Structural Characterization of a K-antigen Capsular Polysaccharide Essential for Normal Symbiotic Infection in Rhizobium sp. NGR234

DELETION OF THE rkpMNO LOCUS PREVENTS SYNTHESIS OF 5,7-DIAzetamido-3,5,7,9-tetraDEOxy-non-2-ULOSonic ACID*

Received for publication, December 22, 2005, and in revised form, May 30, 2006 Published, JBC Papers in Press, June 12, 2006 DOI 10.1074/jbc.M513639200

Antoine J.-L. Le Quéré‡, William J. Deakin‡, Christel Schmeisser§, Russell W. Carlson¶, Wolfgang R. Streit†, William J. Broughton‡, and L. Scott Forsberg‡

From the ‡Laboratoire de Biologie Moléculaire des Plantes Supérieures (LBMPS), Université de Genève, 1292 Genève, Switzerland, the †Institut für Mikrobiologie, Universität Hamburg, 22609 Hamburg, Germany, and the §Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602

Many early molecular events in symbiotic infection have been documented, although factors enabling Rhizobium to progress within the plant-derived infection thread and ultimately survive within the intracellular symbiosome compartment as mature nitrogen-fixing bacteroids are poorly understood. Rhizobial surface polysaccharides (SPS), including the capsular polysaccharides (K-antigens), exist in close proximity to plant-derived membranes throughout the infection process. SPSs are essential for bacterial survival, adaptation, and as potential determinants of nodulation and/or host specificity. Relatively few studies have examined the role of K-antigens in these events. However, we constructed a mutant that lacks genes essential for the production of the K-antigen strain-specific sugar precursor, pseudaminic acid, in the broad host range Rhizobium sp. NGR234. The complete structure of the K-antigen of strain NGR234 was established, and it consists of disaccharide repeating units of glucuronic and pseudaminic acid having the structure →(4)→β-D-glucuronic acid-(1→4)→β-5,7-diacetamido-3,5,7,9-tetraacetoxy-L-glycerol-1-manno-nonulosonic acid. Deletion of three genes located in the rkp-3 gene cluster, rkpM, rkpN, and part of rkpO, abolished pseudaminic acid synthesis, yielding a mutant in which the strain-specific K-antigen was totally absent: other surface glycoconjugates, including the lipopolysaccharides, exopolysaccharides, and flagellin glycoprotein appeared unaffected. The NGRΔrkpMNO mutant was symbiotically defective, showing reduced nodulation efficiency on several legumes. K-antigen production was found to decline after rhizobia were exposed to plant flavonoids, and the decrease coincided with induction of a symbiotically active (bacteroid-specific) rhamnan-LPS, suggesting an exchange of SPS occurs during bacterial differentiation in the developing nodule.

Rhizobium sp. NGR234 is a broad host range endosymbiont able to initiate nitrogen-fixing symbioses with more than 112 genera of legumes (1). A Gram-negative bacterium, it is a member of the α-2 subgroup of the Proteobacteria (2, 3), which includes the Rhizobiaceae, plant pathogens such as Agrobacterium, and phylogenetically related bacteria such as the intracellular animal pathogen Brucella. Most members of this subgroup share an important characteristic, the ability to survive within intracellular host membrane-derived compartments. In the case of rhizobia these are referred to as symbiosomes.

Successful colonization of a legume by a compatible Rhizobium involves an initial exchange of signal molecules, e.g. flavonoids and lipochitoooligosaccharides (Nod factors) leading to the development of unique plant-derived structures (infection threads, symbiosomes, root nodules). Rhizobia migrate through the infection threads and are eventually internalized into the nodule cells, resulting in the formation of symbiosomes, specialized intracellular compartments composed of a plant-derived membrane, which closely surrounds the bacterium (4, 5). Bacteria differentiate into bacteroids within the symbiosome where they actively reduce atmospheric nitrogen to ammonia. Although the early molecular events are well studied, the factors enabling the bacteria to progress within the infection thread, penetrate the root cortical cells, and ultimately survive within the symbiosome compartment are poorly understood (4–6).

Rhizobial surface polysaccharides (SPS)2 exist in close proximity to plant-derived membranes throughout symbiotic infection (4, 5, 7–9). These SPS include exopolysaccharides (EPS),

2 The abbreviations used are: SPS, surface polysaccharides; EPS, exopolysaccharides; KPS, K-antigen polysaccharide; LPS, lipopolysaccharide; sLPS, smooth LPS; Kdo, 3-deoxy-o-manno-oct-2-ulosonic acid; Pse, 5,7-diamino-3,5,7,9-tetraacetoxy-L-glycerol-1-manno-nonulosonic acid (pseudaminic acid); DSS, 2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt; SEC, size exclusion chromatography; DOC, sodium deoxycholate; GC-MS, gas-liquid chromatography-mass spectrometry; TMS, trimethylsilyl; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; 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; ROEY, rotating-frame nuclear Overhauser effect spectroscopy; ORF, open reading frame; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
lipopolysaccharides (LPS), capsular polysaccharides (K-antigens or KPS), and cyclic \(\beta\)-glucans. Most studies have focused on the LPS and EPS, demonstrating that these components are essential for normal nodule development and active nitrogen fixation (\(\text{Ndv}^+, \text{Fix}^+\) phenotype) (4, 6, 7, 9–13). Relatively few studies have examined the K-antigens, and their specific symbiotic role remains unclear (4, 5, 10).

KPS are tightly associated with the rhizobial outer membrane, and thus distinct from the loosely associated, high molecular weight EPS of these bacteria (reviewed in Ref. 4). Rhizobial KPS are strain-specific antigens, and are structurally analogous to the group II K-antigens of \(\text{Escherichia coli}\) in that they are generally acidic, linear polysaccharides having simple (usually disaccharide) repeating units (4, 14). Initial surveys of various rhizobial strains suggested that a common structural motif exists for many of these polysaccharides, in which a 1-carboxy-2-oxo-3-deoxy sugar such as Kdo, or a Kdo analogue (Kdx) alternates with a neutral hexose or uronic acid, yielding linear disaccharide repeats of the type Kdx-Sug, possessing high negative charge density (15, 16). Exceptions to this structural motif have been reported (15, 17–20), and a variety of unusual structural features are now known, including KPS composed of alternating glycosidic and amidic linkages (20). A correlation between KPS structure and host range specificity was recently suggested for some strains of \(\text{Rhizobium fredii}\) (17), although the available structural information was insufficient to allow reliable classification.

