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*

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Received 25 January 1993/Accepted 5 April 1993

The polysaccharide components from cultured cells of *Rhizobium fredii* USDA205 and *Rhizobium meliloti* AK631 were extracted with hot phenol-water and separated by repetitive gel filtration chromatography. Polyacrylamide gel electrophoresis, nuclear magnetic resonance spectrometry, and gas chromatography analyses showed that both of these bacterial species produce unique polysaccharides that contain a high proportion of 3-deoxy-D-manno-2-octulosonic acid (Kdo). These polysaccharides, which constituted a major portion of the extracted carbohydrate, are not excreted into the growth media (i.e., they are not extracellular polysaccharides) and are structurally distinct from the lipopolysaccharides. The primary structure of the preponderant polysaccharide from *R. fredii* USDA205 was determined by high-performance anion-exchange liquid chromatography, nuclear magnetic resonance spectrometry, fast atom bombardment-mass spectrometry, and gas chromatography-mass spectrometry; it consists of repeating units of $[\rightarrow 3)\text{-}\alpha\text{-D-Galp-(1}\rightarrow 5)\text{-}\beta\text{-D-Kdop-(2}\rightarrow)]_n$. This molecule is structurally analogous to the constituents of one subgroup of K antigens (capsular polysaccharides) produced by *Escherichia coli*. Polysaccharides of this type have not previously been identified as components of rhizobial cells. The Kdo-containing polysaccharide from *R. meliloti*, which has not been completely characterized, appears to be structurally related to that of *R. fredii*.

The gram-negative bacteria of the *Rhizobiaceae* family include the nitrogen-fixing symbionts of legumes (*Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* spp.) and the pathogenic *Agrobacterium* genus. These species produce various types of extracellular polysaccharides (EPSs) and cell surface polysaccharides that appear to be important factors in symbiotic and pathogenic effectiveness.

Lipopolysaccharides (LPSs), unique to gram-negative bacteria, are outer membrane inclusions that consist of (i) a glycolipid membrane anchor, which contains characteristic β -hydroxy fatty acids on a glucosamine backbone (lipid A); (ii) a core oligosaccharide that is linked to the lipid A via 3-deoxy-D-manno-2-octulosonic acid (Kdo) and is highly conserved within a species; and (iii) a highly variable O antigen. Polyacrylamide gel electrophoresis (PAGE)-silver stain analyses of rhizobial LPSs frequently result in banding regions (5, 9), indicating that the O antigens of these LPSs may commonly consist of oligosaccharides of specific lengths.

Rhizobia also produce acidic EPSs and cyclic β -glucans. EPSs are secreted compounds and are isolated from the growth media of cultured cells. The EPSs produced by rhizobia are high-molecular-weight heteropolysaccharides composed of small repeating units that contain either uronic acids or noncarbohydrate substituents, such as succinate and pyruvate, as the acidic functions (4, 22, 29). The cyclic β -glucans are commonly harbored within the periplasmic space but may also be secreted; consequently, they are found in both the growth media and the bacterial pellet of cultured cells (42). The role of the β -glucans in symbiosis remains a moot subject.

Numerous studies have shown that bacterial LPS and EPS are important in the infection and nodulation processes; however, the symbiotic phenotype of LPS- and EPS-defective microsymbionts appears to depend on the type of nodule formed by the host.

(i) Inoculation of determinate nodule-forming host plants with rhizobial LPS mutants that are affected in the presence, abundance, or chain length of the LPS O antigen results in the formation of severely underdeveloped nodules (Ndv^-) that do not fix nitrogen (Fix^-) (12, 22, 28). The symbiotic defect of the LPS mutants occurs early in the infection process, during the initial infection thread development; consequently, there is no detectable deposition of bacteria into the nodule cells. The nodulation of indeterminate hosts is also affected by modifications in the LPS, but at a later stage in the infection process. LPS mutants, which produce little or no O antigen, are Fix^- on their indeterminate hosts; however, the symbiotic defect occurs late in the infection process, at the point of bacterial release into the host nodule cells, and there are some endocytosis of the microsymbionts and minor nitrogenase production (6, 22, 31). Thus, the LPS appears to perform different functions in determinate versus indeterminate nodulation.

(ii) The inoculation of indeterminate nodule-forming hosts with EPS^- microsymbionts, which produce no detectable EPS, results in abortive infections (21, 22, 29). In contrast, EPS^- microsymbionts of determinate nodule-forming hosts are $\text{Ndv}^+ \text{Fix}^+$ (2, 13, 18, 22).

(iii) Broad-host-range microsymbionts, which nodulate both determinate and indeterminate hosts, show an EPS requirement on indeterminate hosts but no such requirement on determinate hosts (10, 14).

It is possible that the EPS is rendered unnecessary in

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determinate nodulation systems by the presence of another, hitherto unidentified, bacterial product.

Rhizobium fredii is a microsymbiont of soybeans, which form determinate nodules. Past studies had indicated that the LPS of *R. fredii* USDA205 consisted of 20 to 40% Kdo (8); this report shows that the preponderance of Kdo in the crude extract of *R. fredii* USDA205 is a component of a non-LPS, Kdo-rich polysaccharide (Kdops). This type of polysaccharide, which is novel to rhizobia, is fundamentally distinct from classic LPSs and does not belong to the class of secreted EPSs. The polysaccharide isolated from *R. fredii* USDA205 is structurally analogous to a class of acidic capsular polysaccharides (CPSs) that are commonly found in *Escherichia coli* and are often important in pathogenicity (15). The Kdops from *R. fredii* is also produced by a strain that has been cured of the symbiotic plasmid, demonstrating that the associated genes are not on that plasmid.

