O-Acetylation of the Terminal N-Acetylgalactosamine of the Lipooligosaccharide Inner Core in Neisseria meningitidis

INFLUENCE ON INNER CORE STRUCTURE AND ASSEMBLY*

Received for publication, February 10, 2006, and in revised form, May 8, 2006 Published, JBC Papers in Press, May 9, 2006 DOI 10.1074/jbc.M601308200

Charlene M. Kahler†‡, Shauna Lyons-Schindler†, Biswa Choudhury§, John Glushka§, Russell W. Carlson§, and David S. Stephens¶‡

From the †Department of Microbiology, Monash University, Wellington Road, Victoria 3800, Australia, the §Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602, and the Departments of ¶Medicine and ‡Microbiology and Immunology and Laboratories of Microbial Pathogenesis, Emory University School of Medicine and Veterans Affairs Medical Center, Atlanta, Georgia 30322

O-Acetylation is a common decoration on endotoxins derived from many Gram-negative bacterial species, and it has been shown to be instrumental (e.g. in Salmonella typhimurium) in determining the final tertiary structure of the endotoxin and the immunogenicity of the molecule. Structural heterogeneity of endotoxins produced by mucosal pathogens such as Neisseria meningitidis is determined by decorations on the heptose inner core, including O-acetylation of the terminal N-acetylgalactosamine (GlcNAc) attached to HepII. In this report, we show that O-acetylation of the meningococcal lipooligosaccharide (LOS) inner core has an important role in determining inner core assembly and immunotype expression. The gene encoding the LOS O-acetyltransferase, lot3, was identified by homology to NodX from Rhizobium leguminosarum. Inactivation of lot3 in strain NMB resulted in the loss of the O-acetyl group located at the C-3 position of the terminal GlcNAc of the LOS inner core. Inactivation of either lot3 or lgtG, which encodes the HepII glucosyltransferase, did not result in the appearance of the O-3-linked phosphoethanolamine (PEA) groups on the LOS inner core. Construction of a double mutant in which both lot3 and lgtG were inactivated resulted in the appearance of O-3-linked PEA groups on the LOS inner core. In conclusion, O-acetylation status of the terminal GlcNAc of the γ-chain of the meningococcal LOS inner core is an important determinant for the appearance or exclusion of the O-3-linked PEA group on the LOS inner core and contributes to LOS structural diversity. O-Acetylation also likely influences resistance to complement-mediated lysis and may be important in LOS conjugate vaccine design.

Neisseria meningitidis is the causative agent of epidemic meningitis and fatal septic shock (1). Being an obligate human pathogen, the human nasopharynx is the only known natural environmental niche from which the organism spreads to other human hosts via inhalation of respiratory droplets. In circumstances that are not entirely understood, nasopharyngeal acquisition may also result in invasive meningococcal disease (1, 2). Comparative studies of invasive and carriage isolates of meningococci implicate lipooligosaccharide (LOS) in the attachment and invasion of the nasopharyngeal epithelium (3, 4) and in the pathogenesis of invasive meningococcal disease (5). Meningococcal isolates express 12 immunologically distinct LOS structures (L1–L12) (6–8), which were subsequently shown to correspond to distinct chemical structures (9–13). Examination of carriage and invasive disease isolates by serology indicates that meningococci commonly co-express patterns of LOS immunotypes, with L[1,8], L[3,7,9], and L[2,4] being the most prevalent (3, 4).

Each LOS immunotype structure has a conserved heptose inner core to which α-, β-, and γ-chains are added (see Fig. 1). The length and composition of the α-chain is based upon the phase-variable expression of lgtA, lgtD, and lgtC (for reviews see Refs. 14 and 15). The length and composition of the α- and β-chain extensions from HepII as well as the presence or absence of a γ-chain extension and phosphoethanolamine (PEA) residues on HepII appear to determine immunotype. The presence of the γ-chain extension of α1–3 glucose on HepII, characteristic of L2 and L5 immunotype LOS (Fig. 1), is determined by the phase-variable expression of lgtG (16). In comparison, the presence or absence of the various PEA groups on the inner core is in part determined by whether or not the isolate carries an intact lpt3 or lpt6 gene (17, 18). In some meningococcal strains, O-3-linked PEA is attached to HepII by Lpt3 in the absence of LgtG activity, thus producing the L3 immunotype (17). However, an O-3-linked PEA was not detected in strain NMB, which expresses L[2,4] immunotype LOS and contains an intact lpt3 (18). Ram et al. (19) have proposed that variable additions of PEA to the inner core play an important role in protecting meningococci from complement-mediated lysis, because the O-6-linked PEA groups of L2 and L4
O-Acetylation of \textit{N. meningitidis} Terminal GlcNAc

LOS inner core and the exposed O-3-linked PEA groups of L[1,8] LOS are targets for complement component C4b. Mechanisms that mask these PEA inner core-associated epitopes, such as the co-expression of a long \(\alpha\)-chain, or prevention of the expression of PEA groups on the cell surface, such as competition by the glucosyltransferase, LgtG (17), increase the resistance to complement mediated lysis (19).

Recently, it has become clear that at least some strains of meningococci have the capacity to express all immunotype LOS structures and that complex regulatory networks and structural constraints dictate what pattern of immunotypes are expressed during growth (18). Comparison of the meningococcal LOS inner core structures of different immunotypes reveals that structures without O-3-linked PEA groups are invariably O-acetylated (Fig. 1). We hypothesized that O-acetylation of the terminal LOS inner core \(N\)-acytylgalactosamine in the presence of a lactoneotetraose \(\alpha\)-chain could prevent the enzymatic addition of O-3-linked PEA by Lpt-3 to this substrate. To test this hypothesis, the lipooligosaccharide \(O\)-acetyltransferase (lot3) was identified in \textit{N. meningitidis} strain Z2491 by amino acid homology with a known \(O\)-acetyltransferase, from \textit{Rhizobium leguminosarum} (20).

