm41 serogroup) (A) and *S. fredii* USDA 205 (anti-Rf205 serogroup) (B) after mild acid hydrolysis. The core oligosaccharides elute between 24 and 45 min. Each strain produces serogroup-specific and common oligosaccharides. See the text for details.

with identical retention times, at 30, 32.5, and 35 min. There were at least four Rf205-specific core oligosaccharides at 34.5, 37, 39, and 42 min (the peaks at 39.5 and 43 min are probably from anhydro derivatives of the components that elute just prior to them). The Rm1021 chromatogram contains at least three Rm41-specific peaks, at 25, 27, and 29 min (the peak at 28 min may be a derivative of the component that elutes at 27 min). A similar analysis of the LPS core oligosaccharides from *Sinorhizobium* sp. strain NGR234 (Rf205 serogroup) and *S. fredii* USDA257 (Rm41 serogroup) confirmed that these components (i.e., those that elute prior to 29 min and after 35 min) are specific to the respective serogroups (data not shown). Both of the latter strains also produced each of the common oligosaccharides in similar ratios.

DISCUSSION

Although closely related, *S. fredii*, *S. meliloti*, and *Sinorhizobium* sp. strain NGR234 exhibit distinct patterns of host specificity (7). *S. meliloti* has a relatively narrow host range, consisting solely of indeterminate nodule-forming hosts, including *Medicago* spp., *Melilotis* spp., and *Trigonella* spp. In contrast, *S. fredii* nodulates a diversity of hosts, including both determinate and indeterminate nodule-forming plants (26), yet it does not nodulate the host plants of *S. meliloti*. In addition, *S. fredii*-soybean interactions are strain X cultivar specific (12, 14), and the genetic basis for specificity is partially understood (11, 17, 18, 21). Strain NGR234 can nodulate more than 200 different host plants, including all those that are nodulated by *S. fredii*, except soybeans (26). This diversity in host range among *Sinorhizobium* strains yields an excellent model for the study of the role of the bacterial cell surface in the determination of host specificity.

TABLE 4. K antigens of *Sinorhizobium* spp.

K _R no. ^a	Strain	Complete and partial structures ^b	Reference
K _R 1	<i>S. Fredii</i> USDA205	[→3)-α-D-Galp-(1→5)-β-D-Kdop-(2→) _n	29
K _R 2		[-2-O-MeMann→β-Kdo-] _n	29
K _R 3	<i>S. fredii</i> USDA257	[→3)-β-D-Manp-(1→5)-β-D-Kdop-(2→) _n	9
K _R 4		[→3)-β-D-2-O-MeManp-(1→5)-β-D-Kdop-(2→) _n	9
K _R 5	<i>S. meliloti</i> AK631	[-β-GlcA→Pse5N(β-OH-But)7NAc-] _n	27
K _R 6	<i>S. meliloti</i> NGR247	[-α-Glc→α-NeuNAc-] _n	This report
K _R 7	<i>S. meliloti</i> NGR185	[-β-GlcNAc→β-Kdo-] _n	This report
K _R 8	<i>S. fredii</i> USDA208	[-α-Gal→β-Kdo-] _n	This report
K _R 9	<i>S. fredii</i> USDA201	[-α-Gal→β-Kdo→α-2-O-MeHex→β-Kdo-] _n	This report
K _R 10	<i>Sinorhizobium</i> sp. strain NGR234	[-β-Glc→α-Pse5NAc7NAc-] _n	This report
K _R 11	<i>S. fredii</i> HH303	[Rha, GalA] _n	This report
Consensus structure ^c		[-Sug→Kdx-] _n	

^a Numerical designation of the K antigen.

^b Pse, 5,7-diamino-3,5,7,9-tetraoxynonulosonic acid (pseudaminic acid); Ac, acetyl group; β-OH-But, β-OH-butyl group; Me, methyl group; Sug, any hexose; Kdx, any 1-carboxy-2-keto-3-deoxy sugar. Note that the correct designation, Pse, is used in this report instead of Kdn, which was used in a past report (9).

^c The K antigen from *S. fredii* USDA201 consists of tetrasaccharide repeats [(Sug→Kdx)₂]_n, but conforms to the consensus structure. The only exception to the consensus structure that has been found is from *S. fredii* HH303.

