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.

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**Rhizobium fredii** participates in a nitrogen-fixing symbiosis with soybeans, in a strain-cultivar-specific interaction, and past studies have shown that the cell surface and extracellular polysaccharides of rhizobia function in the infection process that leads to symbiosis. The structural analysis of the capsular polysaccharides (K antigens) from strain USDA257 was performed in this study. The K antigens were extracted from cultured cells with hot phenol-water and purified by size exclusion chromatography. We isolated two structurally distinct K antigens, both containing a high proportion of 3-deoxy-o-manno-2-octulosonic acid (Kdo). The polysaccharides were characterized by matrix-assisted laser desorption ionization–time-of-flight–mass spectrometry, nuclear magnetic resonance spectrometry, and gas chromatography-mass spectrometry analyses. The primary polysaccharide, which constituted about 60% of the K-antigen preparation, consisted of repeating units of mannose (Man) and Kdo, [→3]-β-D-Manp-(1→5)-β-D-Kdop-(2→)], and a second polysaccharide consisted of 2-O-MeMan and Kdo, [→3]-β-D-2-O-MeManp-(1→5)-β-D-Kdop-(2→)]. These structures are similar to yet distinct from those of other strains of *R. fredii* and *R. meliloti*, and this finding provides further evidence that the K antigens of rhizobia are strain-specific antigens which are produced within a conserved motif.

Numerous studies have established a correlation between the specific production of *Rhizobium* lipopolysaccharides (LPS), capsular polysaccharides (K antigens), and extracellular polysaccharides (EPS) and the success of the infection process that leads to the formation of nitrogen-fixing nodules on legumes (reviewed in references 9, 14, and 18). Consequently, the structural nature of these bacterial products may affect the host range of a particular strain. From this point arises a limiting factor in the study of *Rhizobium*-legume interactions: few of the bacterial polysaccharides have been structurally characterized. For example, it has been established that the K antigens are common products of rhizobia and that they are not conserved within a species (19, 23); however, to date, the structure of only one K antigen, from *Rhizobium fredii* USDA205, has been described (22). This report presents the complete structures of two distinct K antigens produced by *R. fredii* USDA257, which is the most thoroughly studied strain of this soybean symbiont.

The interaction between *R. fredii* USDA257 and soybeans is strain-cultivar specific, and the genetic basis for this selectivity is partially understood (1, 2, 12, 13, 17): *R. fredii* USDA257 is unable to nodulate many of the improved cultivars of soybeans, including McCall, unless a mutation is introduced into *nolXWBTUV*, which has homology to *hrp* genes of pathogenic bacteria (17). Importantly, the infection of McCall by the *nol* mutants is subject to nodulation blocking by coinoculation of the mutants with wild-type cells, indicating that strain USDA257 produces a negative factor that normally restricts its host range, due to an inability to complete the infection process on McCall (7, 10). Recently, we have found that each of the extended-host-range mutants (*nolXWBTUV*) exhibits a distinctly modified expression of the cell surface polysaccharides (11), including the K antigens, which may affect the host specificity of the bacterium. The characterization of the wild-type K antigen is, therefore, prerequisite to understanding this phenomenon.

This report shows that *R. fredii* USDA257 produces two distinct K antigens, comprising disaccharide repeating units of 3-deoxy-o-manno-2-octulosonic acid (Kdo)-mannose (Man) and Kdo-2-O-MeMan. A variety of K antigens have now been identified in many strains of *R. fredii* and *R. meliloti*, and all appear to reflect similar motifs yet differ in structural aspects (references 22 and 23 and this report), such as composition, substitution patterns, size range, anomeric configuration, and linkage. These findings may lead to new insights into the structure-function relationship for K antigens, and other polysaccharides, in *Rhizobium*-legume interactions. As the K antigens are now known to be widespread in soil bacteria (19), a suggested index system for the strain-specific capsular polysaccharides of *Rhizobium* spp. is included in Discussion.

**MATERIALS AND METHODS**

*Bacterial growth conditions.* *R. fredii* USDA257 was obtained from S. G. Pueppke (Plant Pathology Department, University of Missouri). Cells were stored at –70°C in 7.5% glycerol, and cultured in yeast extract-mannitol at 28°C, as previously described (21, 22).

