The Structures of the Lipopolysaccharides from *Rhizobium etli* Strains CE358 and CE359

THE COMPLETE STRUCTURE OF THE CORE REGION OF *R. ETLI* LIPOPOLYSACCHARIDES*

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The structural arrangement of oligosaccharides comprising the core region of *Rhizobium etli* CE3 lipopolysaccharide (LPS) has been elucidated through the characterization of the LPSs from two *R. etli* mutants. One mutant, CE358, completely lacks the O-chain polysaccharide, while the second mutant, CE359, contains a truncated portion of this polysaccharide. This structural arrangement of the core oligosaccharides in these LPSs was determined using electrospray ionization mass spectrometry, tandem mass spectrometry, and methylation analysis. Mild acid hydrolysis of the CE359 LPS produces two major core oligosaccharides: a trisaccharide (1) with the structure α-D-Galp-(1→6)-α-D-GalpA-(1→4)-α-D-Manp-(1→5)-Kdop (where Kdop represents 3-deoxy-D-manno-2-octulosonic acid) and a trisaccharide (2) having the structure α-D-GalpA-(1→4)-α-D-GalpA-(1→5)-Kdop. Structure 1 in CE358 LPS lacks the galacturonosyl residue. Glycosyl linkage and tandem mass spectrometry analyses show that the intact LPS core region consists of trisaccharide (2) attached to O-4 of the Kdo residue in tetrasaccharide 1, and that an additional Kdo residue is attached to O-6 of the galactosyl residue of 1.

\[ \text{Kdop-(2→6)-α-D-Galp-(1→6)-[α-D-GalpA-(1→4)-α-D-Manp-(1→5)]-Kdop-(2→4)} \]

**SCHEME 1. *R. etli* core structure.**

The additional terminally linked Kdo residue is not in close proximity to the lipid A moiety, a unique location for a core Kdo residue. The mutant LPS preparations also contain minor LPS species, one of which lacks the Kdo linked to O-6 of the galactosyl residue, another that lacks the galacturonic acid attached to O-5 of Kdo, and a third that lacks two galacturonosyl residues and one Kdo residue. Thus, in addition to lacking both heptose and phosphate, the *R. etli* LPS core region differs substantially from the typical enterobacterial cores. The abundance of galacturonosyl residues in the *R. etli* core might serve as a suitable functional replacement for phosphate, such as would be predicted for Ca²⁺ binding.

Bacteria belonging to the family *Rhizobiaceae* are Gram-negative and are able to form nitrogen-fixing symbiotic relationships with legume plants. The surface polysaccharides, including the lipopolysaccharides (LPSs),¹ are involved in the normal infection process. Mutants that lack the O-chain polysaccharide portion of their LPSs are symbiotically defective in that they are unable to form normal infection threads (1, 2), and/or they cannot invade the root nodule cells (3–5). In addition, it has been shown that structural changes in the LPS occur during symbiotic infection and that most of these changes appear to take place in the O-chain polysaccharide portion of the LPS (6–14). These structural adaptations in response to the environment of the host plant are likely to be important in order for the symbiont bacterium to induce a nitrogen-fixing nodule.

In addition to the importance of determining the symbiotic “virulence” of these bacteria, rhizobial LPSs can have unique structural features compared with the LPSs from enteric bacteria. The most studied LPSs, the topic of this report, are those from *Rhizobium etli* and *Rhizobium leguminosarum*. All of the LPSs examined from wild-type or parent strains of these species are devoid of phosphate. Their lipid A region has a trisaccharide backbone consisting of one each of galacturonosyl (GalA), GlcN, and 2-amino-octulosonosyl (GlcN-onate) residues, the latter two residues being O- and N-acetylated with β-hydroxy fatty acids and one very long chain fatty acid, 27-hydroxyoctacosanoic acid (15). This lipid A also appears not to carry any acylxoyacyl substituents. The core region has been found to consist of two oligosaccharides (16, 17) as shown below.

\[ α-D-Galp-(1→6)-[α-D-GalpA-(1→4)-α-D-Manp-(1→5)]-Kdop \]

**STRUCTURE 1**

\[ α-D-GalpA-(1→4)-[α-D-GalpA-(1→5)]-Kdop \]

**STRUCTURE 2**

* The abbreviations used are: LPS, lipopolysaccharide; GalA, galacturonosyl; Kdo, 3-deoxy-D-manno-2-octulosonic acid; DOC, deoxycholate; PAG, polyacrylamide gel electrophoresis; TEA, triethylamine; GlcN-onate, 2-amino-2-deoxyglucurononic acid; QuiNAc, 2-acetylquinovosamine; HPAEC, high performance anion exchange chromatography; PMAA, partially methylated alditol acetates; GLC-MS, gas-liquid chromatography-mass spectrometry; ESI-MS, electrospray ionization-mass spectrometry; MS/MS, tandem mass spectrometry.

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The core region of *R. etli* and *R. leguminosarum* LPSs does not contain heptose, a common glycosyl residue found in the inner core region of enteric LPSs. Thus, although the individual core components have been isolated and characterized, the arrangement and proportions of these components in the intact LPS from the various *R. etli* strains was unknown and is the subject of this report.