There are many possible functions for the bacterial polysaccharides in symbiosis, such as the promotion of infection thread initiation and development and the elicitation of nodulins, as well as other signal functions. Potential passive functions include the masking of negative determinants and physical protection against harmful plant products. Any such functions, active or passive, may influence compatibility in infection and, ultimately, the host range of the bacterial strain.

It has now been shown that different *Sinorhizobium* strains produce structurally similar, yet chemically distinct, K antigens (Table 4): 10 of the 11 *Sinorhizobium* K antigens that have been partially or completely characterized conform to the consensus structure, Sug-Kdx (where Sug is any hexose and Kdx is any 1-carboxy-2-keto-3-deoxy sugar). Furthermore, *Agrobacterium tumefaciens*, which is closely related to *Sinorhizobium* spp., also produces a polysaccharide that appears to conform to the consensus structure, although it has not been completely characterized (31). Despite the similarities, the K antigens of *Sinorhizobium* spp. are clearly strain-specific antigens. In addition to the differences in glycosyl residue composition, the polysaccharides vary in substitution patterns, linkage points, anomeric configuration, size range, and repeating unit size. One interesting feature that differs among the species is the consistent presence of *N*-acetyl groups in the K antigens of *S. meliloti* strains; these have not been found in *S. fredii* K antigens (Table 4). In addition, it has recently been shown that the K antigens of *Sinorhizobium* spp. are sulfated (27) but the level of sulfation is much lower in normally cultured cells of *S. meliloti* than in *S. fredii*. If the polysaccharides play an active role in the infection process and host specificity, any of the structural features may be important. For example, a very specific size range of EPS II has been found to promote nodulation of alfalfa by *S. meliloti* (10), and a genetic determinant of capsule size range (*rkpZ*) is required for alfalfa nodulation by EPS mutants of *S. meliloti* (32). In addition, specific K-antigen structure appears to be important in early recognition steps in *S. meliloti*-alfalfa symbiosis (1).

The primary function of the K antigens is probably to form a hydrated capsule that surrounds the cell and protects it against abiotic factors, such as desiccation. The advantage of the capsule, as opposed to LPS, is that it may constitute a dense matrix that extends far from the cell surface, with only a fraction of the component molecules in contact with the outer membrane. Present evidence suggests that these capsule "anchors" may be linked to the LPS via disulfide bridges (27); in contrast to

studies of enteric bacteria, no evidence has been found for a lipid moiety on the K antigens of rhizobia. This was also shown in the lack of fatty acid resonances in the NMR spectra presented in this report.

The capsule may also provide protection against other microorganisms, such as phages; in fact, in a recent study it was found that although capsule mutants of *S. meliloti* Rm41 *exoB* (AK631) gained resistance to one of 12 phages tested, they lost resistance to as many as 9 of the 12 (2). This shows that there may be many important functions of the capsule in these soil bacteria and that any functions in nodulation, in relation to structural specificity, may be secondary to those that affect the survival of the cell.

Sinorhizobium spp. are characterized by conserved LPS: the RLPS of 18 of the 19 strains examined are delineated into two major core serogroups (Table 2), which are not correlated with species. Although the RLPS from each serogroup have similar glycosyl residue compositions (Table 3) and contain common core oligosaccharides (Fig. 5), it appears that a difference in the uronic acid content of the serogroup-specific core oligosaccharides is partially responsible for serogroup identity. The presence of the common oligosaccharides in both types of RLPS suggests that there should have been significant cross-reaction in the immunoblot analyses, which was not the case. Therefore, the serogroup-specific core oligosaccharides must be the immunodeterminant components of the RLPS. Present evidence indicates that the serogroup-specific components may be "outer" cores that are at the nonreducing terminus of the RLPS core (27), yielding serogroup specificity despite the presence of common "inner" cores in all *Sinorhizobium* LPS.

The RLPS of *S. meliloti* NRG23 and *S. meliloti* NRG53 gave marginal reactions with anti-Rm41 and probably constitute a third serogroup, and the RLPS of *S. fredii* HH303 was negative with both antisera, suggesting that at least four serogroups exist. Importantly, the subdivision of the LPS into these core serogroups is consistent with relationships based on immunological analyses of whole cells (8, 19, 33). This implies that the RLPS is a major common antigen in *Sinorhizobium* spp. whereas the K antigen is a major strain-specific antigen. This assertion is supported by monoclonal antibody (MAb) studies, performed by Olsen et al. (24), and the subsequent identification of the epitopes in this laboratory (27). The three strain-specific anti-*S. meliloti* MAbs used in that study recognized the K antigens of the homologous strains (and a few others), whereas two of the three common-antigen MAbs recognized the RLPS

of 53 of the 60 strains tested. When the anti-RLPS MAbs were employed in immunoblotting experiments with the strains from this study, they recognized the RLPS of all strains in the anti-Rm41 serogroup (Table 2) except those of *S. meliloti* NRG53 and *S. meliloti* NRG23 (27), confirming that those strains are, in fact, in a distinct RLPS serogroup(s). This also indicated that the Rm41 serogroup is the largest in the genus *Sinorhizobium*.

