Genetic and Structural Characterization of L11
Lipooligosaccharide from \textit{Neisseria meningitidis}
Serogroup A Strains

The lipooligosaccharide (LOS) of immunotype L11 is unique within serogroup A meningococci. In order to resolve its molecular structure, we conducted LOS genotyping by PCR analysis of genes responsible for \(\alpha\)-chain sugar addition (\(lgtA, -B, -C, -E, -H\), and -I) and inner core substituents (\(lgtG, lpt-3\), and \(lpt-6\)). For this study, we selected seven strains belonging to subgroup III, a major clonal complex responsible for meningococcal meningitis epidemics in Africa. In addition, we sequenced the homopolymeric tract regions of three phase-variable genes (\(lgtA, lpt-3\), and \(lpt-6\)) to predict gene functionality. The fine structure of the L11 LOS of each strain was determined using composition and glycosyl linkage analyses, NMR, and mass spectrometry. The masses of the dephosphorylated oligosaccharides were consistent with an oligosaccharide composed of two hexoses, one \(N\)-acetyl-hexosamine, two heptoses, and one KDO, as proposed previously. The molar composition of LOS showed two glucose residues to be present, in agreement with \(lgtH\) sequence prediction. Despite phosphoethanolaminetransferase genes \(lpt-3\) and \(lpt-6\) being present in all seven \textit{Neisseria meningitidis} strains, phosphoethanolamine (PEtn) was found at both O-3 and O-6 of HepII among the three ST-5 strains, whereas among the four ST-7 strains, only one PEtn was found and located at O-3 of the HepII. The L11 LOS was found to be \(O\)-acytelylated, as was indicated by the presence of the \(lot-3\) gene being in-frame in all of the seven \textit{N. meningitidis} strains. To our knowledge, these studies represent the first full genetic and structural characterization of the L11 LOS of \textit{N. meningitidis}. These investigations also suggest the presence of further regulatory mechanisms affecting LOS structure microheterogeneity in \textit{N. meningitidis} related to PEtn decoration of the inner core.

\textit{Neisseria meningitidis} is a major cause of bacterial meningitis and septicemia worldwide, especially in the African “meningitis belt,” where serogroup A has been responsible for most of the recurrent epidemics (1). From an epidemiological perspective, meningococci can be classified into lineages, termed clonal complexes, by multilocus sequence typing based on seven loci. A clonal complex is a group of sequence types (STs)\(^1\) that shares at least four of seven loci in common with a central “ancestral genotype,” after which the complex is named (2). In the last two decades, most serogroup A meningococci isolates have been from the genetic clonal complex termed subgroup III, as determined by multilocus enzyme electrophoresis (3–5). Epidemics have been caused mainly by strains belonging to sequence type 5 (ST-5), ST-7, or ST-2859, as determined by multilocus sequence typing (2–4, 6, 6, 7).

LOS is an important virulence factor for \textit{N. meningitidis}. Meningococcal LOS consists of an oligosaccharide (OS) component linked to lipid A via one of the two 3-deoxy-2 keto-\(\beta\)-manno-octulosonic acid (KDOs). In contrast to the LOS of many enteric bacteria, \textit{N. meningitidis} LOS lacks the highly repetitive sugar side chains but possesses variable OS chains. Meningococcal LOS is heterogeneous, and the expression of LOS in the bacteria is subject to phase variation. \textit{Neisseria meningitidis} isolates express 12 immunologically distinct LOS structures (L1–L12) that were subsequently shown to correspond to distinct chemical structures (8–12). L1–L8 immunotypes are found in group B and group C strains (13, 14), and L9 is shared by group A, B, and C (15). The L10–L12 immunotypes are uniquely associated with serogroup A strains (15).

Each LOS immunotype structure has a conserved heptose inner core to which \(\alpha\), \(\beta\), and \(\gamma\)-chains are added (16, 17). The length and nature of oligosaccharide extension from the proximal heptose residue (HepI) and the presence or absence of inner core substituents on the distal HepII residue defines the immunotype. Multiple genes encoding glycosyltransferases are involved in biosynthesis of meningooccal LOS (Fig. 1), with most located within the genomic regions \(lgt-1\), \(lgt-2\), and \(lgt-3\). The genes responsible for expression of the sugars in the LOS chains are designated \(lgtA, -B, -C, -D, -E, -F, -G\), and \(H\) (16). The genes \(lgtA, lgtD\), and \(lgtC\) are responsible for \(\alpha\)-chain synthesis (18, 19), whereas \(lgtG\) encodes the transferase responsible for the addition of a Glc to position 3 of the distal heptose residue (HepII) (2). The transferase that adds the first sugar to

