Microheterogeneity of *Neisseria* Lipooligosaccharide: Analysis of a UDP-Glucose 4-Epimerase Mutant of *Neisseria meningitidis* NMB

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Neisseria meningitidis is the etiologic agent of epidemic bacterial meningitis. Lipooligosaccharide (LOS) is a principal virulence factor associated with the organism, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of LOS has demonstrated that there is considerable microheterogeneity in the molecule. To begin our understanding of the nature of this heterogeneity, we identified a Tn916-generated LOS mutant of *N. meningitidis* NMB (serotype L3, monoclonal antibodies 3F11⁺, 6B4⁺, and 4C4⁺) that was designated NMB-SS3 (monoclonal antibodies 3F11[−], 6B4[−], and 4C4[−]). The transposon insertion was localized to the amino terminus of the functional copy of the UDP-Glc 4-epimerase gene (*galE*). UDP-Glc 4-epimerase (EC 5.1.3.2) activity was present in *N. meningitidis* NMB but not in NMB-SS3, indicating that the Tn916 insertion had abolished this activity. Mass spectrometric analysis of the LOS from strain NMB revealed multiple species of LOS, which is consistent with extensive microheterogeneity. While the most predominant structure was consistent with a terminal lacto-*N*-neotetrose structure found in other strains of *N. meningitidis*, Galβ1→4GlcNAcβ1→3Galβ1→4Glc→(GlcNAc)→Hep₂PEA→KDO₂ (where Hep is heptose, PEA is phosphoethanolamine, and KDO is 2-keto-3-deoxymannooctulosonic acid), structures containing repetitive hexoses which are not precursors of this structure were also identified. Compositional analysis of LOS from strain NMB-SS3 revealed that there were no galactoses present in the structure. Mass spectrometric analysis of *O*-deacylated LOS revealed the presence of multiple species, with the predominant LOS species in this mutant strain formed by the Hex→(HexNAc)→Hep₂PEA→KDO₂ (where Hex is hexose and HexNAc is *N*-acetylhexosamine) structure. However, LOS structures with repetitive hexoses, e.g., Hex_{*n*}→(HexNAc)→Hep₂PEA→KDO₂ (*n* = 2, 3, or 4), emanating from one or both heptoses were also identified. Since this mutant cannot synthesize UDP-Gal, these structures must be repetitive glucoses. These data suggest that NMB has a glycosyltransferase capable of polymerizing glucose moieties as an alternative biosynthetic pathway to the wild-type lacto-*N*-neotetrose structure.

Meningococcal meningitis and meningococcemia are devastating infections that affect mainly young children and adolescents, and the propensity of *Neisseria meningitidis* to cause epidemics makes it a major worldwide health problem (17). *N. meningitidis* is an encapsulated gram-negative bacterium, and the lipooligosaccharide (LOS) is a major component of the outer membrane and a major virulence factor. It has been shown that the oligosaccharide portion of the LOS mimics human glycosphingolipid antigens found on the surfaces of erythrocytes and other human cells. Composition analyses has indicated that these LOSs are composed of a limited repertoire of sugars, with glucose (Glc) and galactose (Gal) as the only hexoses (Hex) identified (26). Structural analysis has shown that this oligosaccharide can vary, corresponding to serotypically determined L types.

In addition to serotypic differences between strains, there is considerable microheterogeneity of the LOS structures within a single strain, as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of a single strain showing multiple LOS species. Previous studies with

a series of pyocin-derived *Neisseria gonorrhoeae* LOS variants have demonstrated that not all of these LOS species are precursors of the predominant terminal structures (12). For example, the terminal structure of *N. gonorrhoeae* 1291 is Galβ1→4GlcNAcβ1→4Galβ1→3Glc, while the structure of strain 1291b is Galβ1→4Galβ1→3Glc. Since these minor LOS species may play an important role in pathogenesis and/or protective immunity and are difficult to study because they are a minor component of the wild-type LOS repertoire, we have begun to construct defined mutants that express a variant or a more limited number of LOS structures.

In this report, we describe our studies of a Tn916 insertion-derived mutant, NMB-SS3, and map the transposon insertion to the *galE* gene. We extend the observations described by Frosch et al. (5) and Jennings et al. (11) by demonstrating that the meningococcal UDP-Glc 4-epimerase, like the homologous genes from *Saccharomyces fragilis* and *Bacillus subtilis* and unlike the enzyme from mammals, does not have any detectable UDP-*N*-acetylglucosamine (UDP-GlcNAc) 4-epimerase activity associated with the enzyme. Furthermore, we have demonstrated that the standard one-step linked assay for UDP-Glc 4-epimerase, which relies on spectrophotometric measurement of the accumulation of NADH during conversion of UDP-Gal to UDP-glucuronic acid via UDP-Glc, may not be applicable to *N. meningitidis* because of either low enzyme levels or an oxidative sink.

