Structural Characterization of the Primary O-antigenic Polysaccharide of the *Rhizobium leguminosarum* 3841 Lipopolysaccharide and Identification of a New 3-Acetimidoylamino-3-deoxyhexuronic Acid Glycosyl Component

**A UNIQUE O-METHYLATED GLYCAN OF UNIFORM SIZE, CONTAINING 6-DEOXY-3-O-METHYL-D-TALOSE, N-ACETYLQUINOVOSAMINE, AND RHIZOAMINURONIC ACID (3-ACTIMIDOYLMID-3-DEOXY-D-GLUCO-HEXURONIC ACID)**

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*Rhizobium* are Gram-negative bacteria that survive intracellularly, within host membrane-derived plant cell compartments called symbiosomes. Within the symbiosomes the bacteria differentiate to bacteroids, the active form that carries out nitrogen fixation. The progression from free-living bacteria to bacteroid is characterized by physiological and morphological changes at the bacterial surface, a phase shift with an altered array of cell surface glycoconjugates. Lipopolysaccharides undergo structural changes upon differentiation from the free living to the bacteroid (intracellular) form. The array of carbohydrate structures carried on lipopolysaccharides confer resistance to plant defense mechanisms and may serve as signals that trigger the plant to allow the infection to proceed. We have determined the structure of the major O-polysaccharide (OPS) isolated from free living *Rhizobium leguminosarum* 3841, a symbiont of *Pisum sativum*, using chemical methods, mass spectrometry, and NMR spectroscopy analysis. The OPS is composed of several unusual glycosyl residues, including 6-deoxy-3-O-methyl-d-talose and 2-acetamido-2-deoxy-D-quinovosamine. In addition, a new glycosyl residue, 3-acetimidoylamino-3-deoxy-D-glucuronic acid, was identified and characterized, a novel hexosaminuronic acid that does not have an amino group at the 2-position. The OPS is composed of three to four tetrasaccharide repeating units of \( \beta-D-\text{GlcP}-N\text{AnA}-(1→4)\-\beta-D-\text{GlcP}-3\text{NaM}-(1→4)[2-O-\text{Ac}-(3′\-O-Me-\alpha-D-\text{Talp}-(1→3)\-\alpha-L-\text{Fucp}-(1→3)\-\alpha-L-\text{QuiPNaC}-(1→) \). The unique 3-amino hexuronate residue, rizoaminuronic acid, is an attractive candidate for selective inhibition of OPS synthesis.

*Rhizobium leguminosarum* is a Gram-negative endosymbiont that forms a nitrogen-fixing symbiosis with the legume host *Pisum sativum*. Like other Rhizobiaceae, it is a member of the \( \alpha-2 \) subgroup of the Proteobacteria, which includes the phytopathogen *Agrobacterium* and phylogenetically related bacteria such as the intracellular animal pathogens *Bartonella* and *Brucella* (1, 2). A significant feature shared by members of this subgroup is the ability to survive intracellularly within the euca-ryotic host, often surrounded by host membrane-derived compartments, which in the case of rhizobia are termed symbiosomes.

Although the early stages of symbiotic infection have been studied, factors enabling rhizobia to survive within the host cell environment throughout their life cycle are poorly understood (2–7). This is due in part to the difficulty in obtaining sufficient quantities of purified bacteroid mass to allow structural study of components. A model for symbiotic infection begins with a mutual exchange of signal molecules, including *inter alia* plant flavonoids and bacterial lipochitoooligosaccharides, leading to bacterial adhesion to root hairs and the induction of unique plant-derived structures, e.g. root nodules, infection threads, and symbiosomes (2, 8).

Rhizobia migrate through the infection threads and are internalized into the root cortical cells through a process resembling endocytosis. Internalization yields symbiosomes, specialized intracellular compartments composed of a plant-derived membrane that closely surrounds the bacterium. Within the symbiosomes the rhizobia differentiate into bacteroids, the active form that reduces atmospheric nitrogen to ammonia. In the case of *R. leguminosarum*, histochromic and electron micrograph studies have shown that the rhizobial surface is in close proximity to the surrounding plant-derived symbiosome membrane and that contact points appear to exist between the two (2, 9, 10). The lipopolysaccharides (LPS) are major structural and antigenic components of the rhizobial outer membrane, and are suitably located to interact with the plant membrane components and soluble plant products existing in the peribac-
teroid space (2–5, 11, 12). Numerous studies with rhizobial LPS mutants containing structurally defined defects have indicated that a structurally intact LPS, expressed at normal levels, is essential for normal root nodule development and active nitrogen fixation (Ndv⁺, Fix⁺ phenotype) (2, 5, 6, 13–16).

Compositional and immunological studies have shown that in *R. leguminosarum* and the closely related *Rhizobium etli*, LPS/O-antigen epitope expression is modified by environmental factors, including growth at acidic pH or low oxygen concentration, conditions thought to mimic those within the nodule (17–20). In the majority of cases the epitope structures have not been characterized, and it has been difficult to draw precise conclusions about the significance of structural alterations with regard to nodulation efficiency or bacteroid survival. An advance in our understanding of rhizobial LPS structure-function was the observation that the expression of O-antigen/LPS structure can change dramatically upon progression from the free-living state to the bacteroid form. The structural details of this phase shift in LPS surface chemistry were recently described in the *Sinorhizobium sp.* NGR234 model system, where LPS expression shifts from that of a structurally complex, highly branched anionic rough LPS (lacking O-antigen) to an endogenously methylated, hydrophobic, rhamnan O-antigen (attached to a structurally modified core lipid A, *i.e.* a smooth LPS) (6). These structural changes also occur on nitrogen-fixing bacteroids isolated from host *Vigna unguiculata* nodules (6, 15). In the other model system recently studied, that of *R. etli*-Phaseolus vulgaris, the transition from free-living state to bacteroid was accompanied by specific changes in the location and extent of O-methylation of O-antigen, changes that could also result in an alteration of bacteroid surface hydrophobicity (7). These and related studies indicate that a variety of changes in LPS surface chemistry occur during or immediately prior to differentiation of the free-living rhizobia (as exists within the infection threads) into bacteroids (2, 3, 5, 9, 15, 21–23), and the changes appear to be host-symbiont-specific. The significance of these changes is not entirely clear, but may be involved in preparing the bacterial surface for long term survival within the symbiosome by proper interaction with the plant membrane or attenuation of host defense mechanisms (3–5, 18).