*Isolation and purification of the K-antigen polysaccharide.* The extraction and initial purification protocols are presented in detail elsewhere (21, 22). Briefly, cell pellets were extracted with hot phenol-water, and the aqueous phase was fractionated by size exclusion chromatography. The fractions were assayed for Kdo by the thiobarbituric acid assay (25), for uronic acid by the hydroxybiphenyl assay (5), and for neutral sugars by using the anthrone procedure (27). The polysaccharide-containing fractions were pooled, dialyzed, and freeze-dried. K-antigen oligosaccharides were generated by mild acid hydrolysis (1% acetic acid,
10°C, 30 min) and separated by gel filtration on Bio-Gel P-2 (Bio-Rad, Richmond, Calif.), using 1% acetic acid as an eluent.

Polymyxin-agarose affinity chromatography (Detoxi-Gel; Pierce Chemical Co.) was used to remove contaminating LPS. After addition of the sample solution (H2O) the column was washed with H2O (flowthrough fraction), and the LPS was eluted with 1% deoxycholic acid.

PAGE. Aliquots of 2 to 5 µg of polysaccharide extracts were analyzed by deoxycholic acid-polyacrylamide gel electrophoresis (PAGE), using 18% acrylamide; the gels were Acril blue-silver stained as previously described (22).

FAB-MS. Fast atom bombardment-mass spectrometry (FAB-MS) was performed on a ZAB-SE instrument (VG, Manchester, England) in the negative mode with an ionizing voltage of 70 eV and an accelerating voltage of 10 kV, operating at low resolution (ca. 1800). The samples were dissolved in ultrapure H2O, and 1 µl was added to a matrix of thioglycerol (2 µl). The scan range was 300 to 3,000 atomic mass units (amu).

MALDI-TOF-MS. Matrix-assisted laser desorption ionization—time-of-flight—mass spectrometry (MALDI-TOF-MS) analysis was performed on an LDl 1700XN spectrometer (Linear Scientific, Reno, Nev.) in the negative mode. The instrument was operated at an accelerating voltage of 30 kV and an extractor voltage of 9 kV. The sample was ionized with a nitrogen laser (λ = 337 nm) with a pulse width of 3 ns and a 4- to 7.5-µJ pulse. The samples were dissolved in H2O and mixed with a matrix of 100 mM 2,5-dihydroxybenzoic acid in 90% methanol, and the matrix-sample mixture (1 to 2 µl) was applied to the probe and quickly dried under vacuum. The sample solution was serially diluted with matrix to obtain optimal sensitivity. Mass spectra were obtained over a range of 1 to 50,000 amu; between 100 and 250 spectra were acquired and averaged. Polygalacturonic acid was used as a calibration standard.

1H NMR analysis. 1H nuclear magnetic resonance (NMR) analysis was performed on Bruker AM 500 and AM 250 instruments. The samples were dissolved in D2O, and the spectra were obtained at 316 K. Solvent suppression was achieved by low-energy presaturation. The anomic configurations of the Man and 2-O-MeMan residues were established by a 13C-filtered 1D proton experiment.

Glycosyl composition and linkage analyses. Glycosyl residue compositions were determined by gas chromatography-mass spectrometry (GC-MS) analysis of the trimethylsilylated (TMS) methyl glycoside derivatives, using a 30-m DB-1 fused silica column (J&W Scientific, Folsum, Calif.) on a 5890A GC-MS spectrometer (Hewlett-Packard, Palo Alto, Calif.). The location of endogenous silica column (J&W Scientific, Folsum, Calif.) on a 5890A GC-MS spectrom-

RESULTS

Isolation and purification of the Kdo-containing capsular polysaccharide. The aqueous phase of the phenol-water extract from R. fredii USDA257 was fractionated by size-exclusion chromatography on Sephadex G-150 (Fig. 1A). A Kdo-containing component, fractions 38 to 58, was combined to give pool 2 (P2). P2 was then analyzed by PAGE, using Acril blue, a cationic dye, to prestain the gel (Fig. 1A, inset); this showed the presence of non-LPS, acidic polysaccharides that migrated as a series of bands, indicating that the polysaccharide(s) consisted of sequential degrees of polymerization (DP) of a small repeating unit. Although there was a minor contamination from smooth LPS (which contains uronic acid [21, 22]), the uronic acid assay response in fractions 38 to 51 was predominantly a false positive (i.e., a different chromatophore). The uronic acid-positive peak, around fraction 68, contained the rough LPS (not shown). It is important to note that no EPS is found in the extract of the cell pellet, showing that EPS is not tightly associated with the bacterial cell.

