

The *msbB* Mutant of *Neisseria meningitidis* Strain NMB Has a Defect in Lipooligosaccharide Assembly and Transport to the Outer Membrane

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A deletion-insertion mutation in *msbB*, a gene that encodes a lipid A acyltransferase, was introduced into encapsulated *Neisseria meningitidis* serogroup B strain NMB and an acapsular mutant of the same strain. These mutants were designated NMBA11K3 and NMBA11K3cap-, respectively. Neither lipooligosaccharide (LOS) nor lipid A could be isolated from NMBA11K3 although a number of techniques were tried, but both were easily extracted from NMBA11K3cap-. Immunoelectron microscopy using monoclonal antibody (MAb) 6B4, which recognizes the terminal Gal β 1-4GlcNAc of LOS, demonstrated that NMB, NMBcap-, and NMBA11K3cap- expressed LOS circumferentially, while MAb 6B4 did not bind to the surface of NMBA11K3. However, cytoplasmic staining of NMBA11K3 with MAb 6B4 was a consistent observation. Mass-spectrometric analyses demonstrated that the relative amounts of the lipid A-specific C12:0 3-OH and C14:0 3-OH present in the membrane preparations (MP) from NMBA11K3 were substantially decreased (25- and 23-fold, respectively) compared to the amount in MP from its parent strain, NMB. Western blot analyses of MP from NMBA11K3 demonstrated that the levels of porin in the outer membrane of NMBA11K3 were also substantially decreased. These studies suggest that the lipid A acylation defect in encapsulated NMBA11K3 influences the assembly of the lipid A and consequently the incorporation of porin in the outer membrane.

Neisseria meningitidis is one of the leading causes of bacterial meningitis worldwide (1). Meningococcal disease affects mainly children and young adults. The rapid progression of meningococcal disease makes proper diagnosis and subsequent treatment often vital to the survival of infected individuals. If not properly diagnosed and treated, meningococcal infections can lead to shock and death within a matter of hours (35). Due to the detrimental effects caused by these bacteria, a better understanding of meningococcal pathogenesis may prove valuable in the management of systemic meningococcal disease.

One of the major virulence factors of *N. meningitidis* is the capsular polysaccharide. *N. meningitidis* serogroups are based on the capsular polysaccharide. Five serogroups, A, B, C, Y, and W-135, are most often associated with invasive meningococcal strains. Polysaccharide vaccines have been developed for serogroups A, C, Y, and W-135. Additionally, recent work on polysaccharide conjugate vaccines has shown the improved efficacy of these vaccines in infants and young adults (21, 37). Currently a vaccine for serogroup B is not available. The capsular polysaccharide for this serogroup is poorly immunogenic, due to its similarity to human neural adhesion molecules (35).

An additional virulence factor present in *N. meningitidis* is lipooligosaccharide (LOS). LOS is the principal glycolipid present in the outer membrane of *N. meningitidis* and is composed of the oligosaccharide chain extensions, the core, and

the lipid A. The oligosaccharide chain extensions have been shown to play a role in molecular mimicry (8, 19, 34). The lipid A of *N. meningitidis* is similar in structure to lipid A from other gram-negative bacteria (11, 12, 28). The lipid A portion of these bacteria is known to be responsible for many of the adverse effects seen with gram-negative bacterial infections (11, 20).

HtrB and MsbB are the acyltransferases responsible for the addition of the secondary acyl substitutions onto the lipid A (3, 5, 16). Unlike *Escherichia coli*, *N. meningitidis* does not require the presence of the two 2-keto-3-deoxyoctulosonic acid (Kdo) groups for full lipid A acylation (32). Previous work with *htrB* and *msbB* mutants demonstrated that the lipid A portion of their LOS and lipopolysaccharide (LPS) structures were modified (12, 18, 28). These modified forms of LOS and LPS were reduced in their toxicity (9, 16) and in their ability to stimulate cytokine secretion (7, 24). Additionally, the *Haemophilus influenzae* and *Salmonella enterica* serovar Typhimurium *htrB* mutants were reduced in their virulence (9, 16).