The lipid A portion of *R. leguminosarum* 3841 LPS was examined structurally and functionally, and an acpXL mutant was found defective in its ability to transfer long chain fatty acid to lipid A when cultured under normal conditions (24). The mutant was restored in its synthesis of long chain fatty acid–lipid A when grown in the pea nodule environment, indicating that lipid A structure could be influenced by the host (24). Continuing our analysis of the *R. leguminosarum*-Pisum model system, we have characterized the structure of the O-polysaccharide portion of the LPS from the free-living form of *R. leguminosarum* 3841 bv. *viciae*. Like *R. etli*, this bacterium synthesizes a structurally complex, low molecular mass OPS of uniform size, with endogenous O-methylation and O-acetylation contributing to structural heterogeneity. In addition, we have identified a new glycosyl residue, 3-acetimidoylaminol-3-deoxy-D-gluco-hexuronic acid (Glc3NAmA, rhizoaminuronic acid) as a component of the 3841 OPS repeating unit. This is apparently the only known hexosaminuronic acid lacking an amino group at C2. Extensive structural differences between the bacteroid and free-living forms of the *R. leguminosarum* 3841 OPS exist and will be described in a forthcoming report. The availability of the complete genome sequence of *R. leguminosarum* 3841 (25), together with structural information, will help clarify the role of specific LPS structural features in bacteroid adaptation and survival.

**EXPERIMENTAL PROCEDURES**

**Growth of Bacteria**—*R. leguminosarum* strain 3841 was grown in liquid fermentor culture at 28 °C in tryptone/yeast extract (TY) supplemented with Ca²⁺ as described for related rhizobia (12). Cells were pelleted at late log phase (A₅₆₀ = 2.50) and then washed by resuspending in phosphate-buffered saline followed by centrifugation to remove exopolysaccharides and culture media, yielding 648 g (wet weight) of cells per 100 liters of culture.

**Isolation of Lipopolysaccharide and O-polysaccharide**—The washed cells were extracted using a hot phenol/water procedure in which the water layer contained 5 mM EDTA, 0.05% sodium azide, and 50 mM Na₂HPO₄ buffer, pH 7.0, as described previously (11). Lipopolysaccharides (LPSs) were isolated from the resulting water layer using standard chromatographic procedures (21, 26) (details are described in supplemental Fig. S1). The total LPS was subjected to mild hydrolysis in 10 mM sodium acetate buffer, pH 4.5, for 4 h at 105 °C. Lipid A was removed by ultracentrifugation, and the polymeric carbohydrate portion (O-polysaccharide; OPS) was isolated by chromatography using a Bio-Gel P-10 column (45–90 μm, 1.5 × 90 cm). Further procedures describing lipid A and core oligosaccharide removal are in the supplemental material.

**Fractionation of O-polysaccharides by HPLC**—The void volume from the Bio-Gel P-10 column, which contained the total soluble OPSs, was dialyzed, concentrated, and fractionated into separate polysaccharide components using an Asahipak-NH2P-50 4E column (4.6 × 250 mm, 5 μm) equipped with matching guard column (4.6 × 10 mm) and NH2P-LF pre-injector line filter (Shodex, Showa Denko K.K., Tokyo, Japan, distributed by Thomson Instrument Co.). The column was eluted at 0.7 ml/min at 35 °C, with a linear A/B gradient starting at 5% reagent B and ending at 70% B over ~1 h. Reagent A consisted of 75% acetonitrile, 25% water, v/v, and reagent B consisted of 20% acetonitrile, 80% aqueous 1 M ammonium for-

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2 The abbreviations used are: LPS, lipopolysaccharide; OPS, O-polysaccharide; Kdo, 3-deoxy-D-manno-oct-2-ulonic acid; 3MeEDTal, 3-deoxy-3-O-methyltalose; QuiNAC, 2-N-acetamido-2,6-dideoxyglucose (N-acetylquinovosamine); Glc3NAmA, 3-N-acetimidoylaminol-3-deoxy-D-gluco-hexuronic acid; SEC, size exclusion chromatography; GC-MS, gas-liquid chromatography-mass spectrometry; PMMA, partially methylated alditol acetates; TMS, trimethylsilyl; MALDI-TOF, matrix-assisted laser desorption ionization time-of flight; ESI-Q-TOF, electrospray-ionization-quadrupole-TOF; COSY, 1H–1H correlation spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence spectroscopy; HMBC, heteronuclear multiple bond coherence spectroscopy; NOE, nuclear Overhauser effect; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; HPLC, high pressure liquid chromatography.
mate, pH 4.4, v/v. Adjustment of the 1 M aqueous NH₄-formate to pH 4.4 was performed with formic acid prior to adding the acetonitrile. The eluate was split 90:10 between a fraction collector (90%) and an evaporative light scattering detector (10%) (PL-ELS-1000, Polymer Laboratories). The eluate was also monitored by measuring UV absorbance at 215 nm. Chromatography was performed on an AKTA system (Amersham Biosciences) collecting 0.3-ml fractions. Appropriate OPS fractions were combined, dialyzed, concentrated by rotary vacuum evaporation, and subjected to structural analysis.

**De-O-acylation of OPS and Conversion of N-Acetimidoyl to N-Acetyl Groups**—Portions (6–8 mg) of the OPS fractions purified by HPLC were de-O-acylated by treating with 12.5% v/v aqueous ammonia for 12 h at 35 °C (27). This treatment also resulted in quantitative conversion of the N-acetylated OPS samples were subjected to graded acid hydrolysis to establish conditions giving a maximal yield of fairly large oligosaccharides, with minimal yield of monosaccharides and minimal amounts of unhydrolyzed polymer. Typically, for the de-O-acylated polysaccharide, 0.1 M trifluoroacetic acid at 105 °C for 1.5 h was most effective, whereas for the native polymer, which was extensively O-acetylated, 3 h was required to obtain maximum yield of oligosaccharides. The products were evaluated by size exclusion chromatography (SEC) on a Superdex-Peptide HR 10/30 FPLC column (Amersham Biosciences) eluted with 50 mM ammonium acetate, pH 6.0. The oligo- and monosaccharide products were monitored by evaporative light scattering detection with a 9:1 split. Semi-preparative amounts of oligosaccharides were generated by hydrolyzing 2–4 mg of polysaccharide per chromatographic run. Fractions were collected (0.4 ml), and the saccharides were subjected to structural analysis.