Insertional inactivation of lot3 in \textit{N. meningitidis} strain NMB confirmed that this locus encoded the lipooligosaccharide \(O\)-acetyltransferase. In addition, inactivation of both lot3 and lgtG resulted in the appearance of O-3-linked PEA on the LOS inner core of this strain. Therefore, O-acetylation of the terminal \(N\)-acytylgalactosamine residue of the LOS inner core when expressed in combination with a lactoneotetraose \(\alpha\)-chain physically prevents the addition of O-3-linked PEA groups to this site by a functional Lpt-3.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions—Escherichia coli** strain JM109 (21) was used for the maintenance of plasmids. \textit{E. coli} strains were grown in Luria-Bertani (LB) broth (Difco) at 37°C with agitation, or on LB agar plates supplemented with 1.5% agar, and where appropriate, with the following antibiotics: 100 \(\mu\)g/ml ampicillin, 25 \(\mu\)g/ml kanamycin, 150 \(\mu\)g/ml erythromycin, or 10 \(\mu\)g/ml tetracycline. Meningococcal strains were grown on GC agar base (Oxoid) supplemented with 20 mM glucose, 0.43 mM thiamine pyrophosphate chloride, 6.8 mM glutamine, and 12.4 mM Fe(NO\textsubscript{3})\textsubscript{3} in 5% carbon dioxide atmosphere. Liquid cultures were vigorously aerated at 37°C in GC broth with the same supplements and 0.51 M sodium bicarbonate (22). Where appropriate, media was supplemented with 7 \(\mu\)g/ml erythromycin, 1 \(\mu\)g/ml tetracycline, or 40 \(\mu\)g/ml kanamycin.

**DNA Manipulation**—All DNA manipulations were performed by standard methods as described previously (23). Plasmid DNA was purified using the Hi PURE Plasmid Isolation kit from Roche Diagnostics. Restriction endonucleases were purchased from New England Biolabs. DNA sequencing was performed with the BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems) and analyzed on an Applied Biosystems model 3730 DNA analyzer. Oligonucleotide primers were synthesized on an Applied Biosystems 394 oligonucleotide synthesizer. Competent \textit{E. coli} JM109 was prepared by the method described by Chung \textit{et al.} (24). Amino acid sequences were aligned either by ClustalW (www.ebi.ac.uk/clustalw/) (25) or Blast 2 sequences (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) (26).

**Inactivation of the Lipooligosaccharide O-Acetyltransferase (lot3) in Strain NMB**—The \(\text{lot3}\) gene in \textit{N. meningitidis} strain NMB was inactivated by the incorporation of a \(\text{lot3::tetM}\) mutagenic cassette. To construct the \(\text{lot3::tetM}\) cassette, an internal fragment of \(\text{lot3}\) (formerly designated \(\text{NMA2202}\) in \textit{N. meningitidis} strain Z2491) was amplified using the primer pair...
DAP379 (5'-GTCTCGGTGCGGTAAGATAGC-3') and DAP377 (5'-GCAGTAGGAGACGATTACCTCC-3') (Fig. 2). The resulting 1.5-kb PCR product was treated with T4 DNA polymerase and ligated into pHS576 (27) digested with Smal to create pJKD2709. The tetM cassette was released from pJKD2401 by EcoRV digestion and ligated to Smal-digested pJKD2709. Those transfectants that were tetracycline- and chloramphenicol-resistant were assessed by colony PCR for the correct insertion of the tetM cassette into pJKD2709 and were designated pJKD2710. To create NMBlot3, pJKD2710 was transformed in N. meningitidis strain NMB using the protocol of Janik et al. (28), and transfectants were identified by resistance to tetracycline. To ensure these transfectants contained the lot3::tetM cassette in the correct chromosomal location, the lot3 locus was PCR-amplified with DAP371 (5'-CGATTTGTCGCGGAAAGAAACCG-3') and DAP372 (5'-GAAGGCAAGGCAATTGCTTGAGC-3'), located outside the lot3 open reading frame, and the correct mutants were designated NMBlot3 (JKD5172). To create a meningococcal double mutant containing inactivated lot3 and lgtG, the lgtG::kan cassette was introduced into NMBlot3 by transformation with pCK49 (29), to create the strain designated NMBlot3/lgtG (JKD5173).

Isolation of Oligosaccharides—The LOS preparations, prepared as previously described (30), were washed three times with 9:1 ethanol/water (v/v) mixture to remove contaminating phospholipids. The washed LOS preparations were suspended in water and lyophilized. Samples were then subjected to mild acid hydrolysis in 1% aqueous HOAc (v/v) for 2.5 h at 100 °C with constant stirring. The lipid A precipitate that formed during hydrolysis was collected by centrifugation at 3000 × g for 15 min at 4 °C, and supernatants containing the released oligosaccharides (OSs) were decanted and lyophilized. The lyophilized OSs were further purified by gel-filtration chromatography using a Bio-Gel P-4 column (120 × 1 cm, Bio-Rad) and water as eluant.

Glycosyl Composition and Linkage Analysis of the Oligosaccharides—Glycosyl compositional analysis was performed by gas chromatography-mass spectrometry of trimethylsilyl methylglycosides with myoinositol used as an internal standard (31). The samples were methanolized with methanolic 1 M HCl for 80 °C for 18 h. The released monosaccharides were dried under a stream of dry air and acetylated with 3:1:1 methanol/pyridine/acetic anhydride (v/v) at 100 °C for 1 h. After cooling, samples were dried-down and trimethylsilylated with Tri-sil reagent (Pierce) for 30 min at 80 °C. The resulting trimethylsilyl derivatives were analyzed by gas chromatography-mass spectrometry on a Hewlett-Packard HP5890/HP5970 MSD gas chromatograph/mass spectrometer equipped with a Supelco DB-1 fused silica capillary column (30 m × 0.25 mm inner diameter) with helium as the carrier gas.

