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Russell W. Carlson
University of Georgia

Bradley Reuhs
University of Georgia

Tong-Bin Chen
University of Georgia

U. Ramadas Bhat
University of Georgia

K. Dale Noel
Marquette University, dale.noel@marquette.edu

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Lipopolysaccharide Core Structures in *Rhizobium etli* and Mutants Deficient in *O*-Antigen*

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Russell W. Carlson‡§, Bradley Reuhs‡, Tong-Bin Chen‡¶, U. Ramadas Bhat‡‖, and K. Dale Noel**

*From the ‡Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30607 and the **Department of Biology, Marquette University, Milwaukee, Wisconsin 53233*

Lipopolysaccharide (LPS) is a major component of the bacterial outer membrane, and for *Rhizobium* spp. has been shown to play a critical role in the establishment of an effective nitrogen-fixing symbiosis with a legume host. Many genes required for *O*-chain polysaccharide synthesis are in the *lps* α region of the CE3 genome; this region may also carry *lps* genes required for core oligosaccharide synthesis. The LPSs from several strains mutated in the α region were isolated, and their mild acid released oligosaccharides, purified by high performance anion-exchange chromatography, were characterized by electrospray- and fast atom bombardment-mass spectrometry, NMR, and methylation analysis. The LPSs from several mutants contained truncated *O*-chains, and the core region consisted of a (3-deoxy-D-*manno*-2-octulosomic acid) (Kdo)-(2→6)-α-Gal*p*-(1→6)-[α-Gal*p*A-(1→4)]-α-Man*p*-(1→5)-Kdo*p* (3-deoxy-D-*manno*-2-octulosomic acid) (Kdo)pentasaccharide and a α-Gal*p*A-(1→4)-[α-Gal*p*A-(1→5)]-Kdo*p* trisaccharide. The pentasaccharide was altered in two mutants in that it was missing either the terminal Kdo or the GalA residue. These results indicate that the *lps* α region, in addition to having the genes for *O*-chain synthesis, contains genes required for the transfer of these 2 residues to the core region. Also, the results show that an LPS with a complete core but lacking an *O*-chain polysaccharide is not sufficient for an effective symbiosis.

Lipopolysaccharides (LPSs)[1] are important determinants of the surface characteristics and ecology of Gram-negative bacteria. Current understanding of LPS and its biological roles comes mainly from studies of enteric bacteria. To gain a greater appreciation of the role of LPS in nature, it is important to supplement the enteric bacterial paradigm with studies of bacteria in different phylogenetic groups and with distinct physiology. *Rhizobium etli* (1) and *Rhizobium leguminosarum* are closely related species in the α group of the Proteobacteria (2, 3). The biology of these bacteria has been extensively studied, particularly their nitrogen fixing symbioses with certain legumes, and LPS structure is critical in the development of these symbioses. The LPS structure (4) and genetics (5) of several strains of these species are known in some detail.

Structural regions of the LPS from *R. leguminosarum* and *R. etli* have been defined according to fragments released from purified LPSs by mild acid hydrolysis. The structures of the core oligosaccharides from *R. etli* strain CE3, and from *R. leguminosarum* biovar trifolii and bv. viciae (4, 6–10) are:

α-Gal*p*-(1 → 6)-[α-Gal*p*A-(1→4]-α-Man*p*-(1 → 5)-Kdo*p*

STRUCTURE I

and

α-Gal*p*A-(1→4)-[α-Gal*p*A-(1→5)]Kdo*p*

STRUCTURE II

The lipid A portion of these LPSs has a trisaccharide glycosyl backbone consisting of one each of galacturonosyl (GalA), glucosaminosyl (GlcN), and 2-aminogluconosyl (GlcN-onate) residues; the latter 2 residues being *O*- and *N*-acylated with β-hydroxymyristate, -palmitate, -pentadecanoate, -stearate, and 27-hydroxyoctacosanoate (11). Both the lipid A and the core regions are structurally very different from those of the enteric bacteria, in which the lipid A is comprised of an acylated bis-1,4′-phosphorylated β-1,6-glucosamine disaccharide, while the core oligosaccharide usually contains heptose and lacks uronosyl residues. The remaining LPS structural region is the distal *O*-chain polysaccharide that, when present, is the dominant antigen of the LPS and the bacterial cell. *R. leguminosarum* and *R. etli* *O*-chains, as released by mild hydrolysis, are polysaccharides that contain Kdo at their reducing ends (12, 13). This Kdo may be the outermost core glycosyl residue to which the *O*-chain is transferred during the biosynthesis of the LPS.

Synthesis of the core oligosaccharide and *O*-chain portion of the LPS requires *lps* genes from at least five regions of the *R. etli* CE3 genome, as defined by cosmid genetic cloning (5, 14, 15). Most of the genes that have been identified are located in a stretch of the chromosome, termed the *lps* α region (5), in which nine complementation groups have been identified within 18 kilobases of DNA (16) (Fig. 1). It appears that this region carries genes for synthesis of at least the strain-specific *O*-chain glycosyl residues and linkages (17) and at least one gene necessary for core oligosaccharide synthesis as well (9, 18).