**Preparation and Fractionation of Oligosaccharide Subunits**—OPS samples were subjected to graded acid hydrolysis to establish conditions giving a maximal yield of fairly large oligosaccharides, with minimal yield of monosaccharides and minimal amounts of unhydrolyzed polymer. Typically, for the de-O-acylated polysaccharide, 0.1 M trifluoroacetic acid at 105 °C for 1.5 h was most effective, whereas for the native polymer, which was extensively O-acetylated, 3 h was required to obtain maximum yield of oligosaccharides. The progress of the reaction was monitored by 1H NMR and MALDI-TOF MS described below. The reactions were dialyzed versus water, then concentrated by rotary vacuum evaporation, and subjected to analysis.

**Glycosyl Analyses**—Carbohydrate compositions of the OPS and derived fractions were determined by preparing the TMS methylglycosides with GC-MS (electron impact) analysis (13, 30) using a 30-m DB-5 fused silica capillary column (J & W Scientific). Carbohydrate identities and the locations of endogenous O-methyl ether groups. Oligosaccharide subunits derived from the OPS were analyzed as the permethylated oligosaccharide alditols by reduction of the reducing end with NaBD₄, followed by methyl esterification/permethylation (Hakomori method). The products were analyzed by electron impact GC-MS (using SP-23230 and DB-5 columns), and by chemical ionization (CI) MS, using a 30-m DB-1 column with ammonia as reactant gas.

**Mass Spectrometry**—Samples of OPS and derived oligosaccharides were analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry, using a Voyager-DE time of flight (TOF) spectrometer (Applied Biosystems, Boston) in the positive and negative modes, using a matrix of 100 mM 2,5-dihydroxybenzoic acid in 90% methanol. The instrument was operated at an accelerating voltage of 25 kV with extraction delay time of 200 ns. Samples were desorbed with a nitrogen laser (λ = 337 nm) and the detector sensitivity was 1000 mV full scale. Mass spectra were recorded over a m/z range of 500–20,000; spectra are the summation of 200 acquisitions. Maltooligosaccharides (degree of polymerization 3–15) were used for calibration. Electrospray ionization-Q-TOF (ESI-Q-TOF) MS analysis was performed on a Q-TOF II instrument (Micromass, Manchester, UK) equipped with an electrospray source. Samples were infused into the nebulizer at 5 μl/min, using nitrogen as the nebulization gas, and spectra were collected in the positive ion mode. The predicted molecular mass of the various saccharides was calculated using the following average incremental mass values, based on the atomic weights of the elements: hexose, 162.142; Kdo, 220.179; anhydro-Kdo, 202.164; 6-deoxyhexose, 146.143; mono-O-methyl-6-deoxyhexose, 160.170; di-O-methyl-6-deoxyhexose, 174.197; 2-N-acetamido-2,6-dideoxyhexose, 187.196; 3-N-acetamido-3-deoxyhexuronic acid, 217.178; acetimidoyl 41.052; free reducing end, 18.015.

**Nuclear Magnetic Resonance Analyses**—1H spectra and two-dimensional homo- and heteronuclear spectra of the OPS and derived oligosaccharides were recorded at 25 °C on a Varian Inova 600- or 800-MHz spectrometer, using a 5-mm triple probe and the standard Varian software (Varian Medical Systems, Palo Alto, CA). Polymeric samples were analyzed on the 800-MHz instrument, whereas derived oligosaccharides were analyzed on the 600-MHz instrument. Polysaccharides were dissolved in D₂O yielding clear solutions at ~5 mg/ml; spectra were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (δH 0.00 ppm). Oligosaccharides were analyzed at 300–500 μg/260 μl of D₂O using 5-mm symmetrical
RESULTS

Isolation of LPS—The majority of LPS obtained by phenol/water extraction of cultured R. leguminosarum 3841 cells was recovered in the water layer (yield ~257 mg of total LPS/20 g dry cell weight). The LPS was purified as described and analyzed by gel electrophoresis (supplemental Fig. S1). The most abundant component was identified as a smooth LPS (containing O-polysaccharide), having an apparent molecular mass centered around 6,000 Da, of slightly lower mass than that of R. etli LPS (30) (supplemental Fig. S1).

Fractionation, Molecular Mass, and Composition Analysis of OPSs—The polysaccharides derived from water layer LPS were recovered at the void volume of Bio-Gel P-10 as described and analyzed by positive ion ESI-Q-TOF-MS (Fig. 1). Five major polysaccharide components were detected (components A–E) with 64 scans per increment. The anomic configurations of the glycosyl linkages were assigned from carbon-proton coupling constants ($J_{C1,H1}$) measured for the native OPS and derived oligosaccharides by $^1$H-$^1$C HSQC analysis without $^1$C decoupling. Proton-proton coupling constants ($J_{HH}$) were determined where possible from $^1$H spectra, and by excitation of selected protons in a series of one-dimensional TOCSY experiments with mixing times from 0.08 to 0.12 ms.

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R. leguminosarum O-antigenic Polysaccharide Structure

Subsequently, experiments described below revealed the presence of a new glycosyl residue, ultimately resulting in a repeating unit mass of 712, and indicating that components A and B and C and D are in fact the same polysaccharide, each pair differing by a single repeating unit.

The polysaccharide mixture (A–E) was fractionated by HPLC as described (supplemental Fig. S1). Three peaks were obtained, identified by MALDI-TOF MS (supplemental Fig. S2) as peak 1 (component E), peak 2 (components B and D), and peak 3 (components A and C). Glycosyl analysis revealed that HPLC peak 1 (component E) was a neutral polysaccharide composed of xylose, mannose, and glucose, unrelated to the other components (details in supplemental material). HPLC peaks 2 and 3, which included over 90% of the total peak area, contained 3Me6dTal and QuiNAc in a 0.8:1 ratio, along with lesser, non-stoichiometric amounts of (ratios): fucose (0.19), 6-deoxy-3,4-di-O-methyltalose (3.4Me6d-Tal), (range 0.15–0.20), and an unidentified carbohydrate component (0.2), which showed anomalous behavior during all derivatization procedures. The configuration of the 6-deoxy-3-O-methylhexose residue was assigned on the basis of retention times, identical to derivatives prepared from authentic 6-deoxytalose (30, 32) when derivatized by either the TMS methylglycoside, alditol acetate, or partially methylated alditol acetate procedures. Linkage analysis of the peak 2 and peak 3 polysaccharides yielded terminal-3Me6dTal and 3-linked QuiNAc in a 1:1 ratio (not shown).