The unique structural features of the *R. etli* LPSs have also prompted an investigation into the mechanism by which it is synthesized. That work began by determining whether or not *R. leguminosarum* or *R. etli* contained any of the lipid A biosynthetic enzymes found in *Escherichia coli*. It was found that *R. leguminosarum* and *R. etli* contain all of the enzymes necessary to make the *E. coli* lipid A precursor, Kdo<sub>2</sub>lipid-IVa (a di-Kdo-sylated tetraacyl-1,4'-bis-phosphorylated glucosamine disaccharide) (18). Therefore, this component, or a close structural analog, is a common precursor required for the synthesis of *E. coli*, *R. etli*, and *R. leguminosarum* lipids A. This result implies that the biosynthetic steps leading to Kdo<sub>2</sub>lipid-IVa are common to a very wide range of Gram-negative bacteria. The fact that this precursor is essential for cell viability in *E. coli* (19, 20) suggests that it, or a similar rhizobial structural analog, may be required for the viability of the *R. etli* and *R. leguminosarum* cells. The presence of this common lipid A precursor also implied that these rhizobial cells possess unique enzyme activities, which process their Kdo<sub>2</sub>lipid-IVa analog into the mature rhizobial lipid A structure. Several of these enzyme activities have been reported, including a membrane-bound phosphatase that removes the 4'-phosphate from Kdo<sub>2</sub>lipid-IVa and requires the presence of the Kdo residues for maximum activity (21), a second phosphatase that removes the 1-phosphate (22), and a unique acyl carrier protein required for the transfer of the 27-hydroxyoctacosanol residue to the lipid A (23). In addition, a rhizobial-specific mannosyl transferase activity has been identified that transfers mannosyl from GDP-Man to Kdo<sub>2</sub>lipid-IVa (24). It is apparent from these reports that *R. leguminosarum* and *R. etli* contain novel enzymes that can modify the enteric lipid A precursor, Kdo<sub>2</sub>lipid-IVa.

To fully understand how the unique *R. etli* and *R. leguminosarum* LPSs are biosynthesized and to understand their role in determining symbiotic virulence, it is necessary to characterize the structures. Thus, while the types of structures found in the core region, e.g. oligosaccharides 1 and 2, had been determined, as well as the lipid A structure, it was not known how these structures were arranged in the intact LPS. In this report, we describe the structures of the LPS from two *R. etli* mutants, CE358 and CE359, which either lack the O-chain polysaccharide entirely, or contain a truncated version of the O-chain (17). A previous report showed that mild acid hydrolysis of the LPS from CE359 produced core oligosaccharides 1 and 2 as well as monomeric Kdo and galacturonic acid (17). The LPS from CE358 produced the same components except that structure 1 lacked the galacturonosyl residue (17). In this report, the complete structures of the lipid A-core region for the LPSs from these two *R. etli* CE3 mutants are described.

**Experimental Procedures**

**Bacterial Strains**—*R. etli* CE3 (the parent strain) and the *R. etli* mutants CE358 and CE359 were grown in a tryptone/yeast extract supplemented with Ca<sup>2+</sup> as described previously (25). Bacteria were harvested by centrifugation at late log/early stationary phase.

**Lipopolysaccharide Purification**—The parent strain, CE3, and the *R. etli* mutant strains CE358 and CE359, were extracted using the hot phenol-water procedure as described previously (25, 26). Polycrylamide gel electrophoresis (PAGE) in the presence of deoxycholate (DOC) (27, 28) of the water and phenol fractions indicated that LPS was recovered predominantly in the water layer in the case of strain CE3, while the two mutant strains yielded LPS in both the phenol and water layers. Water and phenol layers containing LPS were dialyzed and treated sequentially with ribonuclease, deoxyribonuclease, and proteinase K and then dialyzed and subjected to further purification by either gel filtration chromatography on Sepharose 4B (25, 29) or by affinity chromatography on columns of polymyxin-agarose (Detoxi-Gel, Pierce). For the latter procedure, the created antibodies were used to affinity chromatography as described above and then dialyzed against 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0. Portions of the dialyzed extract (30 ml) were applied to a 10-m1 bed volume column of polymyxin-agarose equilibrated in 50 mM NH<sub>4</sub>HCO<sub>3</sub>. The entire extract was recirculated through the column overnight at a flow rate of 5.0 ml/h. Following recirculation, the flow-through fraction was collected, passed through a single tube, and the column was eluted sequentially with solutions of increasing ionic and/or chaotropic strength to elute the weakly bound non-LPS components. The column was eluted first with 300 mM triethylamine/acidic acid buffer (pH 6.4) containing 10% ethylene glycol, followed by 2.0 M urea in 0.1 M NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0. The LPS was then eluted from the column with 1% sodium DOC in 0.1 M NH<sub>4</sub>HCO<sub>3</sub>, pH 6.0, or with 8.0 M guanidine HCl. Fractions (3 ml) were monitored for LPS and other components by DOC-PAGE analysis with silver staining (28).

Typically, when extracted using the traditional phenol-water procedure, many of the *R. etli* mutants show relatively low yields of LPS compared with that from the parent strain. An alternative extraction procedure was therefore developed and used for the CE358 and CE359 mutant preparations with a setup (30, 31). Typically, 80–100 g (dry weight) of lyophilized cells were extracted with 500 ml of buffer solution with stirring for 1 h at 60 °C. The resulting extract was centrifuged (15,000 × g), yielding a pellet of cell residue and an aqueous supernatant containing LPS, which was dialyzed and treated with enzymes as described above. The aqueous extract was then subjected to polymyxin-agarose affinity chromatography as described above.

**Purification of the Core Oligosaccharides**—Intact LPS was subjected to mild hydrolysis by treating with 1% acetic acid for 1 h at 105 °C (30). The lipid A precipitate was removed by centrifugation, and the supernatant, containing water-soluble core fragments and an O-chain polysaccharide, was analyzed by HPARC on a Carbox PE PA-1 column (Dionex) equipped with a pulse amperometric detection. Separation was achieved using a gradient of 3–80% sodium acetate (1 M) in 100 mM NaOH over 50 min at 1 ml/min (17). The oligosaccharide fractions were collected, passed through Dionex OnGuard H cartridges to remove sodium, and then lyophilized.

**De-O-acylation of *R. etli* Mutant LPS**—Each purified LPS from *R. etli* mutant strains CE358 and CE359 was de-O-acylated with sodium methoxide (0.25 M) at 35 °C for 2 h (15). The reaction mixture was centrifuged (3000 × g), and the supernatant was removed and analyzed for released fatty acids. The precipitate, containing de-O-acylated LPS, was dissolved in water, adjusted to pH 4.0 with dilute acetic acid, and washed two times with hexane/chloroform 1:1 (v/v) to remove residual fatty acids. The resulting de-O-acetylated LPS was then desalted by sequential dialysis (MWCO 1000) and treatment with Dowex 50 or Chelex (bond in the H<sup>+</sup> form) and subjected to electrospray ionization mass spectrometry analysis. In some cases, the de-O-acetylated LPS was converted to the ammonium form (ROO′-NH<sub>3</sub>) by treating with Chelex (NH<sub>4</sub>)<sup>+</sup> to assist in identification of the charge state of ions during ESI-MS analysis.