Due to the importance of the lipid A structure in pathogenesis we wished to explore the possibility that there is an *msbB* homologue in *N. meningitidis*. A gene which showed high similarity to the *htrB* and *msbB* genes from *E. coli* was identified. This gene was cloned, and deletion-insertion mutants were made in *N. meningitidis* encapsulated strain NMB and *N. meningitidis* acapsular mutant strain NMBcap-. These mutants were subsequently designated NMBA11K3 and NMBA11K3cap-, respectively. In this study we report our chemical and immunological analyses of these mutants.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype, relevant phenotype, or selection marker	Source or reference
Strains		
<i>E. coli</i> DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1</i> <i>hsdR17</i> ($r_K^- m_K^+$) <i>phoA supE44</i> $\lambda^- thi-1 gyrA96 relA1$	Invitrogen
<i>N. meningitidis</i> NMB	Wild type, serogroup B	26
<i>N. meningitidis</i> NMBcap-	Δ <i>siaA-siaD</i> , serogroup B	Wyeth-Lederle
<i>N. meningitidis</i> NMBA11K3	Kanamycin resistant, <i>msbB</i> mutant	This study
<i>N. meningitidis</i> NMBA11K3cap-	Kanamycin resistant, <i>msbB</i> mutant, Δ <i>siaA-siaD</i>	This study
Plasmids		
pUC19	Ampicillin, cloning vector	New England Biolabs
pCR2.1	Ampicillin, cloning vector	Invitrogen
pNMBA11pUC19	Ampicillin, <i>msbB</i> PCR product in pUC19 vector	18
pNMBA11K3	Ampicillin, kanamycin, insertion-deletion <i>msbB</i> mutant in pUC19	18

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All bacterial strains and plasmids used in this study are described in Table 1. *E. coli* was grown in Luria-Bertani medium at 37°C and supplemented with appropriate antibiotics. Encapsulated *N. meningitidis* serogroup B strain NMB was isolated from the bloodstream of a patient with meningococcal sepsis. An acapsular NMB mutant (PBCC7232-NMB Δ *siaA-D*) was a gift from Wyeth-Lederle Vaccines and Pediatrics (West Henrietta, N.Y.). This strain was designated NMBcap- for the studies presented here. *N. meningitidis* organisms were grown on gonococcal agar (Becton Dickinson, Sparks, Md.) supplemented with 1% IsoVitalX or on brain heart infusion (BHI) agar (Becton Dickinson) supplemented with 2.5% heat-inactivated fetal calf serum (FCS) at 37°C in 5% CO₂. Liquid cultures of *N. meningitidis* were grown in BHI broth supplemented with 2.5% FCS or in gonococcal broth supplemented with 1% IsoVitalX at 37°C. Kanamycin-resistant *N. meningitidis* was grown on supplemented BHI agar plates or in supplemented BHI broth with 50 μ g of kanamycin/ml. *N. meningitidis* organisms grown in the presence of kanamycin were grown without CO₂. For growth curve cultures, an overnight bacterial culture was used to inoculate a 3-ml culture to an optical density at 600 nm (OD₆₀₀) of 0.05. Cultures were grown at 37°C with agitation. Readings were taken once every hour.

Recombinant DNA and transformation methods. Restriction and modifying enzymes were purchased from New England Biolabs (Beverly, Mass.) and Promega (Madison, Wis.). Standard recombinant DNA protocols were performed as previously described (22). Transformation of *E. coli* with plasmid DNA was done by the CaCl₂ method (6). Transformation of *N. meningitidis* was performed as previously described (27).

DNA isolation. Plasmid DNA was prepared with the QIAprep Spin Miniprep kit or the QIAprep Midiprep kit, according to manufacturer's instructions (Qiagen Inc., Valencia, Calif.). Chromosomal DNA was isolated with the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.).

DNA sequencing and analysis. DNA sequencing reactions were performed by using dye terminator cycle sequencing chemistry with AmpliTaq DNA polymerase and the FS enzyme (PE Applied Biosystems, Foster City, Calif.). The reactions were run on and analyzed with an Applied Biosystems model 373A stretch fluorescence automated sequencer at the University of Iowa DNA Facility. All primers were either commercially available or were purchased from either Genosys Corporation (Aldrich, Milwaukee, Wis.) or IDT Technologies (Coralville, Iowa). Sequence analysis was performed using Assembly LIGN, version 1.0 (Oxford Molecular Group Inc., Oxford, United Kingdom), MacVector (Oxford Molecular Group Inc.), and Wisconsin Package, version 10.0 (Genetics Computer Group, Madison, Wis.).