R. leguminosarum, *R. etli*, and *Bradyrhizobium japonicum* mutants that are deficient in the LPS *O*-chain polysaccharide elicit incomplete infections and root nodule development on their legume hosts (5, 14, 19, 20). Although all LPS mutants that are defective in symbiosis have deficient or altered *O*-chain-containing LPS, it had not been ruled out in previous studies that these mutants might also be defective in the core

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§ To whom correspondence should be addressed. Tel.: 706-542-4439; Fax: 706-542-4412.

¶ Present address: Mayo Clinic, Rochester, MN 55905.

‖ Present address: Miles-Cutter Laboratories, Berkeley, CA 94701.

[1] The abbreviations used are: LPS, lipopolysaccharide; Kdo, 3-deoxy-D-*manno*-2-octulosomic acid; DOC, deoxycholate; PAGE, polyacrylamide gel electrophoresis; HPAEC, high performance anion-exchange liquid chromatography; PMAA, partially methylated alditol acetates; FAB, fast atom bombardment; GLC, gas liquid chromatography; ES, electrospray; MS, mass spectrometry.

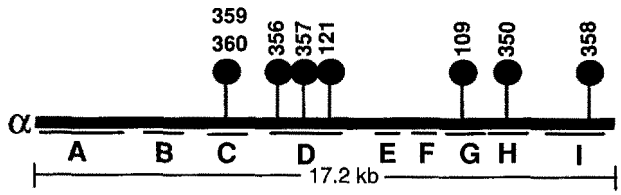

FIG. 1. **The location of the various mutations in the α region of the *R. etli* CE3 genome.** All of the mutants designated by the *filled circle* either lack or have much less than normal LPS I. The *letters* refer to complementation groups, presumably representing operons, defined by Tn5 insertion mutagenesis. The distance shown is between the Tn5 insertions at either end of the region.

oligosaccharides. In fact, the LPSs from two *R. etli* mutants have truncated core structures (9, 18). Therefore, one motive for this study was to determine whether or not an intact core portion of the LPS is sufficient for symbiotic proficiency. Another motive was to infer specific biosynthetic functions for particular genetic loci within *lps* α region by correlating mapped mutations with LPS structural defects. In addition, the results of this study suggest a definition for the biosynthetic core and the linkage between a particular core sugar and the Kdo residue which is at the reducing end of the *O*-chain polysaccharide.

EXPERIMENTAL PROCEDURES

Bacterial Strains—The bacterial strains used in this study are given in Table I. Bacteria were grown in TY medium with added calcium as described previously (12).

LPS Isolation—Bacteria were harvested by centrifugation and the pellets extracted using the hot phenol-water extraction procedure as described previously (12, 21). The LPSs were further purified from the aqueous layer, as described previously (12, 21), by digestion with RNase and DNase, followed by extensive dialysis against deionized water using 12,000–14,000 MWC dialysis tubing, and freeze-dried. The LPS from the parent strain, CE3, had also been further purified by gel-filtration chromatography using Sepharose 4B in and EDTA/triethylamine buffer at pH 7 (12, 22). Separation of higher from lower molecular weight forms of LPS was accomplished by gel filtration chromatography on Sephadex G-150 in the presence of deoxycholate (DOC) as described previously (23–25).

Polyacrylamide Gel Electrophoretic Analysis (PAGE)—PAGE analysis was performed using 18% acrylamide gels with DOC as the detergent (26). The gels were silver stained as described (27).

Isolation of Core Oligosaccharides from the LPSs—Each LPS was dissolved in deionized water (10 mg/ml), acetic acid was added to 1%, and the solution heated at 100 °C for 1 h. This procedure hydrolyzes the ketosidic bond between the polysaccharide Kdo residue and the lipid A, which precipitates (28). The lipid A was removed by centrifugation, and the carbohydrate was further purified by HPAEC on a CarboPac™ PA1 column (DIONEX) using a gradient comprised of 1 M NaOAc (A) and 100 mm NaOH (B); 10% A and 90% B for 10 min and then to 50% A and 50% B by 40 min. The various carbohydrate peaks were collected, the acetate was removed by passage through DIONEX OnGuard H cartridges, and the eluants freeze-dried.

Analysis of the Glycosyl Residues—Glycosyl compositions were determined by the preparation and gas liquid chromatographic (GLC)-mass spectrometric (MS) analysis of alditol acetates, or trimethylsilyl methyl glycosides (29), and glycosyl linkages were determined by methylation analysis using the Hakomori procedure as described by York *et al.* (29). For the isolated core oligosaccharides, it was necessary to reduce the samples with $NaBD_4$ prior to methylation. Also, for certain samples, after methylation, it was necessary to reduce the carboxymethyl groups of the acidic sugars with lithium triethylborodeuteride (Superdeuteride from Aldrich) (29). Alditol acetates of the methylated samples were prepared by hydrolysis, reduction with $NaBD_4$, and acetylation with acetic anhydride in pyridine as described by York *et al.* (29). Combined GLC-MS was performed using an HP5890-5970 GLC-MSD system equipped with a 30-m SP2330 fused silica column from Supelco for the alditol and partially methylated alditol acetates (PMAAs), or with a 30-m DB-1 column from J&W Scientific for trimethylsilyl methyl glycosides.