Minor amounts of several fucose PMAAs derivatives were again detected in amounts not exceeding 15% of the 3-QuiNAc derivative. When the carboxyl-reduced PMAAs were prepared following standard procedures, only these same derivatives were again observed, along with derivatives of 4-linked Kdo. These results were anomalous, because such derivatives in the detected ratios (e.g. the absence of branch point residues) could not yield a polysaccharide. The continued presence of non-stoichiometric amounts of fucose, even after extensive chromatographic purification, and the presence of anomalous derivatives all indicated that an unidentified glycosyl component (or components) was present.

Depolymerization of the OPS and Fractionation of Derived Oligosaccharides—NMR spectroscopy of the primary OPS (HPLC peaks 2 and 3) yielded complex spectra inconsistent with the presence of a diheteroglycan (i.e. one composed only of 3Me6dTal and QuiNAc as suggested by GC-MS analyses). Attempts were therefore made to obtain structural subunits, and the major OPS fractions (e.g. HPLC peak 3, polysaccharides A and C) were subjected to various treatments to effect partial and specific cleavage. Previously it was found that treating R. etli OPS with base (e.g. 0.25 M NaOH, 38 °C, 18 h) yielded oligosaccharides of defined size (primarily tetra- and hexasaccharides), because of β-elimination of uronosyl residues (30). Similar alkali treatments were tried with the 3841 OPS; however, the products consisted mainly of monosaccharides and unsaturated degradation products, too small for structural use. The base lability of the 3841 OPS thus appeared to be even greater than that of R. etli OPS, suggesting the presence of carboxylated or otherwise base-labile residues. However, mild acid was found effective in releasing oligosaccharides of sufficient size to yield useful structural information. Products thus obtained were fractionated on a Superdex FPLC column (supplemental Fig. S3), yielding two major oligosaccharide fractions aligning in size with maltoheptaose (G7) and maltotetraose (G4).

Because it was already known that the polysaccharide contained endogenously O-methylated residues in addition to 6-deoxy and amino sugars (e.g. N-acetylquinovosamine), it appeared likely that the oligosaccharide products were of a lower degree of polymerization than 7 or 4, because the presence of 6-deoxymethyl groups, N-acyl, and O-methyl ether groups all caused a significant increase in the mobility of sugars during SEC compared with the parent sugars (39).

**Structural Analysis of Derived Oligosaccharides and Identification of a New 3-Amino-3-deoxyhexuronic Acid Residue—** Su-perdex fractions aligning with the G7 and G4 standards, and also a lower mass fraction corresponding to monosaccharides, were isolated and analyzed. The smaller saccharide (“G4”), yielded a MALDI spectrum having ions at m/z 381.5, 404.5, and 426.6, suggesting identities of M + H⁺, M + Na⁺, and MNa + Na⁺ adducts (supplemental Fig. S4A). Subjection of G4 to standard methanolysis conditions and analysis of the TMS methylglycosides produced only a small amount of fucose and a much larger amount of an unidentified component with a late retention time in the range of a disaccharide or higher carbon sugar. Similarly, MALDI analysis of the higher molecular mass saccharide (migrating near “G7”) indicated a component 187 mass units higher than the G4 saccharide, suggesting that the G7 compound consisted of G4 linked to QuiNAc (incremental mass 187.2, supplemental Fig. S4B). Composition analysis confirmed this, revealing QuiNAc, in addition to a smaller, non-stoichiometric amount of fucose and a much larger amount of the same late moving unidentified component detected in G4. Assuming that both oligosaccharides contained fucose, it was calculated that G4 must consist of fucose plus an unidentified component having an incremental mass of 217 Da, whereas G7 presumably consisted of this same oligosaccharide linked to QuiNAc. Because a mass of 217 Da is rather low to be a higher carbon glycosyl residue, it was determined that the late moving peak observed during GC-MS analysis was in fact an oligosaccharide that resisted methanolysis, most likely because of the presence of an unidentified residue having a mass of 217 Da. If this residue was glycosidically linked to fucose, the proposed acid stability of the linkage would also account for the curious low recovery of Fuc during composition analysis.

The 1H NMR spectrum of the G4 compound identified three anomic signals in the ratio 1.0:0.8:0.2, suggesting a disaccharide in which the reducing end residue existed in an α/β mixture (supplemental Fig. S5). One N-acetyl signal was assigned (δ1 2.05) indicating that the disaccharide probably contained an amino sugar even though QuiNAc was not detected during composition analysis. Signals for C6 methyl protons were subsequently assigned to fucose on the basis of weak scalar coupling between H5 and H4 protons. A 1H-13C HSQC analysis (Fig. 2 and supplemental Fig. S6) confirmed three anomic centers and a single nitrogen-bearing carbon with distinct upfield shift at δ1 56.76. The 1H-1H COSY (Fig. 2) and TOCSY analyses together with their carbon assignments defined the three glycosyl systems (supplemental Table 1). Fucose was
assigned as the reducing end residue, in α- (δH 5.22, 20%) and β-forms (δH 4.59, 80%). A downfield shift in carbons at δ 81.40 and δ 80.37 was assigned from COSY to C4 of the α- and β-fucose residues, respectively, indicating that Fuc was substituted at O4 by the new amino sugar. This was confirmed by 1H-13C HMBC analysis (Fig. 3), which revealed inter-residue three-bond correlations originating from the anomeric carbon/proton of residue A (Glc3NAcA) to the 4-position of residue B (Fuc) are highlighted by a box. Correlations were strongest for the β-fucose anomer, which constituted 80% of the equilibrium. To increase resolution, the carbon sweep width was set to δ10 – δ20; carbonyl signals thus appear as indicated around δ 65 in this folded spectrum. A correlation between A H5 and A C6 (δC 65.74 = 175.74) defines the C6 carbonyl group of residue A. Other connectivities substantiated this structure, including a three-bond correlation between the H3 ring proton and a carbon at δ 175.10, assigned as the N-acetyl carbonyl carbon, and a two-bond correlation between the acetyl group protons (δH 2.05) and this same carbonyl carbon. Additional multiple bond correlations supporting this structure are evident between H4/C3, H5/C3, H3/C4, H3/C2, and H5/C1. NOEs are represented (from 1H-1H ROESY spectrum, not shown).