A role for K-antigens in symbiotic infection was demonstrated in an \(\text{exoB}^–\) (EPS-deficient) mutant of \(\text{Rhizobium meliloti}\) strain Rm41. Although EPS production is abolished in the mutant (AK631), the K-antigens were expressed at normal levels and were functionally able to compensate for EPS, allowing effective nodule formation (\(\text{Fix}^+\) phenotype) on \(\text{Medicago sativa}\) (alfalfa) (21, 22). Further mutations in genes associated with KPS synthesis produced phenotypes with aborted infection (alfalfa) (21, 22). Further mutations in genes associated with KPS synthesis produced phenotypes with aborted infection. KPS synthesis was specifically blocked, resulting in a defective phenotype with reduced ability to initiate symbiotic infection.

**EXPERIMENTAL PROCEDURES**

**Growth of Bacteria—** \(\text{E. coli}\) recombinants were grown at 37 °C in Luria-Bertani medium (25). NGR234 and its derivatives were raised for 40 h (unless otherwise stated) at 27 °C in tryptone/yeast (TY) (26) or in a minimal medium containing succinate as the sole carbon source (RMS) (27). Gentamycin (Gm), spectinomycin (Sp), rifampicin (Rif), and tetracyclin (Tet) were added when needed to final concentrations of 20, 50, 50, and 15 \(\mu\text{g}\) ml\(^{-1}\), respectively. Flavonoid induction was performed with apigenin as described previously (28). Briefly, \(\text{Rhizobium}\) strains were grown in 100 ml of RMS for 40 h; then the medium was supplemented with 100 \(\mu\)l of 1 mm apigenin in methanol (100 \(\mu\)l of methanol was added to the non-induced cultures). Bacterial strains and plasmids used in this study are listed in the supplemental materials, Table S1.

**Construction of a NGR\(\Delta\text{rk}p\text{MNO}\) Mutant—** The \(\text{rk}p\text{-3}\) locus of \(\text{S. melliloti}\) strain 41 (Sm41)\(^{(3)}\) (GenBank\(^{\text{TM}}\) accession no. AJ245666) contains genes essential for the synthesis of the K-antigen polysaccharides (29). In their report, Kiss et al. (29) showed that some of the genes from the \(\text{rk}p\text{-3}\) region hybridized to DNA of NGR234 suggesting that homologues could be present in the latter. To search for these homologues, the Sm41 \(\text{rk}p\text{-3}\) DNA sequence was blasted against the Genome Survey Sequences database (dbGSS) (NCBI), and sequences originating from NGR234 (30) were retrieved. Among the four sequences obtained, two (GenBank\(^{\text{TM}}\) ID: AZ577715 and AZ577319) had strong homologies to genes (\(\text{rk}p\text{k}\) and \(\text{rk}p\text{Q}\), respectively) involved in the synthesis of K-antigen precursors in Sm41 (29). A \(\text{rk}p\text{k}\)-forward primer (5'-ACTGGTCTG-GCACGAATCCG-3') and the \(\text{rk}p\text{Q}\)-reverse primer (5'-GAGGCCCTTCACGAAATAAACG-3') were used to amplify a 4.4-kb fragment using genomic DNA of NGR234 as the template. The amplified fragment was ligated into EcoRV-restricted pBKs (pALQ01) A 2.2-kb fragment from the latter plasmid was deleted using EcoRV digestion (pALQ02). Later sequencing of the locus showed that the region deleted included \(\text{rk}p\text{M}\), \(\text{rk}p\text{N}\), and the 5'-end of \(\text{rk}p\text{O}\) (Fig. 1 and see text below). After digestion with Smal, a spectinomycin resistance gene (isolated from pH455-Sp) (31) was ligated into the EcoRV site of pALQ02 yielding pALQ03. Finally, the full insert of pALQ03 was excised using XbaI and Sall, then cloned into Xbal/Sall-restricted pJQ2005K (32). The resulting plasmid (pALQ04) was mobilized into NGR234 using a tri-parental mating procedure and the helper plasmid pRK2013 (33). The mating mixture was plated on TY agar containing Rif and Gm, and then on TY plates containing Rif, Sp, and 5% (w/v) sucrose to promote marker exchange. Southern blotting techniques were used to confirm that the deletion in NGR\(\Delta\text{rk}p\text{MNO}\) was correctly inserted.

**Sequence Analysis of the \(\text{rk}p\text{-3}\) Region in NGR234—** The \(\text{rk}p\text{-3}\) region of Sm41 (GenBank\(^{\text{TM}}\) accession no. AJ245666) was blasted against the almost complete NGR234 sequence.\(^{(4)}\) One contiguous (GenBank\(^{\text{TM}}\) accession no. DQ341105) was highly homologous to the \(\text{rk}p\text{ genes}\) and was further annotated. Putative ORFs were predicted using the ERGO suite as well as BioEdit software and BLAST search engines accessible from NCBI. Predicted ORFs were finally translated and blasted against the nonredundant protein data base using the BlastP program (see supplemental materials, Table S2).

\(^{(3)}\) \(\text{Rhizobium}\) and \(\text{Sinorhizobium}\) are synonymous; (Sin)\(\text{Rhizobium mellioti}\) strain Sm41 is strain Rm41.

\(^{(4)}\) W. R. Streit et al., manuscript in preparation.
**Extraction of Cell Surface Polysaccharides and SDS-PAGE**—The bacterial cells obtained by centrifuging 5-ml liquid cultures of RMS were lysed using 100 µl of lysis buffer as described previously (34). Two volumes of sample buffer (120 mm Tris, pH 6.8, 3% (w/v) SDS, 9% (v/v) β-mercaptoethanol, 30% (v/v) glycerol, 0.03% (w/v) bromphenol blue) were then added to the polysaccharide extracts. The final mixtures were separated by SDS-PAGE (15% acrylamide) using 0.2 m Tris (pH 8.9) as the anode buffer while the cathode buffer comprised 0.1 m Tris, 0.1 m Tricine, and 0.1% (w/v) SDS, pH 8.9, at 20 mA. Gels were stained specifically for LPS using periodate oxidation-silver (35), and KPS were visualized by sequential staining with alcian blue-silver (15, 36), with omission of the periodate treatment so that LPS was not detected. These procedures readily distinguish the KPS from LPS; the alcian blue pretreatment is required for KPS visualization, and periodate oxidation is required for LPS staining.

