Functional Relationships of the Genetic Locus Encoding the Glycosyltransferase Enzymes Involved in Expression of the Lacto-N-neotetraose Terminal Lipopolysaccharide Structure in Neisseria meningitidis*

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The biosynthetic function of the lgtABE genetic locus of Neisseria meningitidis was determined by structural analysis of lipopolysaccharide (LPS) derived from mutant strains and enzymatic assay for glycosyltransferase activity. LPS was obtained from mutants generated by insertion of antibiotic resistance cassettes in each of the three genes lgtA, lgtB, and lgtE of the N. meningitidis immunotype L3 strain 3 MC58. LPS from the parent strain expresses the terminal lacto-N-neotetraose structure, Galβ1→4GlcNAcβ1→3Galβ1→4Glc. Mild hydrazine treatment of the LPS afforded O-deacylated samples that were analyzed directly by electrospray ionization mass spectrometry (ESI-MS) in the negative ion mode. In conjunction with results from sugar analysis, ESI-MS revealed successive loss of the sugars Gal, GlcNAc, and Gal in lgt B, lgt A, and lgt E LPS, respectively. The structure of a sample of O- and N-deacylated LPS derived by aqueous KOH treatment of lgt B LPS was determined in detail by two-dimensional homo- and heteronuclear NMR methods. Using a synthetic β-GlcNAc acceptor and a β-lactose acceptor, the glycosyltransferase activities encoded by the lgtB and lgtA genes were unambiguously established. These data provide the first definitive evidence that the three genes encode the respective glycosyltransferases required for biosynthesis of the terminal trisaccharide moiety of the lacto-N-neotetraose structure in Neisseria LPS. From ESI-MS data, it was also determined that the Gal-deficient LPS expressed by the lgt E mutant is identical to that of the major component expressed by immunotype L3 galE-deficient strains. The galE gene which encodes for UDP-glucose-4-epimerase plays an essential role in the incorporation of Gal into meningococcal LPS.

Diseases caused by Neisseria meningitidis remain a signifi-

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The abbreviations used are: LPS, lipopolysaccharide; KDO, 3-deoxy-D-manno-octulosonic acid; PAGE, polyacrylamide gel electrophoresis; LPS-OH, O-deacylated LPS; GLC, gas-liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; HMOC, heteronuclear multiple quantum coherence; PEA, phosphoethanolamine; FCHASE, 5-(fluorescein-carboxamido)-hexanoyl acid succimidyl ester; CE, capillary electrophoresis; MES, 4-morpholinethanesulfonic acid.

19166
and to provide the mechanism by which phase variable expression of the epitope is controlled (18). Mutants generated in each of three genes in the lgtABE locus were implicated by immunological and PAGE analysis of derived LPS to have a role in the synthesis of glycosyltransferase enzymes (18). In this study, the molecular structures for LPS produced by the three lgt mutant strains are determined providing, for the first time, definitive evidence that this genetic locus encodes the glycosyltransferases required for sequential addition of glycoses to the growing lacto-N-neotetraose end group. A relationship between the structures of the LPS elaborated by lgtE gene and the β-1,3-acetylgalactosaminyltransferase activity encoded by the lgtB gene and the β-1,3-N-acetylgalactosaminyltransferase activity of the lgtA gene were unequivocally demonstrated by enzymatic assay using synthetic acceptors.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—The following strains of *N. meningitidis* were used in this study: immunotype L7 strain M982B (NRCC no. 4725); lgt mutant strains Δ3 lgtB, Δ3 lgtA, and Δ3 lgtE; and mutant strains M58B and M58D Gal-E and Gal-I (MC58) as described previously (18). UDP galactose-deficient strains were constructed by insertion of kanamycin-resistance (kan) cassette in the genomic DNA of *N. meningitidis* MC58 and H44/76 (22).

**Preparation of Lipopolysaccharide—** *N. meningitidis* strains were received from microbials on 5% sheep blood agar plates and incubated overnight at 37 °C, and the proceeds of six plates were suspended in 50 ml of Difco Bacto Todd Hewitt broth (Difco). For mutant strains, this culture was used to inoculate 2.5 liters of the same medium containing 50 μg/ml kanamycin (Sigma) and, following incubation at 37 °C for 6–8 h, was used to inoculate 60 liters of the same medium used in a New Brunswick Scientific IF–75 fermenter. Fermenter growth was overnight (approximately 17 h) at 37 °C. The culture was killed by addition of 1% (final concentration) phenol, chilled to 15 °C, and harvested by continuous centrifugation. Wet weight biomass yields were in the order of 3.5 g/liter. *N. meningitidis* strain M982B (immunotype L7) was grown under similar conditions as described previously (26) and purified by repeated ultracentrifugation (105,000 × g, 4 °C, 2 × 5 h).