FIGURE 2. Partial 600-MHz 1H-1H COSY and 1H-13C HSQC spectra of the disaccharide derived from the R. leguminosarum 3841 OPS. The disaccharide was isolated by SEC following mild hydrolytic degradation of the OPS as described. Top panel, partial 1H spectrum; middle panel, COSY; bottom panel, HSQC. The spin system for the unusual 3-aminohexuronate residue (Glc3NAcA) is defined; residue A = βGlc3NAcA; residue B = Fuc; chemical shifts are shown in supplemental Table 1.

FIGURE 3. Partial 600-MHz 1H-13C HMBC spectrum of the disaccharide derived from the RL3841 OPS. The disaccharide was isolated by SEC following mild hydrolysis of the OPS. Inter-residue three-bond correlations originating from the anomeric carbon/proton of residue A (Glc3NAcA) to the 4-position of residue B (Fuc) are highlighted by a box. Correlations were strongest for the β-fucose anomer, which constituted 80% of the equilibrium. To increase resolution, the carbon sweep width was set to δ10 – δ20; carbonyl signals thus appear as indicated around δ 65 in this folded spectrum. A correlation between A H5 and A C6 (δC 65.74 = 175.74) defines the C6 carbonyl group of residue A. Other connectivities substantiated this structure, including a three-bond correlation between the H3 ring proton and a carbon at δ 175.10, assigned as the N-acetyl carbonyl carbon, and a two-bond correlation between the acetyl group protons (δH 2.05) and this same carbonyl carbon. Additional multiple bond correlations supporting this structure are evident between H4/C3, H5/C3, H3/C4, H3/C2, and H5/C1. NOEs are represented (from 1H-1H ROESY spectrum, not shown).
with the suggested incremental mass of 217 mass units for the new residue based on the MALDI MS analysis.

The G4 compound was reduced with borodeuteride at the "reducing end," followed by permethylation and methyl esterification. Analysis of the products by chemical ionization GC-MS yielded the CI spectrum (supplemental Fig. S7) consistent with a permethylated disaccharide having (M + NH₄)⁺ m/z 515, in which the permethylated hexosaminuronic acid occupies the nonreducing end. A separate portion of the disaccharide was reduced at the reducing end, and the products were subjected to the standard methanolysis procedure with preparation of TMS methylglycosides. GC-MS analysis revealed a small amount of fucitol, instead of the previously observed fucose (not shown), confirming that fucose occupied the reducing end.

\(^{1}H-^{1}H\) ROESY analysis of the disaccharide (not shown) identified an inter-residue NOE between H1 of residue A (hexosaminuronic acid), and H4 of both the α/β-anomers of residue B (Fuc). The relative intensity of the NOEs (\(\text{AH1/BH4}\) intense; \(\text{AH1/BaH4}\) weak), was consistent with the relative abundance of the α/β-fucose anomers and confirmed the glycosidic linkage of the hexosaminuronic acid to O4 of Fuc. Intra-residue NOEs were observed for the β-anomer of the fucosyl system between H1/H3, H1/H5, and H3/H5, consistent with axial orientation of these protons and a \(^{1}C\) chair conformation for the fucopyranose residue (assuming an absolute configuration of L-, see below). The large \(J_{1H,1H}\) coupling constants for H1, H2, and H3 (supplemental Table 1) are also consistent with a trans-diaxial arrangement of these protons. For residue A, the location of the N-acetyl group at C3 was substantiated by an NOE between the acetyl group protons and H3. The new residue also showed intense NOEs between H1/H3, H1/H5, H3/H5, and H2/H4, consistent with an axial orientation of protons and the pyranoside \(^{4}C\) chair conformation for the β-anomer, assuming an absolute configuration of D-. The intensity of COSY interactions (Fig. 2) and the scalar \(J_{1H,1H}\) coupling constants (9–10 Hz, supplemental Table 1) indicated that all protons in this system were trans-diaxial, consistent with the glucos-configuration. The anomeric configuration was assigned as β, from the \(J_{C1,H1}\) coupling constant (164.63 Hz, supplemental Table 1), consistent with the \(\delta_{C1}, \delta_{H1}\), and \(J_{1H,1H}\) values. Based on these data, the new amino sugar was identified as 3-acetamido-3-deoxy-D-glucuronic acid (Glc3NACa).

A second set of inter-residue NOEs was observed between AH1 and BH6 of both the α- and β-anomers of residue B (fucose), suggesting that a favored conformation for this disaccharide involved extensive rotation of the glycosidic linkage, such that the fucosyl residue is flipped with respect to Glc3NACa. Examination of model disaccharide libraries (e.g., on line at Glycosciences and at CNRS) shows that the disaccharides β-D-3-acetamido-3-deoxyglucopyranose (1→4)-α-L-fucopyranose, and β-D-glucopyranuronic acid-(1→4)-α-L-fucose, both of which closely approximate the new disaccharide, exhibit several low energy conformers, the lowest having \(\phi = \psi = 99.9^\circ\), bringing Fuc H6 into close proximity with H1 of (GlcA). An analogous NOE, between H1 of Glc3NACa and H6 of Fuc, was consistently observed in the polymeric O-chain as described below.

The \(1H\) spectrum of the larger oligosaccharide (migrating in the G7 region during SEC) revealed four anomeric signals, indicating that it was probably a trisaccharide in which the reducing end existed in α/β equilibrium (supplemental Fig. S6). \(1H-^{13}C\) COSY, TOCSY, \(1H-^{13}C\) HSQC, HMBC, and NOE analyses defined the four glycosyl systems (supplemental Table 1) and identified the trisaccharide as \(\alpha\)-L-QuipNACa-(1→4)-β-D-Glc3NACa-(1→4)-α-L-Fucp-(1→), in which the disaccharide identified above is substituted by a QuiNAc residue. The \(1H-^{13}C\) HMBC spectrum is shown in Fig. 4, and supporting data are described in supplemental Experimental Procedures and Fig. S6.