**Plant Assays**—Seeds of *Flemingia congesta*, *Leucaena leucocephala*, *Pachyrhizus tuberosus*, *Tephrosia vogelii*, and *Vigna unguiculata* were purchased from the suppliers listed in Pueppke and Broughton (1999) (1). The plant tests were performed as described previously (1) and were repeated three times with a minimum of 12 plants for *V. unguiculata* and *T. vogelii* and repeated once for *L. leucocephala*, *P. tuberosus*, and *F. congesta*. To do this, the seeds were surface-sterilized with concentrated H2SO4 and 5% H2O2 before being placed on B&D agar (37) to germinate at 26 °C in the dark. The germinating seeds were planted into sterile vermiculite held in Magenta jars™, inoculated with 107 log-phase cells and grown for 6 weeks.

**Isolation and Purification of Capsular Polysaccharides for Chemical Analyses**—For chemical analyses, cells were cultured at 28 °C in 20 liters of tryptone/yeast extract (TY) supplemented with Ca²⁺ as described for related species (38). At late-log phase (A₆₅₀ = 2.50), the cells were pelleted and then washed by resuspending in phosphate-buffered saline followed by centrifugation to remove EPS. The supernatants, containing EPS, were subjected to glycosyl composition analysis (described previously (34). Two volumes of sample buffer (120 mm Tris, pH 6.8, 3% (w/v) SDS, 9% (v/v) β-mercaptoethanol, 30% (v/v) glycerol, 0.03% (w/v) bromphenol blue) were then added to the polysaccharide extracts. The final mixtures were separated by SDS-PAGE (15% acrylamide) using 0.2 m Tris (pH 8.9) as the anode buffer while the cathode buffer comprised 0.1 m Tris, 0.1 m Tricine, and 0.1% (w/v) SDS, pH 8.9, at 20 mA. Gels were stained specifically for LPS using periodate oxidation-silver (35), and KPS were visualized by sequential staining with alcian blue-silver (15, 36), with omission of the periodate treatment so that LPS was not detected. These procedures readily distinguish the KPS from LPS; the alcian blue pretreatment is required for KPS visualization, and periodate oxidation is required for LPS staining.

**Glycosyl Analyses**—Glycosyl compositions were determined by GC-MS analysis of the trimethylsilyl (TMS) methyl glycoside derivatives (12, 41) using a 30 m DB-5 fused silica capillary column (J&W Scientific/Agilent Technologies, Palo Alto, CA) on a 5890A GC-MSD (Agilent Technologies). Inositol was used as an internal standard, and retention times were compared with authentic standards. The absolute configuration of glucuronic acid residues was determined by GC-MS analysis of the TMS (−)-2-butyl glycoside derivatives (42) of the carbohydrate components and authentic D-GlcA.

**Mass Spectrometry**—Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was performed on a Voyager-DE time of flight (TOF) spectrometer (Applied Biosystems, Boston, MA) 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 100 ns. Samples were desorbed with a nitrogen laser (λ = 337 nm) using a detector sensitivity of 1000 mV FS. Mass spectra were recorded over an m/z range of 500–20,000 and represent the summation of 200 acquisitions. Polygalacturonic acid was used as the calibration standard.

**Nuclear Magnetic Resonance (NMR) Analyses**—1H spectra and all two-dimensional homo- and heteronuclear spectra of the KPS were recorded at 29 °C on a Varian Inova 500 MHz spectrometer using a 5-mm triple probe and standard Varian software (Varian Medical Systems, Palo Alto, CA). The polysaccharide was dissolved and analyzed in D₂O yielding clear solutions at ~5 mg/ml; spectra were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) (δ₄ 0.00 ppm). In most experiments, presaturation was applied to the residual DHO signal. 1H-13C HSQC (43) data were recorded in the absolute value mode with a 4.0 kHz spectral width and a matrix size of 256 × 2048 complex data points with eight scans per increment. 1H-13C TOCSY (44, 45) was recorded with a mixing time of 80 ms and two sets of 256 time increments at eight scans per increment. Carbon-proton one-bond correlations were collected in the 1H-detector mode with a gradientselected 1H-13C HSQC (46, 47) and an acquisition time of 0.2 s, collecting two arrays of 256 increments at 56 scans per increment. The carbon spectral width was 20.1 kHz. 1H-13C HMBC spectra were acquired with 256 × 2048 complex points and 112 scans/increment. The carbon sweep width was 22.6 kHz and acquisition times were 0.35 s (t2) and 0.007 s (t1). For NOE analysis (48), phase-sensitive 1H-13C ROESY was collected with a 300-ms mixing time, with a matrix size identical to that used for COSY and TOCSY experiments, with 48 scans per increment.

**RESULTS**

**The rkp-3 Locus**—Sequence analysis revealed that the rkp-3 region reported by Kiss et al. (29) was also present in the NGR234 genome (Fig. 1 and supplemental materials, Table S2). Although most genes involved in K-antigen synthesis (rkpLM-NOQ) as well as transport-export (rkppRSTI) are syntenous, a few differences were found. First, the putative acetyl transferase encoded by rkpP in Sm41 was not found in the NGR234 rkp-3 locus. Second, the presence of an additional copy of rkpT (rkpT2) was predicted upstream of rkpL. Third, the rkpY gene is...
Rhizobium sp. NGR234 K-antigen Capsular Polysaccharide Structure

Inverted and located upstream of the rkpLMNOQ genes in NGR234. Finally, two regions with high similarities to the probable chain length determinant rkpZ of R. meliloti were found, one of which is highly homologous to a truncated version of rkpZ in Sm1021 (rkpZ22), whereas the other (rkpZ1) is probably non-functional since it contains a frameshift. The high degree of homology of the rkpLMNOQ genes between Rm41 and NGR234 is in accordance with the similar glycosyl composition of the KPS polysaccharides from these strains (29).

Mutation of the NGR234 rkp-3 Locus—To investigate the function of the rkp-3 gene products in symbiosis, the rkpMNO genes were disrupted using an omega cassette (see “Experimental Procedures”). Although shown to be involved in KPS synthesis in Sm41, mutations in some rkp-3 genes can have pleiotropic effects. For example, orthologues of rkpLMNOQ identified in pathogenic bacteria including *Aeromonas* spp. that are required for normal infection alter cell motility (49) (supplemental materials, Table S2). Mutations of the rkpMNOQ orthologues of *A. caviae* reduced its ability to adhere to human epithelial cells, which correlated with a defect in flagellum assembly.