**Deoxycholate-Polyacrylamide Gel Electrophoresis (PAGE)—** Polyacrylamide gel electrophoresis was performed using the buffer system of Laemmli and Favre (27) as modified by Komura and Galanos (28) with sodium deoxycholate as the detergent. Lipopolysaccharide bands were stained and visualized by silver staining as described by Tsai and Frasch (29).

**Partial Acid Hydrolysis—** LPS (25 mg) was hydrolyzed in 1% aqueous acetic acid (5 ml) for 2.5 h at 100 °C, the solution was then cooled (4 °C), and the precipitated lipid A was removed by low speed centrifugation. The supernatant solution was lyophilized, and the water-soluble component was fractionated on a Sephadex G-50 gel filtration column (1.6 × 90 cm, Pharmacia) has been described previously (38).

**Preparation of O-Deacetylated LPS—** LPS was deacetylated with anhydrous hydrazine under mild conditions as described previously (32). Briefly, a sample (14 mg) was treated with anhydrous hydrazine (1 ml) and stirred at 37 °C. After 1 h, the reaction mixture was cooled (0 °C) and hydrazine was destroyed by addition of cold acetone (5 ml) after evaporation under a stream of nitrogen, and the glycoses were determined by gas-liquid chromatography—mass spectrometry (GLC-MS) of their derived alditol acetates as described previously (34). GLC-MS was performed with a Varian ion trap system fitted with a DB-17 fused silica capillary column (0.25 mm × 25 m, Quadrex Corp.) in the electron impact mode with a temperature program starting at 180 °C for 2 min followed by an increase of 5 °C/min to 300 °C.

**Electrospray Ionization-Mass Spectrometry (ESI-MS)—** Samples were analyzed in the negative or positive ion mode on a VG Quattro triple quadrupole mass spectrometer (Fisons Instruments) fitted with an electrospray ion source. Oligosaccharides samples were dissolved in water, this was diluted by 50% with acetonitrile:water (4:4:1, and then the mixture was introduced by direct infusion at 4 μl/min with a Harvard syringe pump 22. The electrospray tip voltage was 3.5 kV, and the mass spectrometer was scanned from m/z 50–2500 with a scan time of 10 s. Data were collected in multichannel analysis mode, and data processing was handled by the VG data system (Masslynx). For MS-MS experiments, precursor ions were selected using the first quadrupole mass analyzer, and fragment ions, formed by collisional activation with argon in the rf-only quadrupole collision cell, were mass analyzed by scanning the third quadrupole. Collision energies were typically 60 eV.

**Nuclear Magnetic Resonance (NMR) Spectroscopy—** NMR spectra were obtained on a Bruker AMX 500 spectrometer using standard Bruker software. Measurements were made at 37 °C on solutions in 0.5 ml of D2O subsequent to several lyophilizations with D2O.

Proton NMR spectra were measured at 500 MHz using a spectral width of 6.0 kHz and a 90° pulse. Proton and 13C chemical shifts were referenced to that of the resonances of internal acetone (δH, 2.22 ppm; δC, 31.07 ppm). Two-dimensional homonuclear correlation experiments (COSY) (35) were measured over a spectral width of 2.3 or 1.3 kHz using data sets of 256 × 2048 points; 32 or 64 scans were acquired, respectively. Spectra were processed in magnitude mode with symmetrization about the diagonal. Two-dimensional nuclear Overhauser effect experiments (NOESY) (36) were performed using a data set of 256 × 2048 points, a spectral width of 2.3 kHz, a 400 ms mixing time and 128 scans.

**Heteronuclear two-dimensional 1H-13C shifts correlations were measured in the 1H-detected mode via multiple quantum coherence (HMQC) with proton decoupling in the 1H domain (37), using data sets of 1024 × 256 points and spectral widths of 4.5 and 13.8 kHz for 1H and 13C domains, respectively; 128 scans were acquired for each t1 value.

**Phosphorous-31 spectra were measured at 202 MHz with a spectral width of 13 kHz and phosphoric acid (85%) was used as the external standard (δP, 0.00 ppm). 1H-31P correlations (HMQC) were made in the 1H-detected mode by using a data matrix of 16 × 1024 points, sweep width of 10 kHz for 31P and 1.3 kHz for 1H, and a mixing time of 60 ms.

**Molecular Biology—** DNA manipulations were performed as described previously (38). A polymerase chain reaction was performed with Pwo polymerase as described by the manufacturer (Boehringer Mannheim). DNA sequencing was performed with an Applied Biosystems model 370A sequencer using the cycle sequencing kit from ABI. The expression vector used for the lgtB gene has been described previously (38).