As the core regions of both RLPS and SLPS from *S. fredii* USDA205 have been shown to be similar (30), the specific recognition of the Rf205 serogroup SLPS by anti-Rm41 is credible evidence that there is limited variation in O-antigen structure among *Sinorhizobium* strains. Also, in both the PAGE and immunoblot analyses, the SLPS, when present, migrated as two distinct bands. Characterization of the two forms of SLPS from *S. fredii* USDA205 has shown that they bear different O antigens. The primary O antigen (upper SLPS banding region) is a glucan, and the secondary O antigen (lower SLPS banding region) is a xylomannan (30). In this regard, *Sinorhizobium* spp. are unusual. The O antigens of most gram-negative bacteria are highly variable, strain-specific surface antigens (37); in this genus, that role is fulfilled by the K antigens. The structural conservation of LPS throughout the genus *Sinorhizobium* suggests that the LPS, in the form found in cultured cells, does not impact on host range, including strain-cultivar specificity. This does not preclude the possibility, however, that the cells may express different forms of LPS in the soil, at the point of infection.

Interestingly, the expression of the two O antigens by *S. fredii* USDA205 is regulated by host-specific compounds (30), and "induced" differences in K antigen expression have been demonstrated in both *S. fredii* and *S. meliloti* (30, 32). Mutations in apparent regulatory genes (*noIXWBTUV*) also result in a modified expression of the LPS and K antigens in *S. fredii* USDA257 (15). In addition, many changes in the expression of *Sinorhizobium* LPS and K antigens are associated with growth under varied abiotic conditions, such as pH or temperature (27). These changes imply that analysis of cultured cells represents an important first step in understanding the role of bacterial surface polysaccharides in symbiosis; however, additional analyses of bacteroides, induced cells, regulatory mutants, and cells grown under different abiotic conditions are clearly required to elucidate the structure-function relationships between the bacterial cell surface and symbiosis.

In light of the complexity of legume-rhizobium interactions, we believe that a comparative analysis of closely related strains of *Sinorhizobium* is a valuable asset in the study of the molecular basis for host specificity. In a similar analysis of LPS and outer membrane proteins from a panel of wild-type strains of *Xanthomonas campestris*, Ojanen et al. (22) illustrated a correlation between host specificity and the pathogen cell surface. Our present and future analyses may yield similar results.

ACKNOWLEDGMENTS

This work was supported by grant MCB-9728564 from the National Science Foundation and by the U.S. Department of Energy-funded Center for Plant and Microbial Complex Carbohydrate Research under grant DE-FG02-93ER-20097 (B.L.R.). S.G.P. was supported by a grant from the USDA/NRI.

We thank Malcolm O'Neill for a critical review of the manuscript.

REFERENCES

1. Bequart-de Kozak, I., B. L. Reuhs, D. Buffard, C. Breda, J. S. Kim, R. Esnault, and A. Kondorosi. 1997. Role of the K-antigen subgroup of capsular polysaccharides in the early recognition process between *Rhizobium meliloti* and alfalfa leaves. *Mol. Plant-Microbe Interact.* **10**:114-123.
2. Campbell, G. R. O., B. L. Reuhs, and G. C. Walker. 1998. Different pheno-

typic classes of *Sinorhizobium meliloti* mutants defective in synthesis of K antigen. *J. Bacteriol.* **180**:5432-5436.