Mass Spectrometry Analysis—Fast atom bombardment-mass spectrometry (FAB-MS) was performed using a VG ZAB-SE instrument at an accelerating voltage of 8 kV. Approximately 2–10 μg of sample was placed on the probe. Thioglycerol was used as the matrix. Electrospray mass spectrometry (ES-MS) was performed using a SCIEX API-III mass analyzer operated in the positive mode with an orifice of 50 V. Samples were dissolved in 20% aqueous acetonitrile and pumped into the mass spectrometer at a rate of 3 μl/min.

NMR—Samples were exchanged several times with D_2O, dissolved in D_2O and analyzed at 295 °K using a Bruker AM500 spectrometer. Chemical shifts were measured relative to the HOD resonance, which, in turn, was measured relative to sodium 3-trimethylsilylpropionate-2,2,3,3-d_4.

TABLE I
Bacterial strains

Strain[a]	Characteristics[b]	Refs.
CE3	*str-1*, Lps$^+$, LPS I, Ndv$^+$, Fix$^+$	34
CE109	*str-1, lps-109*::Tn5, LPS III, Ndv$^-$	14
CE121	*str-1, lps-121*::Tn5, LPS IV, Ndv$^-$	14
CE350	*str-1, lps-3*::Tn5, LPS III, Ndv$^-$	16
CE356	*str-1, lps-7*::Tn5, LPS IV, Ndv$^-$	16
CE357	*str-1, lps-5*::Tn5, LPS IV, Ndv$^-$	16
CE358	*str-1, lps-2*::Tn5, Ndv$^-$	16
CE359	*str-1, lps-359*, Tn5[c], LPS V, Ndv$^-$	30
CE360	*str-1, lps-6*::Tn5, LPS V, Ndv$^-$	16

[a] All strains were derived from wild isolate *R. etli* CFN42.
[b] *str*-1, *ery*-1, and *lps* mutations alter streptomycin sensitivity, erythromycin sensitivity, or lipopolysaccharide; LPS I, III, IV, and V indicate SDS- or DOC-PAGE bands exhibited by the strain in addition to LPS II; Ndv$^-$, elicits incomplete nodule development.
[c] It has not been demonstrated that the Tn5 insertion is responsible for the *lps* mutation of this strain.

RESULTS

PAGE Analysis of R. etli LPSs—The LPSs from *R. etli* CE3 and various mutants were analyzed by DOC-PAGE (Fig. 2). LPS I and LPS II were the major components of the parent (CE3) LPS. The LPS I band was detected in greatly reduced amount in the LPS from CE359 and not detected in any LPSs from the other mutants examined. All of the mutant LPS preparations contained LPS II and, in addition, other low molecular weight forms of LPS designated LPS III (CE350), LPS IV (CE121, CE356, CE357), and LPS V (CE359, CE360). The only exception was the LPS preparation from CE358 which contained only LPS II. These different forms of LPS were distinguishable by their differing abilities to bind four monoclonal antibodies (JIM26, JIM27, JIM28, and JIM29); *i.e.* it was previously reported that LPS IV and V bind all four monoclonals while LPS III binds only JIM26, and LPS II does not bind to any of the monoclonals (30).

Analysis of the R. etli CE3 LPS Oligosaccharides by High Performance Anion-exchange Liquid Chromatography (HPAEC)—Analysis of the mild acid hydrolysate from CE3 LPS by HPAEC showed (Fig. 3*A*) the presence of five components, OS1-OS5. Fractions OS1 and OS2 were identified as monomeric Kdo and GalA, respectively, by comparing retention times to those of authentic standards. The monomeric Kdo, OS1, eluted as several peaks due to the formation of various anhydro forms during the mild acid hydrolysis procedure (31, 32). Subjection of standard Kdo to the mild acid hydrolysis conditions resulted in the same peaks as those observed for OS1 from the LPS samples. Preparation and GLC-MS analysis of the trimethylsilyl methylglycosides of OS2 showed that it was composed of only GalA. Proton NMR analysis of OS3 and 5 showed that their spectra (not shown) matched those published (18) for the major tetra- and trisaccharide components, respectively, from this LPS; therefore, these previously reported structures (Structures I and II shown above) can be assigned to OS3 and OS5. Oligosaccharide OS4 had the same glycosyl composition as the tetrasaccharide, OS3; namely, GalA, Man, Gal, and Kdo. The relative molar ratio of OS2