Prior to further analysis, the intact P2 fraction was subjected to polymyxin-agarose affinity chromatography to remove small amounts of contaminating LPS. The P2 polysaccharides had no affinity for the polymyxin resin, which specifically binds LPS, and eluted readily in the column flowthrough fraction (not shown). Subsequent composition analysis by GC-MS showed that typical EPS components, such as β-OH-fatty acids or glucosamine, were absent in P2.

A portion of P2 was subjected to mild acid hydrolysis, which cleaves the acid-labile ketosidic linkage of Kdo, and the resulting oligosaccharides were fractionated on a Bio-Gel P-2 size exclusion chromatography column. Two components, designated P2-1 and P2-2, eluted in this step (Fig. 1B). Composition analysis by GC-MS showed that P2-1 contained 2-O-MeMan and Kdo in a 1:1 molar ratio and that P2-2 contained Man and Kdo in a 1:1 molar ratio, indicating that each subfraction consisted of disaccharides of hexose and Kdo. This was confirmed by FAB-MS analysis in the negative mode: P2-1 exhibited an ion at m/z 413, which corresponds to the M-1 of a methylhexose-Kdo disaccharide, and peak P2-2 exhibited an ion at m/z 399, which corresponds to M-1 of a hexose-Kdo disaccharide.

MALDI-TOF-MS analysis of fraction P2. The sequential DP nature of the K antigens was confirmed by negative-ion MALDI-TOF-MS analysis of the intact P2 preparation (Fig. 2A). The sequential clusters include pseudo-molecular ions ([M − Na]−, as well as salt adducts [(M + Na)−] and [(M + K)−]. An enlargement of the region between m/z 1,100 and m/z 1,700 is shown in the inset to Fig. 2. The first series of ions is from the oligosaccharides of DP = 3 (i.e., with three disaccharide repeating units). The ion at m/z 1,163 arises from...
the [Man-Kdo]₃ oligosaccharide, and the ion at m/z 1,205 (reported as m/z 1,203.9) arises from the [2-O-MeMan-Kdo]₃ oligosaccharide; the difference of 42 amu represents three times the additional 14 mass units contributed by each methyl substituent. The ions at m/z 1,186 and m/z 1,227 are from Na⁺ adducts.

The second series of ions results from the oligosaccharides of [Man-Kdo]₄, at m/z 1,545, and [2-O-MeMan-Kdo]₄, at m/z 1,601 (reported as m/z 1,602.4). Here, the ions of the Na⁺ adduct (m/z 1,567) and the K⁺ adduct (m/z 1,584) of [Man-Kdo]₄ are discernible. Significantly, no ions that correspond to a variously methylated oligosaccharide (i.e., containing both Man and 2-O-MeMan) were detected; this would be shown by ions at m/z 1,559, m/z 1,573, or m/z 1,587. Hence, R. fredii USDA257 produces two separate K antigens, one containing Man and Kdo and the other containing 2-O-MeMan and Kdo.

**NMR analysis of P2.** ¹H NMR analysis of the intact P2 preparation (Fig. 3) showed only two anomeric proton resonances, at δ 4.75 (Man) and δ 4.6 (2-O-MeMan). The δ values of the hexose anomeric protons were established by NMR analysis of the purified disaccharides (i.e., P2-1 and P2-2 [not shown]). Integration of the anomeric signals indicated a 6:4 ratio of Man:2-O-MeMan, which was in close agreement for the relative areas of the disaccharide peaks shown in Fig. 1B. The resonances at δ 1.75 (H₃α) and δ 2.45 (H₃ε) are indicative of β pyranosyl Kdo (16). This showed that both polysaccharides contain β-linked Kdo. The anomeric configurations of the Man and 2-O-MeMan residues could not be determined from the chemical shift of the anomeric protons; therefore, a ¹³C-filtered 1D proton experiment was performed to establish the ¹³C-¹H coupling constant (data not shown). The ¹³C-¹H coupling constants for both the Man and the 2-O-MeMan residues were 160 Hz, which indicates that these glycosyl residues are β linked.