The motility of NGRΔrkpMNO was compared with NGR234 on swarming agar plates, both strains were equally motile (data not shown) and thus possess functional flagella. Flagella were extracted from the surfaces of both strains, and analysis by SDS-PAGE with silver staining and immunostaining with antibodies raised against rhizobial flagellins (the predominant glycoprotein component of flagella) showed no detectable change in mobility that might have indicated a role of the rkpMNO locus in flagellar glycosylation (see supplemental materials, Fig. S1A). The type III protein secretion system (T3SS) of NGR234 is a major determinant of symbiotic ability (50). Two components of T3SS functionality in NGRΔrkpMNO were also tested: whether secretion still occurred and if pili synthesized by the T3SS (composed predominantly of the protein NopA) were glycosylated by the rkpMNO gene products. Proteins secreted by the T3SS were isolated: immunostaining detected both NopA and NopL in NGRΔrkpMNO demonstrating that the system is still functional. Furthermore, the size (electrophoretic mobility) of NopA was not altered by mutation of rkpMNO (see supplemental materials, Fig. S1B). Thus the motility of NGR234 and glycosylation of its surface proteins appear to be unaffected by mutation of rkpMNO.

**Role of the NGR234 rkp-3 Locus in Polysaccharide Synthesis**—Production of KPS and LPS was examined in both wild-type NGR234 and the NGRΔrkpMNO mutant. As seen in Fig. 2, the mutant totally lacks KPS, which confirms that the rkp-3 genes are functional and effectively involved in the synthesis of the K-antigens. Interestingly, a significant decrease in KPS expression was observed after apigenin induction in the wild-type bacterium. These results differ from those in a previous report (51), which found no differences in the KPS profiles in the presence or absence of apigenin. This could be because of differences in the growth medium used or to longer incubation times (40 h *versus* 24 h in the previous report). To test which of these two possibilities is correct, cells were harvested after 24 h of incubation and the KPS profiles compared by PAGE analysis. Again, the reduction in KPS production was observed but to a lower extent (data not shown). By growing the cells in a rich medium (TY), KPS were unaffected by the addition of apigenin (data not shown) indicating that variations of the growth medium alter the regulation and/or the synthesis of KPS.
Rhizobium sp. NGR234 K-antigen Capsular Polysaccharide Structure

As assessed by SDS-PAGE, the LPS profile of the ΔrkpMNO mutant showed no discernable differences from the parent strain LPS (Fig. 2B, lanes 1 and 3). Like many R. meliloti strains, the parent NGR234 strain has been shown to produce abundant rough-LPS (rLPS, lacking the O-chain), with only trace amounts of smooth-LPS (sLPS, containing O-chain) (15, 40). Addition of apigenin to wild-type NGR234 cultures also induced the production of an unique rhamnan-LPS (Fig. 2B, lane 2 and Refs. 51 and 52). This rhamnan-LPS is the symbiotically active form of sLPS, which carries a linear α(1→3)-linked rhamnan homopolymer O-chain, attached to a structurally modified core-lipid A (52). As shown in Fig. 2B, lane 4, this rhamnose-rich sLPS was also produced by the NGRΔrkpMNO mutant after 40 h of exposure to apigenin.

**Symbiotic Effects of the NGRΔkpMNO Mutant**—The capacities of both the mutant and wild-type bacterium to nodulate *F. congesta, L. leucocephala, P. tuberosus, T. vogelii,* and *V. unguiculata* were compared. Plants that produce compatible interactions with NGR234 (*i.e.* all except *P. tuberosus*) showed a general decrease in plant shoot weight and nodulation ability (Table 1). This reduction of symbiotic efficiency was especially evident for *V. unguiculata* where shoot weight, nodule number and nodule weight were decreased by ~25% (Fig. 3). In the non-compatible interaction between NGR234 and *P. tuberosus,* the lack of KPS did not improve the nodulation capacity of the bacterium.

**Glycosyl Analysis of EPS**—Harvested cells were washed in phosphate-buffered saline as described, and the supernatants, containing EPS, were analyzed by preparing the TMS methyl phosphate-buffered saline as described, and the supernatants, DOC-PAGE analysis of the water layer extracts revealed two distinct K-antigen populations, a dominant higher molecular weight KPS, and a somewhat less abundant lower molecular weight KPS component as described previously (40). All of these KPS stain with alcian blue alone, and with alcian blue followed by silver staining, which is typical of these acidic polysaccharides (15, 52). Both KPS components were separated from the EPS by size exclusion chromatography under dissociative conditions (40), and together these components were subjected to structural analysis without further purification. Approximately 10 mg of total KPS was obtained from 60 mg of water layer extract per chromatographic run (equivalent to 2.8 g of lyophilized cell residue).

**Glycosyl Composition**—Preliminary mineral acid hydrolysis (1 N trifluoroacetic acid 2 h, 105 °C) followed by methanalysis and preparation of the TMS-methyl glycosides revealed the presence of glucuronic acid, in addition to smaller nonstoichiometric amounts of various neutral sugars including (ratios): Man (1.7), Glc (1.0), Gal (0.1), Xyl (1.0), and an O-methyl hexose of undetermined configuration (0.4). The latter components arise from minor polysaccharide components present in the phenol-water extract; presumably these are also surface components. These minor, neutral PS components constitute ~5% of the total carbohydrate, and were not stained by alcian blue or the other staining procedures during PAGE analysis. NMR spectroscopy showed that the primary KPS is composed of glucuronic acid (GlcA) and pseudaminic acid (Pse) in a 1:1 ratio (Fig. 4). The latter glycosyl component is labile to the hydrolytic step and is not readily detected by the TMS methyl glycoside procedure. MALDI-TOF mass spectrometric analysis of the purified KPS confirmed the presence of a hexuronic acid and a nonulosonic acid component (described below). The pattern of TMS (−)-2-butyl glycoside derivatives derived from the KPS matched that obtained from authentic n-GlcA, indicating the absolute configuration of α for the GlcA residues.