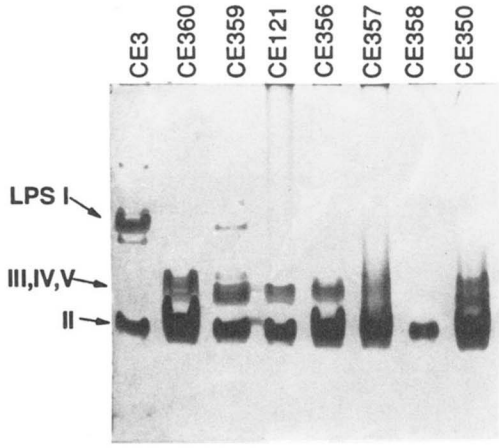

FIG. 2. **The DOC-PAGE profile (silver stain) of LPSs from the various mutants of *R. etli* CE3.** LPS I is the high molecular weight form of the LPS which contains the *O*-chain. LPS II lacks the *O*-chain. LPSs III, IV, and V are various forms of LPS that are distinguished from one another slightly in their electrophoretic mobility, and in their ability to interact with the monoclonal antibodies, JIM26, 27, 28, and 29.

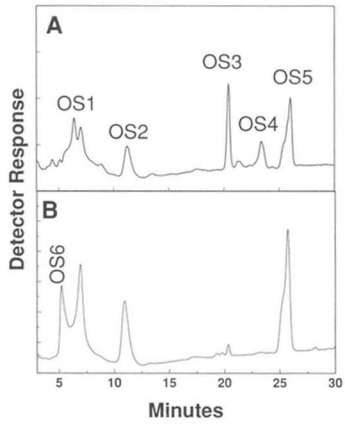

FIG. 3. **Analysis by HPAEC of the LPS core oligosaccharides obtained from *Rhizobium etli* CE3 LPS (*A*), and CE358 LPS (*B*).**

(GalA)/OS3 + OS4 (tetramers)/OS5(trimer) was 1:1:1 and was determined using molar response factors of standard monomeric GalA for OS2 and of a pectic trisaccharide for OS3, OS4, and OS5.

Oligosaccharide OS4 Is a Tetrasaccharide with an Anhydro Kdo Residue at Its Reducing End—As stated above, the glycosyl composition of OS4 was the same as that for OS3. Methylation analysis also showed that OS4 had the same glycosyl linkages as OS3; namely, terminal-GalA/terminal-Gal/4,6-linked Man in a 1:1:1 ratio. The methylation procedure used for this analysis destroyed the Kdo residue and prevented its analysis; however, the various types of Kdo linkages in these LPSs are described below. Analysis by negative FAB-MS of the oligosaccharides prior to purification by HPAEC showed $[M\text{-}H]^-$ ions of m/z 589, 719, and 737. The ions of m/z 589 and 737 are due to the tri- and tetrasaccharides (Structures II and I, respectively). The ion at m/z 719 is consistent with a tetrasaccharide that lacks a water molecule, *i.e.* minus 18 atomic mass units, and could be due a lactone or anhydro version of this molecule. Fractions OS3 and OS4, purified by HPAEC, were reduced with $NaBD_4$, permethylated, and analyzed by ES-MS, Fig. 4, *A* and *B*. Fraction OS3 gave the ions expected for a molecule derived from a tetrasaccharide that contained the expected reducing Kdo pyranose residue; namely, $[M+NH_4]^+$ and $[M+NH_4+Na^+]^+$ of m/z 983 and 1005, respectively. Analysis of OS4, Fig. 4*B*, resulted in ions of m/z 937 and 959, $[M+NH_4]^+$ and $[M+NH_4+Na^+]^+$, respectively. These latter ions were also present in OS3 indicating that this fraction was contaminated with some anhydro or lactone form of the tetrasaccharide. The 46 atomic mass units difference between OS3 and OS4 is not consistent with a lactone which would have been reduced with $NaBD_4$; however, it is consistent with an anhydro-Kdo derivative.

NMR analysis of OS4 gave a complex spectrum (not shown). The complexity of the spectrum may indicate that OS4 contained more than one type of anhydro-Kdo residue. The typical resonances for the methylene protons from the reducing Kdo pyranose residue of OS3 (δ 1.8 and 2.1) were absent. This apparent lack of the Kdo methylene proton resonances can occur for oligosaccharides containing certain anhydro forms of Kdo (33). In a 4,7- or 4,8-anhydro-Kdo residue, the methylene geminal protons are adjacent to a C-2 carbonyl rather than to the hemi-ketal C-2 of a normal reducing Kdo pyranose and are, therefore, quite acidic and easily exchanged with deuterium during preparation of the sample for NMR analysis. Additionally, if not fully exchanged with deuterium, the chemical shifts of such geminal methylene protons are shifted far downfield (*e.g.* to δ 2.90 and 3.3 (33)) compared to their resonances in the tetramer with a normal Kdo pyranose residue (*e.g.* δ 1.8 and 2.1). This combination of deuterium exchange and downfield chemical shift into the region near the glycosyl ring protons, make these methylene protons difficult to observe under the best conditions. Thus, glycosyl composition, glycosyl linkage, mass spectrometry, and NMR analyses support the conclusion that OS4 is a version of the tetrasaccharide that contains either a 4,7- or a 4,8-anhydro-Kdo residue at its reducing end; *i.e.* α-Gal-(1→6)-[α-GalA-(1→4)]-α-Man-(1→5)-[4,7- or 4,8-anhydro]-Kdo.