**Analysis of Glycosyl Residues**—Glycosyl compositions of intact and de-O-acetylated LPS were determined by preparation of the trimethylsilyl methyl glycosides with GLC-MS (electron impact) analysis (31). The purified LPS and de-O-acetylated LPS were subjected to methanalysis in methanolic 1 M HCl at 80 °C for 18 h, N-acetylated, trimethylsilylated, and then analyzed using a 30-m DB-5 fused silica capillary column (J & W Scientific). Linkage analysis of neutral sugars was determined by permethylation (Hakomori method), conversion to the partially methylated alditol acetates (PMAAs) as described previously (31), and GLC-MS analysis using a 30-m SP2330 (Supelco) capillary column. The Kdo and uronic acid linkages were identified by sequential permethylation, reduction of the carboxymethyl groups with lithium triethylborohydride (Superdeuteride, Aldrich), mild hydrolysis (0.1 M trifluoroacetic acid, 100 °C, 30 min) to cleave ketoacid linkages, reduction (NaBD<sub>4</sub>), normal hydrolysis (2 M trifluoroacetic acid, 121 °C, 2 h), and conversion to the PMAAs and GLC-MS analysis (17). The identification of the trimethylsilyl methyl glycoside and PMAA derivatives were also confirmed by chemical ionization, using a 30-m DB-1 column and ammonia as the reactant gas.

**Fatty Acid Analysis**—Ester-linked fatty acids, released from the LPS by de-O-acetylation as described above, were subjected to methanalysis and trimethylsilylation and analyzed by GLC-MS using a 30-m DB-1
RESULTS

Purification of R. etli LPSs and Initial Characterization—The parent strain, CE3, was extracted with the hot phenol/water procedure, and the LPSs were purified by the standard technique of size exclusion chromatography on Sepharose 4B (25, 26, 29). DOC-PAGE analysis showed the presence of typical high molecular weight LPS (LPS I, containing O-chain polysaccharide) and low molecular weight LPS (LPS II, which lacks the O-chain) and various LPSs that carry a truncated O-chain (12, 17). As described in previous reports (12, 17), mutant CE358 produced only one type of LPS, having an electrophoretic mobility identical to LPS II. Mutant CE359 also produced CE359 LPS.

To further improve the yields of mutant LPSs, several alternative extraction procedures were examined that minimized or eliminated the use of phenol. It was found that extraction with a solution of TEA-EDTA-5% phenol (see “Experimental Procedures”) produced relatively high yields of LPS, which could be directly applied to the polymyxin column. Using this extraction procedure combined with polymyxin-agarse chromatography, approximately 45 mg of LPS II could be obtained from 50 g (dry weight) of CE358 cells, and 24 mg of LPS II could be obtained from 50 g (dry weight) of CE359 cells. It was further noted that extraction of the CE359 mutant with the TEA-EDTA protocol yielded preparations highly enriched in LPS II, with essentially no LPS V. In contrast, extraction by the standard phenol/water procedure yielded mixtures of LPS II and V in the phenol layer (as shown in Fig. 1) and preparations enriched in LPS V in the water layer. The different extracts were purified separately by polymyxin affinity chromatography and subjected to structural analysis.

The glycosyl composition of the R. etli strains examined in this study is listed in Table I. The results show the presence of the O-chain glycosyl components in the CE3 parent strain LPS and, to a lesser extent, in the CE359 mutant. The CE358 mutant has an LPS that is devoid of all O-chain glycosyl components. The composition of the O-chain repeating unit of the parent strain has been reported (33, 34). The complete structure of the lipid A moiety of the parent strain, including the fatty acid composition, has been published previously (15), and composition analysis indicates that the mutant LPSs contain a similar lipid A and fatty acid profile; i.e. they contain galacturonic acid, glucosamine, and 2-amino gluconic acid in the lipid A backbone as well as the previously reported 3-hydroxy fatty acids.

column (15). Total fatty acids were released by hydrolysis in 4 M HCl followed by methanolysis, trimethylsilylation, and GLC–MS analysis as above. Amide-linked fatty acids were determined by mild methanolation, trimethylsilylation, and analysis of the resulting N-acylglucosamine methyl glycosides by GLC–MS as described (15, 32).

Mass Spectrometry—Electrospray ionization mass spectrometry (ESI-MS) was performed on a SCIEX API-III triple-quadrupole mass analyzer (PE/SCIEX Thornhill, Ontario, Canada) operated in the positive ion mode with an orifice potential of 50–100 V (15). Spectra were accumulated from 10–60 scans collected over the m/z range 200–2400 with a mass step of 0.2–1.0 atomic mass unit at 1 ms/step. Samples were analyzed at a concentration of 2 μg/μl with a flow rate of 3–5 μl/min using a solution of 15% (v/v) methanol in deionized water containing 0.5% (v/v) acetic acid. Tandem mass spectrometry (MS/MS) was performed on the SCIEX instrument by selecting a parent ion for collision-induced dissociation using argon as the collision gas. The parent ions were detected using the following average incremental mass values, based on the predicted molecular weight, which carries a truncated O-chain (i.e. LPS III, IV, and V) and various LPSs that carry a truncated O-chain (12, 17). As described in previous reports (12, 17), mutant CE358 produced only one type of LPS, having an electrophoretic mobility identical to LPS II. Mutant CE359 also produced CE359 LPS. The glycosyl composition of the R. etli strains examined in this study is listed in Table I. The results show the presence of the O-chain glycosyl components in the CE3 parent strain LPS and, to a lesser extent, in the CE359 mutant. The CE358 mutant has an LPS that is devoid of all O-chain glycosyl components. The composition of the O-chain repeating unit of the parent strain has been reported (33, 34). The complete structure of the lipid A moiety of the parent strain, including the fatty acid composition, has been published previously (15), and composition analysis indicates that the mutant LPSs contain a similar lipid A and fatty acid profile; i.e. they contain galacturonic acid, glucosamine, and 2-amino gluconic acid in the lipid A backbone as well as the previously reported 3-hydroxy fatty acids.