3. Carlson, R. W., U. R. Bhat, and B. Reuhs. 1992. *Rhizobium* lipopolysaccharides: their structures and evidence for their importance in the nitrogen-fixing symbiotic infection of their host legumes, p. 33-44. In P. M. Gresshoff (ed.), *Plant biotechnology and development*. CRC Press, Boca Raton, Fla.
4. Carlson, R. W., and B. S. Krishnaiah. 1992. Structures of the oligosaccharides obtained from the core regions of the lipopolysaccharides of *Bradyrhizobium japonicum* 61A101c and its symbiotically defective lipopolysaccharide mutant, JS314. *Carbohydr. Res.* **231**:205-219.
5. Carrion, M., U. R. Bhat, B. Reuhs, and R. W. Carlson. 1990. Isolation and characterization of the lipopolysaccharides from *Bradyrhizobium japonicum*. *J. Bacteriol.* **172**:1725-1731.
6. Corzo, J., R. Pérez-Galdona, M. León-Barrios, and A. M. Gutiérrez-Navarro. 1991. Alcian Blue fixation allows silver staining of the isolated polysaccharide component of bacterial lipopolysaccharides in polyacrylamide gels. *Electrophoresis* **12**:439-441.
7. deLajudie, P., A. Williams, B. Pot, D. Dewettinck, G. Maestrojuan, M. Neyra, M. D. Collins, B. Dreyfus, K. Kersters, and M. Gillis. 1995. Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium shaeli* sp. nov., and *Sinorhizobium teranga* sp. nov. *Int. J. Syst. Bacteriol.* **44**:715-733.
8. Dowdle, S. F., and B. B. Bohlool. 1985. Predominance of fast-growing *Rhizobium japonicum* in a soybean field in the People's Republic of China. *Appl. Environ. Microbiol.* **50**:1171-1176.
9. Forsberg, L. S., and B. L. Reuhs. 1997. Structural characterization of the K antigens from *Rhizobium fredii* USDA257: evidence for a common structural motif, with strain-specific variation, in the capsular polysaccharides of *Rhizobium* spp. *J. Bacteriol.* **179**:5366-5371.
10. Gonzalez, J. E., B. L. Reuhs, and G. C. Walker. 1996. Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*. *Proc. Natl. Acad. Sci. USA* **93**:8636-8641.
11. Heron, D. S., T. Ersek, H. B. Krishnan, and S. G. Pueppke. 1989. Nodulation mutants of *Rhizobium fredii* USDA257. *Mol. Plant-Microbe Interact.* **2**:4-10.
12. Heron, D. S., and S. G. Pueppke. 1984. Mode of infection, nodulation specificity, and indigenous plasmids of 11 fast-growing *Rhizobacterium japonicum* strains. *J. Bacteriol.* **160**:1061-1066.
13. Jarvis, B. D. W., H. L. Downer, and J. P. W. Young. 1992. Phylogeny of fast-growing soybean-nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*. *Int. J. Syst. Bacteriol.* **42**:93-96.
14. Keyser, H. H., T. S. Hu, B. B. Bohlool, and D. F. Weber. 1982. Fast-growing rhizobia isolated from root nodules of soybean. *Science* **215**:1631-1632.
15. Kim, J. S., and B. L. Reuhs. 1996. Extended host range mutants of *Rhizobium fredii* USDA257 show modified expression of the K antigens and lipopolysaccharides, abstr. L-10. In Abstracts of the 8th International Congress on Molecular Plant-Microbe Interactions.
16. Kim, J. S., B. L. Reuhs, M. M. Rahman, B. Ridley, and R. W. Carlson. 1996. Separation of bacterial capsular and lipopolysaccharides by preparative electrophoresis. *Glycobiology* **6**:433-437.
17. Kovacs, L. G., P. A. Balatti, H. B. Krishnan, and S. G. Pueppke. 1995. Transcriptional organization and expression of *noIXWBTUV*, a locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA 257. *Mol. Microbiol.* **17**:923-933.
18. Krishnan, H. B., and S. G. Pueppke. 1992. Inactivation of *noIC* conditions developmental abnormalities in nodulation of Peking soybean by *Rhizobium fredii* USDA257. *Mol. Plant-Microbe Interact.* **5**:14-21.
19. Krishnan, H. B., and S. G. Pueppke. 1994. Host range, RFLP, and antigenic relationships between *Rhizobium fredii* strains and *Rhizobium* sp. NGR234. *Plant Soil* **161**:21-29.
20. Lerouge, P., P. Roche, C. Faucher, F. Maillet, G. Truchet, J. C. Promé, and J. Dénarié. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* **344**:781-784.
21. Meinhardt, L. W., H. B. Krishnan, P. A. Balatti, and S. G. Pueppke. 1993. Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean *Rhizobium fredii* USDA257. *Mol. Microbiol.* **9**:17-29.
22. Ojanen, T., I. M. Helander, K. Hahtela, T. K. Korhonen, and T. Laakso. 1993. Outer membrane proteins and lipopolysaccharides in pathovars of *Xanthomonas campestris*. *Appl. Environ. Microbiol.* **59**:4143-4151.
23. Olsen, P., M. Collins, and W. Rice. 1992. Surface antigens present on vegetative *Rhizobium meliloti* cells may be diminished or absent when cells are in the bacteroid form. *Can. J. Microbiol.* **38**:506-509.
24. Olsen, P., M. M. Collins, and W. A. Rice. 1994. Reactivity of MAbs raised against whole cells to a panel of *Rhizobium* strains. *Appl. Environ. Microbiol.* **60**:652-661.
25. Petrovics, G., P. Putnoky, B. L. Reuhs, J. S. Kim, T. A. Thorp, D. Noel, R. W. Carlson, and A. Kondorosi. 1993. The presence of a novel type of surface polysaccharide in *Rhizobium meliloti* requires a new fatty acid synthase-like gene cluster involved in symbiotic nodule development. *Mol. Microbiol.* **8**:1093-1094.