This report also presents evidence that a Kdops, which is similar to that of *R. fredii*, is produced by *Rhizobium meliloti* AK631 and Rm41. This polysaccharide has been chromatographically isolated and shown to be distinct from the LPS. *R. meliloti* AK631, an EPS⁻ mutant of Rm41, is an exception to the ostensible requirement for bacterial EPS in indeterminate nodulation systems (discussed above); AK631 is able to effectively nodulate alfalfa, an indeterminate host (27, 37). It has been suggested that the LPS substitutes for the EPS in this symbiotic system (25, 38); however, data from this laboratory, in collaboration with others, indicate that this is not the case. Mutations that affect the production of the Kdops by AK631 also affect the infection of alfalfa by this strain (24a, 29a).

The identification of these previously unreported polysaccharides in rhizobia may aid in the clarification of the distinct roles of bacterially derived polysaccharides in the nodulation of determinate versus indeterminate hosts.

MATERIALS AND METHODS

Organisms and growth conditions. *R. fredii* USDA205 and HC205 were provided by Barry Rolfe (The Australian National University). *R. meliloti* AK631 was obtained from Adam Kondorosi (Centre National de la Recherche Scientifique, Gif-Sur-Yvette, France). *R. meliloti* Rm41 was provided by Dale Noel (Marquette University, Milwaukee, Wis.).

R. fredii USDA205 and HC205 bacterial cultures were grown at 28°C in yeast extract media to an optical density of ca. 0.9 at 620 nm. The bacteria were harvested by centrifugation at 4°C. The bacterial pellets were washed with 1% NaCl, repelleted, washed with H₂O, repelleted, and immediately frozen. The growth and harvest conditions for *R. meliloti* AK631 and Rm41 have been described previously (26).

Extraction and purification. The bacterial polysaccharides were extracted by the hot phenol-water method (36), as modified by Carlson et al. (7); this was followed by exhaustive dialysis of the water layer material against H₂O.

Ultracentrifugation of the *R. meliloti* AK631 water layer was performed at 100,000 × *g* for 4 h at 10°C in H₂O. The supernatant was removed and lyophilized.

The extracted polysaccharides were separated by repeated chromatography on a Sepharose 4B (Sigma, St. Louis, Mo.) column (1 m by 3 cm) in 30 mM triethylamine-10 mM EDTA buffer, pH 7. The column fractions (7 ml) were assayed for the presence of Kdo (thiobarbituric

acid assay), uronic acids, and hexoses by colorimetric analysis (40) and for the presence of LPS by PAGE. PAGE was performed using 15% acrylamide gels with deoxycholic acid as a detergent (19); the gels were developed by the silver staining method of Tsai and Frisch (32). Fractions were pooled, dialyzed against H₂O, and lyophilized.

A second purification step was necessary for the *R. meliloti* AK631 extract; this was performed by chromatography on a Sephadex G150 superfine (Pharmacia, Uppsala, Sweden) column (1 m by 1.5 cm) in a deoxycholic acid buffer (0.2 M NaCl, 1 mM EDTA, 10 mM Tris base, 0.25% deoxycholic acid, pH 9.25). The column fractions were assayed as described above; however, it was necessary to precipitate the deoxycholic acid from fraction aliquots prior to the colorimetric assays. An equal amount of 2% acetic acid was added to each fraction aliquot in a microcentrifuge tube; the samples were then vortexed and centrifuged for 5 min, and the supernatant was removed for the assays. The deoxycholic acid did not affect the PAGE analysis. The pooled fractions from the G150 step were dialyzed against a solution of 4 mM Tris base, 0.25% NaCl, and 10% ethanol for three changes of 10 liters each (this step was critical for removal of the deoxycholic acid); this was followed by exhaustive dialysis against H₂O.

Mild-acid hydrolysis (1% acetic acid, 100°C, 1 h) of intact Kdops preparations from *R. fredii* USDA205 was followed by gel filtration chromatography of the degraded polysaccharides on a Bio-Gel P-2 Minus 400 mesh (Bio-Rad, Richmond, Calif.) column (1 m by 1.5 cm) in 1% acetic acid. The column fractions were assayed for the presence of Kdo and hexose; the peaks were pooled and freeze-dried.

Composition analysis. Composition analysis was performed by gas chromatography-mass spectrometry (GC-MS; Hewlett-Packard 5890A GC-MSD) of the prereduced alditol acetate derivatives (40) on a 30-m Supelco SP2330 fused silica column and of the trimethylsilyl (TMS) methyl glycosides (40) on a 30-m J&W Scientific DB1 fused silica column. Inositol was used as an internal standard, and retention times were compared with authentic standards. The Kdo was a gift of Jerry Ellis (Chemistry Department, Eastern Illinois University).

NMR analysis. ¹H nuclear magnetic resonance (NMR) analyses were performed on a Bruker AM 500, Bruker AMX 600 (Complex Carbohydrate Research Center, University of Georgia) or General Electric QE 300 (University of Illinois) spectrometer in ²H₂O. The measurements were made at 296 or 314 K. The chemical shifts were referenced to acetone at δ 2.24.