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TABLE I

| Glycosyl residue | CE358 | CE359 | CE3 |
|------------------|-------|-------|-----|
| 2,3,4-MeFuc<sup>a</sup> | 0.1   | 0.1   | 0.1 |
| 3-MeHex<sup>a</sup> | 1.0   | 1.0   | 1.0 |
| Fuc              | 0.1   | 0.1   | 0.1 |
| GlcA             | 0.1   | 0.1   | 0.1 |
| Man              | 2.5   | 2.5   | 2.5 |
| Gal              | 3.0   | 3.0   | 3.0 |
| GalNAc           | 0.1   | 0.1   | 0.1 |
| GlcN             | 1.0   | 1.0   | 1.0 |
| GlcN-olate       | 0.3   | 0.1   | 0.1 |

<sup>a</sup> Ratios are normalized to GlcN and are determined from the GLC–MS total ion current peak areas with response factor correction to authentic standards.
Acids (15). One notable difference in the mutant LPSs compared with that from the parent is the significantly lower level of 2-amino-galactose. This may indicate that a significant portion of the mutant LPSs contain molecules that lack the 2-amino-galactonic acid residue normally present in the lipid A.

Analysis of R. etli Mutant CE358 LPS—The glycosyl composition and linkages of the intact LPS from strain CE358 are reported in Tables I and II, respectively. In addition, a portion of the CE358 LPS was subjected to mild acid hydrolysis, and the resulting oligosaccharides were analyzed by HPAEC (Fig. 2A). The results verify an earlier report (17) showing that the CE358 LPS core region consists of two major oligosaccharide components: trisaccharide 2 (shown above) and trisaccharide 3.

\[ \text{structure 3} \]

In addition to trisaccharides 2 and 3, monomeric Kdo and monomeric galacturonic acid were also released from the CE358 LPS during mild hydrolysis (Fig. 2A). These results suggest that the CE358 LPS core region consists of the two oligosaccharides (2 and 3) connected together in an unknown arrangement with additional Kdo and galacturonosyl residues. Alternatively, the individual oligosaccharide components could be carried separately on individual LPS molecules, each having a similar size and DOC-PAGE mobility.

Linkage analysis of the intact CE358 LPS, prior to mild acid hydrolysis, yielded the following PMMA derivatives in a 1:1:2 ratio (see Table II): 1,5,6-tri-O-acetyl-2,3,4,6-tetra-O-methyl-mannitol (m/z 118, 162, 189, and 233), 1,5,6-tri-O-acetyl-2,3,4,5-tetra-O-methyl-galactitol (m/z 118, 162, 189, and 233), and 1,5,6-tri-O-acetyl-2,3,4,5,6-penta-O-methyl-6,2H-octitol (m/z 118, 162, 191, and 235), derived from the trisaccharide terminating in Kdo at the reducing end, a product of the acid hydrolysis conditions (17). In panel A, the a-Galp-(1→4)-Kdo disaccharide is a small component that elutes at approximately 15 min. In panel C, the components eluting between 2 and 5 min and at 13 min are oligosaccharides derived from the truncated O-chain of the CE359 LPS V.

This result indicates that the terminal galactosyl residue of trisaccharide 3 must be substituted at O-6 in the intact LPS by a mild acid-labile residue, presumably Kdo or an oligosaccharide terminating in Kdo at the reducing end. Methylation analysis of the intact CE358 LPS, according to a procedure modified to detect Kdo linkages (see “Experimental Procedures”) gave the PMMA derivatives of terminal-Kdo (1,2,6-tri-O-acetyl-3-deoxy-4,5,7,8-tetra-O-methyl-1,1-2H-octitol (m/z 89, 146, 205, 206, 250, and 366)), and 15,6-linked Kdo (1,2,4,5,6-penta-O-acetyl-3-deoxy-7,8-di-O-methyl-1,1-2H-octitol (m/z 89, 186, 228, 348, and 422)) (Table II and Fig. 3). These results indicate that a Kdo residue must occupy a terminal (nonreducing) location on the majority of the intact LPS molecules. In addition, the 5-linked Kdo residue present in trisaccharide 3 must also...

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**Table II**

| Glycosyl linkage          | Area ratio CE358 | Area ratio CE359 | Area ratio CE359 |
|---------------------------|------------------|------------------|------------------|
| Terminal rhamnose + Fuc   | 0.1              | 0.1              | 0.1              |
| 3-Linked Fuc              | 0.1              | 0.1              | 0.1              |
| 3,4-Linked Fuc            | 0.2              | 0.2              | 0.2              |
| 4-Linked GlcA             | 0.2              | 0.2              | 0.2              |
| Terminal GlcA             | 0.1              | 0.1              | 0.1              |
| Terminal Gal              | 0.1              | 0.1              | 0.1              |
| 3-Linked Man              | 1.0              | 1.0              | 1.0              |
| 6-Linked Man              | 0.2              | 0.2              | 0.2              |
| 3-Linked QuiNAc           | 0.2              | 0.2              | 0.2              |
| Terminal GalA             | 1.0              | 1.0              | 1.0              |
| 6-Linked Gal              | 0.1              | 0.1              | 0.1              |
| 4,6-Linked Man            | 1.0              | 1.0              | 1.0              |
| Terminal Kdo              | 0.1              | 0.1              | 0.1              |
| 4,5-Linked Kdo            | 0.2              | 0.2              | 0.2              |
| 3,5-Linked Kdo            | 1.4              | 1.4              | 1.4              |