A remaining anomaly was the continued low yield of fucose during both the standard methanolsysis and alditol acetate procedures, presumably because of the acid-stable glycosidic linkage of the new amino sugar. With the identification of a hexosaminuronic acid, typified by extreme acid resistance, samples of the disaccharide and OPS were subjected to hydrolysis in 4 or 6 M HCl, and the products were analyzed both as alditol acetates and by methanolysis with conversion to the TMS methylglycosides. Both procedures resulted in high yields of the new monosaccharide, as well as improved recovery of fucose, which, although relatively acid-labile, was nevertheless obtained in higher yields as a result of essentially quantitative cleavage of the linkage. The electron impact-MS of the alditol acetate

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**FIGURE 4. Partial 600-MHz \(^{1}H-^{13}C\) HMBC spectrum of the trisaccharide derived from the RL3841 OPS.** Inter-residue three-bond correlations defining the two glycosidic linkages are underlined (\(\text{GlC3NAc, residue c anomeric proton-residue a carbon-4, etc.}\). Carbonyl carbon signals appear as indicated, around \(\delta_{C} 65.0\) in this folded spectrum. A correlation between a H5 and a C6 (\(\delta_{C} 64.88 \approx 174.88\) defines the C6 carboxyl group. Correlations between \(N\)-acetyl carbonyl carbons and their respective ring protons at residue a H3 and residue e H2 are labeled. Inter-residue NOEs are represented from \(^{1}H-^{1}H\) ROESY, not shown.)
derivative is shown in supplemental Fig. S7, and the GC-MS of the TMS methylglycosides are shown in supplemental Fig. S8. With conditions identified to obtain fucose in reasonable yield, the diastereomeric (-)-2-butyrglycosides were prepared from the disaccharide and OPS. GC-MS analysis identified derivatives identical to those from authentic L-fucose, indicating that fucose was the L-isomer.

Analysis of the De-O-acylated Polysaccharide—Mass spectrometry (Figs. 1 and 5B), and 1H NMR spectra (supplemental Fig. S9) indicated that the native OPS was highly O-acetylated. To collapse the signals, portions of the native OPS (from HPLC peak 3) were de-O-acetylated and analyzed by MALDI-TOF MS. Two major molecular species were detected (Fig. 5A), having pseudomolecular ions at m/z 2352 and 3064, differing by 712 mass units. Each molecular species included a family of ions, differing by ±14 mass units, attributed to O-methyl heterogeneity involving one or more of the endogenously O-methylated glycosyl residues (i.e., 3Me6dTal and 3,4Me6dTal). Other structural features, including two N-acetyl groups (supplemental Table 1) were assigned from the 1H NMR spectrum (supplemental Fig. S9).

Identification of Glycosyl Systems and Linkages—1H-1H COSY, TOCSY, and 1H-13C HSQC analyses identified four major and two minor glycosyl systems comprising the de-O-acylated OPS (supplemental Table 1). A suitable starting point was identification of the three glycosyl residues comprising the component trisaccharide QuiNAc(1→3)Glc3NAcA(1→3)Fuc. The unique chemical shifts of the Glc3NAcA system were identified, followed by QuiNAc; nitrogen-bearing carbons at ΔC 58.96 and 56.00 were assigned to C3 and C2 of Glc3NAcA and QuiNAc, respectively. The C6 carbonyl (8C 176.04) of Glc3NAcA was identified from correlations between H4/C6 and H5/C6 during 1H-13C HMBC anal-
**R. leguminosarum O-antigenic Polysaccharide Structure**

![Diagram](image-url)

**FIGURE 6. Partial 800-MHz $^1$H-$^1$C HMBC spectra of the RL3841 de-O-acylated OPS.** Inter-residue correlations defining the glycosidic linkages between residues d → b, and b → c are underlined, in addition to those previously identified from the oligosaccharide analyses. Several intra-residue correlations supporting the ring system assignments are labeled. Other correlations (not shown) confirmed the identities of all ring systems, including residue a, H5/C6, H5/C4, H5/C1, H4/C6, H4/C5, H4/C3, H3/C5, H3/C4, H3/C2, H3/C1, H2/C1, H3/N-acetate C = O, and N-acetate C = O/N-acetate–C$_\text{H}_3$ residue d, 3-OC$_\text{H}_3$ to C3 (major) and d 4-OC$_\text{H}_2$ to C4 (minor) confirming locations of endogeneous O-methylation. The structure of the tetrasaccharide repeating unit is represented.

The data obtained thus far were consistent with three possible glycosyl sequences for the polysaccharide repeating unit, shown in Structures 1 and 2.

To distinguish these possibilities, inter-residue HMBC correlations were identified between H1 of 3Me6dTal and C3 of Fuc and between C1 of 3Me6dTal and H3 of Fuc, indicating that 3Me6dTal residues were linked to O-3 of Fuc residues (Fig. 6). Another set of inter-residue correlations between Fuc H1-QuinNAc C3 and Fuc C1-QuinNAc H3 identified the remaining linkage, indicating a tetrasaccharide repeating unit with fucose as the branch point residue (in Structure 1). The anomeric configurations of the Fuc and 3Me6dTal residues were assigned as $\alpha$-, based on the $J_{C1,H1}$ values (both > 170 Hz), consistent with $\delta_4$ and $\delta_C$ values (measured in the native OPS; supplemental Table 1). The $\delta_C$ values for the fucosyl carbons are consistent with a pyranosidic form for fucose in the polymer. This was supported by a strong intra-residue NOE between Fuc H3/H5 (Fig. 7).

Two minor glycosyl systems were identified (supplemental Table 1), one was a Kdo residue, which exists as a 2,7-anhydrofuranose and occupies the reducing end of the polysaccharide. A second was identified as a variant of the Glc3NAcA spin system, penultimate to Kdo, glycosidically linked to O4 of the anhydro-Kdo residue. The assignment of these residues from COSY, HSQC, HMBC, and NOE data is described in the supplemental material. The glycosidic sequence at the nonreducing end of the de-O-acyl OPS was deduced from the MALDI spectrum (Fig. 5A). Consecutive losses of 160 and 146 mass units are consistent with loss of 3Me6dTal and Fuc, indicating that the nonreducing end terminates in the sequence 3Me6dTal→Fuc→. The major ion (m/z 2352) is consistent with three repeats of the tetrasaccharide repeating unit (in addition to one anhydro-Kdo residue) for the main de-O-acylated OPS (corresponding to de-O-acylated component A, identified in Fig. 1).