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\(^2\) The abbreviations used are: ST, sequence type; LOS, lipooligosaccharide; OS, oligosaccharide; HexNAc, \(N\)-acetyhexosamine; BisTris, 2-[(bis[2-hydroxyethyl]amino]-2-(hydroxymethyl)propane-1,3-diol; MES, 4-morphohexitol; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometry detection; GC, gas chromatography; HMQC, heteronuclear multiple quantum coherence; TOCSY, total correlation spectroscopy; PEtn, phosphoethanolamine; KDO, 3-deoxy-2-keto-\(\beta\)-manno-octulosonic acid.
The structure of the outer core region of the LOS is encoded by *lgtE* or *lgtH* (allelic variants of the same gene) (19). This addition is usually a Gal residue; however, in the L5 and L10 immunotypes, a Glc is added with the same linkage. It has been reported that there is a single amino acid change from threonine to methionine in all strains in which Glc is added instead of the usual Gal (20). The genes *lpt-3* and *lpt-6* encode the transferases specific for the transfer of a phosphoethanolamine residue to position 3 and 6 of HepII, respectively (21, 22). The gene *lot3* is responsible for O-acetylation of the terminal α-GlcNAc (18). Phase variation mediated by homopolymeric tracts within the coding regions regulates expression of the genes *lgtA*, *lgtD*, *lgtC*, *lgtG*, and *lot3* (16, 23–25).

The structure of L1–L9 LOS has been elucidated by physicochemical methods (8–12, 26). Partial structural information is available for L11 and L10 LOS, which have been shown to predominate among ST-5 and ST-7 strains (4). To date, only one structural study of these LOSs has been reported and was conducted using electrospray ionization-mass spectrometry on O-deacetylated LOS. The proposed composition of the O-deacetylated L11 LOS was as follows: 2 Hex, 1 HexNAc, 2 Hep, 2 KDO, 2 PEtn, and O-deacylated lipid A (27). Neither the nature of the monosaccharides nor the location of the PEtn and O-acetyl substituents on the inner core have been determined.

When analyzed by SDS-PAGE, L11 LOS and L8 LOS bands have been shown to co-migrate and to share some epitopes (28). However, they can be distinguished serologically because L11 LOS does not bind to monoclonal antibody 2-L8 (29). According to Braun *et al.* (30), the α-oligosaccharide chains of L1 and L11 LOS immunotypes are identical, which is not in agreement with the proposed composition and the electrophoretic behavior of L11 LOS because L1 LOS consists of three Hex (two Gal and one Glc), one HexNAc (GlcNAc), two Hep, two KDO, and lipid A. Establishing the structure of the L11 LOS is relevant for the development of LOS-based vaccines against meningococcal disease. A previous study found that patients contracting serogroup A meningococcal disease mounted significant responses against the L11 LOS and that the concentrations of these antibodies were associated with bactericidal activity in serum, considered a correlate of protection against disease (31).

In the present study, our aim was to elucidate the L11 LOS structure of *N. meningitidis* serogroup A strains. To detect potential structural variation, we chose to conduct these studies using several different *N. meningitidis* strains. We selected strains belonging to two different epidemiological lineages, the ST-5 clone (three strains) and the ST-7 clone (four strains). The strains were first characterized according to their LOS genetic repertoire, and thereafter their LOS structures were resolved using glycosyl linkage analysis, NMR spectroscopy, and mass spectrometry.

**EXPERIMENTAL PROCEDURES**

Meningococcal Strains, Media, Growth Conditions, and Chromosomal DNA Isolation—The *N. meningitidis* L11 isolates were selected among the strains previously characterized by Norheim *et al.* (4). The seven meningoococal isolates examined in the present study are listed in Table 1. For chromosomal DNA isolation, bacterial strains were grown on brain heart infusion agar (BD Biosciences) at 37 °C in an atmosphere containing 10% CO₂ for 16 h. Chromosomal DNA meningococcal strains were isolated using a DNA isolation kit for cells (Roche Applied Science) according to the manufacturer’s
recombinations. DNA pellets were resuspended in Tris-EDTA buffer (Invitrogen).

**PCR Amplification of three Genes Involved in LOS Inner Core Biosynthesis:** lpt-3, lpt-6, and lot-3—The presence or absence of each gene was detected by PCR with internal primers using the chromosomal DNA. Primers used for lpt-3 (F2 and H3F) and lpt-6 (0408-3 and 0408-4) detection were described previously by Mackinnon et al. (22) and Kahler et al. (32). Primers for the detection of lot3 genes, 5’lot3 (5’-TTGCCCAAGCGGGTTT-TATAC-3’) and 3’lot3 (5’-CGTTTCTAAGCGGCTGTTCAGTT-3’), were designed with DNASTAR Lasergene® PrimerSelect software (DNASTAR, Madison, WI). Oligonucleotide primers were synthesized by MWG (Eurofins MWG, Ebersberg, Germany).