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TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Characteristic(s)	Reference or source
Strains		
<i>E. coli</i> DH5 α F'	F' <i>thi-1</i> f80 <i>dlacZ</i> Δ M15 Δ (<i>lacZY A-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> ($r_K^- m_K^-$) <i>supE44</i> λ^- <i>gyrA96 relA1</i>	BRL Life Technology, Inc.
<i>E. coli</i> KW251	F' <i>supE44 galK2 galT22 metB1 hsdR2 mcrB1 mcrA</i> (<i>argA81:Tn10</i>) <i>recD1014</i>	Promega
<i>E. coli</i> PL2	<i>thi-1 relA1 galE28</i> λ^- <i>spoT1</i>	1, 28
<i>E. coli</i> INV α F'	F' <i>endA1 recA1 hsdR17</i> ($r_K^- m_K^+$) λ^- <i>supE44 thi-1 gyrA96 relA1</i> f80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR</i>	Invitrogen
<i>N. meningitidis</i> NMB	Wild type, clinical isolate, serotype P1.2,5;L3,7,9	24
<i>N. meningitidis</i> NMB-SS3	Tn916 mutant	23
<i>N. meningitidis</i> FAM20	Wild type, clinical isolate	3a
Plasmids		
pGEM3zf ⁺	Ampicillin	Promega
pGEM7Zf ⁺	Ampicillin	Promega
pCRII	Ampicillin and kanamycin	Invitrogen
pCRII <i>galE</i> -8	Ampicillin and kanamycin	This study
pCRII <i>galE</i> -9	Ampicillin and kanamycin	This study
Oligonucleotides		
Tn916 R end	5'-AAG TGT GAT AAG TCC AG-3'	
SS3 <i>galE</i>	5'-GCC ATC AAG TCG TGA TTT TGG A-3'	

In addition, we have shown that the structures of the mutant LOSs reflect the inability of the *galE* mutant NMB-SS3 to synthesize UDP-Gal. Mass spectrophotometric (MS) analysis revealed structures with repetitive hexoses [Hex_n→(HexNAc)→Hep₂PEA→KDO₂, where *n* = 2, 3, or 4, HexNAc is *N*-acetylhexosamine, Hep is heptose, PEA is phosphoethanolamine, and KDO is 2-keto-3-deoxymannooctulosonic acid] emanating from one or both heptoses, and since this mutant cannot synthesize UDP-Gal, these structures must be repetitive glucoses. This suggested that strain NMB has a transferase capable of polymerizing glucose moieties. On the basis of the observation that minor species with the same molecular weights can be found by MS analysis of LOS from strain NMB, it can be assumed that the mutant NMB-SS3 can also produce these LOS species. These studies show that the analysis of defined mutants in LOS biosynthesis can allow a detailed understanding of the microheterogeneity of *Neisseria* LOSs.

MATERIALS AND METHODS

Materials. Chemicals and antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes and T4 ligase were purchased from GIBCO BRL Life Technology, Inc. (Gaithersburg, Md.) and Promega Co. (Madison, Wis.).

Media and growth conditions. All *Escherichia coli* strains were grown on Luria-Bertani (LB) medium (Difco Laboratories) with antibiotics when applicable. *N. meningitidis* strains were grown on Bacto GC medium (Difco Laboratories) supplemented with 1% (vol/vol) IsoVitalEx (BBL Microbiology Systems, Cockeysville, Md.) and tetracycline when required at 37°C and 5% CO₂.

Bacterial strains, plasmids, and oligonucleotides used in this study. Strains, plasmids, and oligonucleotides used in this study are described in Table 1. *N. meningitidis* strains were tested for the presence of capsules by slide agglutinations and by colony immunoblots with group B-specific anti-capsule antibodies (24). The serotype of *N. meningitidis* NMB was determined to be P1.2,5;L3,7,9.

LOS MAb specificities. Monoclonal antibodies (MAbs) 3F11 and 6B4 are specific for the Galβ1-4GlcNAc epitope (3), and MAb 4C4 recognizes the Glcβ1-4Hep epitope found on *N. gonorrhoeae* 1291c and 1291d (3, 12).

Colony and Western blots (immunoblots). Bacterial colonies were grown overnight and transferred to nitrocellulose disks. The disks were blocked with 3% bovine serum albumin in buffer A (0.01 M Tris-HCl, 0.9% NaCl [pH 7.4]) at room temperature for 1 h. Primary antibody diluted in buffer A was added to the disks and incubated overnight at room temperature. After washing with buffer A, the filters were incubated at room temperature for 1 h in peroxidase-conjugated anti-mouse immunoglobulin M (Kirkegaard and Perry Laboratories, Inc.) at the recommended dilution in buffer A. The disks were washed again with buffer A,

and HRP developer (Bio-Rad) was added to visualize the positive colonies. Western blots were performed as previously described (24).