**NMR Analyses of the KPS and Identification of Glycosyl Residues**—The 1H NMR spectrum (Fig. 4) of the intact KPS (containing both HMW and LMW components) revealed a major resonance at δ 1.11, typical of -CH₃ protons, intense signals at δ 1.95 and δ 2.07 characteristic of acetyl -CH₂ protons, and resonances at δ 1.74 and δ 2.55 characteristic of the H3 ax/eq methylene protons of a 3-deoxy-2-ulosonic acid such as Kdo or higher analogue. A major resonance at δ 4.64 was attributed to the anomeric proton of a β-linked residue (later assigned to GlcA). The *J*₁,₂ coupling (6.5 Hz) is typical of β-linked aldoses, specifically those having a *trans*-dialxial arrangement for H1/H2. Other ring methine protons ranged from δ 3.26 to δ 4.29. A sharp singlet at δ 3.68 arises from -OCH₃ protons of an endogenously O-methylated glycosyl res-

---

**TABLE 1**

Nodulation tests with NGR234 and the rkpMNO mutant

| Host               | NGR234 | ΔrkpMNO |
|--------------------|--------|---------|
|                    | Plant shoot weight | Nodule number | Nodule weight | Plant shoot weight | Nodule number | Nodule weight |
| *V. unguiculata*   | 9146 (639) | 79 (6) | 589 (46) | 7124 (850) | 56 (7) | 461 (64) |
| *T. vogelii*       | 2056 (488) | 8 (4) | 415 (81) | 1768 (174) | 8 (2) | 375 (52) |
| *L. leucocephala*  | 1227 (216) | 20 (5) | 181 (43) | 911 (139) | 17 (4) | 166 (29) |
| *F. congesta*      | 1455 (286) | 108 (22) | nd† | 1124 (183) | 97 (19) | nd |

† Data from three independent replicates.  
‡ Average data are shown with standard deviations in parentheses.  
§ Data from one replicate.  
†† nd, not determined.
idue, a component of a neutral PS contaminant of low abundance. Other signals present at low nonstoichiometric levels included signals at $\delta$ 4.92 and $\delta$ 4.43, which presumably arise from anomeric protons of these PS. The $\delta$ 3.68 as well as other contaminating signals can readily be removed from the primary KPS by ion exchange chromatography (Fig. 4, inset), where the neutral PS migrate in the flow-through fraction.

$^1$H-$^1$H COSY and TOCSY (Fig. 5) analyses provided clear identification of two major glycosyl spin systems in the KPS sample, allowing complete and unambiguous proton assignments. COSY interactions were observed for all nearest neighbor protons in both spin systems, and complete proton assignments are reported in supplemental materials: Table S3, along with visible coupling constants determined from the $^1$H spectrum. Spin system A, identified as belonging to GlcA residues, was identified from COSY interactions between H1 ($\delta$ 4.64) - H2 ($\delta$ 3.26), H2 - H3 ($\delta$ 3.57), H3 - H4 ($\delta$ 4.06), and H4 - H5 ($\delta$ 3.83). TOCSY analyses confirmed strong magnetization transfer between all five protons with large coupling constants characteristic of the gluco-configuration. A suitable entry point for system B was found at the $\delta$ 1.11 signal, and was subsequently identified as the C9 methyl group protons of a deoxynonulosonic acid residue. The H9 protons showed a strong COSY cross-peak to a single proton at $\delta$ 4.14, assigned as H8. Sequential COSY interactions were then identified between H8 - H7 ($\delta$ 4.08), H7 - H6 ($\delta$ 3.90), H6 - H5 ($\delta$ 4.29), H5 - H4 ($\delta$ 4.17), and H4 - H3$_{ax}$ ($\delta$ 1.74). Strong TOCSY interactions were observed between H9/H8/H7/H6, and between H3$_{ax}$/H3$_{eq}$/H4, suggesting axial orientations for H6 and H4. The strength of COSY and TOCSY interactions was comparatively weak between H4/H5/H6 suggesting an equatorial orientation for H5. Selective irradiation of specific ring protons in the nonulosonic acid residue (H6 and H3$_{ax}$) with one-dimensional TOCSY analyses confirmed the member protons of this spin system (not shown).

Carbon assignments from a $^1$H-$^{13}$C HSQC analysis (Fig. 6), confirmed the identity of both glycosyl systems. In spin system A, H1 was found to correlate to a carbon resonance at $\delta$ 103.73 (assigned as the anomeric C1, supplemental materials, Table S3), confirming the $\beta$-pyranosidic form for the glucuronic acid residue. H4 was coupled to a carbon signal at $\delta$ 76.23 (C4). This carbon shift is slightly downfield of that expected for an unsubstituted carbon ($\approx$ $\delta$ 72.5) suggesting this to be the likely site of glycosidic substitution (confirmed by HMBC and NOE analyses, see below). The magnitude of this downfield shift is minimal, presumably due to the absence of an anomeric proton on the nonulosonic acid residue. For spin system B (deoxynonulosonic acid), the HSQC analysis showed carbon-proton cross-peaks for all proton-bearing carbons C3-C9. The identity of this glycosyl residue as a 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acid was established by the distinctive chemical shifts observed for H5/C5 ($\delta_{H}$ 4.29/$\delta_{C}$ 49.30) and H7/C7 ($\delta$ 4.08/$\delta$...
56.26); the upfield carbon shifts are indicative of substitution by nitrogen. Protons H3ax/H3eq were coupled to a carbon resonance at δ 38.14, the carbon shift characteristic of the methylene carbon of a 3-deoxy-2-ulosonic acid structural group. H4 was coupled to a carbon at δ 78.04 (C4), which is downfield from that expected for an unsubstituted C4 hydroxyl (compare δC 66–69 ppm (20, 53–56)), suggesting the likely site of glycosyl substitution. This conclusion was substantiated by a three-bond HMBC correlation from the anomeric proton of the adjoining glycose residue to C4 of this residue, and by strong inter-residue NOEs (discussed below). Additional carbon-proton cross-peaks in the HSQC spectrum included acetyl group protons (-CH3) at δ 2.07 and 1.95, which coupled to carbons at δC 24.98 and 24.96, respectively.

Configuration of the Nonulosonic Acid Residue—The configuration of the deoxynonulosonic acid residue was assigned from JH,H coupling constants, from carbon chemical shifts (δ), and from the intensity of 1H-1H COSY interactions substantiated by intra-residue NOEs. Coupling constants for overlapping protons were determined by selective excitation of distinct ring protons (H6, δ 3.90 and H3ax, δ 1.74) during 1D TOCSY analyses (not shown). The observed small J5,6 coupling (1 Hz), the large J6,7 coupling (9.7 Hz), and the J7,8 value (5.6 Hz) are not in agreement with data reported for three of the four known configurational isomers of the diamino-non-2-ulosonic acid family (legionaminic acid and its epimers, reviewed in Refs. 55 and 57). These coupling constants are consistent however with values reported for the l-glycero-D-manno- isomer (see e.g. Refs. 54 and 58). In particular, a large J3ax,4 (12.2 Hz), and small J4,5 and J5,6 values (both <1 Hz) indicate that proton H4 is axial and that H5 is equatorial, resulting in the lyxo-configuration for carbons C4-C5-C6. The large J6,7 value is also characteristic of isomers containing an axial orientation of the C5 amino group, and reflects the trans-relation-ship for H6-H7 (54, 57), equivalent to the erythro-configuration for carbons C6-C7. In comparison, diamino-nonulosonic acid isomers containing the threo-configuration for C6-C7 exhibit small J6,7 coupling (typ-
Rhizobium sp. NGR234 K-antigen Capsular Polysaccharide Structure

FIGURE 6. Partial 500-MHz $^1$H-$^1$C HSQC spectrum of the Rhizobium sp. NGR234 KPS. One-bond proton-carbon connectivities are shown for all proton bearing carbons in the two glycosyl systems (G1, glucuronic acid H1-C1, etc.). Unlabeled cross-peaks arise from a contaminating polysaccharide, which can be removed by ion-exchange chromatography. Carbon shifts are reported in supplemental materials, Table S3.