Cloning and mutagenesis of the *N. meningitidis msbB* gene. Cloning and mutagenesis of the *msbB* gene was performed as previously described (18). Briefly, the *E. coli htrB* gene was used to search the *Neisseria gonorrhoeae* strain FA1090 sequence at the University of Oklahoma website. The sequence that showed highest homology to the *E. coli* gene was used to design PCR primers. Since the genomes of *N. gonorrhoeae* and *N. meningitidis* are highly homologous, primers gchtrB3 (5'-CAACAGGCGGCGGTGGAACAG-3') and gchtrB4 (5'-TTCCGG CATCCACTCCCTTTG-3') were used for amplification of the *N. meningitidis* strain NMB *msbB* gene. The 1,443-bp PCR product was cloned with the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, Calif.) and was subsequently subcloned into pUC19. This construct was transformed into *E. coli* DH5 α cells (Invitrogen) and was subsequently designated pNMBA11pUC19. Restriction

enzymes *BclI* and *BssHII* deleted 138 bp from the *msbB* gene. A kanamycin resistance cassette was inserted into the modified *msbB* gene, and the resulting construct was designated pNMBA11K3. The proper construct was confirmed by using PCR and restriction enzyme digests. Plasmid DNA from pNMBA11K3 was used for transformation with *N. meningitidis* strains NMB and NMBcap-. Transformants were selected for on BHI plates containing kanamycin.

Southern blot and PCR analyses. Hybridization experiments were carried out according to the manufacturer's protocols. All probes were labeled by either PCR labeling or random labeling with digoxigenin-labeled deoxynucleoside triphosphates (Boehringer Mannheim Corp., Indianapolis, Ind.). Primers gchtrB3 and -4 were used to perform PCRs.

SDS-PAGE of LOS. LOS was isolated from 6 liters of BHI broth supplemented with 2.5% FCS for strains NMB and NMBcap- and 6 liters of BHI broth supplemented with 2.5% FCS and 50 μ g of kanamycin/ml for strain NMBA11K3cap- by using a modified hot-phenol-water preparation (18). Samples were run on a Tris-Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel as previously described by Lesse et al. (13). Silver staining was done according to a previously described protocol (30).

Isolation and SDS-PAGE of MPs. Overnight 10-ml broth cultures were harvested by centrifugation at \sim 3,800 \times g for 10 min. The cell pellet was resuspended in membrane preparation (MP) buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 7.4) and warmed at 56°C for 30 min. Then, cultures were cooled to room temperature. Cultures were passed through a syringe by using needles of different gauges to shear the cells. The process was repeated 10 times for each gauge of needle. This procedure was first performed with a 20-gauge needle, then with a 22-gauge needle, and last with a 25-gauge needle. Sheared cells were centrifuged at 16,000 \times g for 15 min. The supernatant from this spin was centrifuged at 25,000 \times g for 20 min. The supernatant from this spin was centrifuged at 30,000 \times g for 20 min. Finally, the supernatant from the previous spin was centrifuged at 100,000 \times g for 2 h 15 min. All spins were performed at 20°C. The pellet obtained from the last spin was glass-like and was used as the sample for SDS-PAGE. Samples were separated on a 4 to 20% Tris-Tricine gradient gel (13). Equal quantities of proteins were loaded onto each gel, as determined by spectrophotometric readings. Coomassie blue staining and silver staining were performed according to previously described protocols (17, 30).

Western blot analyses of LOS and MPs. Western blot analyses were performed as previously described by Towbin et al. (29). MAb 6B4, which recognizes the terminal Gal β 1-4GlcNAc moiety of the oligosaccharide chain extension (2), was utilized to detect LOS. The porin antibody 3H1 was a gift from Milan Blake (Baxter Hyland Immuno, Columbia, Md.). The blots were developed by using the Super Signal West Pico chemiluminescent substrate according to the manufacturer's instructions (Pierce, Rockford, Ill.).

SEM and TEM analyses. For microscopy studies, all bacterial strains were grown in BHI broth to an OD₆₀₀ of 0.8 to 1.0. Samples for transmission electron microscopy (TEM) analysis were placed in an equal volume of 4% paraformaldehyde (final concentration, 2%). Samples for scanning electron microscopy (SEM) analysis were settled onto silicon wafers and fixed in 2% paraformaldehyde. Cells for immuno-TEM were dehydrated by using a standard graded-ethanol series, followed by embedment in London Resin White (Ted Pella Inc., Redding, Calif.). For resolution of cell membrane morphology by TEM, bacterial cells were treated with osmium according to standard protocols and then dehydrated through graded ethanols and embedded in Epon acrylic resin. Thin sections of embedded cells were mounted on nickel grids. The Epon-embedded