The PCR was performed with 5 μl of 10× PCR buffer without magnesium, 1.5 μl of 50 mM MgCl₂, 1 μl of 10 mM dNTP mixture (Invitrogen), 10 μM of each primer, 0.2 μl of Platinum TaqDNA polymerase (Invitrogen), 100 ng of chromosomal DNA, and nuclease-free H₂O (Qiagen, Courtaboeuf, France) in a final volume of 50 μl. PCR cycles of amplification were adapted for each gene according to the manufacturer’s recommendations. PCR products were analyzed by electrophoresis on 1.5% agarose gels and stained with SYBR Gold (Invitrogen).

**Sequencing of Homopolymeric Tracts in Phase-variable Genes and Sequencing of lgtH**—Homopolymeric tract regions of lgtG and lot3 were amplified by PCR using primers chosen in flanking regions (Table 2). Oligonucleotide primers were designed using Lasergene® PrimerSelect software (DNASTAR) and were synthesized by MWG (Eurofins MWG). PCR primers for the detection of the lgtG gene were synthesized with PGEX tags and lot3 PCR primers with M13 tags. The lgtH gene was sequenced to determine the function of the related transferase responsible for the addition of the first sugar on the α-chain. When a Thr is found at position 153, lgtH encodes for a galactosyltransferase, whereas when a Met is found at that position, lgtH encodes for a glucosyltransferase. In these studies, 421-bp fragments of lgtH coding for the mutation T153M were amplified using primers P9 and P24, as described by Zhu (19).

The PCR mixtures comprised 10 μl of 5× Expand HiFiPLUS reaction buffer, 1 μl of 10 mM dNTP mixture (Invitrogen), 2 μl of 10 μM of each primer, 0.5 μl of Expand HiFiPLUS enzyme blend (Roche Applied Science), 100 ng of chromosomal DNA, and nuclease-free H₂O (Qiagen, Courtaboeuf, France) in a final volume of 50 μl. PCR cycles of amplification were adapted for each gene according to the manufacturer’s recommendations and were performed on a GeneAmp® PCR System 9700 (Applied Biosystems, Carlsbad, CA). PCR products were purified by QIAquick® spin columns (Qiagen), and then analyzed by electrophoresis on 1.5% agarose gels and stained with SYBR® Gold (Invitrogen).

**Table 3**

**Primer Sequences for PCR amplification of homopolymeric tracts of phase-variable genes lgtG and lot3**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’→3’</th>
<th>Strain</th>
<th>Position on the genea</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGEX5P-lgtG</td>
<td>GGCGCTGCAAGCGCCACCTTGTTGATTTACGCGAACCCCTCTCATCT</td>
<td>Forward</td>
<td>281–301</td>
<td>bp</td>
</tr>
<tr>
<td>PGEX3P-lgtG</td>
<td>CCGGAGCTCTCGATGGTACAGGAGCGAATTTTCCCAGGTTTGTG</td>
<td>Reverse</td>
<td>518–538</td>
<td>bp</td>
</tr>
<tr>
<td>lgt3 seq-lot3</td>
<td>GCTTTCTAACAGCAGACCGGCGCTCTCTAGAATAACGGAAG</td>
<td>Forward</td>
<td>1040–1059</td>
<td>164 bp</td>
</tr>
<tr>
<td>lgt3 rev-lot3</td>
<td>AACAGCTATGACCTAGGGATTTTCCGCGAACAG</td>
<td>Reverse</td>
<td>1184–1203</td>
<td>164 bp</td>
</tr>
</tbody>
</table>

**Table 3**

**Probes for sequencing homopolymeric tracts of phase-variable gene lgtG and for sequencing the lgtH region involved in LgtH glycosyltransferase function**

<table>
<thead>
<tr>
<th>Gene</th>
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<tbody>
<tr>
<td>lgtG</td>
<td>PGEX-5P</td>
<td>Forward</td>
<td>IRD-700</td>
<td>GGCGCTGCAAGCGCCACCTTGTTG</td>
</tr>
<tr>
<td>lgtG</td>
<td>PGEX-3P</td>
<td>Reverse</td>
<td>IRD-800</td>
<td>CCGGAGCTCTCGATGGTACAGGAGCG</td>
</tr>
<tr>
<td>lot3</td>
<td>M13 seq</td>
<td>Forward</td>
<td>IRD-700</td>
<td>GCTTTTTGAGTCGAGAC</td>
</tr>
<tr>
<td>lot3</td>
<td>M13 rev</td>
<td>Reverse</td>
<td>IRD-800</td>
<td>AACAGCTATGACCTAGGGATTTTCCGCGAACAG</td>
</tr>
<tr>
<td>lgtH</td>
<td>lgtH</td>
<td>Reverse</td>
<td>IRD-700</td>
<td>GGATCCCTGGCTCGGTTTTCG</td>
</tr>
</tbody>
</table>

a Position on the sequence of reference strain MC58 (accession number AED02998).