FIGURE 7. Partial 500-MHz $^1$H-$^1$C HMBC spectrum of the Rhizobium sp. NGR234 KPS. Two inter-residue connectivities (underlined) indicate the two glycosidic linkages of the repeating unit, ($p$C4-8H1, pseudaminic acid $C$4-glucuronic acid H1, etc.) Significant intra-residue interactions, both 2- and 3-bond, are also labeled. Unlabeled cross-peaks arose from a contaminating polysaccharide.

ically <2 Hz) (53, 58) reviewed in Ref. 55. Accordingly, these data indicate that the residue is of the $L$-glycero-1-manno-configuration and is thus $5,7$-diacetamido-3,5,7-tetraideoxy-$L$-glycero-1-manno-non-2-ulosonic acid (pseudaminic acid, Pse), or its enantiomer. Studies with the model compounds $N$-acetylthreonine and $N$-acetylassallo-threonine, as well as with synthetic pseudaminic acid isomers have shown that the chemical shift of $C_9$ is influenced in a predictable manner by the configuration at $C_8$ (53, 54, 57). Isomers containing the $threo$-configuration for C7-C8 exhibit a $C_9$ resonance consistently downfield (C9 $\delta_c$ ~ 20.0 ppm) relative to the erythro-forms (C9 $\delta_c$ ~ 16.3 ppm for the free monosaccharide, and 17.7 ppm for glycosidically linked residues in polysaccharides). In the case of the NGR234 KPS, the chemical shift of C9 ($\delta_c$ 18.46) suggested the erythro-configuration for carbons C7-C8 based on comparison with literature values for 4-O-substituted Pse residues glycosidically linked in polysaccharides (53).

The chemical shift difference (0.81 ppm) measured between H3eq and H3ax is relatively large, and is consistent with the axial orientation of the vicinal C1 carboxyl for glycosides of 3-deoxy-2-ulosonic acids (54, 58, 59). Smaller differences (< 0.6 ppm) for H3eq/H3ax are characteristic of the equatorial orientation of the C1 carboxyl (53, 59). The absolute values of the chemical shifts of both H3eq and H3ax are also consistent with reported data for the axial orientation of the carboxyl (54) but not with an equatorial orientation (53). The chemical shift of the anomeric carbon (C2) is also dependant on the orientation of the C1 carboxyl, and the observed value (C2 $\delta_c$ 102.3) is in virtual agreement with the axial orientation of COOH in polysaccharides having this configuration (53, 54, 57, 58). This differs from Pse residues containing equatorial COOH, in which C2 ranges from approximately $\delta_c$ 96 – 99 ppm (20, 53, 57). In addition, comparisons of deoxynonulosonic acids from various polysaccharides have shown that the chemical shift of C6 in such residues is influenced in a predictable manner by the C1 carboxyl orientation. Residues containing equatorial COOH show C6 resonances around $\delta_c$ 71 – 73 (20, 53, 54), while those having axial COOH show C6 approx 2 – 3 ppm downfield ($\delta_c$ 73 – 75) (53, 54, 57, 58) similar to that reported here for the NGR234 KPS ($\delta_c$ 76.2). Accordingly, all data indicate the axial orientation for the COOH, and the Pse residue is thus the $\beta$-anomer.

Identification of Glycosyl Linkages—$^1$H-$^1$C HMBC analysis (Fig. 7) revealed two prominent inter-residue correlations, indicating the presence of two glycosidic linkages and the existence of a disaccharide repeating unit. A three-bond correlation from the anomeric carbon (C2) of Pse to H4 of GlcA indicated that Pse is glycosidically linked to C4 of GlcA residues. A second inter-residue correlation from C4 of Pse to the anomeric proton (H1) of GlcA confirmed that GlcA is linked glycosidically to C4 of Pse. Significant intra-residue HMBC correlations were identified between C1/H2, C6/H5, C3/H2, and C3/H4 of GlcA, and between C7/H9, C8/H9, C1/H3a, C5/H6, and C4/H5 of Pse, confirming these assignments. Two-bond HMBC correlations were observed between the acetate methyl protons at $\delta_h$ 1.95 and acetate carbonyl at $\delta$ 176.68, and between acetate $\delta_h$ 2.07 and its carbonyl carbon at $\delta$ 177.58. A weak three-bond correlation between the acetate carbonyl carbon at $\delta$ 177.58 and the H5 ring proton of Pse ($\delta$ 4.29) was observed (supported by NOEs described below). The structure of the KPS disaccharide repeating unit is thus ((4)-$\beta$-$p$-GlcA-(1$\rightarrow$4)-$\beta$-Pse5Ac7Ac-2$\rightarrow$)n, and is represented in Fig. 8. This structure completes and corrects the partially determined structure proposed in earlier work (15).

$^1$H-$^1$H ROESY and NOESY experiments supported the aforementioned conclusions and provided additional structural and configurational information (Fig. 9). For the GlcA spin system, strong intra-residue NOEs were observed between H1/H3/H5 and between H2/H4, consistent with axial orientation of these protons and the $\delta C_1$ chair conformation of the glucopyranosyl uronate residues. For the Pse spin system, the
axial orientation of H4 assigned from $J_{H,H}$ values was substantiated by a strong NOE between H3$_{eq}$/H4 (Fig. 9B), whereas an NOE between H3$_{ax}$/H4 was not observed, suggesting a closer proximity of H4 to H3$_{eq}$ than to H3$_{ax}$. Strong intra-residue NOEs were also observed between H5/H6 and H5/H4 (Fig. 9A), providing evidence for an eq-ax relationship for these three protons, consistent with the conclusions drawn from $J_{H,H}$ measurement and COSY/TOCSY analysis. Other significant NOEs existed between the exocyclic protons H9/H8/H7, and H9/H6, H8/H6, and H7/H5 (Fig. 9B). NOEs were also observed in ROESY analysis between the acetate methyl protons at $\delta$ 1.95 and the Pse H5 ring proton (δ 4.29), and between the acetate protons at δ 2.07 and the Pse H7 ring proton (δ 4.08) (Fig. 9B), providing further evidence that both amino groups were N-acetylated.