PAGE of polysaccharides. Purified polysaccharides and crude extracts were analyzed by PAGE using 18% acrylamide gels with deoxycholic acid as the detergent (19). The gels were silver stained (32) or Alcian blue-silver stained (11). In the latter procedure the gels were incubated with 0.5% Alcian blue in 2% acetic acid for 30 min prior to fixing and silver staining.

HPLC analysis and purification of *R. fredii* USDA205 Kdops. High-performance liquid chromatography (HPLC) analysis of oligosaccharides from *R. fredii* was performed by anion-exchange chromatography on a Dionex Metal-free BioLC chromatograph (Dionex Corp., Sunnyvale, Calif.) with a CarboPac PA1 column (4 by 250 mm) using a pulsed amperometric detector with a gold working electrode (Dionex Corp.). The detector was operated at 1,000-nA sensitivity with the following pulse potentials and durations for detection of carbohydrates: 0.05 V, 500 ms; 0.60 V, 80 ms; and -0.60 V, 50 ms. The response time of the detector was

FIG. 1. (A) Sepharose 4B gel filtration (30 mM triethylamine–10 mM EDTA) elution profile of hot phenol-water extract (water phase) from *R. fredii* USDA205. The fractions were assayed for the presence of Kdo (●; A_{550}), uronic acids (▲; A_{520}), and hexoses (+; A_{620}). The presence of LPS in the column fractions was determined by PAGE analysis (inset); the gel shows alternate fractions from 36 to 72. LPS was not detected by PAGE analysis after fraction 72. (B) Bio-Gel P-2 gel filtration (1% acetic acid) elution profile of the mild-acid-degraded polysaccharides from peak 4B-3 (A). The fractions were assayed for the presence of Kdo and hexoses. The FAB-MS mass spectra of the peak pools are shown in the inset: P2-2, upper spectrum; P2-1, lower spectrum. The ion at m/z 413 corresponds to $M-H^+$ of the 2-*O*-methylmannose-Kdo disaccharide. The ion at m/z 399 corresponds to the $M-H^+$ of the galactose-Kdo disaccharide.

set to 1 s. The eluents were prepared with ultrapure water, carbonate-free liquid 50% NaOH, and $CH_3COONa \cdot 3H_2O$ (Baker analyzed HPLC reagent); they were then filtered (0.2- μ m-pore-size Nylon-66 membranes) and degassed with helium (eluent degas module; Dionex Corp.) prior to use. Eluent A consisted of 100 mM NaOH, and eluent B consisted of 1 M sodium acetate in 100 mM NaOH. The gradient was produced by a linear variation of the proportion of eluent B, as follows: 0 min, 10% B; 3 min, 10% B; 10 min, 20% B; 25 min, 100% B; 50 min, 100% B; 55 min, 10% B; 60 min, 10% B. The flow rate was 1 ml/min at ambient temperature. Postcolumn addition of 400 mM NaOH at 0.6 ml/min with a pressurized reagent delivery system (Dionex Corp.) improved detector response and stabilized the baseline. All samples were dissolved in ultrapure water and filtered through 0.2- μ m-pore-size Nylon-66 Microfilterfuge tubes (Rainin Instrument Co., Woburn, Mass.). Preparative HPLC was performed on the same instrument with a CarboPac PA1 semipreparative column (9 by 250 mm) (Dionex Corp.). The eluents were A, H_2O (ultrapure water) and B, 1 M sodium acetate. The proportion of eluent B was varied as follows: 0 min, 10% B; 3 min, 10% B; 40 min, 60% B; 45 min, 100% B; 50 min, 10% B. The flow rate was 5 ml/min at ambient temperature. The detector and postcolumn NaOH were used only in the initial runs to optimize the conditions; during the collection of the oligosaccharides the detector was bypassed and the eluent was sent directly to a fraction collector. The individual fractions were tested for purity by rechromatographing an aliquot of each under the analytical conditions described above, after dilution with H_2O (1:10). The fractions were pooled and desalted by passage through OnGuard sample pretreatment cartridges (Dionex Corp.) prior to further analysis.

FAB-MS. Fast atom bombardment-mass spectrometry (FAB-MS) was performed on a VG ZAB-SE instrument in the negative mode, with an accelerating voltage of 70 eV, operating at low resolution (ca. 1:800). The samples were

dissolved in ultrapure H_2O , and 1 μ l was added to a matrix of thioglycerol (2 μ l). The scan range was 100 to 1,000 m/z for disaccharide analysis and 300 to 3,000 m/z for analysis of the nondegraded Kdops. The detected ions were compared with the predicted average-mass ions of the nonresolved high-mass ion clusters by using the CARBOMASS (41) software.

Structural analysis. The galactosyl linkage was determined by the Hakomori method, as described by York et al. (40). The partially methylated alditol acetates were analyzed on a Hewlett-Packard 5890A GC-MSD instrument with a 30-m Supelco SP2330 fused silica column. The Kdo linkage was identified by prereduction of the galactose-Kdo disaccharide, followed by per-*O*-methylation and analysis by GC-MS.

Glycosyl configuration. The enantiomeric configuration of the galactosyl residue was determined by GC analysis of the TMS (+)-sec-butyglycosides of the polysaccharide and an authentic D-(+)-galactose standard (42). The Kdo was assumed to be in the D configuration, as is commonly reported.