Hot-phenol LOS extraction. LOS was purified from *N. meningitidis* by a modified hot-phenol method (27) as follows. Overnight bacterial cultures from GC plates were washed twice in 1× phosphate-buffered saline (supplemented with 0.15 mM CaCl₂ and 0.5 mM MgCl₂) and were incubated at 4°C overnight with stirring in the presence of lysozyme (1 mg/ml). The following day, the mixture was subjected to RNase and DNase digestion (1 μg of each per ml) at 37°C for 30 min to degrade nucleic acids. The digested product was extracted once with an equal volume of hot acid-phenol (90% at 65°C) and reextracted once with an equal volume of water (65°C). After dialyzing the supernatant against water to remove the phenol, the LOS was repeatedly washed by centrifugation (105,000 × *g*; 2 h; room temperature), and the final pellet was dissolved in water and lyophilized. LOS was then subjected to electrophoresis on 13.1% polyacrylamide gels as described previously (15) and was visualized by silver staining as described elsewhere (25).

Preparation and MS of O-deacylated LOS. Approximately 2 mg of LOS from strain NMB or NMB-SS3 was O-deacylated according to the procedure described by Helander et al. (8). The resulting O-deacylated LOS sample was then analyzed by electrospray ionization-MS (ESI-MS) in the negative-ion mode on a G/Fison Bio-Q triple quadrupole as described in detail elsewhere (7). The experimentally determined average molecular weights were then searched for matching monosaccharide compositions assuming a base molecular weight of 953.0089 for the conserved diphosphoryl diacyl lipid A moiety (7). The interval average mass values used in this program for the monosaccharides were as follows: Hex, 162.1424; HexNAc, 203.195; Hep, 192.1687; KDO, 220.1791; and PEA, 123.0483.

Monosaccharide composition analysis. O-Deacylated LOSs from both strains NMB and NMB-SS3 were analyzed for their Glc, Gal, and hexosamine contents by high-pH anion-exchange chromatography as previously described (18). Briefly, approximately 100 μg of O-deacylated LOS was hydrolyzed in 6 N HCl acid for 4 h at 100°C. The samples were lyophilized, a small aliquot of resuspended solution was injected onto a Dionex PA-1 column, and the monosaccharides were separated by using a standard gradient with pulsed amperometric detection (18).

Recombinant DNA and transformation methods. All recombinant DNA techniques were performed as outlined elsewhere (21). Transformations of *N. meningitidis* were performed as previously described (24). Electroporations were carried out with a GIBCO-BRL Cell-Porator under recommended conditions.

DNA sequencing and analysis. DNA was sequenced by the dideoxy method (22) with the Sequenase sequencing kit, version 2.0 (U.S. Biochemicals). DNA sequences were analyzed with the Genetics Computer Group package (University of Wisconsin [2]).

Hybridizations. Oligonucleotide hybridizations of Southern blots were carried out with ³²P-end-labeled probes at the desired temperatures in 4× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA), 2× Denhardt's solution, 20 mM Na₃P₂O₇, and 0.2% SDS. The filters were washed twice at the hybridization temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–20 mM Na₃P₂O₇–0.1% SDS for 15 min, which was followed by

FIG. 1. Silver-stained SDS-PAGE of purified LOSs. Lanes: A, *N. gonorrhoeae* 1291; B, *N. meningitidis* NMB; C, *N. meningitidis* NMB-SS3. The NMB LOS migrates at 4.5 kDa, whereas the mutant LOSs migrate at approximately 3.4 kDa because of their truncation. The higher-migrating band above the major band in lane C is likely due to nonspecific addition of Glc to the growing LOS chain by glycosyltransferases (see text).

two washes in $0.1\times$ SSC-20 mM $\text{Na}_3\text{P}_2\text{O}_7$ -0.1% SDS for 15 min. Plaque and colony lift hybridizations were performed as described elsewhere (21).

Preparation of cell extract. Cell extracts for the enzyme assays were prepared from overnight cultures on agar plates. Cells were resuspended in 1 ml of 0.1 M glycine (pH 8.7) to an optical density at 650 nm of approximately 1.5. After washing twice with ice-cold 0.1 M glycine, the cells were spun down and resuspended in 500 μl of 0.1 M glycine buffer (pH 8.7). The suspension was sonicated twice for 10 s each time in ice water at 80 W, with a 50-s interval. The cell debris was pelleted at $15,800\times g$ for 30 min in a microcentrifuge at 4°C , which was followed by a second centrifugation step at $100,000\times g$ for 30 min at 4°C where indicated. The supernatants were immediately assayed for enzyme activity.