A strong inter-residue NOE was observed between GlcA H1 and Pse H4, confirming the HMBC correlation (GlcA H1/Pse C4) and the 1→4 linkage between these residues (Fig. 9A). The GlcA H1 proton also showed a strong inter-residue NOE with Pse H5, but not with Pse H3$_{eq}$. Interestingly, a series of weak inter-residue NOEs suggested considerable inter-residue interactions in the overall polysaccharide, as opposed to a highly extended polysaccharide conformation. These NOEs included: Pse H3$_{ax}$/GlcA H4, and Pse H8/GlcA H4 (observed in ROESY and NOESY experiments), and between Pse H3$_{eq}$/GlcA H5, Pse H5/GlcA H3, and Pse H5/GlcA H5 (observed in NOESY but not in ROESY).

MALDI-TOF Mass Spectrometry—Mass spectrometric analysis in the negative ion mode revealed a cluster of ions centered around 8,000 mass units having a sigmoidal distribution, showing that the KPS polysaccharides were polydisperse. A lower molecular weight polysaccharide population was also evident centered around 4,100 mass units (Fig. 10). The mass differences between the peaks in each ion family were primarily 176 and 316 mass units, because of the incremental masses of hexuronic acid and pseudaminic acid, respectively. Size heterogeneity within each ion family was thus caused by different degrees of polymerization of the repeating units, in addition to the presence or absence of either of the two monosaccharide residues. Lability of the ketosidic linkages during polysaccharide isolation presumably contributed to this heterogeneity, to an unknown degree.

DISCUSSION

Genes involved in the synthesis of the K-antigens (KPS) have been well characterized in _Rhizobium meliloti_ strain Rm41 (29, 60, 61). Three gene clusters, _rkp-1_, _rkp-2_, and _rkp-3_, are reportedly involved in the synthesis and/or export of KPS, and these same three loci are present in the closely related strain NGR234. As shown by Kiss et al. (29), the _rkp-3_ locus comprises ten open...
Rhizobium sp. NGR234 K-antigen Capsular Polysaccharide Structure

FIGURE 10. Negative ion MALDI-TOF mass spectrum of K-antigen polysaccharide from Rhizobium sp. NGR234. Ion clusters show mass differences of 316 and 176 mass units attributed to the incremental masses of pseudaminic and glucuronic acid. A higher molecular weight population of K-antigens centered around 8,000 mass units with sigmoidal distribution was also observed (not shown).

reading frames, and six of these (rkpLMNOPQ) appear to encode proteins specifically dedicated to the synthesis of the strain-specific KPS sugar precursors in Rm41. The present study demonstrates that selective inhibition of KPS synthesis in strain NGR234 reduced its symbiotic efficiency on several legume hosts, including V. unguiculata, a preferred host of the parent strain (Fig. 3). Inhibition of KPS synthesis was accomplished by construction of a mutant containing deletions in three of the aforementioned genes, rkpMNO, located in the rkp-3 locus (Fig. 1). EPS production and composition was unaltered after mutation of rkpMNO. LPS synthesis also appeared to be unaffected in the NGRΔrkpMNO mutant as assessed by DOC-PAGE analysis; synthesis of the primary KPS was totally blocked (Fig. 2). Bacterial motility, flagellin glycoprotein, and components of the type III secretion system also were not reported for the latter (15, 29). A notable difference in the two operons is the absence of rkpP located at the center of the rkp-3 genes. EPS synthesis is reported to contain Pse as the (Kdx) component. The R. meliloti strain Rm41 KPS is composed of N-(3-hydroxybutyryl)-Pse, in unknown linkage/anomericity along with GlcA (15, 29). The only other known occurrence is in S. fredii strain HH103, which produces a unique homopolymeric KPS composed entirely of α-7-(3-hydroxybutyramido)-Pse in alternating amidic and glycosidic linkage; the latter occurs between the hemiketalic hydroxyl of Pse residues and the 3-hydroxyl of the N-acyl substituents. A third known example of a nonulosonic acid component in a rhizobial KPS is that of R. meliloti strain NRG2427, which contains NANA linked to glucose (15). A fourth R. meliloti strain, Rm1021, produces a KPS that does not fit the structural template, a homopolymer of β-(2→7)-linked Kdo (18).

Recent structural studies have identified an alternative KPS structural motif, consisting of the disaccharide (hexose-hexuronic acid) (15, 17, 19). Three R. fredii strains, HWG35, B33, and HH303, share this motif and are characterized by the ability to nodulate both Asian and American soybean cultivars (Nod1 Fix1 phenotypes). Four other R. fredii strains conform to the original Kdx-Sug motif, and these can only nodulate the Asian cultivars (1, 17, 64). Thus, the majority of R. fredii strains examined to date align with one of these two structural motifs; further structural information is needed before a true correlation can be established however.

As with enteric and pathogenic bacteria, many Rhizobium strains have been found to synthesize more than one type of KPS (4, 14–16). This is not surprising and probably reflects the need for a high degree of structural variability in response to changing environmental conditions. Relevant issues thus include determining which of the multiple forms are biologically relevant, under what conditions, and what factors regulate differential KPS expression. Studies have shown that the expression of rhizobial KPS is modified by abiotic factors including temperature and pH (4), and by plant-derived factors, including flavonoids. Apigenin and soybean root extract were found to cause an up-regulation of the secondary KPS in R. fre-
Rhizobium sp. NGR234 K-antigen Capsular Polysaccharide Structure

dii USDA205 (65), and alfalfa root extract evoked an increase in all KPS components in R. meliloti AK631 (the natural host-symbiont pairs) whereas soybean root extract had no effect on the latter (21). Here we present the structure of the major KPS molecular species (when grown in complex medium). Under these conditions however, we noted that NGR234 produces at least two other surface polysaccharides (not including EPS) in addition to the primary KPS. These include a homopolymer of Kdo (minor amounts were detected during electrospray mass spectrometry) which qualifies structurally as a secondary KPS, and at least one neutral PS composed of xylose, mannose, and endogenously O-methylated hexoses. Whether the synthesis of these latter PS is up-regulated at some point during the course of symbiotic infection is not known, nor is the biological role of these PS. Similar neutral polysaccharides have been noted in R. meliloti strains (21). Preliminary results indicate that the ratio of primary KPS to these secondary PS changes significantly during laboratory culture under different growth conditions; the regulation and structural analysis of these secondary PS is currently under study.