**Preparation of Fluorescin-labeled Aminophenylglycosides—** p-Aminophenylglycoside (10 mg) (Sigma) was dissolved in 0.5 ml of 0.2 M triethylamine acetate buffer, pH 8.2. 5-(Fluorescein-carboxamido)-hexafluoropropanoic acid succimidylester (10 mg, single isomer) (FCHASE, Molecular Probes) was dissolved in 0.5 ml of methanol and added to the aminophenylglycoside solution. The mixture was stirred in the dark for 3 h at room temperature and then dried in a Savant Speedvac. The dry mixture was resuspended in 200 μl of 50% acetonitrile and spotted on a 1-mm thick 20 cm × 20-cm Silica 60 TLC plate (E. Merck). The TLC plate was developed with the following solvent system: ethyl acetate/methanol/water/acetic acid 7:2:1:0.1. After air drying in a fume hood, the bright yellow product was scraped off the plate and eluted with five 10-ml washes of distilled water. The water eluates were pooled, and the product was concentrated and desalted by binding to a Sep-Pak C18 reverse phase cartridge. After washing the cartridge with 20 ml of water, the product was eluted in 1–3 ml of 50% acetonitrile. The product was quantitated by spectrophotometry with E399 = 68,000 M–1 cm–1.

**Capillary Electrophoresis—** Capillary electrophoresis (CE) was performed with a Beckman P/ACE 5510 equipped with an Argion ion laser-induced fluorescence detector, λ = 488 nm. The capillary was a standard 75 μm × 50-cm bare silica, with the detector at 47 cm. The capillary was conditioned before each run by washing with 0.2 M NaOH

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for 2 min, water for 2 min, then 20 mM sodium dodecyl sulfate/25 mM sodium tetraborate, pH 9.4, for 2 min. Samples were introduced by pressure injection for 2–5 s, and the separation was performed at 12 kV, 53 μA. Peak integration was performed with the Bedeck System Gold (version 8) software.

Enzyme Assays—Cell extracts were prepared using an Avestin B3 Emulsiflex cell disrupter (Avestin Ottawa, Ontario). The clarified cell extracts were centrifuged at 100,000 g for 2 min, water for 2 min, then 20 mM sodium dodecyl sulfate/25 mM sodium tetraborate, pH 9.4, for 2 min. Samples were introduced by pressure injection for 2–5 s, and the separation was performed at 12 kV, 53 μA. Peak integration was performed with the Bedeck System Gold (version 8) software.

Enzyme Assays—Cell extracts were prepared using an Avestin B3 Emulsiflex cell disrupter (Avestin Ottawa, Ontario). The clarified cell extracts were centrifuged at 100,000 g for 1 h to pellet the cell membrane. Glycosyltransferase reactions were performed at 37°C in 20-μl volumes and contained MES buffer 50 mM, pH 6.7, 10 mM MnCl₂, 5 mM dithiothreitol, 10 mM labeled acceptor, 1 mM UDP-Gal or 1 mM UDP-GlcNAc as donor, and various amounts of enzyme, either from crude bacterial extracts or extracts of recombinant E. coli with the cloned lgtB gene. The reactions were terminated by the addition of an equal volume of 2% SDS and heat to 75°C, for 3 min. These samples were then diluted appropriately in water prior to analysis by CE.

After the reaction the FCHASE-aminophenylglycosides were bound to a Sep-Pak C₁₈ reverse phase cartridge, desalted by washing with water and then eluted in 50% acetonitrile. After drying under vacuum, the samples were dissolved in water, and glycosidase assays were performed as described by the enzyme manufacturer (Oxford Glycosystems). These samples were then diluted with water and again analyzed by CE.

**RESULTS**

Lacto-N-neotetraose-deficient mutant strains of *N. meningitidis* were constructed by insertion of kanamycin resistance cassettes into the lgtABE locus (mutants lgtA, lgtB, and lgtE) and the galE gene (mutant galE) of the L3 immunotype strain (lgt) MC58 (18, 22). LPS was obtained from fermenter-grown cells by extraction with aqueous phenol (26) followed by ultracentrifugation of the dialyzed and concentrated aqueous phase. Meningococcal LPS, representative of the parent strain, has been reported (5) to comprise of β-galactose (Gal), α-glucose (Glc), 2-amino-2-deoxy-D-glucose (GlcN), L-glycerol-α-manno-heptose (Hep), and 3-deoxy-α-manno-octulosonic acid (KDO) in the molar ratio of 2:1:4:2:2. Compositional analysis of lgtB and lgtA LPS revealed the presence of these constituent sugars, but significantly less Gal was detected in both LPS samples as well as a diminished amount of GlcN in lgtA LPS. Quantitative analysis of the respective core oligosaccharides indicated the presence of equal molar amounts of Glc and Gal. Gal was not detected among the complete acid hydrolysis products of lgtE and galE LPS indicating the absence of Gal in these LPS samples.

Deoxycholate-PAGE analysis of the LPS from the immunotype L3-derived mutants revealed single-band patterns that were faster migrating than meningococcal LPS containing the complete lacto-N-neotetraose epitope (Fig. 1). The observed electrophoretic mobilities of the bands correspond to low molecular weight LPS composed of a lipid A and a core oligosaccharide. LPS from lgtA, lgtB, and lgtE showed consecutively faster relative mobilities than that of immunotype L7 LPS consistent with successive sugar deletions in the core oligosaccharide regions (Fig. 1). galE LPS gave a more diffuse band that exhibited similar electrophoretic mobility to lgtE LPS. LPS from the *N. meningitidis* immunotype L7 strain M982B has been shown (9) to contain the immunotype L3 basal structure with complete expression of the lacto-N-neotetraose epitope.