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**Figure 2**

Analysis by HPAEC of the core components released by mild acid hydrolysis of the R. etli mutant LPSs. A, R. etli CE358 LPS; B, CE359 LPS II; C, CE359 LPS V. The structures of tetrasaccharide 1 and trisaccharides 2 and 3 are described in the Introduction and under “Results.” AnhydTetra, the tetrasaccharide 1 containing an anhydro-Kdo residue at the reducing end, a product of the acid hydrolysis conditions (17). In panel A, the a-GalpA-(1→4)-Kdo disaccharide is a small component that elutes at approximately 15 min. In panel C, the components eluting between 2 and 5 min and at 13 min are oligosaccharides derived from the truncated O-chain of the CE359 LPS V.
be substituted at O-4 in the intact LPS, since a derivative arising from 5-linked Kdo, detected during methylation of oligosaccharide 3, was not observed during methylation of the intact LPS. In the intact LPS, this 5-linked Kdo residue of oligosaccharide 3 could be substituted at O-4 by either the Kdo residue of oligosaccharide 2 (yielding 4) or by a single terminal Kdo residue (yielding the alternative structure 5).

$$\text{Kdo-(2\rightarrow6)-}\alpha-D-\text{Galp-(1\rightarrow6)-}\alpha-D-\text{Manp-(1\rightarrow5)-Kdp}(2)$$

\[ \alpha-D-\text{GalpA-(1\rightarrow4)}\{\alpha-D-\text{GalpA-(1\rightarrow5)}\}-\text{Kdp}(2) \]

\[ \begin{align*}
\text{Structure 4} \\
\text{Structure 5}
\end{align*} \]

Other alternative structures would also fit these data, particularly if the two core components, oligosaccharides 2 and 3, were carried separately on different LPS molecules.

Methylation analysis also yielded several minor derivatives, detected at nonstoichiometric levels, presumably arising from small populations of molecules due to glycosyl heterogeneity. These minor derivatives arise from 4-linked Kdo (i.e. $1\text{,}2\text{,}4\text{,}6$-tetra-$O$-acetyl-3-deoxy-5,7,8-tri-$O$-methyl-1,1,2$^3$H-octitol ($m/z$ 89, 205, 278, 320, and 394)), 4,6-di-$O$-substituted mannose (1,4,5,6-tetra-$O$-acetyl-2,3-di-$O$-methyl-mannitol ($m/z$ 118 and 261)), and terminal galactose (1,5-di-$O$-acetyl-2,3,4,6-tetra-$O$-methyl-galactitol ($m/z$ 118, 161, 162, and 205)). As discussed further below, each of these minor derivatives corresponds to minor LPS species identified by mass spectrometry. Thus, the derivatives obtained during linkage analysis of the intact CE358 LPS are consistent with the glycosyl compositions and core oligosaccharide structures determined previously; however, the multiplicity of these derivatives and the overall similarity of the core components detected during HPAEC analysis precluded unambiguous structural assignments for the intact LPS without the aid of molecular mass information.

To distinguish between the alternative structures, a portion of the intact CE358 LPS was subjected to de-$O$-acylation and ESI-MS analysis. The positive ion ESI-MS spectrum is shown in Fig. 4, and the major molecular ions are listed along with their proposed formulas in Table III.
others in the spectrum are doubly charged protonated molecular ions of the general formula (M + 2H)\(^{2+}\). The charge state and type of cationization were readily determined by examination of the incremental differences between various ions in the spectrum and by observing the alterations in m/z values produced during controlled cationization with the addition of ammonium or sodium to the sample. The molecular mass of this species (1918.4 atomic mass units) was then arrived at by multiplying the m/z value by the number of charges (z) and subtracting an equivalent number of protons. This molecular species does not include the 2-amino gluconosyl residue of the lipid A, indicating that it is either absent in a significant portion of the LPS, as indicated from the composition results (Table I) discussed above, or is somewhat labile to the alkaline de-O-acylation conditions. The lability of this residue was indicated in the previously reported fast atom bombardment mass spectrum of the de-O-acylated lipid A from the *R. etli* parent strain (15), in which the major ion observed was that of the molecule without 2-amino gluconate. The major de-O-acylated CE358 LPS carries the 14-carbon chain length fatty acid (\(\beta\)-hydroxyamyrinic acid) in amide linkage to the glucosamine residue of the lipid A backbone. This is in agreement with fatty acid analysis of the CE358 LPS, which shows that the amide-linked fatty acids consist of \(\beta\)-hydroxyamyrinate, \(\beta\)-hydroxy palmitate, and \(\beta\)-hydroxystearate in a 7.6:1.0:1.2 ratio. A similar ratio, in which \(\beta\)-hydroxyamyrinic acid was the predominant amide-linked fatty acid, was observed for the parent strain lipid A (15, 35). A family of minor ions in the spectrum also arises from this same molecule, due to lactone oxonium ion formation with charge retention on the nonreducing end sequences: Kdo-Hex-Hex (m/z 545), Kdo-Hex (m/z 383), Kdo (m/z 221), [GalA]_3 Kdo (m/z 573), and GalA-Kdo (m/z 397).

The major molecular ion at m/z 960.2 (M, 1918) and its associated ions clearly indicate that the major form of the CE358 de-O-acylated LPS carries both core oligosaccharides 2 and 3 together in the same molecule. Molecular ions for individual LPS molecules carrying either trisaccharide 2 or trisaccharide 3 alone were not observed, and such molecules therefore do not contribute to the glycosyl heterogeneity of the CE358 LPS preparation. To distinguish between the resulting structural alternatives (i.e. structures 4 or 5 above), the major molecular ion (m/z 960.2) was selected as parent ion for collision-induced dissociation using the electrospray instrument and argon as collision gas. The resulting fragment ions (Fig. 5) consisted of both Y-type sequence ions (36) resulting from glycosidic bond cleavages, with charge retention on the reducing end moiety, and the complementary B-type sequence ions. The Y-type ions demonstrate the sequential loss of terminal Kdo (yielding m/z 850.2, 1698.4 atomic mass units), followed by loss of hexasaccharide (yielding m/z 769.2, 1536.4 atomic mass units), indicating that Kdo indeed occupies a terminal (nonreducing) position and that a hexosyl residue occupies the penultimate position. These results, in combination with the methylation data, clearly support the core arrangement shown in 4, and indicate that the major de-O-acylated LPS from CE358 has the structure indicated in Fig. 5.