RESULTS

***R. fredii* USDA205. (i) Extraction, purification, and initial analysis.** Cultured cells of *R. fredii* USDA205 were extracted by the hot phenol-water method, and the H_2O layers were chromatographed on a Sepharose 4B gel filtration column. The fractions were assayed by colorimetric and PAGE techniques. The resultant Sepharose 4B column profile and the silver-stained PAGE gel of those fractions that contained LPS are shown in Fig. 1A and the inset. This protocol was repeated with three separate cultures of bacteria with consistent results. One peak, 4B-3, contained a high concentration of Kdo; however, this peak did not contain LPS, as determined by PAGE (Fig. 1A and inset). The elution of the LPS coincided with the elution of uronic acids, which are components of the LPS (discussed below), and preceded the

elution of peak 4B-3. Although LPS was not detected by PAGE analysis in any fractions after 72, peak 4B-3, which had previously been reported as the LPS (8), was rechromatographed under identical conditions to ensure complete removal of LPS. The elution point of the rechromatographed material was identical to that of peak 4B-3 (data not shown).

The peak 4B-3 pool, which accounted for ~25 to 30% of the recovered water layer extract (wt/wt), was then analyzed by GC-MS of the TMS methyl glycoside and alditol acetate derivatives. Only three glycosyl components were detected in this pool: galactose (Gal), Kdo, and 2-*O*-methylmannose. The molar ratio of the hexosyl components to Kdo was 1:1. The alditol acetate derivative of 2-*O*-methylmannose was identified from the mass spectrum and by comparison of the retention time with those of partially methylated alditol acetates of a mannose standard. The galactose and Kdo were identified by comparison with authentic standards. The methanolysis-TMS procedure used for carbohydrate analysis also results in the formation of the methyl esters of fatty acids and the β -*O*-TMS methyl esters of β -hydroxy fatty acids, which are constituents of rhizobial lipid A (39); fatty acid methyl esters were not detected in the analysis of peak 4B-3. The ratio of galactose to 2-*O*-methylmannose in the 4B-3 pools, as determined by GC analysis, varied between 8:1 and 10:1 for the different isolates (see "NMR analysis" for corroborative evidence).

The composition of peak 4B-2 was also analyzed for comparison; peak 4B-2, which contains LPS, is composed of glucose, glucuronic acid, galacturonic acid, galactose, mannose, xylose, glucosamine, and Kdo in a molar ratio of 12:1:1:1:2:0.5:0.5:1.5. These components were identified by comparison with authentic standards. The methyl esters of fatty acids and the β -*O*-TMS methyl esters of β -hydroxy fatty acids were detected as major components of peak 4B-2; however, they could not be quantified because of a lack of standards. Peaks 4B-1 and 4B-4 contain LPS, neutral polysaccharides, and phosphorylated β -glucans (29a; they were not examined further for this report).

A portion of the 4B-3 (Fig. 1A) pool was subjected to mild-acid hydrolysis (34), and the degraded polysaccharides were chromatographed on Bio-Gel P-2; this resulted in the elution of two components (Fig. 1B). The peaks were pooled and analyzed by GC-MS techniques. Peak P2-1 contained 2-*O*-methylmannose and Kdo in a 1:1 molar ratio. Peak P2-2 contained galactose and Kdo in a 1:1 molar ratio. These samples were then analyzed by FAB-MS in the negative mode; the results are shown as the inset to Fig. 1B. Peak P2-1 exhibited an ion at m/z 399, which corresponds to the $M-H^+$ of a galactose-Kdo disaccharide, and peak P2-2 exhibited an ion at m/z 413, which corresponds to $M-H^+$ of a 2-*O*-methylmannose-Kdo disaccharide. This supported the composition data given above.

(ii) **NMR analysis.** The 1H NMR spectrum of the nondegraded 4B-3 (Fig. 1A) pool is shown in Fig. 2. The chemical shift (δ), δ 5.08, and coupling constant (J), $J = 3.93$ Hz, of the anomeric proton of the galactosyl residue demonstrate that it is in the α configuration (1). The δ 's of the Kdo methylene (H3) resonances, δ 1.8 (H3_a) and δ 2.4 (H3_e), demonstrate that the Kdo is in the β pyranosyl configuration (20); i.e., there is a δ separation of >0.5 ppm. The sample was run at a high temperature in order to shift the H₂O resonance upfield and allow the 2-*O*-methylmannose anomeric proton to be resolved, δ 4.8 ($J < 2.0$ Hz) (1). The δ 's of the hexosyl anomeric resonances were verified from the spectra of the purified disaccharides (data not shown). Integration showed that the galactose/2-*O*-methylmannose

anomeric proton ratio was ~10:1, which is consistent with the composition data (discussed above). The characteristic resonances of lipid A fatty acids are not present in this spectrum, which is further evidence that this polysaccharide is not LPS. The simplicity of this 1H NMR spectrum, i.e., the galactose H1 and Kdo H3 resonances, indicates that there is one type of galactosyl linkage and one type of Kdo linkage.

The 2-*O*-methylmannose-Kdo polysaccharide, which is a minor component of peak 4B-3 (Fig. 1A), was separated from the Gal-Kdo polysaccharide in subsequent HPLC preparations (data not presented) and was not analyzed further for this study.