Treatment of the LPS samples with anhydrous hydrizine under mild conditions afforded water-soluble O-deacylated samples that were suitable for mass spectral analysis by ESI. In the last few years, ESI-MS has proved to be a valuable tool for structural analysis and for probing structural heterogeneity of low molecular weight LPS (39–42). ESI-MS, recorded in the negative ion mode, for the lgt mutant O-deacylated LPS (LPS-OH) are shown in Fig. 2. For each sample, the mass spectrum is dominated by molecular peaks corresponding to doubly and triply deprotonated ions arising from a single molecular species and this is in agreement with the deoxycholate-PAGE results. For the lgtB LPS-OH sample (Fig. 2A), triply [M – 3H]⁻ and doubly [M – 2H]²⁻ charged ions were observed at m/z 876.1 and m/z 1314.3, respectively. These data indicate that the
composition of the major O-deacylated LPS species (Mz, 2631) contains one less hexose residue than that predicted for the complete lacto-N-neotetraose epitope of immunotype L7 LPS-OH (i.e. Mz, 2793). The MS-MS spectrum of tgt B LPS-OH produced by low energy collisional activation of either the triply or doubly deprotonated ion afforded a major fragment ion at m/z 951 arising from cleavage of the KDO-β-glucosamine bond in which the ketosidic oxygen is retained by the O-deacylated lipid A forming a Y-type fragment ion (43) (data not shown). Cleavage of this linkage in LPS-OH to yield the O-deacylated lipid A singly charged ion appears to be the dominant fragmentation pathway in the negative ion mode (40, 42). The mass of this fragment is in accord with the proposed structure of N. meningitidis lipid A (10). The ESI-MS and MS-MS data are consistent with the tgt LPS containing a truncated lacto-N-neotetraose chain (GlcN-Gal-Glc) attached to the basal inner core region of the molecule and this was confirmed by a detailed NMR analysis of the LPS backbone oligosaccharide (see below). ESI-MS analysis of tgt A and tgt E LPS-OH gave triply and doubly charged ions of lower mass (Fig. 2, B and C) corresponding to consecutive loss of hexosamine (GlcN) and a hexose (Gal) in the respective LPS samples. As expected (18, 22), the gal E LPS-OH sample gave an ESI-MS that was similar to that of tgt E (data not shown). In addition to this major LPS-OH species (Mz, 2265.9), the ESI-MS of the gal E sample revealed a minor component (5–10% of the total) containing one extra hexose residue (Mz, 2427.6) which was indicated to be Glc from sugar analysis. The ESI-MS data and the proposed compositions are summarized in Table I and the structural relationships are presented in Fig. 3.

Removal of ester and amido linked fatty acid groups of the tgt B LPS derived by treating it with strong alkali according to established procedures (33) afforded a backbone oligosaccharide sample. ESI-MS of the LPS backbone oligosaccharide sample, so obtained, in the negative ion mode revealed abundant triply [M – 3H]+ and doubly [M – 2H]+ charged ions at m/z 682.7 and 1024.5 corresponding to a decasaccharide trisphosphate (Hex3–Hex2–Hex1–KDO2–(H2PO3)3) as the major molecular species (Mz, 2051.2). Correspondingly, the sample ions gave at m/z 513.7, 684.7, and 1026.4 from [M + 4H]+, [M + 3H]+ and [M + 2H]+ multiply charged protonated ion species (Mz, 2050.9) in the positive ion spectrum. MS-MS of the doubly deprotonated ion at m/z 499.1 from the glucosamine disaccharide bisphosphate derived from the lipid A portion of the molecule pointing to the presence of a single phosphate substituent in the core oligosaccharide region. A comparison of these results with the ESI-MS data obtained for tgt B LPS-OH (Table I) clearly indicates loss of ethanolamine from the PEA moiety, which is known to be substituted at O-3 of the penultimate heptose in immunotype L3 LPS (9). This most likely occurred under the alkaline KOH conditions used in the deacylation procedure. Elimination of ethanolamine accompanied by phosphate migration in Hae-mophilus influenzae LPS under these reaction conditions has been recently observed.

The sequence of the glycosyl residues within the oligosaccharide component of the tgt B LPS was confirmed by NMR spectroscopy (45). This was achieved by measurement of nuclear Overhauser effects (NOEs) between protons on contiguous residues in the backbone oligosaccharide sample and required initial complete assignment of ring 1H resonances.