**Preparation of OS and Dephosphorylated OS (deP-OS) from LOS**—OS was purified from broth-grown cells by the hot phenol/water extraction procedure (35). Purified LOS was thereafter diluted in lysing buffer (XT Sample Buffer 4× from Bio-Rad), and 0.2 μg of each LOS was subjected to SDS-PAGE on a 12% BisTris gel (NuPAGE® gel, Invitrogen) in MES-SDS running buffer (Invitrogen) at 150 V for 1.5 h. After fixing the gel, LOS profiles were detected by silver staining using the method described by Tsai and Frasch (36).

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**Probes for sequencing homopolymeric tracts of phase-variable gene lgtG and for sequencing the lgtH region involved in LgtH glycosyltransferase function**

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<td>Forward</td>
<td>IRD-700</td>
<td>GCTTTTTGAGTCGAGAC</td>
</tr>
<tr>
<td>lot3</td>
<td>M13 rev</td>
<td>Reverse</td>
<td>IRD-800</td>
<td>AACAGCTATGACCTAGGGATTTTCCGCGAACAG</td>
</tr>
<tr>
<td>lgtH</td>
<td>lgtH</td>
<td>Reverse</td>
<td>IRD-700</td>
<td>GGATCCCTGGCTCGGTTTTCG</td>
</tr>
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<td>AACAGCTATGACCTAGGGATTTTCCGCGAACAG</td>
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<td>lgtH</td>
<td>lgtH</td>
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<td>IRD-700</td>
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for analysis, with the exception of NMR, in which oligosaccharides were dissolved in D₂O.

Matrix-assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry of LOS and deP-OS—Intact purified LOS were analyzed by MALDI-TOF mass spectrometry using an AUTOFLEX II mass spectrometer (Bruker Daltonik SA, Wissembourg, France) in linear mode. Each LOS suspension (1 mg/ml) was mixed in a 1:2 (v/v) ratio with 20 mM sodium acetate buffer, pH 5.5, to which 2,5-dihydroxybenzoic acid in matrix solution. Spectra were acquired in negative acquisition mode. DeP-Os were analyzed by MALDI-TOF using an AUTOFLEX II mass spectrometer in reflector mode. The deP-OS samples (500 μg/ml) were mixed in a 1:1 (v/v) ratio with 10 mg/ml 2,5-dihydroxybenzoic acid in matrix solution. Spectra were acquired in positive acquisition mode.

Compositional Analysis by High Performance Anion Exchange Chromatography with Pulsed Amperometry Detection (HPAEC-PAD)—Trifluoroacetic acid hydrolysis and HPAEC-PAD analyses were performed as described previously (37). Briefly, samples (10 μg) of the LOSs were hydrolyzed in 2 N trifluoroacetic acid at 121 °C for 2 h. The acid was removed under a stream of nitrogen, and the samples were redissolved in water (400 μl) prior to analysis. Chromatography of the samples was performed using a Dionex ICS2500 ion chromatography system (Dionex, Sunnyvale, CA) coupled to an AS50 autosampler. Separations were performed on a CarboPac PA1 (4 × 250 mm) analytical column (Dionex) with a guard column (4 × 50 mm). Pulsed amperometric detection was used incorporating a quadruple-potential waveform. Data were collected and analyzed on computers equipped with Dionex Chromel™ software (Dionex). Monosaccharide standards used were Glc and GlcN (Sigma).

Glycosyl Linkage Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)—Glycosidic linkage analysis was performed by GC-MS on permethylated alditol acetate derivatives of deP-OS obtained by methylation according to the method of Kim et al. (38). The methyl ether compounds were analyzed by GC as the partially methylated and acetylated alditols obtained after trifluoroacetic acid hydrolysis (2 N trifluoroacetic acid at 121 °C for 2 h), reduction with sodium borohydride, and peracetylation.

For these studies, a Varian 1200 GC-mass spectrometer (Varian, Palo Alto, CA) was used with a 30 m × 0.25-mm VF 5 MS capillary column. The gas chromatography conditions were as follows: helium carrier gas (Alphagaz 2 grade, Air Liquide), flow rate 1 ml/min. Oven conditions included an initial temperature of 120 °C for 1 min, 3 °C/min to 240 °C, injector temperature 250 °C, MS transfer line temperature 300 °C. MS acquisition was done from m/z 60–400 in electron impact mode. Identification of compounds was based on comparison with standard maltose-producing T-Glc and 4-Glc.