Linkage analyses were carried out by the slurry NaOH method modified from that of Ciucanu and Kerek (32). Samples were dissolved in 0.5 ml of Me2SO by stirring overnight at room temperature under an N2 atmosphere. After dissolution, a freshly prepared slurry of NaOH in Me2SO was added (0.5 ml), and the reaction mixture was stirred for 2 h at room temperature. Methylation was performed by the sequential addition of iodomethane (250 ml followed by 100 ml) at 30-min intervals. The permethylated monosaccharide was extracted into the organic phase (dichloromethane) after partitioning the reaction mixture between water and dichloromethane. The organic phase was then removed by evaporation under a stream of N2. The permethylated OS was further purified using a Sep-Pak C18 cartridge to remove any remaining Me2SO. The permethylated OS was hydrolyzed with 4 M trifluoroacetic acid (100 °C, 6 h), reduced with NaBH4, and acetylated, and the resulting partially methylated alditol acetates were analyzed by gas chromatography-mass spectrometry using an SPB capillary column (25 m × 0.25 mm, from Supelco) and DB-1 capillary column.

Mass Spectrometry—Oligosaccharides were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) using a 4700 Proteomics Analyzer instrument (Applied Biosystems) in reflectron mode. The OS samples were dissolved in water (1 mg/ml) and mixed in a 1:1 (v/v) ratio with 0.5 M 2,5-dihydroxybenzoic acid in methanol matrix solution. Spectra were acquired in both the positive and negative acquisition modes.

NMR Spectroscopy—NMR spectra were collected on Varian Inova500 and 600 spectrometers using standard software supplied by Varian. The samples were exchanged several times with D2O (99.8% Aldrich), and final measurements were made in 0.5-ml D2O solutions (100% D, Cambridge Isotope Laboratories) at 25 °C. Proton NMR spectra were measured at 600 MHz using spectral width of 8 kHz, and the data were processed with HOD signal referenced at 4.78 ppm on proton scale. The correlated spectroscopy (gCOSY) spectra were measured over a spectral width of 2.25 kHz using a dataset of (t1 × t2) points with 16 scans. The total correlated spectroscopy (TOCSY) spectra were collected using the same sized data set with 32 scans with a mixing time of 80 ms. For the heteronuclear single quantum coherence (HSQC) experiment, the spectral widths in the proton and carbon dimensions were 2.2 and 13.9 kHz, respectively, and 96 scans were acquired. One-dimensional 31P NMR spectra were done at pH 7.0 using a Varian Inova500 instrument with a broadband probe adjusted to 202.38 MHz. Proton-decoupled 31P spectra were acquired with a spectral width of 10 kHz calibrated with phosphoric acid (85%) as the external standard (δp = 0.0 ppm). The two-dimensional proton-detected H1-31P heteronuclear multiple bond quantum coherence (HMQC) and HMQC-TOCSY experiments were performed using the standard pulse sequence supplied by Varian. The HMQC spectrum was collected using a data set of 128 × 2048 (t1 × t2) points with a total of 32 scans. The measurements of HMQC and HMQC-TOCSY spectra were done using a J1H,P coupling value of 12.0 Hz. The mixing time used for HMQC-TOCSY spectrum was 60 ms, and a total of 64 scans was collected. The spectral widths in 31P-1H HMQC and HMQC-TOCSY experiments were set to 5 kHz in the phosphorus dimension and 2.25 kHz in the proton dimension, respectively.

RESULTS
Identification of the Lipooligosaccharide C-3 O-Acetyl Transferase Gene (lot3) in N. meningitidis Strain NMB—Meningococcal LOS immunotypes L2 and L4 are O-acetylated at the C-3...
location of the phosphoethanolamine (PEA) groups. The minor OSs from NMBlot3 were also analyzed by glycosyl composition analysis and mass spectrometry. The results showed that the minor OSs consisted of glycinylated versions of the major OS structure (i.e. the ion masses were 57 mass units greater than those of the major OS), as well as the presence of detectable amounts of sialic acid monosaccharide that was presumably released from the α-chain during mild acid hydrolysis.

The major OSs from the LOSs of NMB, and NMBlot3 were then analyzed by MALDI-TOF MS, and the resulting spectra are shown in Fig. 3 (A and B), respectively. The molecular ions observed for the OS from NMBlot3 (m/z 1804, 1822, 1826, 1844, and 1866 (Fig. 3B)) are all consistent with sialated and anhydro-sodiated forms of a single oligosaccharide structure. These masses are 42 mass units less than the corresponding ion masses for the OS from NMB (Fig. 3A). This result is consistent with the NMBlot3 LOS having a structure that is the same as reported for NMB LOS (36) with the notable exception that the NMBlot3 OS lacks an acetyl group. Previous work on the NMB LOS had reported that the OS was acetylated at O-6 of the GlcNAc residue terminally linked to O-2 of HepII (36).