As would be expected, the 1H detected 13CNMR spectrum of the backbone oligosaccharide derived from the tgt B LPS sample showed resonances in the low field region (90–105 ppm), corresponding to the anomic carbons from eight aldose residues. In addition, diagnostic signals from the methylene carbons of two KDO residues were observed at 35.1 and 35.7 ppm. The 1H NMR spectrum showed characteristic resonances (34, 46) in the high field region from the H-3 methylene protons from the two ω-linked-KDO residues at 1.84 ppm (δ, 1H, H-3ax), 2.00 ppm (δ, 1H, H-3eq) and 2.15 ppm (δ, 2H, H-3eq/H-3ax). The low field region of the 1H spectrum (5.8–4.4 ppm) was complex indicating the sample to be a mixture of two related decasaccharides. The anomic region of the two-dimensional 1H–13C correlation map was especially revealing since a doubling of anomic 1H signals from several residues in the inner core oligosaccharide region was readily discernable (Fig. 4). An approximate 60/40 ratio of two decasaccharides is indicated from estimation of the area of related anomeric 1H signals, e.g. ω-GlcN anomeric protons at 5.42/5.40 ppm. The proton resonances were assigned by two-dimensional homonuclear correlation (COSY) (45) and the component monosaccharide units were identified from the 1H chemical shift (9, 47) and coupling constant values (48). The chemical shift data (Table II) is consistent with each ω-sugar residue being present in the pyr-
anomeric ring form. Further evidence for this was obtained from
intraresidue NOE data (Table III) which also served to confirm
the anomeric configurations of the linkages.

Proton spin-systems corresponding to the monomeric com-
ponents from an α-glucosamine (α-GlcN), two heptoses (α-Hepl,
α-Hepl I), a β-glucose (β-Glc), and a β-galactose (β-Gal) in each of
the two decasaccharides were identified (Table II). In addition
to the two KDO residues, unique single spin-systems were
identified for the two glucosamine residues in the deacylated
lipid A region (α-GlcN-P, β-GlcNII) and for the terminal
β-linked glucosamine (β-GlcNII). Correlation of the H-2 re-
sonances with the directly attached 13C resonances (HMOC
experiment), which occurred in the 13C-chemical shift region
(50–60 ppm) diagnostic of amino substituted carbons, con-
formed the identity of the GlcN residues.

A comparison of the 1H NMR data for the residues listed
in Table II revealed significant differences in chemical shifts
and coupling patterns for the 1H resonances associated with the
residues assigned to α-Hepl. This was readily apparent from the
downfield shifted values for α-Hepl I H-3 (4.41 ppm, 3J H-P ~
10 Hz) and α-Hepl I* H-4 (4.45 ppm, 3J H-P ~ 10 Hz), and is
attributed to phosphate substitution at different sites in the
two oligosaccharides. In the 31P NMR spectrum, three signals
were observed at 1.37, 0.55 and –1.84 ppm, of which the latter
two showed strong correlations in the 1H-31P correlation ex-
periment to H-4 of β-GlcNII and H-1 of α-GlcN-P, respectively,
confirming the presence of monophosphate groups at the cor-
responding positions in the GlcNβ1→6GlcN moity. As expected
for the deacylated oligosaccharide derived from the par-
ent strain LPS (9), a strong correlation was observed between
α-Hepl I H-3 (4.41 ppm) and one of the 31P resonances (0.55
ppm) in the 1H-31P correlation map indicating substitution by
phosphate of the C-3 position. In the related oligosaccharide,
the phosphate substituent is located at the C-4 position of the
heptose as indicated by the occurrence of a strong correlation
between α-Hepl I* H-4 (4.45 ppm) and the 31P signal at 1.37
ppm.

The two decasaccharides were shown to have identical sugar
residue sequences from transglycosidic NOE measurements.
NOE connectivities were observed between anomeric and ag-
lyconic protons on contiguous residues (Table III). Thus, the
occurrence of NOEs between the proton pairs β-GlcN I H-1/β-
Gal H-3, β-Gal H-1/β-Glc H-4, β-Glc H-1/α-Hepl I H-4, and
α-Hepl/KDO H-5 established the partial sequence of the main
chain: GlcNβ1→3Galβ1→4Glcβ1→4Heplα1→5KDOI.

In N. meningitidis LPS (5), Hepl forms a branch point to
which the disaccharide GlcNα1→2Hepl I is attached. This was
confirmed for Iglt B LPS from the observed NOEs between
α-GlcN H-1/α-Hepl I H-2 and α-Hepl I H-1/α-Hepl I H-3 protons.
KDO in the main chain (KDOI) is known (49, 50) to be substi-
tuted at O-4 by a second unit (KDOII) and to link the core
oligosaccharide to the putative O-deacylated lipid A. The 1H
NMR and ESI-MS data is in accord with this inference and,
as expected, a transglycosidic NOE is observed between H-1of
β-GlcNII and the H-6/H-6 proton pair of α-GlcN-P in the
deacylated lipid A region. The structures of the two decasac-
charides are depicted in Table II.