**NOE Analysis and Conformational Features of the De-O-acyl OPS**—The $^1$H-$^1$H ROESY spectrum of the de-O-acylated OPS yielded inter-residue NOEs from all anomer protons (Fig. 7), confirming the linkages and glycosidic sequence assigned from HMBC correlations. Several longer range, inter-residue NOEs provided additional insight into several aspects of polysaccharide conformation. An NOE (supplemental Fig. S10) between A1/B6 was analogous to that observed in the oligosaccharides, indicating that a favored conformation exists in which the fucosyl residue is flipped over with respect to Glc3NAcA. Another NOE was observed between B4/D5 (Fuc H4-3Me6dTal H5) requiring that the plane of the 3Me6dTal ring is rotated out of the plane of, and nearly perpendicular to, the Fuc ring. Models show that only $\alpha$-linked-3Me6dTal residues, and not $\beta$-, could form such an NOE with Fuc, consistent with the anomeric assignment from $J_{C1,H1}$ and $\delta_C$ values (supplemental Table 1). Moreover, models show clearly that if the absolute configuration of the $\alpha$-3Me6dTal residue was $\beta$-, then formation of a B4/D5 NOE would necessitate an additional NOE between B3/D3; however, the latter was not observed. Only the $\alpha$-d-
isomer of 3Me6dTal would allow the formation of the B4/D5 NOE without the accompanying B3/D3 NOE, indicating that the α-3Me6dTal residues have the α-absolute configuration. Another interesting feature is the arrangement of the side chain 3Me6dTal residues. An inter-residue NOE was observed between the N-acetyl group methyl protons of residue C (QuiNAc) and H5 of residue A (Glc3NAcA). The presence of this NOE, and an NOE between C1/A4, would suggest that the two N-acetyl groups (of residues A and C) be on “opposite sides” of the polymer, with residue A flipped with respect to residue C (Fig. 7). This arrangement would necessitate that the side chain 3Me6dTal residues be distributed on “alternating sides” of the polysaccharide. Models also show that only the L-configuration for QuiNAc would allow the simultaneous formation of these two NOEs (assuming the α-configuration for Glc3NAcA).

Localization of O-Acetyl and N-Acetimidoyl Groups and NOE Analysis of the Native OPS—The presence of N-acetimidoyl groups in the native PS was evident from the characteristic δC and δH values for this moiety (C = O, 166.84; CHα, 2.25/2.21, see supplemental Table 1). HMBC correlations between the carbonyl carbon and H3 of residue A, and between this carbonyl carbon and the methyl group protons (δH 2.25/2.21) allowed assignment of the N-acetimidoyl group to C3 of residue A (Glc3NAcA). Confirmation of the presence and location of this moiety was obtained from the large upfield shift of the nitrogen-bearing carbon (C3), from δc 62.81 in the native PS to 58.96 in the de-O-acylated PS (supplemental Table 1). The mild de-O-acylation conditions typically cause quantitative conversion of N-acetimidoyl to N-acetyl groups, and the resulting carbon shift is typically −3 ppm or more (28, 29, 40).

Comparison of the native spectra with those of the de-O-acylated OPS revealed four O-acetyl groups, identified from the downfield δH shift of four ring protons into the anomeric region, indicative of de-shielded protons attached to carbons bearing the O-acetyl groups. COSY and HSQC analyses (supplemental Fig. S11) indicated that most of the shifted protons were attached to C2 of 3Me6dTal residues, indicating that each repeating unit was O-acylated at C2 of the 3Me6dTal residue in the fully acetylated molecular species. A downfield shift (δH 1–3 ppm) was observed for each C2 carbon of the 3Me6dTal residues, relative to those of the de-O-acylated OPS (supplemental Table 1), reflecting the expected α-effect because of acylation (41). Also consistent with C2 as the site of O-acylation was a substantial upfield shift of the anomeric carbon of 3Me6dTal in the native OPS compared with that of the de-O-acylated OPS, indicative of the β-effect because of O-acylation at C2 (41). The assignment of downfield shifted protons to H2 of 3Me6dTal residues was supported by NOEs between H1/H2 and H2/H3 of this residue (residue D) in the native OPS (supplemental Fig. S12). Heterogeneity in each glycosyl system was observed in the native OPS, particularly for the terminal 3Me6dTal residues (supplemental Table 1). The occurrence of three distinct sets of 2-O-acetyl-3Me6dTal δH/δC signals probably originates from the three repeating units, each unit giving rise to a slightly different magnetic environment for the side chain 3Me6dTal residues. Bulky O-acetyl groups at C2 presumably contribute to this heterogeneity. A minor set of C2-shifted protons was assigned to fucosyl residues (supplemental Table 1), indicating that one or two of the fucosyl residues in the linear portion of the polysaccharide can also be 2-O-acylated; however, the random or specific locations...
of O-acetylated fucose (i.e. which repeating units) was not assigned. Further insight into the degree of O-acetyl heterogeneity in the native polysaccharide can be deduced from the MALDI mass spectrum (Fig. 5B), which indicates a maximum of four major molecular species reflecting varying degrees of O-acetylation.

The glycosyl sequence of the native OPS was confirmed by inter-residue dipolar correlations from all anomic protons (supplemental Fig. S12) and by inter-residue $^1$H-$^{13}$C HMBC couplings. A notable difference between the native and de-O-acetylated polysaccharides was the presence of an unusual “capping” residue at the nonreducing end of the former, identified from COSY, TOCSY, HSQC, and HMBC analyses as a 4,6-dideoxy-4-formamido-hexopyranose of unidentified configuration (residue E, supplemental Table 1 and Fig. 5B). This terminal residue and the residue to which it is glycosidically linked are apparently cleaved from the polysaccharide during de-O-acetylation. Additional details identifying this residue are in supplemental materials. The complete structures of the OPSs from the free-living forms of R. leguminosarum 3841 and R. etli CE3 are compared in Fig. 8.

FIGURE 8. Glycosyl sequence of the R. leguminosarum 3841 OPS and comparison with the OPS from R. etli CE3. Shown are the primary O-polysaccharides produced by the free-living forms of both species. Both polysaccharides are O-acetylated and O-methylated, particularly on the side chain 6-deoxytalosyl residues. Essentially 100% of side chain 6dTal residues are methylated at O3 in both polysaccharides; 15–20% of these are also methylated at O4 in the RL3841 OPS. In RL3841, approximately ≥80% of the 3Me6dTal residues, and 20% of the interior fucosyl residues, are acetylated at O2, as estimated from mass spectrometry and NMR data. The main component of the RL3841 OPS consists of three tetrasaccharide repeating units; the main component of the R. etli CE3 OPS contains five trisaccharide repeating units (30). Inter-residue conformational aspects are not represented in these diagrams.