NMR Spectroscopy—NMR spectroscopy experiments were performed using a 500-MHz Bruker Avance DRX spectrometer (Bruker Biospin, Wissembourg, France). Spectra were measured for solutions at 25 °C in D₂O and pD = 7.0 using phosphoric acid (2%) as an external standard for 31P (δ = 0.00 ppm) and TSP as an external reference for 1H NMR (δ = 0.00 ppm). The measurement of two-dimensional heteronuclear multiple quantum coherence (HMQC) 1H/13C spectrum was done using an J1H,13C coupling value of 7 Hz, and the HMQC-total correlation spectroscopy (TOCSY) was performed using a mixing time of 200 ms with the same coupling J1H,13C.

RESULTS

Characterization of the LOS Genes of the L11 Strains—The genes (lgtA, -B, -C, -E, -H, lgt-2 (lgtF), lgt-3 (lpt-6 and lgtG), lpt-3, lpt-6, and lot3) were analyzed by PCR (Table 4). For the L11 strains, the presence of lgtA, lgtF, lgtH, and lgtG and absence of lgtC and lgtE genes have been shown previously (4). The lgtH gene in the L11 strains encodes a predicted protein with a Met, rather than a Thr, at position 513, and lgtH gene with Met at position 153 transfers a Glc residue to the glucose linked to heptose I in the α-chain in place of a Gal residue (20).

The presence of both lpt-3 and lpt-6 genes indicates that all seven strains are genetically able to add O3 and O6 PEtn to HepII. In addition, homopolymeric tracts of phase-variable genes (lgtA, lgtG, and lot3) were determined to predict their phase status (Table 4). lgtA was present in all seven strains and...
showed evidence of phase variation, as described previously (4). No evidence of phase variation for the lgtG gene was detected because for all of the strains tested, the gene was predicted to be in phase “off,” indicating that those strains would not be able to incorporate a Glc residue on HepII. Similar conclusions were reached for the lot3 gene; however, in this case, the phase was “on” for all seven strains, indicating that an O-acetyl residue could be added to the GlcNac.

Taken together, these results showed that the LOS gene repertoire of the seven N. meningitidis strains studied was identical for 8 of 10 genes. Moreover, these strains did not differ in their genetic ability to add PEtn and O-acetyl residues to the LOS inner core.

L11 LOS Characterization by SDS-PAGE and Determination of the Carbohydrate Composition by MALDI-TOF MS Analysis—LOSs of the seven L11 strains were analyzed by SDS-PAGE, and their electrophoretic patterns were compared with those of purified L3, L6-like (LOS from lgtB mutant of strain MC58), and L8 LOS. In agreement with the report by Kim et al. (27), silver-stained gels showed that LOS L11 had the same electrophoretic mobility as LOS L8 (Fig. 2), which suggests that the L11 and L8 LOSs have the same molecular weight. No reactivity was observed with an anti-L8 monoclonal antibody by immunoblot (data not shown).

The seven purified LOS preparations were hydrolyzed under mild acid conditions to isolate the oligosaccharides from the lipid A. They were treated subsequently with HF to remove the PEtn groups and were then analyzed by MALDI-TOF MS. Each oligosaccharide was detected as a series of sodium adduct molecular ions. A major peak was detected at m/z 1154.4 for all of the seven dephosphorylated OS (results not shown), which is consistent with a dehydrated oligosaccharide composed of 2 Hex, 2 Hep, 1 HexNac, and 1 KDO residue (the second branched KDO is hydrolyzed under the conditions used to release the oligosaccharide from lipid A). Another peak was detected at m/z 1176.4 (+42.0) that could be assigned to the same glycoform carrying an O-acetyl group. O-Acetylation of LOS was confirmed by 1H NMR analysis of the OS as described below.

In summary, the seven purified L11 LOSs all showed an electrophoretic profile and carbohydrate composition similar to those previously observed for L8 by Kim et al. (27, 29).

Monosaccharide Composition Analysis of L11 LOS by HPAEC-PAD and 1H NMR—To further characterize the structural features conferring serospecificity to L11 LOS, monosaccharide analysis was performed by HPAEC-PAD. Chromatograms of the seven hydrolyzed L11 LOSs were compared with the chromatogram of hydrolyzed L8 LOS (Fig. 3). In contrast to the L8 LOS chromatogram, the L11 chromatograms showed the absence of Gal and indicated that an additional Glc was present instead of Gal.