Enzyme assays. Descriptions of the standard linked assays (one step and two step) for UDP-Glc 4-epimerase used in this study have been published elsewhere (6). For analysis of the UDP-sugars in high performance anion-exchange chromatography coupled with conductimetric detection (HPAEC-CD), the UDP-Glc 4-epimerase assay was performed in the absence of UDP-Glc dehydrogenase. After a 20-min incubation period, the reaction mix was frozen in dry ice-isopropanol bath. The proteins in the reaction mix were precipitated by thawing the frozen pellet in 2 volumes of ice-cold 100% ethanol, were pelleted, and were washed with 70% ethanol at -20°C . The supernatants were pooled, lyophilized, and resuspended in water for analysis in HPAEC-CD. The UDP-GlcNAc 4-epimerase activity was assayed by similar modification of the assay described by Oguchi and Oguchi (16).

Detection of UDP-monosaccharides. Detection of UDP-sugars was performed as described elsewhere (9).

Selection on Gal-supplemented media. The FAM20 *galE* gene was PCR amplified and cloned into the cloning vector pCRII (Invitrogen) in both orientations. The recombinant plasmids pCRII*galE*-8 and pCRII*galE*-9 were electroporated into *E. coli* PL-2, a *galE* mutant. Kanamycin-resistant transformants were plated on M9 minimal medium agar containing 0.2% glycerol and thiamine, and supplemented with 5 mM galactose and 50 μg of kanamycin per ml as required. Fresh overnight single colonies on LB plates were used to inoculate the appropriate M9 plates. The inoculated M9 plates were incubated at 37°C overnight and scored on the following day for growth.

Nucleotide sequence accession number. The sequence of the 1.4-kb fragment described below has been submitted to GenBank and assigned accession number U19895.

RESULTS

Screening of *N. meningitidis* Tn916-NMB mutants for modified LOS structures. A tetracycline-resistant mutant of strain NMB (MAbs 3F11⁺, 6B4⁺, and 4C4⁻), which we designated NMB-SS3, was identified. This mutant reacted with MAb 4C4 and not with MAbs 3F11 and 6B4 (24). The LOS from this mutant was isolated, and SDS-PAGE analysis showed that the molecule was truncated and migrated at approximately 3.4 kDa (Fig. 1). To assess the genetic linkage between the Tn916 insertion and the altered LOS phenotype, back transforma-

FIG. 2. Mapping of Tn916 in the chromosome of SS3. Lanes: A and D, *Sau*3AI digest of parent strain NMB; B and E, *Sau*3AI digest of mutant strain SS3; C, *Sau*3AI-digested pAM120 containing Tn916. Lanes A and B were probed with random primer-labeled pAM120, and lanes C to E were probed with an oligonucleotide probe specific for the right end of Tn916. The single band migrating at 1.2 kb in lane E (arrow) demonstrates the presence of a single copy of Tn916 in the chromosome of SS3. The upper band in lane C is due to partial digestion of pAM120.

tions were performed with genomic DNA from NMB-SS3. A transformation frequency of $10^{-4}/\mu\text{g}$ of DNA per recipient (a rate which is highly suggestive of homologous recombination and which is 10^3 -fold greater than the transposition frequency of Tn916 from its donor plasmid) for the conversion of strain NMB to a mutant LOS phenotype was observed. SDS-PAGE analysis and Western blots of LOSs from back transformants were identical to those for the LOSs from original mutants, further suggesting that the mutant LOS phenotype was due to the insertion of Tn916 into the LOS biosynthesis region of NMB and was not due to phase variation. Failure to detect NMB-SS3 colonies that were negative for MAb 4C4 from colony blots of approximately 5,000 clones further corroborated the idea that the mutant LOS phenotype associated with NMB-SS3 was due to transposon insertion and not to LOS phase variation.

Mapping of the Tn916 insertion in NMB-SS3. To map the insertion of Tn916 in NMB-SS3, we probed a *Sau*3AI digest of genomic DNA from NMB-SS3 with an oligonucleotide (Table 1) from the right end of Tn916 (Tn916 R end). The probe identified a single band migrating at 1.2 kb (Fig. 2) in the NMB-SS3 DNA digest, indicating that only a single copy of the transposon had inserted into the genomic DNA. This band was cloned into pGEM7Zf⁺ after fragment enrichment and sequencing of this insert showed that the fragment contained 188 bp of meningococcal DNA.