The ESI-MS spectrum of the de-O-acylated CE358 LPS shows, in addition to the m/z 960.2 ion family, several doubly charged molecular ions of minor intensity at m/z 1048.2 (M₂, 2094.4), 872.2 (M₂, 1742.4), 850.2 (M, 1698.2), and 674.0 (M, 1346.0) (see Table III). These minor ions show that there is glycosyl heterogeneity in the CE358 LPS. The m/z 1048.2 ion is consistent with an additional galacturonosyl residue. The presence of this additional residue is supported by the HPAEC analysis of the mild acid released oligosaccharides (Fig. 2A), which shows the presence of a small amount of tetrasaccharide 1, containing galacturonic acid linked to O-4 of the mannosyl residue. As discussed above, methylation analysis (Table II) shows small amounts of 4,6-linked mannoside, which also supports the presence of this structure. The m/z 872.2 ion arises from a structure that lacks one galacturonosyl residue. Again, such a structure is supported by the presence of a small amount of 4-linked Kdo (Table II and Fig. 3) and the HPAEC analysis (Fig. 2A), which indicates a small amount of an oligosaccharide with a retention time corresponding to a disaccharide of \(\alpha\)-GalpA-(1→4)-Kdo. Mild acid hydrolysis of *R. etli* LPS has been shown previously to release a small amount of this \(\alpha\)-GalpA-(1→4)-Kdo disaccharide (17). The minor ion m/z 850.2 indicates the presence of LPS molecules lacking one Kdo residue. Methylation analysis (Table II) again shows the presence of minor amounts of terminally linked galactose, indicating that a small percentage of LPS is present that lacks the Kdo residue that is terminally linked to O-6 of this galactosyl residue. Finally, the minor ion at m/z 674.0 is consistent with the absence of two galacturonosyl and one Kdo residues. This minor species could lack the trisaccharide 2 unit; however, since methylation analysis of this LPS does not reveal any 5-linked...
Kdo, a more likely possibility for the arrangement of this minor species is as follows.

$$\alpha-D-Galp-(1\rightarrow6)-\alpha-D-Manp-(1\rightarrow5)-Kdo \rightarrow Kdo-(2)$$

**Structure 6**

Thus, both methylation analysis of the CE358 LPS and HPAEC analysis of the core components support the identification of minor molecular species having the mass assignments indicated by ESI-MS analysis (Fig. 4 and Table III). That these minor molecular ions result from true glycosyl heterogeneity in the LPS rather than ion fragments formed during electrospray is further supported by the fact that fragmentation of individual maltooligosaccharide standards was not observed under identical ionization conditions. The structures of the minor de-O-acylated CE358 LPS species are shown in Fig. 4.

**Analysis of the R. etli Mutant CE359 LPS**—As described above, mutant CE359 produces two forms of LPS, i.e. LPS II and LPS V, as shown in PAGE analysis (Fig. 1). However, the relative proportion of these two bands varies considerably depending on the extraction procedure as well as on the particular cell preparation. Fig. 1 shows the LPS pattern typically observed from the phenol layer of phenol/water extracts. The water layer of such extracts is often enriched in LPS V, while LPS II-enriched preparations are readily obtained by extraction using the TEA-EDTA protocol. Glycosyl composition analysis (Table I) shows that in addition to the core region glycosyl components found in CE358, the CE359 LPS contains small amounts of glycosyl residues that are typical of the parental O-chain polysaccharide (i.e. fucose, 3-O-methylrhamnose, glucuronic acid, N-acetylgalactosamine, and additional mannos). This result, together with the increased electrophoretic mobility compared with the higher molecular weight LPS I of the parent strain, shows that CE359 LPS V contains a truncated version of the O-chain and is consistent with previous reports showing that LPS V binds monoclonal antibodies that are specific for the O-chain (12).

A portion of the CE359 LPS preparation containing both LPS II and V (Fig. 1) was de-O-acylated, converted to the ammonium form (ROO$^-\mathrm{NH}_4^+$), and analyzed by ESI-MS. The spectrum, Fig. 6, demonstrated the presence of two major families of ions consistent with the presence of the two LPS forms, LPS II and LPS V, as revealed by DOC-PAGE (see Fig. 1). The doubly charged molecular ions are of the general formula (M + 2$\mathrm{NH}_4^+$)$^{2+}$. The ion at $m/z$ 1065.0 thus indicates an (experimental) mass of 2093.9 atomic mass units for the LPS II, and the ion $m/z$ 1554.0 yields an experimental mass of 3071.9 atomic mass units for LPS V. Minor molecular species were also detected, and those results are described below.
Mild acid hydrolysis and HPAEC analysis of the CE359 LPS II obtained by TEA-EDTA extraction yielded five core components with identical retention times to those released from the CE358 core although in different proportions (Fig. 2B). The CE359 LPS II contained relatively equal amounts of oligosaccharides and only small amounts of trisaccharide, which shows the presence of a small amount of terminally linked galactose, supporting the presence of a minor LPS species lacking the Kdo that is terminal with HPAEC analysis (Fig. 2). This minor amount of terminally linked galactose, supporting the minor LPS species at $M_r$ 2094. However, as with the CE358 LPS, 5-linked Kdo was not observed during methylation analysis of CE359 LPS, and therefore the absence of trisaccharide 2 would not appear likely. The minor $m/z$ 762 ion may be a fragment ion or, alternatively, could arise from additional core region heterogeneity as follows.

\[
\text{α-D-Galp-(1→6)-α-D-Manp-(1→5)-Kdo} \rightarrow 4) 4) \uparrow \uparrow \uparrow \alpha-\text{GalpA-1} \text{ Kdo-2}
\]