Glycosyltransferase activity of the Iglt B gene was established
using a fluorescence-labeled synthetic acceptor in a capillary
electrophoresis-based assay. Cell extracts of MC58 (L3 immu-
notype strain φ3) and the Iglt B mutant were used in glycossy-
transferase assays with FCHASE-aminophenyl-β-GlcNAc as
an acceptor molecule. Capillary electrophoresis analysis of the
reaction mixture from MC58 is shown in Fig. 5. Three major
peaks were observed in the reaction mixture from MC58. The
fastest migrating peak (peak 1) was identified as FCHASE-
aminophenyl-β-LacNAc as its migration time (12.9 min.) is
identical to authentic FCHASE-β-LacNAc (data not shown). In
addition, this peak is sensitive to β-galactosidase as shown in
Fig. 5B. The second peak having a migration time of 13.1 min
corresponded to FCHASE-aminophenyl-β-GlcNAc. The third
peak at 14.6 min results from the action of an endogenous
hexosaminidase activity present in the extracts. The appear-
ance of peak 2 was dependent on the addition of UDP-Gal and
MnCl2 to the reaction mixture and the presence of β-linked
GlcNAc as an acceptor. This same enzyme activity could be
expressed in E. coli carrying the Iglt B gene in an expression
vector (data not shown). The cell extracts of the Iglt B mutant
strain failed to catalyze addition of β-Gal to the GlcNAc accep-
tor (data not shown).

A similar analysis was performed for the function of the IgltA
gene. Cell extracts of MC58 had β-N-acetylglucosaminyltrans-
ferase activity when FCHASE-aminophenyl-β-lactose was used
as an acceptor and UDP-GlcNAc was used as a donor. The
product peak from this reaction was also shown to be sensitive
to β-N-acetylhexosaminidase (data not shown). Cell extracts of
the Iglt A mutant were unable to transfer β-GlcNAc to the
lactose acceptor.

DISCUSSION

The IgltABE genetic locus is required for the biosynthesis of
the lacto-N-neotetraosyl terminal LPS structure in N. meningi-
tidis (18). Insertion mutants have been constructed in each of
the three genes in the IgltABE locus of the immunotype L3
strain (φ3) of MC58. In previous work, immunological and
PAGE analysis of LPS from mutants Iglt B, Iglt A, and Iglt E
suggested structural alterations in lacto-N-neotetraosyl
epitope, but the determination of the chemical structures of
the LPS core oligosaccharide regions had not been reported. To
uniquely assign functions to IgltABE locus, determination of
the detailed structure of the LPS core oligosaccharide regions
was necessary. In this study, the complete molecular structure
of deacylated Iglt B LPS was determined by electrospray
mass spectrometry and detailed NMR analytical methods.
A structural model from this LPS is shown in Fig. 6.

The structure of the N. meningitidis immunotype L3 LPS
has been determined in detail (9). In complete expression of
the L3 immunotype, the terminal β-galactose of the lacto-N-neo-
tetraosyl epitope is capped by α-2,3-linked sialic acid residues.
**TABLE II**
Proton chemical shifts and coupling constants for the deacylated backbone oligosaccharides derived from N. meningitidis lgt B LPS

| Proton resonance | β-GlcN | β-Gal | β-Glc | α-Hepl | α-HeplII | α-GlcN | KDOII | KDOII | β-GlcNII | α-GlcN-P |
|------------------|--------|-------|-------|--------|----------|--------|-------|-------|----------|----------|
| H-1              | 5.00   | 4.54  | 4.50  | 4.58   | 4.59     | 5.29   | 5.28  | 5.11  | 5.52     | 5.42     |
| (J,2,3)          | 8.1    | 8.9   | 8.9   | 8.9    | 8.3      | 8.2    | 8.2   | 7.2   | 8.5      | 8.4      |
| H-2              | 3.12   | 3.71  | 3.72  | 4.12   | 4.15     | 4.53   | 3.86  | 3.38  | 3.38     | 3.16     |
| (J,2,3)          | 9.9    | 10.0  | 9.5   | (—)    | (—)      | (—)    | (—)  | (—)   | (—)      | (9.6)    |
| H-3              | 3.70   | 3.90  | 3.64  | 4.09   | 4.11     | 4.41   | 3.92  | 3.99  | 2.00     | 3.91     |
| (J,3,4)          | 10.5   | 3.4   | 9.8   | (—)    | (—)      | (—)    | (—)  | (—)   | (—)      | (10.2)   |
| H-4              | 3.50   | 4.19  | 3.64  | 4.29   | 4.25     | 4.08   | 3.59  | 3.52  | 4.22     | 3.95     |
| (J,4,5)          | (—)    | (—)   | (—)   | (—)    | (—)      | (—)    | (—)  | (—)   | (—)      | (9.9)    |
| H-5              | 3.51   | 3.71  | 3.53  | 4.18   | 4.28     | 3.81   | 3.89  | 3.90  | 4.28     | 3.63     |
| H-6              | (—)    | 3.80  | (—)   | 4.08   | 4.10     | (—)    | (—)  | (—)   | (—)      | 4.12     |
| H-7              | (—)    | (—)   | (—)   | (—)    | (—)      | (—)    | (—)  | (—)   | (—)      | (—)      |
| H-8              | (—)    | (—)   | (—)   | (—)    | (—)      | (—)    | (—)  | (—)   | (—)      | (—)      |

* Data recorded on the left are for the deacetylated backbone oligosaccharide (9). Data on the right are for proton resonances shifted (±0.01 ppm) in the deacetylated backbone oligosaccharide which carries a phosphate substitute at the O-4 position in α-Hepl.