Characterization of the Core Oligosaccharides Purified from the LPSs of R. etli Mutants—Fig. 3*B* shows the HPAEC profile of the mild acid hydrolysates from mutant CE358. The HPAEC profiles for strains CE350, CE357, CE356, CE121, CE359, and CE360 were identical to that of CE3 (Fig. 3*A*). For strain CE358, OS3 and 4 were replaced by OS6. The relatively short retention time of OS6 indicates that it is not as acidic as the other oligosaccharide components. This was confirmed by chemical analysis which is described further below.

As in the CE3 parent LPS, the GalA/tetramer/trimer ratio for all the mutant LPSs, except that from CE358 in which OS6 replaces OS3 and OS4, is 1:1:1. Thus, of the strains examined in this report, only strain CE358 (from complementation group I) appears to be altered in the core oligosaccharides that are released from the LPS by mild acid hydrolysis.

Negative ion FAB-MS analysis (spectrum not shown) of the mild acid hydrolysate of CE358 LPS gave two major ions; one of $[M\text{-}H]^-$ m/z = 589 which was due to the $GalA_2Kdo$ trisaccharide (OS5, Structure II), and one of $[M\text{-}H]^-$ m/z = 561 which was consistent with a trisaccharide consisting of 2 hexosyl (Hex) and 1 Kdo residues, Hex_2Kdo. These results were confirmed by ES-MS analysis of the reduced permethylated oligosaccharides, Fig. 5.

The Hex_2Kdo trisaccharide (OS6) was isolated by HPAEC and its structure deduced by glycosyl composition, linkage, and NMR analyses. Composition analysis showed that it consisted of Gal, Man, and Kdo. Glycosyl linkage analysis of the neutral sugars showed the presence of terminal Gal and 6-linked Man. The proton NMR spectrum of OS6 (spectrum not shown) matched that previously reported for a Hex_2Kdo trisaccharide from another α region mutant, CE109 (18). This result was consistent with the fact that HPAEC analysis of the mild acid hydrolysate of CE109 LPS also showed the presence of OS6 (data not shown). Thus NMR, FAB-MS, and HPAEC analyses strongly suggest that OS6 from CE358 has the same structure