26. **Pueppke, S. G., and W. J. Broughton.** Symbiotic specificity in the legume-*Rhizobium* symbiosis: *Rhizobium* sp. NGR234 and *R. fredii* USDA257 have exceptionally broad, interrelated host ranges. *Mol. Plant-Microbe Interact.*, in press.
27. **Reuhs, B. L.** Unpublished data.
28. **Reuhs, B. L.** 1996. Acidic capsular polysaccharides (K antigens) of *Rhizobium*, p. 331–336. In G. Stacey, B. Mullin, and P. M. Gresshoff (ed.), *Biology of plant-microbe interactions*. International Society for Molecular Plant-Microbe Interaction, St. Paul, Minn.
29. **Reuhs, B. L., R. W. Carlson, and J. S. Kim.** 1993. *Rhizobium fredii* and *Rhizobium meliloti* produce 3-deoxy-D-manno-2-octulosonic acid-containing polysaccharides that are structurally analogous to group II K antigens (capsular polysaccharides) found in *Escherichia coli*. *J. Bacteriol.* **175**:3570–3580.
30. **Reuhs, B. L., J. S. Kim, A. Badgett, and R. W. Carlson.** 1994. Production of the cell-associated polysaccharides of *Rhizobium fredii* USDA205 is modulated by apigenin and host root extract. *Mol. Plant-Microbe Interact.* **7**:240–247.
31. **Reuhs, B. L., J. S. Kim, and A. G. Matthyse.** 1997. Attachment of *Agrobacterium tumefaciens* to carrot cells and *Arabidopsis* wound sites is correlated with the presence of a cell-associated, acidic polysaccharide. *J. Bacteriol.* **179**:5372–5379.
32. **Reuhs, B. L., M. N. V. Williams, J. S. Kim, R. W. Carlson, and F. Côté.** 1995. Suppression of the Fix⁻ phenotype of *Rhizobium meliloti* *exoB*⁻ by *lpsZ* is correlated to a modified expression of the K polysaccharide. *J. Bacteriol.* **177**:4289–4296.
33. **Sadowsky, M. J., B. B. Bohlool, and H. H. Keyser.** 1987. Serological relatedness of *Rhizobium fredii* to other rhizobia and to the bradyrhizobia. *Appl. Environ. Microbiol.* **53**:1785–1789.
34. **Truchet, G., P. Roche, P. Lerouge, J. Vasse, S. Camut, F. De Billy, J.-C. Promé, and J. Dénarié.** 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. *Nature* **351**:670–673.
35. **Tsai, C., and C. E. Frisch.** 1982. A sensitive silver stain for detecting lipo-polysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**:115–119.
36. **Weissbach, A., and J. Hurwitz.** 1958. The formation of 2-keto-3-deoxyheptanoic acid in extracts of *Escherichia coli* B. *J. Biol. Chem.* **234**:705–709.
37. **Whitfield, C., and M. A. Valvano.** 1993. Biosynthesis and expression of cell-surface polysaccharides in Gram-negative bacteria. *Adv. Microb. Physiol.* **35**: 135–246.
38. **York, W. S., A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim.** 1985. Isolation and characterization of plant cell walls and cell wall components. *Methods Enzymol.* **118**:3–40.