**TABLE III**
Proton NOE data for the deacylated backbone oligosaccharides derived from N. meningitidis lgt B LPS

| Anomeric proton | Intraresidue NOE | Transglycosidic NOE | Partial sequence |
|-----------------|------------------|---------------------|-----------------|
| ppm             | ppm              |                     |                 |
| 5.00  (β-GlcNII)| 3.70  (H-3), 3.51 (H-5) | 3.90  (H-3 of β-Gal) | GlcNβ1→3Gal |
| 4.54  (β-Gal)   | 3.90  (H-3), 3.71 (H-5) | 3.64  (H-4 of β-Glc) | Galβ1→4Glc |
| 4.50  (β-Glc)   | 3.90  (H-3), 3.71 (H-5) | 3.59  (H-4 of β-Glc) | Glcβ1→4Glc |
| 4.58  (β-Glc*)  | 3.64  (H-3), 3.53 (H-5) | 4.29, 4.08  (H-4, H-6 of α-Hepl) | Glcβ1→4Hepl |
| 4.59  (β-Glc*)  | 3.69  (H-3), 3.60 (H-5) | 4.25, 4.10  (H-4, H-6 of α-Hepl) | Glcβ1→4Hepl |
| 5.29  (α-Hepl)  | 4.12  (H-2)      | 4.27  (H-5 of α-KDOII) | Heplα1→5KDOII |
| 5.28  (α-Hepl)  | 4.15  (H-2)      | 4.28  (H-5 of α-KDOII) | Heplα1→5KDOII |
| 5.42  (α-GlcN)  | 3.38  (H-2)      | 4.53  (H-2 of α-Hepl) | GlcαN1→2Hepl |
| 5.40  (α-GlcN)  | 3.38  (H-2)      | 4.38  (H-2 of α-Hepl) | GlcαN1→2Hepl |
| 5.41  (α-HeplI) | 4.53  (H-2)      | 4.09, 4.12  (H-3, H-2 of α-Hepl) | Heplα1→3Hepl |
| 5.32  (α-HeplI) | 4.38  (H-2)      | 4.11, 4.15  (H-3, H-2 of α-Hepl) | Heplα1→3Hepl |
| 4.84  (β-GlcNII)| 3.91  (H-3), 3.63 (H-5) | 4.29, 3.81  (H-6, H-6 of α-GlcN-P) | GlcNβ1→6GlcN-P |
| 5.75  (α-GlcN-P)| 3.48  (H-2)      |                     |                 |

LPS of other N. meningitidis immunotypes, notably L7, also express the lacto-N-neotetraose epitope, but not the sialylated variant. LPS from IgB only differs from that of the L7 immunotype in that it lacks the terminal β-galactose residue of the lacto-N-neotetraose epitope (9) and this is consistent with the observed inability of this mutant strain to bind the type-specific L3 monoclonal antibody Mn4A8-B2 (18). A comparison of O-deacylated LPS derived from IgB with those from IgA and IgE by ES-MS revealed further sugar truncations in the lacto-N-neotetraose epitope arising from respective loss of β-GlcNAc and β-GlcNAc-β-Gal residues. It was further established that the IgE LPS is identical to the major LPS component expressed by the galE mutant, a galactose deficient LPS resulting from inactivation of UDP-Glc-4-epimerase which is required for synthesis of UDP-Gal (22).

The structural data for the mutant LPS dearly indicate that the lgtABE locus encodes the glycosyltransferases for the biosynthesis of lacto-N-neotetraose terminal epitope. A mutation in the lgtE gene affords the truncated LPS containing a 1,4-linked β-Glc terminal group. Thus, the sugar specificity of the lgtE gene is capable of adding β-Gal in a 1,4-linkage to this β-Glc to form the terminal lactose structure. Thus, the lgtE gene encodes for a β-galactosyltransferase. It is worthy to note that the structure of the lgt
relationships of meningococcal glycosyltransferases