These results were further confirmed by 1H NMR studies where deP-OS of L8 and L11 LOSs were analyzed, and the respective proton spectra were compared (Fig. 4). In the spectrum of the deP-OS isolated from strain MK499/03 LOS, we observed a resonance signal at 3.32 ppm that is absent in deP-OS L8 spectrum and that is characteristic of the H-2 proton of the additional Glc (39). Conversely, the anomeric resonance assigned to H-1 β-Gal at 4.48 ppm on the L8 spectrum was absent in the L11 spectrum. In this spectrum, a signal at 4.55 ppm could be assigned to the anomeric proton of the additional Glc with a β linkage due to its observed chemical shift and its high J1,2 (~8 Hz) coupling constant. From these results, we could conclude that L11 LOS can be distinguished from L8 LOS by the absence of the terminal β-Gal of the α-chain and the presence of an additional β-Glc.

Glycosyl Linkage Analysis of the deP-OS by GC-MS—To determine the location and linkage pattern of the second Glc of L11 LOSs, GC-MS analysis was performed on permethylated alditol acetate derivatives of deP-OS isolated from MK499/03 LOS (results not shown). In these studies, the derivative characteristic of terminal Glc and a derivative of disubstituted Glc were observed. By comparison of the retention times of maltose derivatives and by identification of specific ion fragments, the GC-MS analysis revealed the presence of a 4-Glc. Therefore, we could deduce that L11 LOS contains a disaccharide β-Glc(1→4)β-Glc. Because the main difference observed in L8 and L11 1H NMR spectra was the resonance signals assigned to the α-chain terminal hexose residue, we concluded that this disaccharide is located at O-4 of HepII and constitutes the α-chain oligosaccharide of L11 LOS.

Analysis of PEtn Substitution by MALDI-TOF Mass Spectrometry and NMR—MALDI-TOF MS analysis of the ST-7 LOS revealed major peaks at m/z 3434.3 and 3557.3, and these were consistent with the following compositions: Hex2HexNac2Ac2Hep2PEtn2KDO2Lipid A and Hex2HexNac2Ac2Hep2PEtn2KDO2Lipid A, respectively (Fig. 5).
Additionally, a +57 peak was observed for each spectrum except for the MK802/02 LOS that showed glycoforms with a glycine moiety.

L11 strains produced LOS that were mono- or disubstituted with PEtn. Interestingly, the relative abundance of each phosphorylated LOS population was observed to vary relative to the particular N. meningitidis strain tested. The relative intensity of the signals showed that ST-7 strains produced more LOS with one PEtn, and ST-5 strains produced more LOS with two PEtn moieties from the different patterns of oligosaccharide phosphorylation seen in the $^{31}$P NMR studies. One-dimensional $^{31}$P NMR spectra (Fig. 6) of Z1054 OS, representative of ST-5 strains, revealed two peaks at $-0.67$ and $-0.12$ ppm and suggest that its LOS contains two PEtn groups (40). Two similar peaks at lower intensity were observed in the one-dimensional $^{31}$P NMR spectrum of OS from MK499/02 (a representative of the ST-7 strains). In addition to these two resonances, a major resonance was detected on the latter spectrum at $-0.50$ ppm, which would correspond to an LOS possessing a single PEtn group. The exact location of the PEtn was further elucidated by two-dimensional $^{31}$P-1H NMR experiments on MK499/02 because it contains two glycoforms, the major one that contains a single PEtn and a minor glycoform that contains two PEtn moieties.

The $^{31}$P-1H HMOC spectrum showed that the phosphorus atom at $-0.50$ ppm was associated with the O-CH$_2$ protons of the PEtn group at 4.15 ppm and to the H-3 proton of HepII at 4.41 ppm (data not shown). These correlations are characteristic of PEtn substitution at O-3 of HepII (10). The two minor peaks detected at $-0.67$ and $-0.12$ ppm were both correlated to O-CH$_2$ of PEtn and to H-3 and H-6 of HepII (4.41 and 4.56 ppm), respectively. These results are consistent with an OS containing two PEtn groups, at O-6 and O-3 of the HepII residue (40). Furthermore, a $^{31}$P-1H HMOC-TOSCY analysis of MK499/02 OS (Fig. 7) showed that the $^{31}$P signals at $-0.67$ and $-0.12$ ppm are both associated with the anomeric proton at 5.77 ppm, characteristic of H-1 HepII with two PEtn, and the $^{31}$P signal at $-0.50$ ppm is associated with the anomeric proton at 5.67 ppm, characteristic of H-1 HepII with one PEtn at O-3. These results further suggest that the major OS of the MK499/02 strain con-
tains a structure with one PEtn group located on the O-3 of HepII and that a minor OS has a structure containing two PEtn groups located on the O-3 and O-6 of HepII. In summary, our NMR and MALDI-MS analyses showed that the four ST-7 strains produced a LOS mainly with one PEtn group at O-3 of HepII, whereas the three ST-5 strains produced a LOS with two PEtn groups at O-3 and O-6 of HepII.