This conclusion was based on comparison of one-dimensional TOCSY NMR spectra of the NMB OS taken before and after hydrogen fluoride treatment, which removes PEA as well as the O-acetyl group from the OS. This work on the NMBlot3 LOS required that we re-examine the location of this O-acetyl group in the NMB LOS, as well as confirm its absence in the NMBlot3 LOS. This was accomplished by examining the OS from NMB and from NMBlot3 by gradient correlation spectroscopy (gCOSY), total correlation spectroscopy (TOCSY), and gradient heteronuclear single quantum coherence spectroscopy (gHSQC). From these experiments, it was possible to make assignments for the majority of the protons for both the NMB OS (Table 1) and the NMBlot3 OS (Table 2). Comparison of the assignments for these OSs showed that the α-GlcNAc residue in NMB OS resonates at d 5.17, which is considerably downfield from the H3 resonance of this residue in NMBlot3 OS, which resonates at d 3.85. This downfield shift is consistent with O-acetylation at this position in NMB OS and a lack of this acetylation in the NMBlot3 OS. A TOCSY spectrum of the NMB OS showing the coupling of the H1 (d 5.28), H2 (d 4.19), H3 (d 5.17), and H4 (d 3.68) protons of the α-GlcNAc residue is given in Fig. 4.

The presence and location or absence of O-acetyl groups can also be observed in the gHSQC spectra of the NMB and NMBlot3 OS samples (Fig. 5, A and B, respectively). The NMB OS gHSQC spectrum (Fig. 5A) shows two N-acetyl groups (carbons) at d 2.04 and 2.06 (d 22.5), and the O-acetyl methyl protons (carbon) at d 2.11 (d 20.6). However, only two N-acetyl methyl protons (carbons) are observed in the NMBlot3 OS spectrum (Fig. 5B) at d 2.11 (d 22.5) and d 2.04 (d 21.9). The NMBlot3 OS lacks the O-acetyl resonances. The gHSQC spectrum of the NMB OS also contributed to assigning the location of the O-acetyl group to O-3 of the α-linked GlcNAc residue. From the gCOSY spectrum (not shown), the anomic proton of the α-GlcNAc residue (d 5.28) was shown to be coupled
The gHSQC spectrum (Fig. 5A) shows that this H2 is coupled to the C2 at δC 51.9, which confirms that this is the α-GlcNAc residue. These data, together with the TOCSY data (see Fig. 4), allowed the assignment of the resonances for the GlcNAc residue that are given in Table 1. From these assignments, it was shown that the H3 of this GlcNAc is at δH 4.19.
O-Acetylation of N. meningitidis Terminal GlcNAc

5.17 and is coupled to a carbon at δC 74.0 (see Fig. 5A). This H3/C3 coupling is clearly absent in the gHSQC spectrum of the NMBlot3 OS (Fig. 5B). Thus, these NMR data support the mass spectrometric data, which show that the OS from NMBlot3 LOS lacks an O-acetyl group, and that the O-acetyl group on NMB LOS is located at O-3 of the α-GlcNAc residue and not at O-6 as previously reported (36).

O-Acetylation of the Terminal GlcNAc Residue of Meningococcal LOS Influences PEA Additions to the Inner Core—The meningococcal LOS inner core structure is masked by the additions to HepII, which are highly variable and heterogeneous (14). One of these additions, the presence of the γ-chain extension of α1–3 glucose on HepII, characteristic of L2 and L5 immunotype meningococcal LOS, is determined by the phase-

| TABLE 1 | Proton chemical shift values for the OS from NMB LOS (partial assignment) |
| Residue | H1 | H2 | H3 | H4 | H5 | H6a,b | H7a,b |
|---------|----|----|----|----|----|--------|--------|
| α-d-D-HepII (A) | 5.77 | 4.25 | 4.23 | 4.17 | 3.76 | 4.57 | 3.83, 3.90 |
| α-d-Glc (B) | 5.43 | 3.6 | 3.72 | 3.48 | ND | ND | ND |
| α-d-GlcNAc (C) | 5.28 | 4.19 | 5.17 | 3.68 | ND | ND | ND |
| α-d-D-HepI (D) | 5.09 | 4.18 | 4.16 | 4.08 | ND | ND | ND |
| β-d-GlcNAc (E) | 4.72 | 3.81 | 3.74 | 3.59 | ND | ND | ND |
| β-d-Glc (F) | 4.56 | 3.44 | 3.64 | 3.56 | ND | ND | ND |
| β-d-Gal (G) | 4.48 | 3.55 | 3.67 | 3.93 | ND | ND | ND |
| β-d-Gal (H) | 4.43 | 3.59 | 3.75 | 4.16 | ND | ND | ND |

ND, not detected.

α Indicates the position of the O-acetyl group.

| TABLE 2 | Proton chemical shift values for the OS from NMBlot3 OS (partial assignment) |
| Residue | H1 | H2 | H3 | H4 | H5 | H6a,b | H7a,b |
|---------|----|----|----|----|----|--------|--------|
| α-t-D-HepI (A) | 5.71 | 4.18 | 4.21 | 4.16 | 3.73 | 4.56 | 3.74, 3.82 |
| α-t-Glc (B) | 5.41 | 3.58 | 3.72 | ND | ND | ND | ND |
| α-t-GlcNAc (C) | 5.18 | 3.89 | 3.85 | 3.48 | 3.86 | 3.9 | ND |
| α-t-D-HepI (D) | 5.09 | 4.17 | 4.11 | ND | ND | ND | ND |
| β-t-GlcNAc (E) | 4.72 | 3.82 | 3.76 | 3.59 | ND | ND | ND |
| β-t-Glc (F) | 4.56 | 3.44 | 3.65 | 3.56 | ND | ND | ND |
| β-t-Gal (G) | 4.48 | 3.55 | 3.68 | 3.94 | 3.56 | 3.44 | ND |
| β-t-Gal (H) | 4.46 | 3.59 | 3.75 | 4.16 | 3.92 | ND |

ND, not detected.