(iii) **HPLC analysis and purification; FAB-MS analysis of the Gal-Kdops.** The peak 4B-3 (Fig. 1A) Kdops pool was analyzed by FAB-MS; the result is shown in Fig. 3A. Successive degrees of polymerization (DPs) of up to six Gal-Kdo subunits were plainly detected by the FAB-MS as the ions at m/z 781.2, m/z 1,163.6, m/z 1,545.4, m/z 1,928.7, and m/z 2,310.2. These mass ions were compared with the predicted ions from the CARBOMASS software (41) for resolved low-mass ions and nonresolved high-mass ion clusters: each mass ion corresponds to the mass_{(dimer)ⁿ} - [$1 + (H_2O)_n - 1$] (n = number of repeating units) of the sequential DPs of the Gal-Kdo subunits within the limits of resolution of the instrument, as calibrated for this study. Several more sequential ions of this series were detected in subsequent analyses; however, the signal-to-noise ratios were too low for unambiguous assignment. Mass ions that corresponded to sequential DPs of the 2-*O*-methylmannose-Kdo polysaccharide component of peak 4B-3 were scarcely detectable above the noise (data not shown).

The purified Gal-Kdo disaccharide (peak P2-2; Fig. 1B), which gave a FAB-MS ion at m/z 399 (described above), was chromatographed on a Dionex HPLC with a CarboPac PA-1 anion-exchange column; from this the retention time of the disaccharide was determined. The nondegraded polysaccharide preparation was then chromatographed on the Dionex HPLC. The result was an anion-exchange chromatogram characteristic of acidic polysaccharides of consecutive DPs (23) with the first peak corresponding to the Gal-Kdo disaccharide (Fig. 3B).

The individual HPLC peaks were collected from the Dionex HPLC with a semipreparative column to determine the correlation between the HPLC chromatogram (Fig. 3B) and the FAB-MS spectrum (Fig. 3A). The samples were eluted without NaOH to prevent base-catalyzed degradation. The isolated oligosaccharides were rerun on the Dionex HPLC, using the analytical column and conditions, to compare them with the peaks in Fig. 3B and analyzed by FAB-MS to compare the mass ions with those in Fig. 3A; the HPLC chromatogram and FAB-MS spectrum of the Gal-Kdo hexasaccharide are shown as insets in Fig. 3B and A, respectively. The first six peaks from Fig. 3B were identified by this protocol. From these data it was determined that the successive HPLC peaks correspond to the successive FAB-MS mass ions and therefore to consecutive DPs of the Gal-Kdo disaccharide subunit.

(iv) **Structural analysis.** The galactosyl linkage was determined by per-*O*-methylation, hydrolysis, reduction, and acetylation of the HPLC-purified oligosaccharides, followed by GC-MS analysis. The GC retention times were compared with those of the partially methylated alditol acetates of a galactose standard. The electron impact mass spectrum of the major galactitol peak was that of a 3-linked hexopyranosyl, i.e., 2,4,6-tri-*O*-methylhexitol acetate, identified by pri-

FIG. 2. ^1H NMR spectrum of the peak 4B-3 pool (Fig. 1A) from *R. fredii* USDA205. The measurement was made at 314 K. The resonances at δ 1.8 and δ 2.4 are characteristic of the methylene protons of β -linked Kdo; the resonances at δ 5.0 and δ 4.7 are due to the anomeric protons of galactose and 2-*O*-methylmannose, respectively. The δ of the galactosyl anomeric proton demonstrates that it is in the α configuration. The inset shows the ^1H NMR spectrum of the equivalent 4B pool from the phenol-water extract of *R. fredii* HC205, which has been cured of the symbiotic plasmid; the measurement was made at 296 K; consequently, the 2-*O*-methylmannose anomeric resonance was not resolved from the solvent signal.

mary fragments at m/z 118, m/z 161, m/z 234, and m/z 277. Terminal galactopyranose was also detected, i.e., 2,3,4,6-tetra-*O*-methylhexitol acetate, identified by the primary fragments at m/z 118, m/z 161, m/z 162, and m/z 205. This indicated that a galactosyl residue occupied the terminal, nonreducing position of the purified oligosaccharide.

The Kdo residue was extremely labile under the conditions used for methylation analysis, and its linkage could not be unambiguously determined by this method; therefore, the purified Gal-Kdo disaccharide (peak P2-2; Fig. 1B) was reduced with sodium borodeuteride, per-*O*-methylated, and analyzed by GC-MS. The chromatogram exhibited only one peak (data not shown). The mass spectrum of the derivatized galactosyl-Kdo disaccharide is shown in Fig. 4A; Fig. 4B depicts the expected fragmentation pattern of a sodium borodeuteride-reduced, per-*O*-methylated hexosyl-(1 $\rightarrow$ 5)-Kdo disaccharide.

The galactosyl residue was found to be in the D configuration, as determined by GC analysis of the TMS (+)-sec-butylglycosides of the polysaccharide and an authentic D-(+)-galactose standard.

The primary structure of the Gal-Kdo molecule from *R. fredii* USDA205, based on the data presented above, is [$\rightarrow$ 3]- α -D-Galp-(1 $\rightarrow$ 5)- β -D-Kdop-(2 $\rightarrow$) $_n$. A diagram of this structure is presented in Fig. 5.

(v) **PAGE analysis of purified polysaccharides.** Kdops and LPS (peaks 4B-3 and 4B-2, respectively; Fig. 1A) and crude extract were analyzed by PAGE with deoxycholic acid as a detergent (19). The resultant gels were stained by two different procedures (Fig. 6). The silver staining technique of Tsai and Frisch (32) stained only the LPS (Fig. 6A), whereas the Alcian blue-silver staining technique of Corzo et al. (11) stained both the LPS and the Kdops (Fig. 6B). Alcian blue is a cationic dye that is effective for staining polyanionic polysaccharides (33). The LPS of *R. fredii* gives two banding regions, which is common in PAGE studies of rhizobial LPSs. In contrast, the Kdops appears as a ladder-like banding pattern after Alcian blue-silver staining. The ladder pattern displayed by the Kdops is indicative of an incremental series of repeating-unit DPs; a similar result has been seen in the PAGE-Alcian blue analysis of partially digested hyaluronic acid (33).