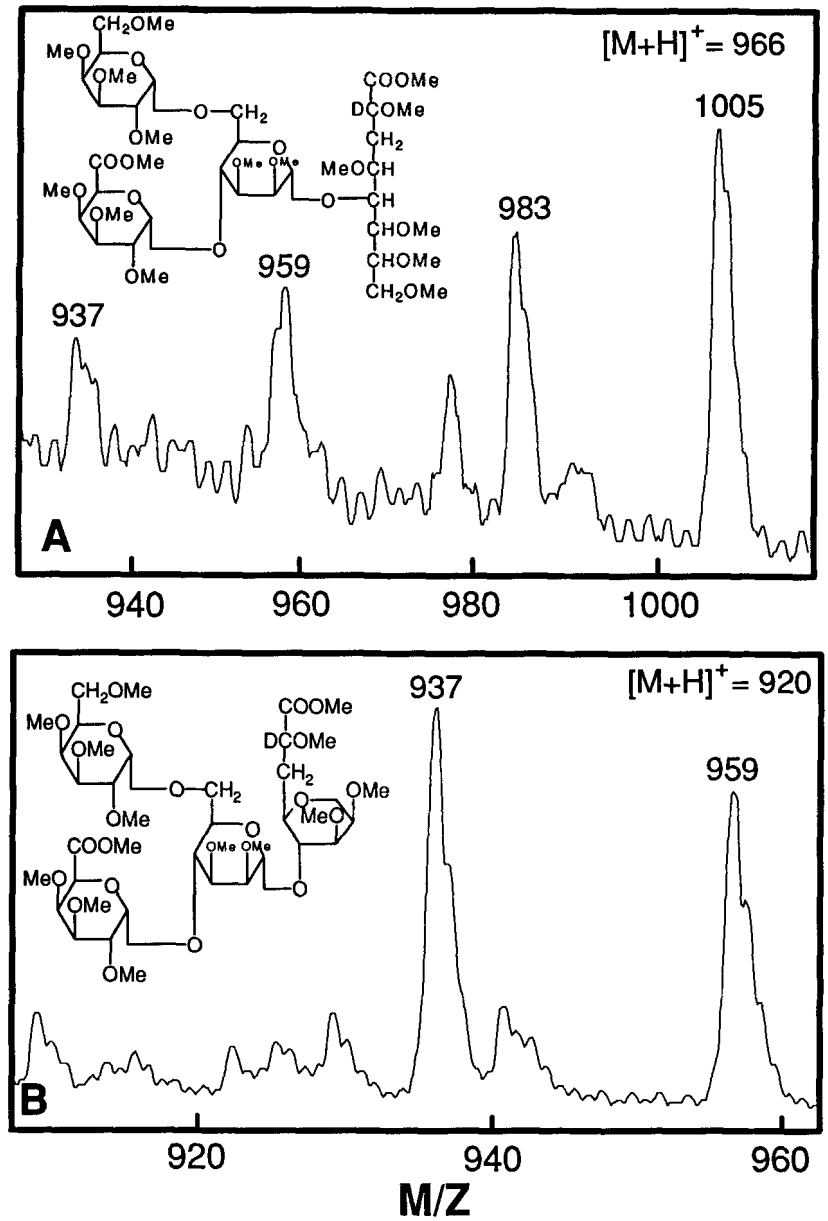

FIG. 4. **Analysis by ES-MS of the purified OS3 (*A*), and OS4 (*B*), after reduction with $NaBD_4$ and permethylation.**

as that previously reported (9, 18) CE109 trisaccharide; namely, α-Gal-(1→6)-α-Man-(1→5)-Kdo.

Analysis of the Intact LPSs from the R. etli Mutants—The above results showed that only one mutant, CE358, was altered in the core oligosaccharides released by mild acid hydrolysis of its LPS. However, it was necessary to examine the mutant LPSs prior to mild acid hydrolysis in order to determine if there were other differences that may not have been detectable due to the mild acid hydrolysis conditions.

Small amounts of the LPSs were methylated and carboxymethyl reduced. This was followed by the preparation and GLC-MS analysis of the PMAAs. Fig. 6 shows the results for the LPSs from strains CE350, 357, and 358 which represent the three types of LPSs observed. The LPSs from the other mutants had the same glycosyl linkages as shown for strain CE357. Peak 1, which was present only in the LPS from strain CE350, had a retention time and fragmentation pattern consistent with the PMAA of terminal Gal (m/z = 205, 162, 118, 161). Peak 2 was present only in the LPS from strain CE358 and was due to the PMAA of 6-linked Man (m/z = 233, 189, 162, 118). Peak 3, in the case of CE350 LPS, was due to the PMAA of terminal GalA (m/z = 235, 191, 162, 118) in which the carboxyl group had been reduced with $NaBD_4$ prior to hydrolysis and acetylation. Minor ions of m/z = 233 and 189 were present in peak 3 from CE350 LPS and indicated the presence of small amounts of 6-linked Gal. In the case of the LPSs from CE358 and CE357, as well as the other remaining mutant LPSs, terminal galactose (peak 1) was not detected, and peak 3 consisted of a mixture of the PMAAs derived from 6-linked Gal and terminal GalA; m/z = 235 (233), 191 (189), 162, 118. The ratio of the 233:235 (or 189:191) ion intensities is somewhat reflective of the 6-linked Gal/terminal GalA ratio and was 0.077, 0.15, and 0.23 for the LPSs from CE350, CE357, and CE358, respectively. The larger ratio for CE358 compared with that for CE357 LPS was consistent with the fact that the former LPS lacks one of the GalA residues. Peak 4 was the PMAA of 4,6-linked Man (m/z = 261,118) and was found in the LPSs from all the mutants except CE358 which contained only 6-linked Man (peak 2).

With one exception, these data are consistent with structures of the oligosaccharides released by mild acid hydrolysis. The exception is that the Gal residue in the mild acid released

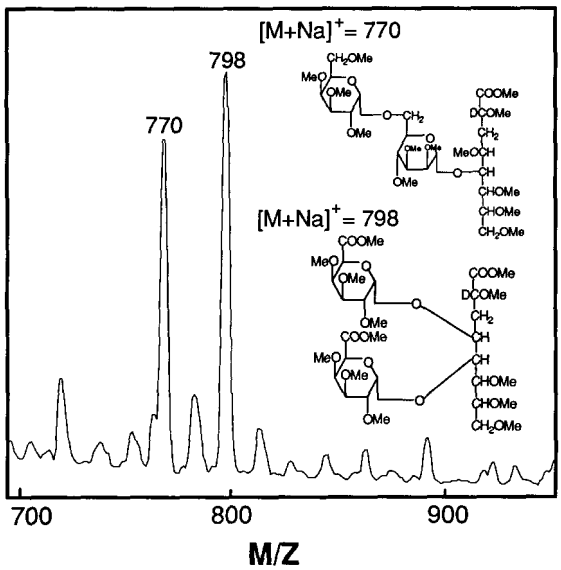

FIG. 5. **Analysis by ES-MS of the core oligosaccharides released from CE358 LPS by mild acid hydrolysis.** The sample was reduced with $NaBD_4$, and permethylated prior to analysis.