TABLE 2. HPAEC-CD analysis of UDP-Glc 4-epimerase activity in *N. meningitidis* NMB and NMB-SS3^a

Extract	UDP-Gal (nmol)	UDP-Glc (nmol)	% Conversion
G4E ^b	1.7	7	80.9
PL-2	8.9	<0.05	<0.6
NMB	6.3	1.6	20.2
NMB-SS3	8.5	<0.05	<0.6

^a Starting concentration in the assay at time zero was 300 μ M (180 nmol). The assays were repeated three times with similar results. Data from one experiment are shown. Detection limit of UDP-Glc is at least 50 pmol (9).

^b Purified yeast UDP-Glc 4-epimerase (Sigma Chemicals).

The 1.2-kb fragment and a 20-mer oligonucleotide derived from the 188-bp meningococcal sequences at the junction (SS3 *galE* [Table 1]) were subsequently used to identify a λ -EMBL3 clone from a genomic DNA library of *N. meningitidis* FAM20. We sequenced a 1,400-bp region containing the SS3 *galE* oligonucleotide probe, and the DNA sequence data showed an open reading frame of 339 amino acids with a nucleic acid sequence nearly identical to that of previously cloned *galE* genes from other strains of *N. meningitidis* (5, 11). Jennings et al. (11) and Frosch et al. (5) reported the presence of an open reading frame with similarity to the *E. coli* UDP-*N*-acetylmanosamine (UDP-ManNAc) 2-epimerase gene just downstream of the meningococcal *galE* gene. We also detected a partial open reading frame with similarity to the UDP-ManNAc 2-epimerase gene just downstream of the FAM20 *galE* gene.

UDP-Glc 4-epimerase activity in NMB and NMB-SS3. Sequence data from the right junction in NMB-SS3 showed that the transposon had inserted immediately downstream of the start codon in the *galE* gene, replacing the ATG start codon with a TAA termination codon. Thus, the transposon had displaced the promoter, the ribosome-binding site, and the start codon, potentially abolishing transcription and/or translation of the epimerase gene. The predicted effect of this mutation would be a reduction in or the absence of UDP-Glc 4-epimerase activity in the mutant strain NMB-SS3.

To determine whether the mutant strain NMB-SS3 had UDP-Glc 4-epimerase activity, we assayed crude cell extracts for the enzyme activity. Our initial attempts with the standard one-step assay (6) by detection of NADH at 340 nm failed to show epimerase activities in both NMB and NMB-SS3 extracts. The absence of UDP-Glc 4-epimerase activity did not appear to be due to inactivation of enzymes during preparation of the extracts, since the extracts contained measurable phosphoglucosyltransferase activity (data not shown).

In order to assay for epimerase activity in our crude cell extracts, we modified the enzyme assay procedure and utilized HPAEC-CD to detect conversion of UDP-Gal to UDP-Glc. Crude cell extract from strain NMB had UDP-Glc 4-epimerase activity, as indicated by a 20.2% conversion rate (Table 2). In contrast, extract prepared from mutant NMB-SS3 showed no epimerase activity, suggesting that the transposon insertion had abolished the expression of a functional UDP-Glc 4-epimerase enzyme. This difference is not due to protein concentrations in the assays, since equal amounts of protein from each extract were added. The assay was repeated three times with extracts prepared on different days and showed similar results.

In *E. coli* and yeasts, the epimerase enzyme is responsible for converting exogenous Gal to Glc and, therefore, has a K_{eq} [UDP-Glc]/[UDP-Gal] of 3.5. However, in *N. meningitidis*, the enzyme is most likely involved in generating Gal from Glc to serve as the substrate in LOS biosynthesis and thus may have

a very different K_{eq} . To determine the K_{eq} [UDP-Glc]/[UDP-Gal] of the meningococcal enzyme, the extract from *E. coli* PL-2::pCRII*galE*-9 was used in the UDP-Glc 4-epimerase assay modified for HPAEC-CD analysis by using UDP-Glc and UDP-Gal as the substrates. After a 30-min incubation at 37°C, the K_{eq} [UDP-Glc]/[UDP-Gal] values were determined to be 3.5 with UDP-Glc as the substrate and 3.3 with UDP-Gal as the substrate. This assay was repeated with similar results. This suggests that enzyme kinetics favors formation of UDP-Glc.

Complementation of *galE28* in *E. coli* PL-2. The wild-type meningococcal *galE* gene was subcloned into the plasmid vector pCRII (Invitrogen) in both orientations, (in pCRII*galE*-8, the *galE* gene is colinear with the *lacZ* promoter) and was introduced into the *galE*-negative background of *E. coli* PL-2. We assayed the crude cell extracts prepared from the transformants for UDP-Glc 4-epimerase activity by the one-step UDP-Glc 4-epimerase assay (6). The assays showed that UDP-Glc 4-epimerase activity was present in the transformant harboring the plasmid pCRII*galE*-9, but not in pCRII*galE*-8 or in the parent strain PL-2. Growth on Gal-supplemented M9 minimal medium agar (Fig. 3) showed that the parent strain PL-2 failed to grow in the presence of Gal, whereas growth of PL-2::pCRII*galE*-9 was not inhibited. This strongly suggested that the open reading frame disrupted by Tn916 insertion encoded the functional meningococcal UDP-Glc 4-epimerase.