In many PAGE studies of anionic polysaccharides, such as hyaluronic acid and CPSs, the detergent (which prevents aggregation of LPS) is omitted. The Kdops, LPS, and crude extract of *R. fredii* were also analyzed by PAGE in the absence of deoxycholic acid (data not shown); the banding pattern produced by the Kdops was independent of the presence of the detergent, whereas the LPS was not detected in gels that did not contain deoxycholic acid, as expected.

FIG. 3. FAB-MS mass spectrum (A) and Dionex HPLC chromatogram (B) of undegraded peak 4B-3 pool (Fig. 1A) from *R. fredii* USDA205. The Dionex HPLC retention time of the Gal-Kdo disaccharide (8 min) was determined from the chromatogram of the purified disaccharide (P2-2, Fig. 1B, and data not shown). The insets of panels A and B show the FAB-MS mass spectrum and Dionex chromatogram, respectively, of the HPLC-isolated hexasaccharide. From these data it was concluded that successive HPLC peaks represent consecutive DPs of the Gal-Kdo polysaccharide. See the text for details.

Although these results sustained the conclusion that the isolated Kdops is not a glycolipid, the usefulness of this technique in this investigation was limited by the fact that it did not allow for the detection of both Kdops and LPS in a given pool.

The PAGE banding pattern of the Kdops is greatly supportive of the FAB-MS and Dionex data (discussed above). The lane that contained crude extract (i.e., both LPS and Kdops) clearly shows that the Kdops is not detected by silver staining alone.

***R. fredii* HC205.** Cultures of *R. fredii* HC205 (cured of the *sym* plasmid) were grown, extracted, purified, and analyzed by the same protocol as *R. fredii* USDA205; the Sepharose

FIG. 4. (A) Electron impact mass spectrum of the sodium borodeuteride-reduced, per-*O*-methylated galactosyl-Kdo disaccharide from *R. fredii* USDA205; (B) predicted electron impact fragmentation pattern of a sodium borodeuteride-reduced, per-*O*-methylated hexosyl-(1→5)-Kdo disaccharide. The primary fragment at m/z 381 is the indicative ion. Each mass ion difference of -32 represents a loss of methanol.

4B column profile and the composition analysis of the Kdops from HC205 showed no significant differences from those of USDA205. In addition, the ^1H NMR spectrum of the Kdops pool from HC205 was identical to that of *R. fredii* USDA205 (Fig. 2, inset). This spectrum was acquired at a lower temperature than was that of the *R. fredii* USDA205 Kdops; consequently, the 2-*O*-methylmannose anomeric proton resonance is not resolved from the solvent signal. The analysis of *R. fredii* HC205 demonstrated that the genes involved in

FIG. 5. Primary structure of the novel Kdops from *R. fredii* USDA205. HPLC data have shown the presence of >30 DPs of the $[\rightarrow 3)\text{-}\alpha\text{-D-Galp-(1}\rightarrow 5)\text{-}\beta\text{-D-Kdop-(2}\rightarrow)]_n$ repeating unit (Fig. 3B). PAGE data indicate a much greater degree of polymerization (see Fig. 6B).

FIG. 6. Silver-stained (A) and Alcian blue-silver-stained (B) PAGE gels (18% acrylamide gels with deoxycholic acid as the detergent) of polysaccharides from *R. fredii* USDA205. Lanes: 1, phenol-water extract (water layer); 2, peak 4B-3 pool (Fig. 1A); 3, peak 4B-2 pool (Fig. 1A).

the synthesis of the Kdops are not located on the symbiotic plasmid.

***R. meliloti*.** Figure 7A shows the Sepharose 4B chromatography column profile of the ultracentrifugation supernatant of the hot phenol-water extract (water phase) of *R. meliloti* AK631. The bar above the profile shows those fractions that contained LPS, as determined by silver-stained PAGE gels. In later experiments the ultracentrifugation step was found to be unnecessary and was omitted.

Peak 4B-1 was further purified by gel filtration chromatography on a Sephadex G150 column in the presence of deoxycholic acid (Fig. 7B); deoxycholic acid had previously been used in chromatography to prevent LPS aggregation (24). PAGE analysis showed that only one peak (G150-2) contained LPS (bar). No LPS was detected in peak G150-1 by this method. Peak G150-1 eluted in the void volume of this column, indicating that it contained relatively high-molecular-weight material compared with the LPS; this conclusion was supported by the PAGE data (see Fig. 9, described below). These data show that the presence of the deoxycholic acid in the eluent is necessary to prevent LPS aggregation and subsequent coelution with higher-molecular-weight polysaccharides.

The composition of peak G150-1, as determined by GC-MS analysis of the TMS methyl glycosides, was xylose, mannose, glucose, and Kdo in a molar ratio of 1:1:1:5. Fatty acid methyl esters (indicative of LPS) were not detected in peak G150-1; however, they were a major component of peak G150-2.