sections were stained with 5% uranyl acetate and lead citrate for contrast. Samples for SEM and immuno-TEM were treated with neuraminidase (1 U/ml; Oxford GlycoScience, Novato, Calif.) for 2 h at 37°C prior to labeling with MAb 6B4. Following overnight incubation with the primary antibody, specimens were incubated with goat anti-mouse IgM conjugated to gold beads, either 12-nm gold bead-conjugate (Jackson ImmunoResearch, West Grove, Pa.) for TEM or 25-nm gold bead-conjugate for SEM (EMS, Ft. Washington, Pa.). The TEM samples were finally counter-stained with 5% uranyl acetate. Immunolabeled samples for SEM were incubated in 2.5% glutaraldehyde to cross-link the antibodies and then processed by using a standard graded-ethanol series. These samples were carbon coated before being viewed on an S-4000 scanning electron microscope (Hitachi, Mountain View, Calif.). TEM samples were viewed with a Hitachi H-7000 transmission electron microscope. All samples were viewed with microscopes located in the Central Microscopy Research Facility at the University of Iowa.

GC/MS analysis of membrane fatty acids. The MPs from strains NMB, NMBA11K3, NMBcap-, and NMBA11K3cap- were treated with 0.5 ml of 10% (wt/wt) BF₃-methanol (Supelco, Bellefonte, Pa.) and heated at 100°C for 6 h. Samples were allowed to cool to room temperature and then were treated with 0.5 ml of saturated NaCl solution, followed by 0.5 ml of high-performance liquid chromatography grade hexanes (Aldrich, St. Louis, Mo.). After the samples were vortexed and centrifuged, the organic layers were removed and transferred to clean vessels. The aqueous layers were then extracted a second time with 0.5 ml of hexanes. The combined organic layers were evaporated to dryness under a stream of nitrogen and later redissolved in hexanes for gas chromatography/mass spectrometry (GC/MS) analysis. Samples were analyzed with a Hewlett-Packard 5890 gas chromatograph interfaced with a VG70SE mass spectrometer. The gas chromatograph was equipped with an on-column injector (J & W Scientific, Folsom, Calif.), and samples were separated on a 30-m by 0.25-mm BPX70 column with a 0.25- μ m film thickness (SGE, Inc., Austin, Tex.). The initial oven temperature, 100°C, was held for 5 min, and then data acquisition was started and the samples were eluted by using a temperature gradient from 100 to 220°C at 4°C/min. The carrier gas was helium at \sim 6 lb/in². Relative peak areas were measured from the total ion chromatograms for each run and normalized to the C16:0 component.

RESULTS

Cloning and mutagenesis of the *N. meningitidis msbB* gene.

The *N. meningitidis msbB* gene was amplified by PCR and cloned. This gene has been previously shown to be able to complement for both the temperature sensitivity and the LPS phenotype of an *E. coli htrB* mutant (18). A deletion-insertion mutation was generated in the *N. meningitidis msbB* gene and the resulting construct was designated pNMBA11K3.

Transformations of pNMBA11K3 into *N. meningitidis* strains NMB and NMBcap- were performed as previously described (27). Selection for transformants was done on plates containing kanamycin. Southern blot analyses and PCR demonstrated that the proper mutations had been incorporated into the *N. meningitidis* NMB and NMBcap- genomic DNA (data not shown). The resulting transformants were designated NMBA11K3 and NMBA11K3cap-, respectively. These mutations should not have a polar effect on downstream genes. First, a kanamycin cassette, which has previously been shown to produce nonpolar mutations (15), was utilized to construct the pNMBA11K3 mutant. Second, the *msbB* gene is not part of an operon. We sequenced over 200 bp of DNA downstream from the *msbB* gene, and no open reading frames were found. In addition, the annotated sequence from *N. meningitidis* strain MC58 indicates that the closest gene is almost 300 bp downstream from the *msbB* gene, and it is transcribed in the opposite orientation. Proper capsule expression phenotypes for the four different strains were confirmed by using MAb 2-2-B, a serogroup B-specific capsular MAb, which was a gift from