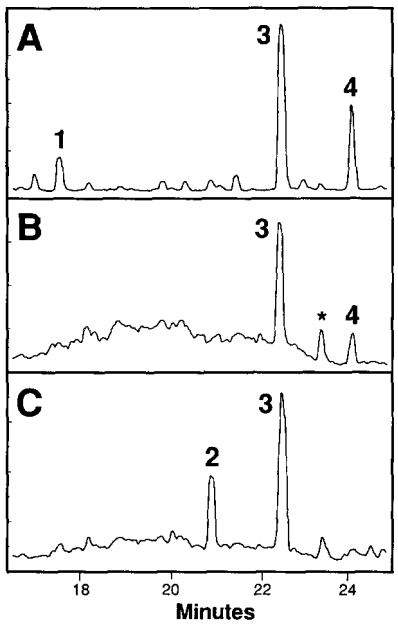

FIG. 6. **The GC profiles of the partially methylated alditol acetates derived from the LPSs from CE350 (*A*), CE357 (*B*), and CE358 (*C*).** Comparison of the retention times to authentic standards and the mass spectral data show that *peak 1* is the PMAA of terminal Gal, *peak 2* of 6-linked Man, *peak 3* of a mixture of 6-linked Gal and terminal GalA, and *peak 4* of 4,6-linked Man. *Peak* * was identified as phthalate, a common contaminant. The remaining small peaks observed are most likely other non-carbohydrate contaminants.

oligosaccharides is terminally linked while it is 6-linked in the intact LPSs, except for that from CE350 in which it is largely terminally linked. Thus, in these LPSs, except for the LPS from CE350, the Gal of the core tetrasaccharide has a mild acid labile residue, presumably Kdo, attached at *O*-6. Since it is known that the *O*-chain polysaccharide purified by mild acid hydrolysis has a Kdo residue at its reducing end, it is likely that it is this residue which is attached to *O*-6 of Gal in these intact LPSs.

The linkages of the Kdo residues for several of the LPSs, *e.g.* from strains CE357 and CE358, were determined by methylation, carboxymethyl reduction (lithium triethylborodeuteride), mild acid hydrolysis (0.1 M trifluoroacetic acid at 80 °C for 30 min), reduction (sodium borodeuteride), normal acid hydrolysis (2 M trifluoroacetic acid at 121° for 2 h), reduction (sodium borodeuteride), and preparation of the PMAAs. Three types of Kdo residues were found in these LPSs; terminally linked Kdo (primary fragments of *m/z* 89, 205, 206, 250, and 366), 5-linked Kdo (*m/z* 89, 206, and 394), and 4,5-linked Kdo (*m/z* 89, and 422). The 4,5- and 5-linked Kdo residues are consistent with the tri- and tetrasaccharide core oligosaccharide structures, and the terminal Kdo is presumably due, in part, to the Kdo residue that is attached to *O*-6 of the core tetrasaccharide Gal residue. Thus, it is likely that the core structure for the LPSs from CE3 and its mutants, except for CE350, contains terminal Kdo attached to *O*-6 of the Gal residue.

In summary, these methylation results from the intact LPSs showed (*a*) that the intact core region of these LPSs contain a Kdo residue attached to *O*-6 of the Gal residue, (*b*) that this Kdo residue is the likely site of *O*-chain attachment, and (*c*) that the LPS from mutant CE350 lacks this Kdo residue.

Alditol acetate analysis of the intact LPSs from CE350, 356, 357, 359, 360, and 121 show that they all contain small amounts of glycosyl residues previously reported (12) to be in the *O*-chain polysaccharide, *e.g.* GlcA, methylated Rha, Fuc, methylated Fuc, and quinovosamine (Qvn). Since PAGE and immunoblot analyses (30) showed that the monoclonals only bind to LPS I, III, IV, or V, and not to LPS II, these results indicate that the minor amounts of LPS III, IV, and/or V that are present in these mutants are forms of the LPS that contain various truncated *O*-chains. This was confirmed by purifying a small amount of LPS IV from strain CE121 using gel filtration chromatography in the presence of DOC. Analysis of this LPS IV showed the presence of GlcA/2-*O*-MeRha/Fuc/2,3-di-*O*-MeFuc/Man/Qvn = 1:1:1:1:1:1. The LPS from the parent strain, CE3, has these sugars in roughly a 4:4:4:1:1:1 ratio with the exception that a 2,3,4-tri-MeFuc residue replaces the 2,3-di-*O*-MeFuc residue. The repeating unit of this *O*-chain is comprised of GlcA/2-*O*-MeRha/Fuc in a 1:1:1 ratio.[2] Thus, the reduced level of these sugars in LPS IV and its faster PAGE mobility compared with that for LPS I are consistent with the concept that LPS IV has a truncated *O*-chain.

DISCUSSION

The results described above suggest that the "rough" LPSs (*i.e.* LPS II) from the parent and mutant strains have the structures shown in Fig. 7. The site of attachment of the core region to the lipid A, and the lipid A structure, have been described in a previous report (11). The LPS II core region is comprised of the previously reported tetra- and trisaccharide molecules (9, 18) (Structures I and II), with a Kdo residue linked to *O*-6 of the tetrasaccharide Gal residue. This Kdo residue may be the site of *O*-chain attachment.