A mutant LPS is identical to that elaborated by immunotype L3 strain (5). Correspondingly, the Igta mutant containing a functional Igta gene is capable of adding β-GlcNAc in a 1,3-linkage to the terminal β-Gal of the lactose epitope. It follows that the Igta gene encodes the specific β-N-acetylglucosamine transferase for synthesis of the GlcNAcβ1→3Gal terminal unit. Finally, the parent immunotype L3 strain (MC58), which contains a functional Igta gene, is capable of elaborate the lacto-N-neotetraose unit which indicates that this gene encodes the β-galactosyltransferase for catalyzing addition of the 1,4-linked β-Gal to the terminal β-GlcNAc. The function of this gene was firmly established by demonstrating β-galactosyltransferase enzyme activity with a synthetic β-GlcNAc acceptor. Correspondingly, the β-N-acetylglucosaminyltransferase activity encoded by Igta was confirmed with a synthetic β-lactose acceptor, whereas experiments to assay transferase activity of the Igte gene using a synthetic β-Glc acceptor were unsuccessful. It is likely that the latter enzyme has a more stringent acceptor specificity and requires β-Glc linked to heptose, precluding recognition of our synthetic acceptor.

Identification and characterization of the Igta genetic locus was first reported (25) in N. gonorrhoeae and it was postulated that the genes within the locus encoded for glycosyltransferases involved in the biosynthesis of lacto-N-neotetraose and its GalNAc containing analogue in gonococcal LPS. In meningococci, the IgtaBE locus contains three genes which are homologous to the gonococcal Igta, Igte, and Igte genes (18). The role of these genes in meningococcal LPS phase variation has been demonstrated (18) and it was recently shown (51) that LPS phase variation in N. gonorrhoea occurs by a similar genetic mechanism. The evidence presented in the present study unequivocally demonstrates the glycosyltransferase functions of this Neisseria gene locus. The site where the specific transferases function in the biosynthesis of the lacto-N-neotetraose epitope is shown in Fig. 6.

Recently Lee et al. (52) reported that a meningococcal gal E mutant (strain NMB-SS3) expresses glycosyltransferase activity capable of adding one or two additional glucose residues to the immunotype L2 inner core LPS structure, and it was suggested that this provided an alternative biosynthetic pathway to the normal wild-type lacto-N-neotetraose structure. The structure of the LPS of immunotype L2 is similar to that of immunotype L3 in that it contains the lacto-N-neotetraose oligosaccharide epitope and the sialylated analogue, but differs in the inner core region where it contains an additional α-Glc moiety that is 1,3-linked to HeplII (8). In the present study, the gal E mutant (NCI 3 strains MC58 and H44/76) produced, in addition to the expected LPS, (GlcNAcβ1→3Gal→4Hep→2PEA→KDO2→LipidA), a minor amount of a second LPS species containing one additional glucose (<10% by ESI-MS). The extra glucose containing species, (GlcNAcβ1→3Gal→4Hep→2PEA→KDO2→LipidA), was not detectable by ESI-MS in the LPS-OH sample obtained from the Igte mutant, a strain known to contain a mutation in a single gene (22); only the GlcNAcβ1→3Gal→4Hep→2PEA→KDO2→LipidA-OH species was observed (Figs. 2C and 3). These data are consistent with either the galactosyltransferase encoded by the Igte gene possessing the capability to mediate the addition of β-Glc to the Glcβ1→4Hep acceptor at low levels, or the presence of a meningococcal glycosyltransferase which manifests itself when there is a build up of this acceptor.

A mutant LPS is identical to that elaborated by immunotype L8 (5). Correspondingly, the Igta mutant containing a functional Igta gene is capable of adding β-GlcNAc in a 1,3-linkage to the terminal β-Gal of the lactose epitope. It follows that the Igta gene encodes the specific β-N-acetylglucosamine transferase for synthesis of the GlcNAcβ1→3Gal terminal unit. Finally, the parent immunotype L3 strain (MC58), which contains a functional Igta gene, is capable of elaborating the lacto-N-neotetraose unit which indicates that this gene encodes the β-galactosyltransferase for catalyzing addition of the 1,4-linked β-Gal to the terminal β-GlcNAc. The function of this gene was firmly established by demonstrating β-galactosyltransferase enzyme activity with a synthetic β-GlcNAc acceptor. Correspondingly, the β-N-acetylglucosaminyltransferase activity encoded by Igta was confirmed with a synthetic β-lactose acceptor, whereas experiments to assay transferase activity of

3 ESI-MS and sugar analysis of LPS-OH from a meningococcal gal E mutant derived from immunotype L2 (35E5) constructed by insertion of kanR cassette intro the galE gene of strain 35E (22), gave the expected species, GlcNAcβ1→3Gal→4PEA→KDO2→LipidA-OH (M, 2427.6) as the major component, together with a small amount (10%) of the species containing one additional glucose (M, 2599.9). At variance with results reported for strain NMB-SS3 (52), LPS-OH species containing further additions of glucose residues were not detectable (A. Martin, D. Krajcarski, and J. C. Richards, unpublished results).
gene with that enzymatic activity using a synthetic glycosyltransferase acceptor. We are now in the process of characterizing this enzyme.

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