α Indicates the position of the O-acetyl group.
variable expression of lgtG (16). In some meningococcal strains, O-3-linked PEA is attached to HepII by Lpt3 in the absence of LgtG activity, thus producing the L3 immunotype (17). However, in strain NMB, which contains an intact Lpt3, inactivation of lgtG did not result in the appearance of O-3-linked PEA on the inner core of the LOS (18). Conversely, O-3-linked PEA is an inner core decoration in mutants of strain NMB, which lacked the addition of the α-chain (18, 37, 38). These results suggested that the conformation adopted by the inner core with the addition of the α-chain, in combination with the O-acetylation of the terminal GlcNAc residue, precluded the addition of O-3 PEA groups by Lpt3. To test this important hypothesis, which has implications for immunotype switching in vivo, a mutant strain in which both lgtG and lot3 were insertionally inactivated, designated NMBlot3/lgtG, was analyzed for the appearance of O-3 PEA groups on the inner core in the presence of an intact α-chain. Purification of the OSs by gel-filtration chromatography using Bio-Gel P2 resulted in one major and additional minor OSs from NMBlot3/lgtG. The major OS from NMBlot3/lgtG had the same glycosyl residue linkages as those for NMBlot3 OS with the exception that terminally linked Glc was absent; this result is consistent with the mutation in lgtG.

The major OSs were analyzed by MALDI-TOF MS, and the resulting spectrum is shown in Fig. 3C. The mass spectrum of the OS from NMBlot3/lgtG shows two clusters of ions. Each cluster represents a single structure. The ions of the first cluster (m/z 1765, 1783, 1787, 1805, 1809, 1827, 1831, and 1849) are all consistent with various sodiated and anhydro-sodiated forms of a structure that, compared with the NMBlot3 OS structure, lacks one hexosyl residue and contains an additional (i.e. for a total of two) PEA group. The second cluster ions (m/z 1642, 1660, 1664, 1682, 1686, and 1704) are consistent with sodiated and anhydro-sodiated forms of a structure that, compared with the NMBlot3 OS structure, lacks a hexosyl residue. This missing hexosyl residue is consistent with the methylation data showing that this OS lacks a terminally linked Glc; i.e. the terminal Glc that is linked to O-3 of HepII. In addition to the glycosyl linkage and MS data showing that the NMBlot3/lgtG OS lacks both the terminal α-Glc residue and the O-acetyl group, the lack of both these moieties was also confirmed by NMR analysis. Comparison with the spectra for NMB and NMBlot3 OSs (Fig. 5, A and B, respectively), the spectrum for the NMBlot3/lgtG OS (Fig. 5C) lacks the anomeric H/C signal for the α-Glc residue (residue B), and lacks the downfield H/C resonance for the terminal α-GlcNAc as observed for the NMB OS (Fig. 5A). As observed for NMBlot3, the minor OSs from NMBlot3/lgtG consisted of glycinylated versions of the major OS. These results support the conclusions that the NMBlot3/lgtG mutant, which, in addition to missing the O-acetyl group, lacks the terminally linked Glc attached to O-3 at HepII, was able to express a major structure containing two PEA groups attached to HepII.

Location of LOS Inner Core PEA Groups Influenced by O-Acetylation—To determine the location of the PEA groups on NMBlot3 and NMBlot3/lgtG OSs, two NMR experiments were performed. The first was a $^{31}$P-1H HMQC experiment, and the spectra for the NMBlot3 and NMBlot3/lgtG OSs are shown in Fig. 6. Fig. 6A shows that the phosphorous atom of the PEA group was correlated to the H-6 proton of HepII and to the -OCH$_2$- methylene protons of the PEA group. These results were consistent with NMBlot3 OS containing a single PEA group attached to O-6 of HepII. The spectrum of NMBlot3/lgtG OS shown in Fig. 6B was consistent with an OS that contains two PEA groups. For one PEA group the phosphorous atom was correlated to H-6 of HepII and to -OCH$_2$- of PEA, and for the second PEA group the phosphorous atom was correlated to H-3 of HepII and to -OCH$_2$- of PEA. These results are consistent with the NMBlot3/lgtG OS containing two PEA groups, at O-6 and O-3 of the HepII residue. The locations of the PEA groups were further confirmed by $^{31}$P-1H HMQC-TOCSY analysis. The spectrum for the NMBlot3 OS is shown in Fig. 7A and shows that the phosphorous of the PEA group is correlated to H-6, H-5, H-7a, and H-7b of HepII and to the -O-CH$_2$- methylene and -CH$_2$-NH$_2$ methylene protons of the PEA group. The spectrum for the NMBlot3/lgtG OS (Fig. 7B) showed that the phosphorus of one PEA group was correlated with HepII and...
PEA methylene protons consistent with a location at O-6 of HepII as just described for the NMBlot3 OS, whereas the phosphorus of the second PEA group was correlated to H-3, H-2, H-4, and H-5 of HepII and to the protons of both of the PEA methylene groups. These results confirm that the major OS from NMBlot3/lgtG LOS contains structures with two PEA groups located at O-3 and O-6 of HepII, as well as with a single PEA group located at O-3 or O-6 of HepII. Thus, in strain NMB, O-3-linked PEA groups appear on the LOS inner core only in the absence of both O-3-linked glucose at HepII and O-acetylation of the terminal GlcNAc. This is consistent with the hypothesis that in the presence of a full-length α-chain and O-acetylation of terminal GlcNAc of the inner core, Lpt3 is unable to gain access to HepII of the inner core.