Localization of O-Acetyl Substituent by NMR—MALDI-TOF MS analysis of deP-OS showed that the L11 LOSs were O-acetylated. Results of $^1$H NMR analyses also showed a resonance signal characteristic of the O-acetyl group at 2.20 ppm (data not shown). Heterogeneity of the H1 HepII signal due to partial O-acetylation indicated the presence of O-acetylated and non-O-acetylated forms of OS as was seen previously in MALDI-TOF spectra.

To attempt to localize the O-acetyl group, a one-dimensional TOCSY experiment was performed on the oligosaccharide from strain MK499/03 isolated after mild acid hydrolysis, using a selective excitation of H-1 $^1$H9251-GlcNAc at 5.21 ppm. The spectrum showed the scalar correlations up to H-6 (Fig. 8). The results of the present studies clearly showed a resonance assigned to H-3 (3.75 ppm) that is characteristic of unsubstituted protons, indicating the absence of O-acetyl group at O-3. In contrast, the three proton resonances assigned to H-5, H-6a, and H-6b showed downfield chemical shift values, indicating acetylation at O-6. These results are in agreement with previous reports of $^1$H NMR assignment studies that showed meningococcal LOS to be acetylated at O-6 of GlcNAc (41, 42).

DISCUSSION

Previously published studies employing a combination of chemico-physical analyses have shown that N. meningitidis LOS immunotypes L1–L9 possess specific oligosaccharide structures (8–12, 26, 42). Furthermore, many reports have described the genetic basis of LOS biosynthesis, which enable to some extent the prediction of LOS immunotypes (18, 19, 32, 43, 44). To date, there have been very few studies aimed at fully characterizing the genetics of L11 LOS biosynthesis (4) or its chemical structure (27, 29). We report here the results of the first study to compare the genetic repertoire and the LOS structure of multiple strains of N. meningitidis subgroup III, the primary subgroup responsible for serogroup A meningococcal disease in the African meningitis belt (7). To assess the reliability of genetic prediction of LOS structure, we had prior to this work analyzed in our laboratory 30 strains corresponding to different immunotypes by both genetic and biochemical methods. We were able to show that the genetic prediction fits with the phenotypically expressed structure for 26 of the 30 strains studied (data not shown).

The structure of the OS isolated from L11 immunotype LOS is shown in Fig. 9. Our results indicate that despite genetic heterogeneity in loci involved in α-chain elongation (lgt genes) (Table 4), the seven selected L11 strains produce LOSs that all have the same α-chain oligosaccharide. This OS consists of a glucosyl disaccharide $^n$Glc-(1→3)$^n$Glc linked to O-4 of HepI. This structure is explained by the presence of the $lgtH3$ allele encoding a glucosyl transferase instead of a galactosyltransferase due to Thr$^{153}$ substitution. In a previous study by Norheim et al. (4), among 14 L11 strains studied, the $lgtH3$ allele was found on seven other ST-7 strains and on four of six ST-5 strains, indicating its predominance among the L11 strains. The link between the presence of the methionine residue instead of the threonine at position 153 and the glycosyltransferase function of the encoded protein was
reactivity between L5 and L11 LOS has been reported. Indeed, whereas L5 LOS has no PEtn substitution but a glucose residue at O-3 of HepII in the inner core, the seven L11 LOS all contain at least one PEtn at O-3 of HepII, which would be predicted due to the presence of the lpt3 gene and the phase OFF of the lgtG gene.

Although the lpt6 gene was also present in the seven strains analyzed, only four strains added PEtn at the O-6 position of HepII. The dissubstitution of HepII has only been reported for mutant strains producing a truncated α-chain LOS (40) and for the L7 strain M986 (46). However, from the early 1990s, studies on neisserial LOS have suggested the presence of a second PEtn molecule in the core OS of Neisseria LOS without localization (47, 48). Accordingly, lpt3 and lpt6 genes were shown to be both present in 35% of strains from a collection of 188 N. meningitidis strains screened for the presence of these genes, in agreement with previously published data (21).

Mass spectrometry analysis of LOS from selected strains possessing both genes has shown that the addition of PEtn-3 and PEtn-6 is stochiometric in some strains and not in others. Surprisingly, it appeared that the patterns of inner core PEtn substitution of L11 strains differ according to the sequence type because both PEtn-3 and PEtn-6 are present in ST-7 LOS, and only PEtn-3 is present in ST-5 LOS. A recent study suggested that the absence of di-PEtn-substituted LOS in strains containing both lpt3 and lpt6 genes does not result from the presence of PEtn at one position of HepII precluding the addition of PEtn to the other position (49). To date, the factors influencing the addition of one or both of the PEtns in meningococcal LOS are not known.