**Complete characterization of the K antigens.** The hexosyl linkages were determined by GC-MS analysis of the per-O-
methylated alditol acetates. The mass spectrum of the major mannitol peak was that of a 3-linked hexopyranose, i.e., 2,4,6-tri-O-methylhexitol acetate, identified by primary fragments at m/z 118, m/z 161, m/z 234, and m/z 277, indicating that both the Man and the 2-O-MeMan are 3 linked. Terminal mannopyranose, i.e., 2,3,4,6-tetra-O-methylhexitol acetate, was also detected, identified by the primary fragments at m/z 118, m/z 161, m/z 162, and m/z 205. The Kdo residue was labile to the hydrolysis steps in methanol analysis, and its linkage could not be unambiguously determined by this method; therefore, the disaccharides resulting from mild acid hydrolysis of fraction P2 were reduced with sodium borodeuteride, per-O-methylated, and analyzed by GC-MS. Only one permethylated oligosaccharide eluted (data not shown), showing that both hexosyl residues were linked to the same position on Kdo. The mass spectrum of the derivatized mannosyl-Kdo disaccharide is shown in Fig. 4A; the spectrum is consistent with the expected fragmentation pattern of a borodeuteride-reduced, per-O-methylated hexosyl-1,5-Kdo disaccharide (Fig. 4B).

The Kdo residue was found to be a β enantiomer, as determined by GC-MS analysis of the TMS (+)-sec-butylglycosides of the polysaccharide and an authentic β-(-)-Man standard. There was no 2-O-MeMan standard available for a similar analysis of that component.

K-antigen structures. The primary structure of the Man-Kdo K antigen from R. fredii USDA257, based on the data presented above, is \([\rightarrow\beta-3-\beta-d-Manp-(1\rightarrow5)-\beta-d-Kdop-(2\rightarrow)\]. A diagram of this structure is presented in Fig. 5. The 2-O-MeMan-Kdo K antigen has the same structure (if the 2-O-MeMan is assumed to be β), with CH₃ at R₁.

**FIG. 4.** (A) Electron impact mass spectrum of the per-O-methylated diglycosyl alditol derived from the P2 polysaccharides by mild acid hydrolysis, NaB₂H₄ reduction, and per-O-methylation. (B) The predicted e.i. fragmentation pattern of a sodium borodeuteride-reduced, per-O-methylated hexosyl-(1\rightarrow3)-Kdo disaccharide. The primary fragment at m/z 381 is the indicative ion. Each mass ion difference of –32 represents a loss of methanol.

**FIG. 5.** Primary structure of the repeating unit of the K antigens (P2) from R. fredii USDA257. The major polysaccharide (R₁ = H) contains Man and Kdo and comprises about 60% of fraction P2. The minor polysaccharide component (R₁ = CH₃) contains 2-O-MeMan and Kdo and comprises about 40% of P2.

**DISCUSSION**

This report presents the structures of the second and third K antigens isolated from soil bacteria (22), and the first to be characterized from an important model strain of rhizobia. The two K antigens produced by R. fredii USDA257 comprise disaccharide repeating units of Man-Kdo and 2-O-MeMan-Kdo. These polysaccharides are structurally similar to, yet distinct from, the K antigen first isolated from R. fredii USDA205 (22) (Table 1). In addition, recent data indicate that the major capsular polysaccharide from R. mellioti AK631 contains hexose and a variant of Kdo (20) and that it is substituted with acetate and β-hydroxybutyrate (strain AK631 also produces a secondary, Kdo-containing polysaccharide). Other R. mellioti strains have also been found to produce K antigens containing other Kdo-like sugars (23). These findings suggest a conserved motif for capsule structure in R. fredii and R. mellioti: small repeats containing 1-carboxy-2-keto-3-deoxy (i.e., Kdo-like) sugars and hexose. There is, however, a significant degree of variation within this motif, in composition, linkage, substitution patterns, and size range.