The results described above also show that mild acid hydrolysis releases monomeric GalA from all of the LPSs. Thus, the core region must contain a "GalA-1 → *X*" in which *X* is an unidentified substituent or chemical environment which renders the GalA glycoside bond labile to mild acid. The mechanism by which monomeric GalA is released from the LPS by mild acid hydrolysis is not yet understood.

The precise arrangement of the core components in the intact LPS is not known. It is possible that the tri- and tetrasaccharides are present on two different types of LPS molecules. However, their presence in approximately a 1:1 ratio in both the parent and in all of the mutant LPSs indicates that there may be a single LPS species which contains all of the core elements. Further work is in progress to structurally charac-

[2] R. W. Carlson and U. R. Bhat, unpublished data.

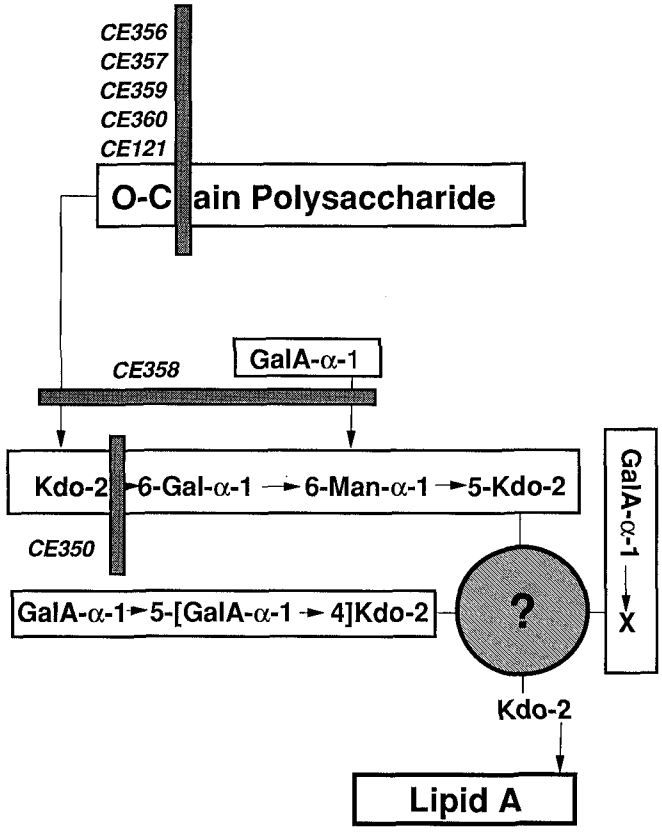

FIG. 7. **A schematic representation showing the possible structure of the LPS from *R. etli* CE3 and its various mutants.** Mutant CE358 lacks the GalA residue α-linked to *O*-4 of Man, as well as the *O*-chain polysaccharide. Mutant CE350 lacks the Kdo residue attached to *O*-6 of Gal. However, this mutant is somewhat leaky in that a small percentage of its LPS contains this Kdo residue as well as a truncated portion of the *O*-chain polysaccharide. Mutants CE356, CE357, CE359, CE360, and CE121 all contain a complete core region as well as various truncated versions of the *O*-chain polysaccharide. The *shaded circle* indicates that it is not yet known how these various core oligosaccharides are linked together in the complete molecule. The *X* represents a moiety or chemical environment which renders the GalA glycoside bond labile to mild acid. The core region is attached via a mild acid labile substituent (presumably Kdo) to *O*-6 of the lipid A GlcN residue (11).

terize the intact core region of these LPSs.

The two mutants which vary in their core structures are CE350 and CE358. The core region from strain CE350 lacks the Kdo residue that is normally attached to O-*6* of Gal, and the core region from strain CE358 lacks the GalA residue that is normally attached to O-*4* of the Man residue. The missing Kdo residue in CE350 LPS suggests that the defective gene in this mutant may encode a specific CMP-Kdo transferase. It should be noted that glycosyl linkage analysis of the CE350 LPS suggests that a small portion of the Gal residues has Kdo at *O*-6. Thus, this mutation does not lead to a complete lack of this Kdo residue. That some of the CE350 LPS molecules have this Kdo residue, and that a truncated version of the *O*-chain is attached to that residue, is supported by the fact that CE350 LPS preparations have LPS III, contain small amounts of *O*-chain sugars, and bind the JIM26 monoclonal antibody. On the other hand the LPS from strain CE358 seems to consist only of LPS II, and completely lacks *O*-chain sugars, suggesting that (*a*) the GalA residue that is missing from the *O*-4 position of the Man residue may be required for transfer of *O*-chain to the core region, and (*b*) that the defective gene in CE358 may encode a UDP-GalA transferase.

Other than those of CE350 and CE358, LPSs analyzed in this study have all of the core components and contain various forms of truncated *O*-chain as evidenced by the presence of typical *O*-chain glycosyl residues. Since all of these mutants are symbiotically defective (14, 16, 30), it is apparent that a complete core region alone is not sufficient for *R. etli* to effectively nodulate its legume host.

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