### Table IV

| Observed ion $^a$ | Experimental mass | Proposed formula $^b$ | Predicted ion $^c$ |
|------------------|------------------|----------------------|-------------------|
| 762 ± 0.5        | 1522 ± 1         | 1522(4H)$_2$         | 762.2             |
| 938              | 1874             | 1874.7(4H)$_2$       | 938.3             |
| 960              | 1918             | 1918.7(4H)$_2$       | 960.4             |
| 1039             | 2076             | 2076.8(4H)$_2$       | 1039.4            |
| 1040             | 2094             | 2094.8(4H)$_2$       | 1040.4            |
| 1057             | 2095             | 2094.8(NH$_3$)$_2$   | 1057.0            |
| 1065             | 2094             | 2094.8(NH)$_2$       | 1065.5            |
| 1062             | 2122             | 2122.9(4H)$_2$       | 1062.5            |

$^a$ The maximum accuracy of each ion assignment is ±0.5 atomic mass unit, which results from the incremental scan step setting of 1.0 atomic mass unit used during acquisition.

$^b$ The calculated mass for the proposed LPS species (see "Results"). The charge state and type of cationization were determined as described in the text.

$^c$ The predicted doubly charged ion based on the calculated mass.

The types of de-O-acylated structures obtained from the CE359 LPS II fraction are summarized in Fig. 7. The CE359 LPS V-enriched fraction, obtained from water layers of phenol/water extracts, was also further characterized. Mild acid hydrolysis of the LPS V and HPAEC analysis yielded the profile in Fig. 2C. The results show that tetrasaccharide 1, trisaccharide 2, and monomeric galacturonic acid are again major core components as with the CE359 LPS II. However, a decrease in monomeric Kdo was observed in comparison with that released from CE359 LPS II or that from CE358 LPS. In addition, several new components, not found in CE359 LPS II, were observed during HPAEC analysis (Fig. 2C). These new components have mobilities identical to those of O-chain oligosaccharides derived from the LPS of the parent strain after mild acid hydrolysis (not shown). Composition analysis of these oligosaccharides showed that they contain glycosyl residues characteristic of the O-chain polysaccharide (i.e., mannose, N-acetylglucosamine, fucose, 3-O-methylrhamnose, glucuronic acid, and Kdo). Methylation analysis of the intact CE359 LPS (Table II) also showed the presence of O-chain glycosyl linkages, namely terminal rhamnose, 3,4-linked fucose, 4-linked glucuronic acid, 3-linked mannose, 3-linked N-acetylglucosamine, and 4-linked Kdo. These derivatives were also observed during methylation analysis of the intact parent strain LPS (Table II) and of the intact purified O-chain, recovered after mild hydrolysis of the parent strain LPS (results not shown).

ESI-MS analysis of the de-O-acylated CE359 LPS V produced the positive ion spectrum shown in Fig. 8, and the molecular ions and proposed compositions are listed in Table V. The sample yields doubly charged protonated molecular ions ([M + 2H]$^{2+}$) at $m/z$ 1048, indicating a mass of 2094.8 atomic mass units. This species is 176 mass units greater than the major CE358 LPS, indicating the presence of an additional galacturonic acid residue on the CE359 LPS II. This is consistent with HPAEC analysis (Fig. 2), which shows that tetrasaccharide 1, carrying the GaLA residue, is a major core component of the CE359 LPS II in place of trisaccharide 3, which lacks this GaLA residue and is found in the CE358 LPS II. Methylation analysis of CE359 LPS confirmed these results in that the major mannose residue was 4,6-linked mannose instead of the 6-linked mannose residue found in CE358 LPS. MS/MS analysis of the $m/z$ 1048 ion (data not shown) supported the same glycosyl sequence as that observed for CE358 LPS with the exception that CE359 LPS II contains the additional galacturonic acid residue linked to the O-4 of mannose.

Glycosyl heterogeneity in the CE359 LPS II family was evident from several minor ions in the ESI-MS spectrum (Fig. 7, Table IV), at $m/z$ 960 ($M_r$ 1918), at $m/z$ 938 ($M_r$ 1874, and at $m/z$ 762 ($M_r$ 1522). The $M_r$ 1815 LPS is a minor species that lacks a galacturonic acid residue and is likely to have the same structure as the major LPS species described above for CE358; i.e., the core region lacks the GaLA residue attached to O-4 of mannose. This is supported by methylation analysis of the CE359 LPS, which, in addition to 4,6-linked mannose, shows small amounts of 6-linked mannose (Table II), and by HPAEC analysis (Fig. 2B), which shows the presence of a small amount of trisaccharide 3. The $M_r$ 1874 LPS species is 220 mass units less than the major CE359 LPS II species and therefore lacks a Kdo residue. Methylation analysis of the CE359 LPS yielded a minor amount of terminal linked galactose, supporting the presence of a minor LPS species lacking the Kdo that is terminal with HPAEC analysis (Fig. 2). This minor amount of terminal linked galactose, supporting the minor LPS species at $M_r$ 2094. However, as with the CE358 LPS, 5-linked Kdo was not observed during methylation analysis of CE359 LPS, and therefore the absence of trisaccharide 2 would not appear likely. The minor $m/z$ 762 ion may be a fragment ion or, alternatively, could arise from additional core region heterogeneity as follows.

\[
\text{α-D-Galp-(1→6)-α-D-Manp-(1→5)-Kdo} \rightarrow 4) 4) \uparrow \uparrow \uparrow \alpha-\text{GalpA-1} \text{ Kdo-2}
\]
The complete core region structure of the *R. etli* LPS is shown in Fig. 9, which shows the structural arrangement of the core oligosaccharides isolated previously, together with the proposed structure for the truncated O-chain. Fig. 9 also indicates the structural defects found in the LPSs from various *R. etli* mutants based on the results reported here and on previous reports (16, 17, 37).