An interesting feature of K-antigen expression by R. fredii USDA257 is the production of two distinct polysaccharides, one of which contains a methyl ether group on the hexosyl...
component (i.e., 2-O-MeMan). *R. fredii* USDA205 also produces two K antigens, one with galactose and one with 2-O-MeMan (22). There are two possible explanations for this phenomenon: (i) each cell has the ability to produce both polysaccharides, and (ii) individual cells, which can produce one or the other, are present in the same culture. Past work supports the former explanation, as the strains are frequently reisolated from single colonies (20). In addition, it has been shown that a variation in culture conditions will favor the production of a specific polysaccharide (discussed below).

Another important aspect of K-antigen expression is the fact that these polysaccharides are extracted from the cell pellets of cultured rhizobia; this is in stark contrast to EPS, which is isolated from the growth media of cultured cells. In no case have we detected any EPS in the cell pellet extract of *R. fredii* or *R. mellioti* (references 21 to 24 and this report), which suggests that these two classes of polysaccharides are fundamentally distinct in nature; i.e., the K antigens are capsular polysaccharides, whereas the EPS is an excreted slime polysaccharide, not a capsular component. In addition, the K antigens are strain-specific antigens, whereas the EPS are conserved amongst different strains (25).

The elucidation of the K-antigen structures has led to new insights and questions about the molecular interaction between *Rhizobium* and legumes. For example, NMR analysis (Fig. 3) showed that the K antigens produced by *R. fredii* USDA257 carried no common substitutions, such as acetate or pyruvate, and this was also found to be the case with the K antigen of *R. fredii* USDA205 (22). These groups are very stable, and they would not be removed during the extraction procedure. In contrast, four K antigens from *R. mellioti* AK631 (22), *R. mellioti* NRG247, *R. mellioti* NRG185, and *Rhizobium* sp. NGR234 have been partially characterized (23); each is structurally distinct, yet all have been found to carry acetyl substituents. Interestingly, the latter strains possess the *nodL* gene, which is involved in the acetylation of the Nod factors, whereas *nodL* is not present in *R. fredii*, and it does not produce O-acetylated Nod factors (6). A study is ongoing to determine if there is a correlation between the acetylation of the Nod factor and the acetylation of the K antigens, and if this might affect the interaction with the respective host plants.

There is substantial evidence for an active function for some *Rhizobium* polysaccharides, including EPS and K antigens, in the infection process (as opposed to a simple masking effect): EPS I-mediated infection of alfalfa by *R. mellioti* Rm1021 is dependent not only on the production of the EPS but also on the specific structure and DP; mutants which fail to succinate the EPS I are unable to infect the host plant (15), and a DP of four octasaccharide repeats (i.e., 32 sugars) appears to be the active size range (3). In addition, a recent report demonstrated that exogenous EPS II of *R. mellioti* can promote infection by EPS mutants on alfalfa at very low concentrations (<10⁻¹¹ M) and that the activity is specific to a DP of ca. 16 disaccharide repeats (i.e., 32 sugars) of EPS II (8). Other studies have shown that the capsular polysaccharides of *R. mellioti* AK631 elicit early recognition responses in alfalfa, via the induction of genes in the flavonoid pathway (4), and that the K-antigen-mediated infection of alfalfa by *R. mellioti* AK631 is dependent on the size range (24).

Conversely, host plant-derived compounds have been shown to influence the expression of the K antigens by *R. fredii* and *R. mellioti* (21, 24). Agpinen, a flavonoid inducer of the *R. fredii nod* genes, and host (soybean) root extract cause the upregulation of the secondary, 2-O-MeMan-containing K antigen produced by *R. fredii* USDA205 (21); alfalfa root extract evoked an overall increase in K-antigen production by *R. mellioti* AK631, a microsymbiont of alfalfa, whereas the soybean root extract had no effect (24). These observations show that the K antigens are involved in a complex, and specific, interaction between the host plant and the microsymbiont during the infection process. Consequently, structural studies on the K antigens produced by wild-type strains and host range mutants, such as the *nodXWBTU* mutants of *R. fredii* USDA257, are necessary to dissect this interaction.

The K antigens are common products of rhizobia, and as different strains produce similar yet distinct K antigens, it is helpful to give a specific designation to each polysaccharide (Table 1). This will be particularly useful in future studies of structural genes associated with K-antigen production.

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