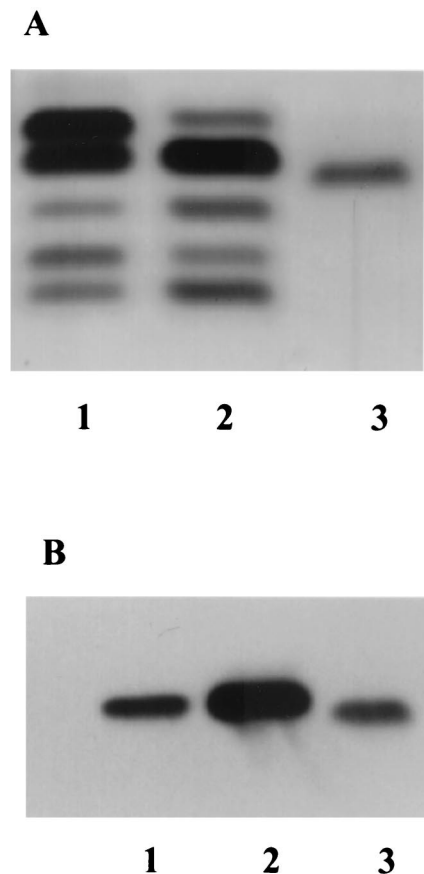


FIG. 1. Characterization of NMBA11K3cap- LOS by SDS-PAGE and Western blot analyses. (A) Silver staining analysis of an SDS-PAGE gel. Lane 1, NMB LOS; lane 2, NMBcap- LOS; lane 3, NMBA11K3cap- LOS. The sialylated LOS (top band) is absent from the NMBcap- LOS because the sialylation genes were deleted in this strain. A different glycoform of LOS is visible in the NMBcap- LOS sample where the sialylated LOS band would normally migrate. (B) Western blot analysis with MAb 6B4. Lane 1, NMB LOS; lane 2, NMBcap- LOS; lane 3, NMBA11K3cap- LOS.

Wendell Zollinger (Walter Reed Army Institute of Research, Silver Spring, Md.) (data not shown).

Comparison of growth rates of *N. meningitidis* strains NMB, NMBcap-, NMBA11K3, and NMBA11K3cap-. To determine whether the mutation in the *msbB* gene had any effect on the growth rate of the bacteria, growth curves were determined. These growth curves demonstrated that there was no difference in the growth rates of strains NMB, NMBcap-, and NMBA11K3cap- (data not shown). However, there was a reduction in the growth rate of strain NMBA11K3 compared with those of the other strains. In the first 8 h of growth, NMBA11K3 reached 50% of the density of the other strains, and after 24 h growth was reduced by approximately one-third. The growth curves were performed three times, and the results of all three experiments were consistent.

Characterization of the LOS by SDS-PAGE and Western blot analysis. Silver staining showed that the NMBA11K3cap- LOS migrated through the gel slightly faster than the NMB and NMBcap- LOS (Fig. 1A) and that the NMBA11K3cap- LOS stained brown instead of black. This staining pattern was