Several mechanisms involving KPS function could account for the reduced nodulation efficiency of the NGR234 kkpMNO mutant. As with enterobacterial CPS, it is generally considered that a major role of rhizobial KPS is to form a protective layer against abiotic environmental stress (e.g. dehydration), from phage adsorption, and from plant defense response mechanisms (7, 8, 29, 66). In this sense, the role of KPS is one of passive protection. Accumulating evidence also indicates an active, signaling role for KPS in both survival and in establishing symbiotic infection. Purified KPS (isolated from an Rm41 mutant) when applied to M. sativa (alfalfa) leaves, induced a specific and rapid induction of plant genes involved in the isoflavonoid pathway (phytoalexin synthesis), indicating that KPS are specifically monitored by the plant via an active recognition mechanism (67). KPS was found to functionally substitute for EPS in an exoB (EPS deficient) mutant of Rm41, resulting in a delayed but functional nodulation (21, 22). The ability of KPS to compensate for EPS was found to be dependent on the molecular weight range of the KPS (21), suggesting specific recognition mechanisms. In the R. meliloti Rm41/M. sativa system, derivatives of Rm41 defective in KPS synthesis yielded symbiotic phenotypes having aborted (shorter) infection threads, with higher rates of aberrant morphologies compared with the parent strain (9). Recently, S. fredii HH103 mutants defective in KPS synthesis were found to have impaired symbiotic efficiency on soybean cultivars (13).

Previous immunological and histochemical analyses have indicated that surface polysaccharide expression is reduced (compared with free-living undifferentiated cells) or absent on the bacteroid surface in both indeterminate and determinate nodule-forming hosts (68, 69). In support of these observations, our results show that KPS production is significantly reduced as a result of flavonoid induction (Fig. 2). Recently, we have shown that the major surface polysaccharide present on flavonoid-induced NGR234 cells is not a capsular polysaccharide, but a rhamnan O-antigen, carried on a structurally modified core-lipid A anchor (i.e. a bacteroid-specific LPS) (52). These observations indicate that a major change in bacterial surface chemistry occurs during, or immediately prior to differentiation of the free living rhizobia (within the infection threads) into bacteroids (51, 52, 70, 71). The loss of K-antigen polysaccharide of high negative charge density, accompanied by the appearance of a neutral and relatively hydrophobic 6-deoxyhexose-containing O-chain implies a significant increase in surface hydrophobicity when the bacterium resides within the symbiosome. Similar molecular changes toward greater hydrophobicity, have been reported during bacteroid development for R. leguminosarum, pea and bean interactions (71). Such changes may be necessary to promote bacteroid survival, or for proper interaction with the plant-derived symbiosome membrane. Analysis of these Rhizobia–plant interactions provides a useful model for the study of intracellular pathogens.

Acknowledgments—We thank Florencia Ares-Orpel for help with this work and to John Glushka for assistance with NMR analyses. We thank Dora Gerber for general support, Olivier Schumpp for many interesting discussions, and Birgit Scharf for the gift of the anti-flagella antisemur. The Complex Carbohydrate Research Center was supported in part by Department of Energy Grant DE-FG02-93ER20097.

REFERENCES
1. Pueppke, S. G., and Broughton, W. J. (1999) Mol. Plant-Microbe Interact. 12, 293–318
2. Bhat, U. R., Carlson, R. W., Busch, M., and Mayer, H. (1991) Int. J. Syst. Bacteriol. 41, 213–217
3. Bhat, U. R., Mayer, H., Yokota, A., Hollingsworth, R. I., and Carlson, R. W. (1991) J. Bacteriol. 173, 2155–2159
4. Kappenberg, E. L., Reuhs, B. L., Forberg, L. S., and Carlson, R. W. (1998) in The Rhizobiales (Spinak, H. P., Kondorosi, A., and Hoooykaas, P. J., eds) pp. 119–154, Kluwer Academic Publishers, Dordrecht, Boston, London
5. Broughton, W. J., Jabbouri, S., and Perret, X. (2000) J. Bacteriol. 182, 5641–5652
6. Mathis, R., Van Gijssem, F., De Rycke, R., D’Haeze, W., Van Maelseaeke, E., Anthonio, E., Van Montagu, M., Holsters, M., and Vereecke, D. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2655–2660
7. Becker, A., Fraysse, N., and Sharypova, L. (2005) Mol. Plant-Microbe Interact. 18, 899–905
8. D’Haeze, W., and Holsters, M. (2004) Trends Microbiol. 12, 555–561
9. Pellock, B. J., Cheng, H.-P., and Walker, G. C. (2000) J. Bacteriol. 182, 4310–4318
10. Fraysse, N., Couderc, F., and Poinot, V. (2003) Eur. J. Biochem. 270, 1365–1380
11. Niehaus, K., Lagaes, A., and Puhler, A. (1998) Mol. Plant Microbe Interact. 11, 906–914
12. Forsberg, L. S., Noel, K. D., Box, J., and Carlson, R. W. (2003) J. Biol. Chem. 278, 51347–51359
13. Parada, M., Vinardell, J. M., Oller, F. J., Hidalgo, A., Gutierrez, R., Buendia-Claveria, A. M., Lei, W., Margaret, I., Lopez-Baena, F. J., Gil-Serrano, A. M., Rodriguez-Carvajal, M. A., Moreno, J., and Ruiz-Sainz, J. E. (2006) Mol. Plant-Microbe Interact. 19, 43–52
14. Reuhs, B. L., Carlson, R. W., and Kim, S. J. (1993) J. Bacteriol. 175, 3570–3580
15. Reuhs, B. L., Geller, D. P., Kim, J. S., Fox, J. E., Kolli, V. S. K., and Pueppke, S. G. (1998) Appl. Environ. Microbiol. 64, 4930–4938
16. Forsberg, L. S., and Reuhs, B. L. (1997) J. Bacteriol. 179, 5366–5371
17. Rodriguez-Carvajal, M. A., Rodriguez, J. A., Soria-Diaz, M. E., Tejero-Mateo, P., Buendia-Claveria, A., Gutierrez, R., Ruiz-Sainz, J. E., Thomas-Oates, J., and Gil-Serrano, A. M. (2005) Biomacromolecules 6, 1448–1456
18. Fraysse, N., Lindner, B., Kaczynski, Z., Sharypova, L., Holst, O., Niehaus, K., and Poinot, V. (2005) Glycoconj. J. 15, 101–108
19. Rodriguez-Carvajal, M. A., Tejero-Mateo, P., Espartero, J. L., Ruiz-Sainz,