**DISCUSSION**

Lipooligosaccharide structure and variability in *N. meningitidis* has been shown to be important for the pathogenesis of this organism. An examination of various LOS immunotyped meningococcal strains by Jennings *et al.* (15) demonstrated that one mechanism of LOS phase variation was due to changes in the length of homopolymeric tract regions of lgtA, lgtC, and lgtG (Table 3). Interestingly, the most frequently detected reversible LOS immunotype switching event has been that of L[1,8] to L[3,7,9], since a mixture of these structures is expressed in many isolates (3, 4). Although immunotype switching involving L2 and L4 immunotypes is less frequent, it does occur with two natural events of L[1,2] (4) and L[2,3] (15) immunotype interconversions being described. Our study indicates that the LOS structural changes resulting in conversion between immunotype pattern expression of L[2,4] and L[3,7,9] LOS is in part dependent upon the phase-variable expression of the lipooligosaccharide O-acetyltransferase encoded by lot3.

An L3 immunotype is distinguished from an L2 LOS immunotype by four structural changes to the HepII of the inner core: the loss or exclusion of O-3-linked PEA group from HepII, the addition of O-3-linked glucose by LgtG, the addition of O-6-linked PEA group to the terminal GlcNAc. Mackinnon *et al.* (17) demonstrated that expression of LgtG was sufficient to exclude the addition of O-3-linked PEA groups from the LOS inner core of L3 immunotype strains and ascribed this dominant effect to increased efficiency for this site by LgtG. However, inactivation of LgtG and Lpt6 in strain NMB, which expresses a mixture of L2 and L4 LOS immunotypes, does not result in the appearance of an L3 immunotype by four structural changes to the HepII of the inner core: the loss or exclusion of O-3-linked PEA group from HepII, the addition of O-3-linked glucose by LgtG, the addition of O-6-linked PEA group to the terminal GlcNAc.

The presence of lot3 has been established in representative meningococcal strains expressing all twelve immunotypes (7). O-Acetylation of the chemical structures for immunotypes L1, L2, L4, L5, and L6 has been established (see Fig. 1 for references).

### TABLE 3

Expression matrix for meningococcal LOS immunotypes

| Strain       | Immunotype |
|--------------|------------|
| NMB          | L2         |
| M981         | L5         |
| M992         | L6         |
| NMBlot3/Llot3 | L1         |

**Strain Immunotype LOS biosynthesis genes**

| Strain | Immunotype | lgtA⁺ | lgtC⁺ | lgtG⁺⁺⁺ | lpt3⁺⁺⁺ | lpt6⁺⁺⁺ | lot3⁺⁺⁺ |
|--------|------------|------|------|-------|--------|--------|--------|
| 126E   | L1         | A    | P⁺   | A     | P      | A      | P⁺    |
| NMB    | L2         | P⁺   | A    | P⁺    | P⁺    | P⁺    | P⁺    |
| M981   | L5         | P⁺   | A    | P⁻    | A      | P⁺    |
| M992   | L6         | P⁺   | A    | P⁻    | A      | P⁺    |

"A" indicates absent, "P" indicates present. **"-" indicates phase off, and "⁺⁺⁺" indicates phase on.**

The presence of O-Acetylation of the chemical structures for immunotypes L1, L2, L4, L5, and L6 has been established (see Fig. 1 for references).
(lot3) was identified by homology to a known LOS O-acetyltransaferase from *R. leguminosarum*. Inactivation of lot3 in strain NMB resulted in the loss of the O-acetyl group located on the terminal GlcNAc of the LOS inner core. We found that O-acetylation of the GlcNAc residue is at O-3 as previously assigned by Kogan et al. (11). The proton spectrum of the NMB OS in a previous report (36) clearly shows that it is identical to that obtained for our current analysis of NMB OS and has the downfield \( \alpha \)-GlcNAc H3 resonance at \( d \) 5.17 due to O-acetylation at that position. The O-6 assignment in the earlier report was made by comparing one-dimensional TOCSY spectra before and after HF treatment of the OS. The fact that this treatment removes both O-acetyl and PEA groups and the rather poor quality of the one-dimensional TOCSY spectra resulted in the incorrect assignment of the O-acetyl group. Therefore, the lot3 locus in strain NMB has been designated "lot3" indicating the linkage of the O-acetyl group at the O-3 position of the terminal GlcNAc residue in the L2 immunotype structure.

The O-3-linked PEA groups did not appear on the LOS inner core of NMB\( lot3 \) mutants due to the dominant effect by LgtG as observed by Mackinnon (17). Inactivation of lgtG in the absence of Lot3 activity in strain NMB resulted in the appearance of O-3-linked PEA groups on the inner core, supporting the hypothesis that the O-acetyl group was responsible for obstructing access of Lpt3 to the LOS inner core in the absence of O-3-linked glucose. Based upon previous observations on the effect of O-acetylation on the conformation of *Salmonella typhimurium* O-antigen (39) the most likely mechanism appears to be that the meningococcal LOS undergoes a conformational change upon O-3 acetylation of GlcNAc, which, in the presence of the lactoneotetraose \( \alpha \)-chain, prevents access to the inner core by Lpt3. A comparison of lot3 genes found in the meningococcal genome databases with that of strain NMB indicates that a short polymeric G-tract is present in the central region of the open reading frame. The point mutation inactivating lot3 in strain MC58, which expressed L3 immunotype LOS, was found in this region; however, whether the length of the polymeric tract undergoes phase variation at a detectable rate remains unknown.