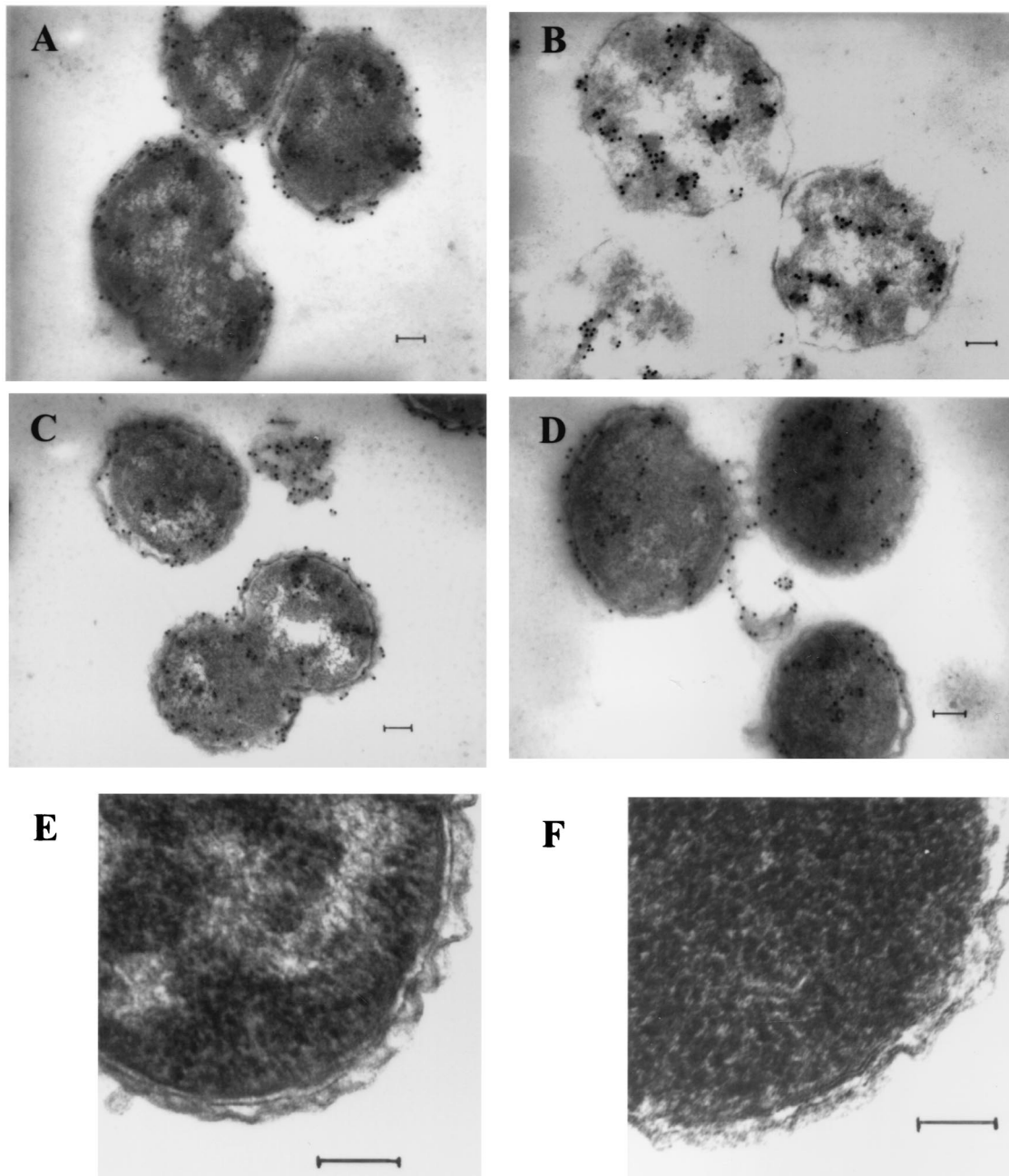


FIG. 2. Examination of MAb 6B4-immunolabeled *N. meningitidis* using TEM analyses. (A) NMB; (B) NMBA11K3; (C) NMBcap-; (D) NMBA11K3cap-; (E and F) Epon-embedded sections showing the structure of the bacterial cell membranes of NMB (E) and NMBA11K3 (F). Scale bars, 1 μ m (A to D) and 100 nm (E and F).

consistent with previous reports of LPS and LOS isolated from *htrB* and *msbB* mutants (12, 18, 23). Western blot analysis using LOS isolated from NMB, NMBcap-, and NMBA11K3cap- was performed. The blot showed that MAb 6B4 bound to all of the LOS samples tested (Fig. 1B). These results indicated that the oligosaccharide portion of the NMBA11K3cap- LOS was intact. LOS could not be purified from NMBA11K3 by using phenol-water, proteinase K, and petroleum ether-phenol extraction methods. Subsequent MPs from NMBA11K3 failed to reveal the presence of LOS bands.

TEM analyses of MAb 6B4-immunolabeled NMB, NMBA11K3, NMBcap-, and NMBA11K3cap-. Figure 2 shows micrographs representative of each of the samples. Immunoelectron micrographs of NMB, NMBcap-, and NMBA11K3cap- (Fig. 2A, C, and D, respectively) show the typical diplococcus structure of *N. meningitidis*. These micrographs also show structurally intact membranes and an electron-dense cytoplasm. The NMBA11K3 sample (Fig. 2B) shows bacteria that still have the coccoid shape but that are somewhat larger than the bacteria from the NMB, NMB cap-, and NMBA11K3cap- samples.