During our UDP-Glc 4-epimerase enzyme assays with crude cell extracts from NMB and NMB-SS3, we noticed that the NMB extract showed the ability to convert UDP-*N*-acetyl-galactosamine (UDP-GalNAc) to UDP-GlcNAc at low levels. To determine whether the meningococcal UDP-Glc 4-epimerase was a dual functional enzyme like the mammalian homologs (14, 19) or whether a second enzyme was responsible for the activity, we assayed the crude cell extract from *E. coli* PL-2::pCRII*galE*-9 for UDP-GalNAc 4-epimerase activity. HPAEC-CD analysis of reaction end products showed that the meningococcal UDP-Glc 4-epimerase was not able to carry out the conversion of UDP-GalNAc to UDP-GlcNAc in an *E. coli* PL-2 background (Table 3).

LOS compositional and MS analysis. To further examine the NMB-SS3 LOS, the purified LOS was first subjected to SDS-PAGE. The silver-stained gel showed that the NMB LOS contained a major doublet band and a number of minor faster-migrating species and that the NMB-SS3 LOS contained a major band that migrated faster than the major bands in NMB LOS (Fig. 1).

Hot-phenol-purified LOSs from NMB and NMB-SS3 were analyzed for their monosaccharide compositions and their molecular weights by ESI-MS after conversion to their water-soluble *O*-deacylated derivatives (Table 4). Monosaccharide composition analysis of the *O*-deacylated LOS showed a Gal-Glc-GlcNAc mole ratios of 1/1.5/2.9 for the parental NMB strain and of 0/1/2 for the NMB-SS3 mutant strain. It is clear from the composition data that the LOS from mutant NMB-SS3 does not contain any Gal but does contain GlcNAc. This observation is in line with the absence of UDP-Glc 4-epimerase in NMB-SS3. UDP-Glc 4-epimerase is required for the conversion of UDP-Glc to UDP-Gal, the activated form of Gal needed as the substrate by the galactosyltransferase.

From the ESI-MS analysis, both the wild-type NMB and NMB-SS3 LOSs appeared heterogeneous (Fig. 4). Minor species of NMB and NMB-SS3 LOSs were not initially detected by SDS-PAGE. The most likely reason for this is that they were below the level of detection by the silver-staining procedure compared with the higher mass resolution and dynamic range of the ESI-MS analysis. However, overloading of SDS-PAGE gels has demonstrated LOS species comparable in size to those

FIG. 3. Growth of *E. coli* PL-2 transformed with the meningococcal *galE* gene. The parent strain PL-2 grew only on nonselective minimal medium agar plates, whereas the transformant containing the plasmid pCRIIgalE-9 grew on all minimal medium agar plates. The minimal medium plates were supplemented with thiamine (–), 50 µg of kanamycin per ml (K), 5 mM Gal (G), and 5 mM galactose and 50 µg of kanamycin per ml (GK).

found by ESI-MS analysis. In Table 4, several of the LOS species which were identified have similar M_r s in both NMB and NMB-SS3. These include the species with M_r s of 2,590 and 2,752, which suggests that NMB-SS3 (and possibly NMB) expresses a transferase capable of adding successive glucoses. The proposed LOS structures from strains NMB and NMB-SS3 are shown in Fig. 5, along with the previously published oligosaccharide structure of the L3 serotype described by Jennings et al. (10).

DISCUSSION

A Tn916 insertion in *N. meningitidis* NMB-SS3 was localized to the 5' end of an open reading frame in meningococcal chromosomal DNA. A search of the databases with the amino acid sequence of the open reading frame showed the polypeptide as a potential UDP-Glc 4-epimerase. The cell extracts

from both the parent and the mutant were assayed for UDP-Glc 4-epimerase activity by HPAEC-CD analysis, which showed that mutant NMB-SS3 did not contain any detectable levels of UDP-Glc 4-epimerase activity. Compositional analysis of LOSs from strains NMB and NMB-SS3 failed to detect galactose in the LOS from the mutant, thus correlating the absence of UDP-Glc 4-epimerase activity in the mutant with the absence of Gal in the LOS. The putative meningococcal *galE* gene was cloned into UDP-Glc 4-epimerase-deficient *E. coli* PL-2 and transformed *E. coli* PL-2 from a Gal-sensitive to a Gal-tolerant phenotype. Analysis of the cell extracts from strain PL-2 and the transformant confirmed the presence of UDP-Glc 4-epimerase activity in the Gal-tolerant transformant.