The lot3 locus has been found in all meningococcal strains examined to date, including isolates expressing all twelve immunotype structures (data not shown). The intact lot gene was found in those strains expressing immunotype LOS structures with an O-acetyl group (Fig. 1 and Table 3). From these data we predict that immunotypes L9–L12 are also O-acetylated, indicating that this substitution is common among all immunotypes with the exception of L3 and L7. Based upon the structures for LOS immunotypes L1–L8, and the data derived from mutating the LOS biosynthesis pathways in strains NMB and MC58, the following conclusions can be drawn regarding the expression of the O-3 PEA group on the LOS inner core of meningococcal strains. In meningococcal isolates expressing a LOS structure with a short \( \alpha \)-chain (such as L1 and L8), O-3 PEA groups can be added to the inner core by Lpt3 regardless of the O-acetylation state of the terminal GlcNAc of the \( \gamma \)-chain (18). In comparison, the spectrum of decorations added to the LOS inner core of meningococcal isolates expressing an \( \alpha \)-chain consisting of lactoneotetraose is determined by the O-acetylation status of the terminal GlcNAc. When lot3 is not expressed in the presence of a lactoneotetraose \( \alpha \)-chain, the phase-variable expression of lgtG and the presence or absence of functional lpt3 and lpt6 determines which decorations are added to the inner core (Table 3). When lot3 is expressed in the presence of a LOS structure with a full-length \( \alpha \)-chain, even if the strain contains an active lpt3, only the O-6-linked PEA groups and O-3-linked glucose residues can be added to the LOS inner core. We cannot completely exclude the possibility that the absence of Lot may affect the stability or function of Lpt3. However, it appears unlikely that Lot and Lpt3 form a complex, because the predicted structures of these proteins indicate that they reside in separate cell compartments (cytoplasm and periplasm, respectively, data not shown).

The biological relevance of these findings is of considerable interest. Ram et al. (19) have suggested that there is a strong positive selection pressure for the expression in invasive meningococcal disease of LOS structures with a lactoneotetraose chain and an inner core decorated with an O-3 PEA group, because this structure is unavailable for binding to the complement factor C4b. The O-3 PEA group results in increased resistance to killing by human serum during bloodstream dissemination. The identification of lot3 and the effect on the exclusion of the addition of O-3 PEA groups to the LOS inner core also suggest that there may be biological selection for preventing or masking the appearance of O-3-linked PEA groups on the surface of meningococci. LOS functions as an adherence and cell invasion ligand (3, 4, 40, 41), in addition to influencing these events by obstruction of Opa adhesins (42). Alternatively, the effect on the presence of O-3 PEA groups may be an indirect consequence of immunological selection directed at the O-acetyl group on the LOS inner core. Investigations of the immunological responses generated by meningococcal LOS immunotypes have shown that the O-acetylated L2 and L5 structures induced higher antibody titers than do non-O-acetylated L[3,9] structures (43). Polyclonal antiserum raised against L2 and L5 immunotypes was bactericidal, but immunotype-specific, whereas the response elicited by L[3,9] immunotypes recognized common epitopes preserved between L2 and L[3,9] (44) but was not bactericidal.

Present strategies for the development of LOS glycoconjugates as meningococcal vaccine candidates are providing preliminary evidence that such vaccines will yield cross-protective immunity against meningococci (45). Because the majority of *N. meningitidis* isolated from invasive disease cases express the L[3,7,9] immunotype LOS (3, 4), and because the expression of the L[3,7,9] immunotype LOS by *N. meningitidis* may increase resistance to complement-mediated lysis during disseminated disease (19), most of this work has focused upon utilizing an L3 LOS. However, studies assessing the distribution of LOS immunotypes expressed in meningococcal disease isolates indicate that at least one-quarter of strains causing invasive meningococcal disease express L[2,4], and these strains may utilize other mechanisms for resistance to complement mediated lysis (46). Based upon our current and previous observations of strain NMB (29), the steps involved for conversion among immunotypes include: the phase-variable expression of lgtA, the carriage of an active lot3, the acquisition of islets carrying lpt6 and
O-Acetylation of N. meningitidis Terminal GlcNAc

IgtG, and the phase variable and regulated expression of IgtG. The mechanisms of LOS assembly and the basis of immunotype switching are important in both understanding meningococcal pathogenesis and for LOS vaccine design. O-Acetylation of meningococcal LOS represents a potential mechanism for immune escape from a vaccine targeting only L3 immunotype LOS, and therefore multiple endotoxin structures may be required for an effective meningococcal endotoxin-based vaccine.

Acknowledgement—We thank L. Pucko for help with manuscript preparation.