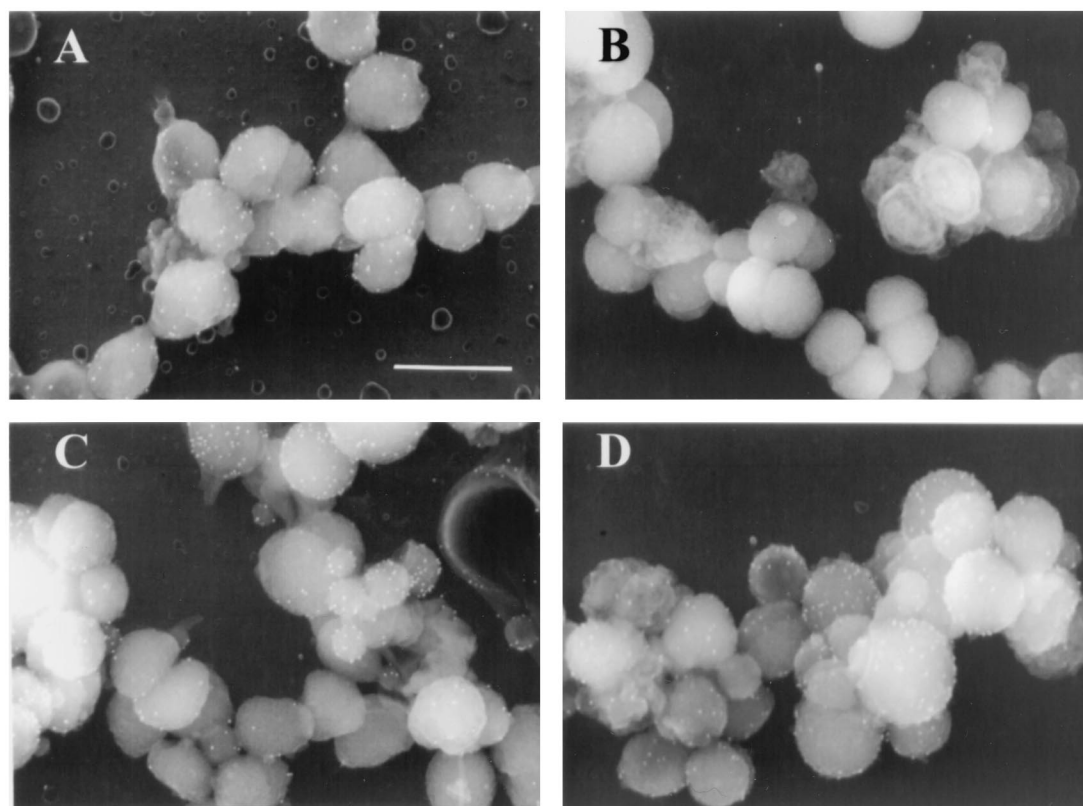


FIG. 3. SEM analyses of MAb 6B4-immunolabeled *N. meningitidis*. (A) NMB; (B) NMBA11K3; (C) NMBcap-; (D) NMBA11K3cap-. Scale bars, 100 nm.

Additionally, there appear to be patches, instead of an even distribution, of electron-dense material in the cytoplasm. Immunoelectron micrographs of NMB, NMBcap-, and NMBA11K3cap- (Fig. 2A, C, and D, respectively) show the meningococci were labeled circumferentially with MAb 6B4. The immunoelectron micrograph of NMBA11K3 (Fig. 2B) shows the meningococci were labeled with MAb 6B4 predominately in the cytoplasm of the bacteria, with essentially no MAb 6B4 label present on the outer membrane. Higher-power electron microscopy revealed that both NMB and NMBA11K3 had evidence of bilamellar outer membranes (Fig. 2E and F, respectively).

SEM analyses of MAb 6B4-immunolabeled NMB, NMBA11K3, NMBcap-, and NMBA11K3cap-. Figure 3 shows micrographs representative of data collected from each of the samples. All strains showed the typical diplococcus shape when viewed by SEM. NMB, NMBcap-, and NMBA11K3cap- (Fig. 3A, C, and D, respectively) showed surface labeling with MAb 6B4. MAb 6B4 did not label the surface of NMBA11K3 (Fig. 3B).

SDS-PAGE and Western blot analyses of MPs from NMB, NMBcap-, NMBA11K3, and NMBA11K3cap-. Both silver staining and Coomassie blue staining demonstrated that there were differences in the components of the MP from NMBA11K3 compared with those from NMB, NMBcap-, and NMBA11K3cap- (Fig. 4). Silver-staining analysis showed that there was no detectable LOS present in the NMBA11K3 sample (Fig. 4A). Western blot analysis utilizing MAb 6B4 con-

firmed the absence of a full-length LOS structure in the MP from NMBA11K3 (Fig. 5B). Examination of the MP also suggested that there were decreases in the levels of at least two other components of the outer membrane, those with molecular masses of ~42 and ~35 kDa, in NMBA11K3 compared with levels in its parent strain, NMB. Western blot analyses determined that the proteins with altered levels were PorA and PorB, respectively (Fig. 5A). The expected molecular masses of these proteins are approximately 42 kDa for PorA and 35.7 kDa for PorB.