The results described in the present study clearly show that the previously reported core oligosaccharides from *R. etli* CE3 (16, 17), i.e. structures 1 and 2, are carried together on a single LPS molecule and are arranged as shown in Fig. 9. The data do not support the idea that there are two separate LPS species, one with structure 1 as the core region, and a second with structure 2, in which the former contains the O-chain polysaccharide. The results also show that the mutant LPSs examined in this report contain a number of minor LPS species due to heterogeneity in both the core region, particularly in the case of CE358 LPS, and in the truncated O-chain of CE359 LPS V. In addition, it was previously shown that mild acid hydrolysis of the LPSs from *R. leguminosarum* bv. trifolii and viciea strains yields core oligosaccharides identical to those obtained from *R. etli* CE3 LPS (16, 17, 38–40), strongly suggesting that the *R. leguminosarum* LPS core region is identical to that shown in Fig. 9 for *R. etli*. The identity of the *R. leguminosarum* LPS core with that of *R. etli* CE3 is also supported by the fact that monoclonal antibodies to the core region of an *R. leguminosarum* bv. viciea LPS cross-react with equal affinity to the LPSs from numerous strains of *R. leguminosarum* bv. trifolii, bv. viciea, and *R. etli* CE3 (41). Thus, it is proposed that this core structure is common to the LPSs from strains of *R. leguminosarum* and *R. etli*.

The core region of *R. etli* LPS differs in several respects from the typical cores of enterobacterial LPS, specifically in the absence of both heptose and phosphate, and notably in the location of a Kdo residue in the O-chain attachment region, removed four residues distally from the lipid A moiety. This Kdo residue serves as the linking residue between the O-chain and the core region. Mild acid hydrolysis of *R. etli* LPS and isolation of the resulting O-chain by size exclusion chromatography indicate that this 4-linked Kdo residue occupies the reducing end of the polysaccharide and is readily reduced with borodeuteride to a stable 4-linked Kdo alditol. As the structure of the complete *R. etli* LPS carbohydrate backbone becomes more clear, it appears that both an inner and outer core region can be distinguished, analogous to the enterobacterial LPS. The *R. etli* and *R. leguminosarum* common inner core contains Kdo, mannose, galactose, and an abundance of galacturonic acid, the latter probably serving as a substitute for phosphate found in the enteric LPS core regions. The resulting inner core would have a high negative charge density, allowing the LPS to stabilize in the membrane in the presence of Ca\(^{2+}\) and other divalent cations, such as proposed for the enteric LPS inner core (42, 43). It is well documented that pectic polysaccharides rich in GalA exhibit a high degree of cross-linking in the presence of Ca\(^{2+}\) (44). The outer core region of the *R. etli* LPS is the O-chain attachment region and consists of one residue each of 3-linked mannosyl, 3-linked N-acetylenolaminosyl, and 4-linked Kdo residues. This region contains considerably less hexose than the hexose-rich outer cores found in many enteric LPS. We suggest that the *N*-acetylenolaminosyl residue may be a signal for proper O-chain attachment or elongation, analogous to the role proposed for fructose and sedoheptulose in *Vibrio cholerae* LPS (45, 46) or for the core modifications

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proposed for *Salmonella* and *Shigella* (47, 48). Other structural features of the core region are undoubtedly required for *O*-chain attachment in *R. etli* and *R. leguminosarum* and are currently under study in other *R. etli* mutants.

Fig. 9 also shows the proposed structure of the truncated *O*-chain in the LPS from mutant CE359. This structure is based on the ESI-MS data and linkage analysis of the de-*O*-acylated LPS described in this report as well as on unpublished data for the *O*-chain repeating unit from the parent strain. The single residues of 3-linked mannose and 3-linked *N*-acetylquinovosamine are not part of the repeating unit and, as discussed above, are defined as an outer core region. Both residues are carried in the polysaccharide portion released by mild acid hydrolysis of the parent strain LPS; the relative order of these residues with respect to the reducing end 4-linked Kdo residue will be determined by MS/MS analysis of the *O*-chain polysaccharide. While the order of these residues is not known with certainty, preliminary results indicate that a portion of the reducing Kdo residues are destroyed during mild acid hydrolysis and that subsequent reduction of the *O*-chain polysaccharide with boro(deuteride) yields the deuterated alditols of Kdo and, to a lesser extent, QuiNAc, suggesting a penultimate location for this residue as indicated in Fig. 9.

Previous *1H* NMR analysis (16, 37) of the core oligosaccharides 1 and 2 has shown that all of the glycosyl residues, with the exception of the Kdo residues, are *α*-linked as shown in Fig. 9. The anomeric configurations of the Kdo residues have not yet been determined in the intact LPS; however, they are shown as *α*-anomers, analogous to the Kdo of LPS from enteric bacteria. The configurations of the lipid A glycosyl residues were previously determined by *1H* NMR (15). The anomeric configurations of the *O*-chain glycosyl residues are based on preliminary results obtained from chromium trioxide oxidation and will be described in more detail in a forthcoming report on this polysaccharide.

An interesting feature of these *R. etli* LPSs is the fact that mild acid hydrolysis releases monomeric galacturonic acid. In a previous report (17), it was suggested that this may be due to an additional galacturonosyl residue (*i.e.* additional to those found in structures 1 and 2) that is attached in some acid-labile manner to the core region. However, the ESI-MS results reported here clearly show that the de-*O*-acylated LPSs from

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CE358 and CE359 do not contain any additional galacturonosyl residues. Furthermore, the alkaline conditions of the de-O-acetylation experiment do not cause the release of galacturonic acid from the LPS (data not shown). Thus, it is concluded that the CE3 lipid A-core structure shown in Fig. 9 serves as the foundation for the attachment of the O-chain polysaccharide. In order for symbiosis to be successful, it is necessary that the O-chain be polymerized (i.e., it cannot be a truncated version) and that the polymerized O-chain be present in sufficient amounts (i.e., a sufficient percentage of the LPS molecules must contain the O-chain polysaccharide). In addition, it is known that modification to the O-chain occurs during symbiotic infection. Knowledge of this LPS structure will greatly facilitate our further characterization of the LPS modifications required for symbiosis.

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