We determined the K_{eq} [UDP-Glc]/[UDP-Gal] for the meningococcal UDP-Glc 4-epimerase to be approximately 3.5. This indicated to us that the enzyme is much more efficient at generating UDP-Glc than UDP-Gal. It appears to contradict the purpose of the enzyme, i.e., to generate UDP-Gal to serve as the substrate in LOS biosynthesis. This result is not surprising, since a UDP-Glc 4-epimerase enzyme that is too efficient at generating UDP-Gal would be detrimental to the bacteria. This bias toward UDP-Glc could indicate that the enzyme is expressed constitutively to compensate for the observed bias toward Glc or that the expression is regulated such that the enzyme is produced at high levels only during LOS biosynthesis. We favor the former scenario, since presumably the LOS is constantly being turned over and thus

TABLE 3. HPAEC-CD analysis of UDP-GlcNAc 4-epimerase activity in *N. meningitidis* NMB and *E. coli* PL-2::pCRIIgalE-9^a

Extract	UDP-GalNAc (nmol)	UDP-GlcNAc (nmol)	% Conversion
NMB	4.1	1.3	23.6
PL-2::pCRIIgalE-9	5.4	<0.1	<1.8

^a Starting concentration in the assay concentration at time zero, 100 µM (50 nmol). Assays were repeated three times with similar results. Data from one experiment are shown. Detection limit of UDP-GlcNAc is at least 100 pmol (9).

TABLE 4. Molecular weight analysis of *O*-deacylated LOS by ESI-MS for NMB and NMB-SS3^a

LOS	Observed M_r	Calculated M_r	Relative ion abundance ^b	Proposed composition ^c
NMB major <i>O</i> -acylated LOS ^d				
A	2,304.8	2,305.17	23	Hex ₂ HexNAc ₁ Hep ₂ KDO ₂ lipid A
B	2,428.0	2,428.22	26	Hex ₂ HexNAc ₁ Hep ₂ PEA KDO ₂ lipid A
C	2,590.0	2,590.36	13	Hex ₃ HexNAc ₁ Hep ₂ PEA KDO ₂ lipid A
D	2,751.8	2,752.5	26	Hex ₄ HexNAc ₁ Hep ₂ PEA KDO ₂ lipid A
E	2,793.2	2,793.55	16	Hex ₃ HexNAc ₂ Hep ₂ PEA KDO ₂ lipid A
F	2,832.6	2,832.65	17	Hex ₄ HexNAc ₂ Hep ₂ KDO ₂ lipid A
G	2,912.7	2,912.63	43	Hex ₄ HexNAc ₂ Hep ₂ P KDO ₂ lipid A
H	2,955.4	2,955.7	100	Hex ₄ HexNAc ₂ Hep ₂ PEA KDO ₂ lipid A
I	3,035.1	3,035.68	30	Hex ₄ HexNAc ₂ Hep ₂ P PEA KDO ₂ lipid A
J	3,120.6	3,123.91	21	NeuAc Hex ₄ HexNAc ₂ Hep ₂ KDO ₂ lipid A
K	3,204.0	3,203.89	43	NeuAc Hex ₄ HexNAc ₂ Hep ₂ P KDO ₂ lipid A
L	3,246.3	3,246.96	93	NeuAc Hex ₄ HexNAc ₂ Hep ₂ PEA KDO ₂ lipid A
M	3,326.7	3,326.93	30	NeuAc Hex ₄ HexNAc ₂ Hep ₂ P PEA KDO ₂ lipid A
NMB SS3 major <i>O</i> -deacylated LOS ^e				
A	2,305.0	2,305.17	45	Hex ₂ HexNAc ₁ Hep ₂ KDO ₂ lipid A
B	2,428.3	2,428.22	100	Hex ₂ HexNAc ₁ Hep ₂ PEA KDO ₂ lipid A
C	2,590.5	2,590.36	77	Hex ₃ HexNAc ₁ Hep ₂ PEA KDO ₂ lipid A
D	2,751.0	2,752.50	8	Hex ₄ HexNAc ₁ Hep ₂ PEA KDO ₂ lipid A

^a Results from one experiment.^b Relative ion abundances were determined from summation of both triply and doubly charged ions.^c Compositions are based on molecular weight searches with a computer algorithm.^d Composition analyses indicate that all Hex = Glc and Gal and HexNAc = GlcNAc.^e Composition analyses indicate that all Hex = Glc and HexNAc = GlcNAc.

LOS biosynthesis genes would be expressed at all times, albeit at a basal level. The kinetics of UDP-Glc 4-epimerase activity observed here does not exclude the possibility of further regulation imposed on the basal expression levels. It would be interesting to determine the level of transcription of the *galE* gene in meningococci under various conditions to better understand the control of UDP-Gal levels in *N. meningitidis*.