REFERENCES

1. Yazdankhah, S. P., and Caugant, D. A. (2004) J. Med. Microbiol. 53, 821–832
2. Neal, K. R., Nguyen-van-Tam, J. S., Slack, R. C., Kaczmarski, E. B., White, A., and Ala’Aldeen, D. A. (1999) Epidemiol. Infect. 123, 507–509
3. Jones, D. M., Borrow, R., Fox, A. J., Gray, S., Cartwright, K. A., and Poolman, J. T. (1992) Microb. Pathog. 13, 219–224
4. Scholten, R. J., Kuipers, B., Valkenburg, H. A., Dankert, J., Zollinger, W. D., and Poolman, J. T. (1994) J. Med. Microbiol. 41, 236–243
5. Moller, A. S., Bjerre, A., Brusletto, B., Joo, G. B., Brandtzaeg, P., and Kierulf, P. (2005) J. Infect. Dis. 191, 768–775
6. Mandrell, R. E., and Zollinger, W. D. (1977) Infect. Immun. 16, 471–475
7. Tsai, C. M., Mocca, L. F., and Frasch, C. E. (1987) Infect. Immun. 55, 1652–1656
8. Zollinger, W. D., and Mandrell, R. E. (1977) Infect. Immun. 18, 424–433
9. Difabio, J. L., Michon, F., Brisson, J., and Jennings, H. J. (1990) Can. J. Chem. 68, 1029–1034
10. Gamin, A., Beurret, M., Michon, F., Brisson, J. R., and Jennings, H. J. (1992) J. Biol. Chem. 267, 922–925
11. Kogan, G., Uhrin, D., Brisson, J. R., and Jennings, H. J. (1997) Carbohydr. Res. 298, 191–199
12. Michon, F., Beurret, M., Gamin, A., Brisson, J. R., and Jennings, H. J. (1990) J. Biol. Chem. 265, 7243–7247
13. Pavliak, V., Brisson, J. R., Michon, F., Uhrin, D., and Jennings, H. J. (1993) J. Biol. Chem. 268, 14146–14152
14. Kahler, C. M., and Stephens, D. S. (1998) Crit. Rev. Microbiol. 24, 281–334
15. Jennings, M. P., Srikhanta, Y. N., Moxon, E. R., Kramer, M., Poolman, J. T., Kuipers, B., and van der Ley, P. (1999) Microbiology 145, 3013–3021
16. Banerjee, A., Wang, R., Uijson, S. N., Rice, P. A., Gotschlich, E. C., and Stein, D. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10872–10877
17. Mackinnon, F. G., Cox, A. D., Pleshed, J. S., Tang, C. M., Makepeke, K., Coull, P. A., Wright, J. C., Chalmers, R., Hood, D. W., Richards, J. C., and Moxon, E. R. (2002) Mol. Microbiol. 43, 931–943
18. Kahler, C. M., Datta, A., Tseng, Y. L., Carlson, R. W., and Stephens, D. S. (2005) Glycobiology 15, 409–419
19. Ram, S., Cox, A. D., Wright, J. C., Vogel, U., Getzlaff, S., Boden, R., Li, J., Pleshed, J. S., Meri, S., Gulati, S., Stein, D. C., Richards, J. C., Moxon, E. R., and Rice, P. A. (2003) J. Biol. Chem. 278, 50853–50862
20. D’Haeze, W., and Holsters, M. (2002) Glycobiology 12, 798–1058
21. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119
22. Morse, S. A., and Bartenstein, L. (1974) Proc. Soc. Exp. Biol. Med. 145, 1418–1421
23. Kahler, C. M., Carlson, R. W., Rahman, M. M., Martin, L. E., and Stephens, D. S. (1996) J. Bacteriol. 178, 1265–1273
24. Chung, C. T., Niemela, S. L., and Miller, R. H. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2172–2175
25. Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. (2003) Nucleic Acids Res. 31, 3497–3500
26. Tatusova, T. A., and Madden, T. L. (1999) FEMS Microbiol. Lett. 174, 247–250
27. Takeshita, S., Sato, M., Toba, M., Masahashi, W., and Hashimoto-Gotoh, T. (1987) Gene (Amst.) 61, 63–74
28. Janik, A., Juni, E., and Heym, G. A. (1976) J. Clin. Microbiol. 4, 71–81
29. Tseng, Y. L., Datta, A., Ambrose, K., Lo, M., Davies, J. K., Carlson, R. W., Stephens, D. S., and Kahler, C. M. (2004) J. Biol. Chem. 279, 35053–35062
30. Kahler, C. M., Carlson, R. W., Rahman, M. M., Martin, L. E., and Stephens, D. S. (1996) J. Bacteriol. 178, 6677–6684
31. York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., and Albersheim, P. (1985) Methods Enzymol. 118, 3–40
32. Ciucau, I., and Kerek, F. (1984) Carbohydr. Res. 131, 209–217
33. Firmin, I. L., Wilson, K. E., Carlson, R. W., Davies, A. E., and Downie, J. A. (1993) Mol. Microbiol. 10, 351–360
34. Davis, E. O., Evans, I. J., and Johnston, A. W. (1988) Mol. Gen. Genet. 212, 531–535
35. Warren, M. J., Roddam, L. F., Power, P. M., Terry, T. D., and Jennings, M. P. (2004) FEMS Immunol. Med. Microbiol. 41, 43–50
36. Rahman, M. M., Stephens, D. S., Kahler, C. M., Glushka, J., and Carlson, R. W. (1998) Carbohydr. Res. 307, 311–324
37. Monteiro, M. A., Fortuná-Nevin, M., Farley, J., and Pavliak, V. (2003) Carbohydr. Res. 338, 2905–2912
38. Lee, F. K., Stephens, D. S., Gibson, B. W., Engstrom, J. J., Zhou, D., and Apicella, M. A. (1995) Infect. Immun. 63, 2508–2515
39. Kim, M. L., and Sluach, J. M. (1999) FEMS Immunol. Med. Microbiol. 26, 83–92
40. Harvey, H. A., Jennings, M. P., Campbell, C. A., Williams, R., and Apicella, M. A. (2001) Mol. Microbiol. 42, 659–672
41. Plant, L., Sundqvist, J., Zughair, S., Lovkvist, L., Stephens, D. S., and Jonsson, A. B. (2006) Infect. Immun. 74, 1360–1367
42. Virji, M., Makepeke, K., Peak, I. R., Ferguson, D. J., Jennings, M. P., and Moxon, E. R. (1995) Mol. Microbiol. 18, 741–754
43. Jennings, H. J., Lugowski, C., and Ashton, F. E. (1984) Infect. Immun. 43, 407–412
44. Verheul, A. F., Booms, G. J., Van der Marel, G. A., Van Boom, J. H., Jennings, H. J., Snipe, H., Verhoef, J., Hoogerhout, P., and Poolman, J. T. (1991) Infect. Immun. 59, 5366–5373
45. Cox, A. D., Zou, W., Gidney, M. A., Lacelle, S., Pleshed, J. S., Makepeke, K., Wright, J. C., Coull, P. A., Moxon, E. R., and Richards, J. C. (2005) Vaccine 23, 5046–5054
46. Jarva, H., Ram, S., Vogel, U., Blom, A. M., and Meri, S. (2005) J. Immunol. 174, 6299–6307