O-chains that have been characterized, such as that from Sinorhizobium sp. NGR234, contain endogenously O-methylated L-rhamnose (6), and the O-chain from Mesorhizobium loti NZP2213 contains O-acetylated-Tal and 6-deoxy-2-O-methylhexose residues may confer a degree of hydrophobicity on these glycans, a property that could influence bacteroid surface chemistry and help facilitate symbiotic infection.

In addition to the primary OPS, the free living form of R. leguminosarum 3841 also produces a secondary polysaccharide (Fig. 1 and supplemental Fig. S1), composed of xylose, mannose, and glucose. This neutral polysaccharide is eventually separated from the primary OPS by HPLC (supplemental Fig. S1). This xylomannan shows limited affinity to polymyxin-agarose, and preliminary results indicate that it is not attached to a “normal” rhizobial lipid A but rather is associated with an acyl glycerol moiety.\(^3\) Interestingly, the relative abundance of these

\(^3\) L. S. Forsberg and R. W. Carlson, unpublished data.
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two polysaccharides is modified by external physiological conditions. In free living cells cultured under normal conditions, the xylomannan constitutes only 5% (supplemental Fig. S1) of the total PS isolated from the LPS fractions, the other 95% is the primary OPS. When cells are grown at pH 5 (conditions intended to mimic those within the symbiosome), the xylomannan becomes the dominant PS, constituting >95% of the total polysaccharide components. In an earlier report (18), composition analysis of LPS-associated carbohydrates extracted from cultured R. leguminosarum 3841 cells showed an increase in xylose and mannose when grown under the physiological extremes; however, it was not known if this represented the appearance of a new polysaccharide or a structural alteration to a single PS. This study shows that the source of xylose, mannose, and glucose is indeed a separate polysaccharide, and preliminary results indicate that a similar or identical xylomannan is also synthesized by R. leguminosarum 3841 bacteroids isolated from P. sativum nodules. The structure of this putative “bacteroid-specific” xylomannan, its regulation, and relation to lipid A-core is currently under study.

Recent studies have succeeded in isolating bacteroids in sufficient quantity and purity to allow direct examination of bacteroid-specific LPS, providing new insight into the nature of the bacteroid surface and the role of LPS in bacteroid survival and adaptation. In the Sinorhizobium sp. NGR234, Vigna unguiculata model system, the transition from free living cells to bacteroids is accompanied by a shift in LPS surface chemistry, from a rough LPS lacking O-antigen in the vegetative state to a smooth LPS composed of a rhamnan O-antigen attached to a structurally modified core-lipid A (6). The endogenously methylated rhamnan homopolymer is relatively hydrophobic, and the modified core region lacks the acidic sugars commonly found in the antigenic outer core of LPS from free living sinorhizobia. This “phase shift” in LPS surface chemistry may promote proper interaction between the bacteroid and the surrounding symbiosome membrane or attenuate the host innate immune response in some way, possibly as a mimic of host structural modifications.

The complete genome of strain RL3841 was recently sequenced (25), and preliminary analyses indicate there appear to be two major gene clusters devoted to O-chain/LPS biosynthesis, one of which is chromosomal and the other located on plasmid pRL9, distinct from the symbiosism plasmid pRL10. The particular features of this polysaccharide, specifically uniform size and low molecular mass, appear to be most compatible with the “monomeric” biosynthetic mechanism, in which individual monosaccharide residues are transferred consecutively from the glycosyl donor (XDP-sugar) to the nonreducing end of the growing chain (reviewed in Ref. 49). This mechanism has been associated with the uniformly sized OPSs synthesized by certain strains of Escherichia coli, Rhodospirillum rubrum, and others (49). This type of discrete size OPS is frequently encountered in bacteria possessing an ordered surface typified by a crystalline glycoprotein surface layer (i.e. S-layer), including diverse Gram-negative eubacteria (50). R. leguminosarum/R. etli are not known to possess an S-layer; however, the presence of OPS of uniform size could allow the ordered assembly of other surface components or promote interaction with plant-derived symbiosome membranes, either through multiple weak (e.g. hydrophobic) interactions, or via specific plant receptors such as lectins (51, 52).

Unlike rhizobial capsular polysaccharides, which often have a high negative charge density (21, 53), surveys of O-chain structures from diverse species seem to suggest that highly negatively charged OPS are not a particularly favored structural
feature (54, 55). A maximum charge density of one negative residue per every three repeating unit residues is common, and the incorporation of additional negative residues is frequently compensated (55) by the introduction of a positive charged group, e.g. ethanolamine. The rhizobial O-chain biosynthetic machinery seems to be particularly stringent in this regard. Among published rhizobial structures, neutral OPS that have some degree of hydrophobicity appear to be favored, and residues imparting net negative charge are either absent (6, 16) or when present are blocked by esterification (as in R. etli CE3) or neutralized with a positive substituent (N-acetimidoyl group, shown here for R. leguminosarum) to yield the zwitterion. At normal physiological pH, at or around neutrality, it would be expected that the RL3841 OPS has no net charge, and this is the form believed to be expressed while the bacteria adhere and colonize the plant surface. Following internalization, the bacteroid surface/O-antigen could encounter acidic pH, such as the symbiosome compartment (2, 22). If the pH approaches the pKₐ value of the uronic acid carboxyl (e.g. pH 4.0), a net positive charge could exist on these OPS and at the bacteroid surface; the OPS could behave transiently as a polycationic species, forming an electrostatic barrier that would repel cationic antimicrobial peptides (4, 44).

Hexosaminuronic acids occur rarely, and the absence of an amino group at C2 is a structural feature that raises biosynthetic questions. Several 2-amino (56, 57) and 2,3-diaminomannuronic acid forms believed to be expressed while the bacteria adhere and colonize the plant surface. Following internalization, the bacteroid surface/O-antigen could encounter acidic pH, such as the symbiosome compartment (2, 22). If the pH approaches the pKₐ value of the uronic acid carboxyl (e.g. pH 4.0), a net positive charge could exist on these OPS and at the bacteroid surface; the OPS could behave transiently as a polycationic species, forming an electrostatic barrier that would repel cationic antimicrobial peptides (4, 44).

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