In humans and other higher mammals, the *galE* gene encodes a second 4-epimerase enzyme activity, the one responsible for interconversion of UDP-*N*-acetylglucosamine and UDP-GalNAc (UDP-GlcNAc 4-epimerase) (14, 19). In the yeast *Saccharomyces fragilis* and the gram-positive bacterium *Bacillus subtilis*, the two epimerase activities appear to reside in two distinct loci (4, 20). In our UDP-GlcNAc 4-epimerase activity assay, the cell extract from *N. meningitidis* NMB showed enzyme activity while the extracts from *N. meningitidis* NMB-SS3 and *E. coli* PL-2::pCRIIgalE-9 did not. This result suggested that the meningococcal enzyme may not be capable of catalyzing both 4-epimerase activities. It is possible that the UDP-GlcNAc generated by NMB extract may be due to the presence of UDP-ManNAc 2-epimerase activity and contaminating UDP-ManNAc in our assay. It has been reported that the *E. coli* UDP-ManNAc 2-epimerase has a K_{eq} of 9 ([UDP-GlcNAc]/[UDP-ManNAc]), indicating that the enzyme is much more efficient at generating UDP-GlcNAc than the mannosamine nucleotide sugar (13). The source of this enzyme activity could be an open reading frame that is located immediately downstream of the *galE* gene separated by a short spacer (5, 11, this study) which showed a high degree of amino acid similarity to the *E. coli* UDP-ManNAc 2-epimerase enzyme. In NMB-SS3 extract, this activity was not detected, perhaps because of a polar effect the transposon insertion had on the expression of the UDP-ManNAc 2-epimerase and the low level of contaminating UDP-ManNAc. These two conditions could have contributed to concentrations of UDP-GlcNAc that were below the sensitivity of the detection system employed here. Alternatively, an inhibitor of the UDP-GlcNAc 4-epimerase

activity may exist in the *E. coli* extract, and this may have prevented formation of UDP-GlcNAc.

The ESI-MS analysis of NMB-SS3 LOS showed LOS species with multiple hexose moieties substituted on the Hep₂-KDO₂-lipid A core structure (Fig. 5). However, since composition analysis indicated that only glucose was present, this either suggests that a transferase capable of linking successive glucoses is present in this strain or suggests a lack of substrate specificity by the galactosyltransferase responsible for addition of a Gal residue to terminal Glc in a growing LOS chain. Nonspecific transfer of glucose by a galactosyltransferase could reflect the increased levels of UDP-Glc in the cytoplasm in the absence of UDP-Glc 4-epimerase activity. Alternatively, the addition of multiple Glc units of 3 to 4 may be an alternate biosynthetic pathway, since a Hex₄→HexNAc₁→Hep₂→PEA→KDO₂ lipid A LOS species (D) also appeared in the wild-type strain that is not an intermediate in the synthesis of the LOS containing lacto-*N*-neotetrose. Complete studies of the chemical structure of the NMB-SS3 and NMB LOSs are necessary before any definitive conclusions can be reached concerning these possibilities. It is interesting that while NMB was serotyped as an L3 strain, the molecular weight of the most abundant structure gives a composition that is most consistent with the L2 serotype. The L2 and L3 structures primarily differ by a Hex on the second Hep residue. The phosphorylation pattern for the L3 serotype has not been determined, and the role that this may play in immunologic analysis is not known.

Numerous attempts to determine the UDP-Glc 4-epimerase activity levels in *N. meningitidis* NMB by the one-step standard assay failed, and we utilized HPAEC-CD chromatography to detect the conversion of UDP-Glc to UDP-Gal. The lack of sensitivity shown by the one-step level may be a cumulative effect of low enzyme levels in the cell extracts and perhaps the presence of an oxidative sink that can rapidly convert NADH back to NAD⁺, resulting in no net increase of A_{340} . To rule out the possibility of contaminating membrane oxidases, we

FIG. 4. Negative-ion ESI-MS mass spectra of *O*-deacylated LOSs from *N. meningitidis* NMB (A) and NMB-SS3 (B). See Table 4 for molecular weights and proposed composition analyses.

FIG. 5. Comparison of the *N. meningitidis* L3 serotype (A), NMB (B), and NMB-SS3 (C). The extra Hex identified on the NMB LOS species has been placed on the second Hep as has been reported for the L2 serotype. See Table 4 for molecular weights.

removed the contaminating outer and inner membranes from *N. meningitidis* NMB extract by centrifugation at $100,000 \times g$ for 30 min at 4°C but were unable to detect formation of UDP-Glc by the one-step assay (data not shown). This observation suggests that the oxidases are cytoplasmic or perhaps that the amount of NADH generated in the assay may be below the level of detection by the spectrophotometer.

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