Among the seven strains studied, two strains, MK802/02 and Z1054, displayed the lgtB gene, whereas the other did not. Therefore, from a genetic perspective, both of those strains had the ability to add a galactose residue on the glucosyl disaccharide β-Glc-(1→4)-β-Glc, as did the L10 strains and L5 strains. In the Norheim study, 14 L10 strains were analyzed, and all of them displayed an lgtB gene, whereas only four of 38 L11 strains displayed this gene (4). The structure of an L10 strain (strain 7880) was described by Tsai et al. (50) and was shown to be able to extend the α-chain with a galactose residue on the glucosyl disaccharide. We did not detect the production of L10 LOS in addition to L11 LOS for either strain MK802/02 or strain Z1054. These results suggest that in addition of the lgtB presence, other mechanisms may regulate the expression of either the l10 or the l11 immunotype. Questions generated by the present study include whether or not the culture medium employed may influence this elongation despite the fact that lgtB is not described as being phase-variable and whether allelic

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structure of the N. meningitidis L11 LOS

variation of the lgtB gene could lead to different enzymatic specificities.

Structural microheterogeneity due to the absence or the presence of glycine and O-acetyl groups within each LOS molecule was detected. Except for the MK802/02 LOS spectrum, each of MALDI-TOF MS spectra of native LOS revealed glycoforms with a glycine moiety (m/z + 57).

The exact position of this residue has not been definitively elucidated; however, it is likely that it is present at O-7 on HepII (45). Also, 1H NMR and MALDI-TOF MS spectra of the L11 elucidated; however, it is likely that it is present at O-7 on HepII/m forms with a glycine moiety (each of MALDI-TOF MS spectra of native LOS revealed glycoecule was detected. Except for the MK802/02 LOS spectrum, at the O-6 position of under the mild hydrolytic conditions used to obtain the -acetylated forms of LOS. However, it is possible that O-acetyl groups (such as glycine) could be partially removed under the mild hydrolytic conditions used to obtain the oligosaccharides.

Most N. meningitidis LOS immunotypes have been described as having O-acetyl groups at O-3 of the inner core α-GlcNAc (8–10, 43) and with a characteristic H-3 proton resonance of this residue at 5.17 ppm (8, 43). The one-dimensional TOCSY spectrum of MK499/02 OS clearly showed the α-GlcNAc H3 resonance at 3.75 ppm, indicating the absence of O-acetyl group at this position. However, the downfield H6 resonances of the same residue above 4 ppm would indicate that the O-acetyl group is located at position 6 of α-GlcNAc. Although the lot3 gene has been described as being responsible for the addition of acetyl at O-3 of α-GlcNAc (43) in an L2 strain (strain NMB), the same strain was analyzed for its structural LOS composition and was shown to display an O-acetyl group at the O-6 position of α-GlcNAc (41). For the L11 strains analyzed in the present study, although lot-3 is present and its phase was shown to be ON, no O-acetyl at position 3 was found. In addition, the L11 strain 126E has been reported bearing O-acetyl at position 6 of α-GlcNAc (41). Altogether, these studies indicate that lot-3 may not be the sole gene responsible for O-acetyl group addition on LOS. In the Lgt-3 locus, containing both lgtG and lpt6 genes, an additional gene, NMA0407, encodes for a putative O-acetyl transferase (21). In all of the seven L11 strains studied, this gene is present according to PCR analysis (data not shown) and could be responsible for the addition of the O-acetyl group at the O-6 position of α-GlcNAc. Further studies are needed to assess this hypothesis.

To our knowledge, this is the first study that describes the complete structure of the L11 LOS and the first time that LOS structure determinations were made based on the analysis of more than a single strain. Although the genetic analysis predicted three possible α-chain structures for all of the seven L11 strains studied, structural analysis demonstrated that L11 strains only produce a short common α-chain. These studies emphasize the need to determine LOS structure on multiple N. meningitidis strains in order to detect microheterogeneity. These studies also suggest the existence of additional regulatory mechanisms affecting LOS structure microheterogeneity in N. meningitidis. Therefore, the use of genetic analyses alone to predict LOS structure may not accurately reflect the phenotypic variability observed in these studies.

With the elucidation of the structure of the L11 LOS immunotype, a more comprehensive structural profile of the LOS involved in disease isolates can now be established. This may provide valuable insight into the structural basis of the N. meningitidis immunotyping system, which could be used in formulating LOS-based vaccines against meningococcal meningitis.

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