The non-LPS, Kdo-rich component (G150-1) was analyzed by ^1H NMR spectroscopy (Fig. 8B). The NMR spectrum contains the H3_a (δ 1.75) and H3_e (δ 2.55) resonances of β -linked Kdo (discussed in the *R. fredii* NMR results above); the fact that they are broad and poorly resolved indicates that the Kdo is a component of a large polysaccharide. The signal at δ 2.1 is in the δ region where the methyl protons of acetyl substituents resonate, and the signals at δ 2.37, δ 1.22, and δ 1.11 appear to be due to methylene and methyl groups of deoxy-sugars or noncarbohydrate substituents. The spectral region at δ 3 to 4.5 contains the glycosyl ring proton resonances.

The ^1H NMR spectrum of isolated LPS from AK631 (G150-2 from Fig. 7B) is shown in Fig. 8A. This spectrum

FIG. 7. (A) Sepharose 4B gel filtration elution profile of the ultracentrifugation supernatant of the hot phenol-water extract (water phase) from *R. meliloti* AK631; (B) Sephadex G150 (gel filtration) elution profile of the *R. meliloti* AK631 peak 4B-1 pool (A). The G150 eluent contained deoxycholic acid to prevent LPS aggregation. The fractions from both preparations were assayed for the presence of Kdo. Abs, absorbance. Bars show those fractions in which LPS was detected by PAGE; no LPS was detected in peak G150-1.

differs significantly from that of the Kdops (Fig. 8B): (i) the glycosyl ring proton region is clearly dissimilar (δ 3 to 4.5); (ii) the resonances at δ 2.37, δ 2.1, δ 1.22, and δ 1.11 are minor signals (indicating that the LPS is not completely free of the Kdops); (iii) the LPS spectrum contains resonances in the anomeric proton region (δ 4.5 to 5.2) that are not present in the spectrum of G150-1; and (iv) the characteristically broad lipid A fatty acyl resonances at δ 1.1 to 1.3 and δ 0.7 to 0.9 (the fatty acyl methylene and methyl protons, respectively) are present only in the LPS spectrum.

These NMR spectra demonstrate that the Kdops and the LPS from *R. meliloti* AK631 are distinct polysaccharides.

Figure 9 shows a PAGE gel of purified polysaccharides and crude extract from *R. meliloti* AK631 and the crude extract from Rm41. The gels were stained by silver stain (panel A) and Alcian blue-silver stain (panel B). The purified

FIG. 8. ^1H NMR spectra of peaks G150-2 (LPS) (A) and G150-1 (Kdops) (B) from *R. meliloti* AK631. See the text for details.

LPS from AK631 was detected by both staining procedures, as it was with *R. fredii*, and appears as two banding regions. The purified Kdops was stained only by the Alcian blue-silver stain procedure, as was the case with the Kdops from

R. fredii USDA205. The crude extracts from AK631 and Rm41 contain both LPS and Kdops.

The low mobility of the Kdops indicates that it has a high molecular weight range; this supports the NMR and G150 chromatography data, discussed above.

The PAGE banding pattern produced by the Kdops was independent of the presence of deoxycholic acid (data not shown), as it was with the Kdops from *R. fredii* USDA205 (discussed above), which is further evidence that it is not a glycolipid.

The data presented in this section clearly demonstrate that *R. meliloti* AK631 and Rm41 produce a non-LPS, Kdops that can be chromatographically separated from the LPS. The structure of this polysaccharide is currently under investigation.

DISCUSSION

The results of this study show that the majority of the Kdo in the crude polysaccharide extract of *R. fredii* USDA205 is not from the LPS, as previously reported. The data presented demonstrate that the preponderance of Kdo is, in fact, a component of a separate, and structurally distinct, type of polysaccharide that had not been previously identified as a constituent of rhizobia. The primary structure of the major polysaccharide from *R. fredii* USDA205 has been determined; it consists of repeating units of $[\rightarrow 3)\text{-}\alpha\text{-D-Galp-(1}\rightarrow 5)\text{-}\beta\text{-D-Kdop-(2}\rightarrow)]_n$. The NMR data (Fig. 2) clearly show that this polysaccharide carries no substituents, such as a

FIG. 9. Silver-stained (A) and Alcian blue-silver-stained (B) PAGE gels of polysaccharides from *R. meliloti* AK631 (A and B) and Rm41 (B). Lanes 1, phenol-water extract from AK631; lanes 2, purified Kdops (G150-1) from AK631; lanes 3, purified LPS (G150-2) from AK631; lane 4, phenol-water extract from Rm41. The Alcian blue-silver-stained gel (B) shows that the G150-2 LPS pool (lane 3) is not completely free of Kdops; this is consistent with the NMR data (Fig. 8A and text).

lipid anchor. The polysaccharide from *R. fredii* USDA205, as extracted into the water layer by the protocol used in this investigation, is heterogeneous in size, chromatographing on the Dionex HPLC as a series of sequential DPs, from 1 to >30, of the galactosyl-Kdo repeating unit. The composition analysis of the LPS (peak 4B-2; Fig. 1A) shows that the Kdops is not a lipid A-free degradation product of LPS. The production of the Kdops by *R. fredii* HC205, a strain that has been cured of the symbiotic plasmid, demonstrates that the genes required for the synthesis of the polysaccharide are not on that plasmid.

It is probable that the Kdops that has been isolated from *R. fredii* USDA205 by the hot phenol-water method is not the *in vivo* form of the polysaccharide. The ketosidic bond formed by Kdo is often very labile under even mildly acidic conditions (34); therefore, the hot phenol-water extraction conditions (i.e., pH and temperature) could partially degrade this polysaccharide. Similar problems have been reported in work with Kdo-containing CPSs from *E. coli* (16). In recent studies with *R. fredii* USDA205, more-moderate extraction conditions (i.e., neutral pH and low temperature) have been employed. When that extract is analyzed by PAGE, the result is a diffuse, low-mobility banding region for the Kdops, rather than a ladder pattern; however, after a brief pretreatment of the extract with dilute acid, the ladder pattern is once again apparent (29a).

A second Kdo-rich component from *R. fredii* USDA205 copurified with the galactose-Kdo polysaccharide on the Sepharose 4B gel filtration column. The structure of this minor component, which consists of 2-O-methylmannose-Kdo subunits, is under investigation.

R. meliloti, the microsymbiont of alfalfa, has significant DNA homology with *R. fredii* (17, 30). The LPS from *R. meliloti*, like that of *R. fredii*, had been reported to contain an abundance of Kdo: >50% of the total carbohydrate (4). The data presented in this report demonstrate that *R. meliloti* AK631 and Rm41 produce a non-LPS Kdops that can be chromatographically separated from the classic LPS. The composition of the LPS from *R. meliloti* AK631 has recently been elucidated (29a); the LPS contains <10% Kdo. A similar result has been obtained in studies of the LPS from *R. meliloti* 10406 (35). In contrast to the LPS composition, the carbohydrate composition of the purified Kdops from *R. meliloti* AK631 consists of ~60% Kdo; therefore, as in the case of *R. fredii*, the LPS is not the primary source of the Kdo found in the extracts of *R. meliloti* AK631. The structure of the Kdops from *R. meliloti* AK631 is currently under investigation.

R. meliloti Rm41 (from which AK631 is derived) is an exception to the ostensible requirement for EPS in indeterminate nodulation (27, 37). Data from this laboratory, in collaboration with others, indicate that it is the Kdops, not the LPS, as previously suggested (25, 38), that may substitute for the EPS in this system. These studies have demonstrated a correlation between the production of the Kdops by *R. meliloti* AK631 and the successful nodulation of the indeterminate host plant by that strain (24a, 29a).

The Kdops from *R. fredii* is structurally analogous to a class of polysaccharides found in *E. coli*: K antigens (Kapselantigene) are acidic polysaccharides that form capsules over the surface of the bacterial cells and are often important in pathogenicity; one subgroup of the K antigens is classified according to the presence of Kdo as the principal acidic component. In studies of *E. coli*, these CPSs are distinguished from LPS by the fact that Kdo accounts for 40 to 60% of the total mass of the former as opposed to <10% of

the latter (15); this is consistent with the data on the *R. fredii* and *R. meliloti* polysaccharides. The Kdo-containing K antigens commonly consist of linearly arranged, dimeric or, less frequently, trimeric, repeating units of Kdo and neutral hexoses, pentoses, aminohexoses, or methylpentoses in various linkages (15). The reported K-antigen structures are cognate with the structure of the *R. fredii* Kdops described in this report (as well as the 2-O-methylmannose-Kdo component currently under investigation). The high proportion of Kdo in the *R. meliloti* polysaccharide is also similar to that of the K antigens.

The PAGE banding patterns exhibited by the Kdo-containing polysaccharides from both *R. meliloti* AK631 and *R. fredii* USDA205 are similar to those commonly seen in PAGE analyses of CPSs (degraded and/or undegraded); the PAGE banding patterns of the rhizobial polysaccharides are independent of the presence of deoxycholic acid in the gel.

The similarity of the rhizobial, Kdo-containing polysaccharide to the K antigens of *E. coli* suggests that they may be surface components and consequently that they may play a role in the nodulation processes. Other evidence also suggests that they may be surface polysaccharides with some biological function: (i) a distinct correlation has been shown between the production of the Kdops from *R. meliloti* AK631 (discussed above) and the effective nodulation of the host plant by that microsymbiont (24a, 29a), and (ii) immunological studies have shown that the polysaccharides from *R. meliloti* AK631 and *R. fredii* USDA205 are surface antigens (24a, 29a). It is possible that in the determinate nodulation of soybean by *R. fredii* the Kdops performs a function that renders the EPS unnecessary.

There is abundant evidence that similar polysaccharides may be produced by several other species of rhizobia (29a).

Agrobacterium tumefaciens, another member of the *Rhizobiaceae*, is closely related to *R. meliloti*; they produce similar β -glucans and EPSs and have significant DNA homology (3). This laboratory has obtained evidence that a non-LPS, Kdo-containing polysaccharide from *A. tumefaciens* is fractionated into the water phase of phenol-water extracts; this component is separated from the LPS by chromatography and gives a PAGE pattern that is similar to that of the Kdops from *R. fredii*, which is stained only by the Alcian blue-silver staining procedure (29a). The nature of this polysaccharide is presently under investigation.

Research with *R. fredii* in this laboratory is currently focused on the characterization of the 2-O-methylmannose-Kdo component, the *in vivo* localization of the Gal-Kdo Kdops, and the possible symbiotic functions that these polysaccharides may have. The structure of the polysaccharide from *R. meliloti* AK631 is also under investigation.

ACKNOWLEDGMENTS

The assistance of Malcolm O'Neill throughout the course of this study was invaluable and greatly appreciated.

This work was supported in part by NIH grant GM395832RWC and by the U.S. Department of Energy-funded Center for Plant and Microbial Complex Carbohydrate Research under grant DE-FG09-87ER13810.

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