MS analyses of MP fatty acids from NMB, NMBcap-, NMBA11K3, and NMBA11K3cap-. Fatty acid methyl esters were prepared from the MPs and were subsequently analyzed by GC/MS (Fig. 6 and Table 2). All of the expected fatty acids were present in each sample; however, the relative abundances of the fatty acids detected from the samples varied. For both the encapsulated and acapsular strains, the hydroxylated fatty acids derived exclusively from lipid A (C12:0 3-OH and C14:0 3-OH) had lower relative abundances in the *msbB* mutants than in the parental strains. This phenomenon was most dramatic for the encapsulated strains, where the levels of C12:0 3-OH and C14:0 3-OH were 25- and 23-fold higher, respectively, in the NMB sample than in the NMBA11K3 sample. The NMBcap- sample had C12:0 3-OH and C14:0 3-OH levels that were both approximately twofold higher than those of the NMBA11K3cap- sample. Additionally, the C12:0 fatty acid, which is also found in lipid A, was recovered in lower relative abundance in the *msbB* mutants than in their respective pa-

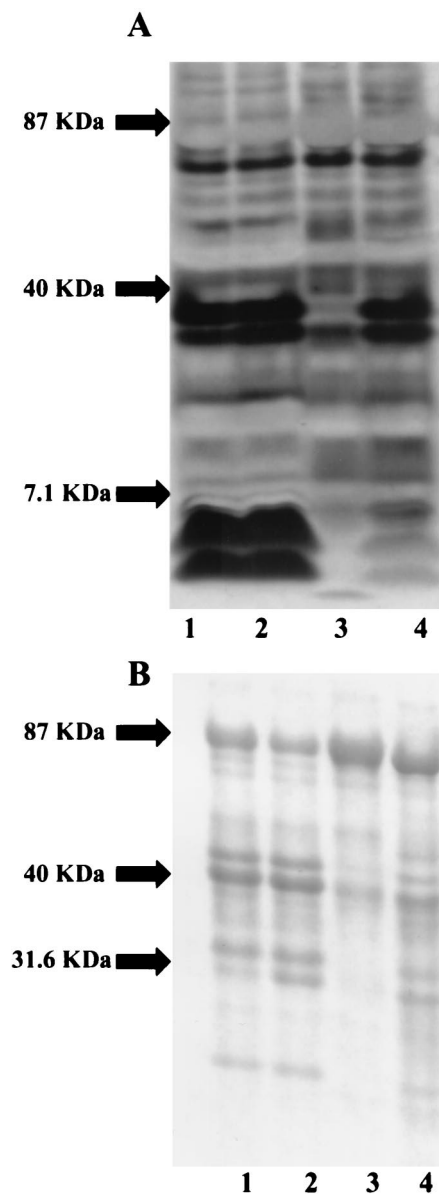


FIG. 4. Silver staining (A) and Coomassie blue staining (B) analyses of MPs from *N. meningitidis* strains NMB (lane 1), NMBcap- (lane 2), NMBA11K3 (lane 3), and NMBA11K3cap- (lane 4).

rental strains (Table 2). Compared to the lipid A fatty acid ratios in the NMBcap- strain, the relative amounts of C12:0, C12:0 3-OH, and C14:0 3-OH detected in the NMBA11K3cap- strain suggest the loss of a single C12:0 fatty acid from the lipid A structure in the *msbB* mutant. This observation is consistent with molecular mass measurements of the intact lipid A from NMB, NMBcap-, and NMBA11K3cap- obtained by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS, which showed a shift to lower mass (~182 Da, corresponding to the loss of a lauric acid residue) for the *msbB* mutant (data not shown). No LOS could be isolated from the NMBA11K3 mutant strain; therefore, MALDI-TOF analysis of the intact lipid A was not possible. These data demonstrate that the relative abundance of the lipid A fatty acids present in

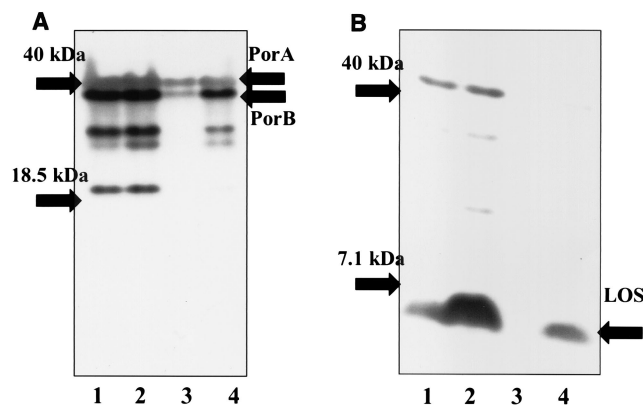


FIG. 5. Western blot analyses of MPs from *N. meningitidis* strains NMB (lanes 1), NMBcap- (lanes 2), NMBA11K3 (lanes 3), and NMBA11K3cap- (lanes 4). (A) MAbs 3H1 for porin; (B) MAbs 6B4 for LOS. Expected molecular masses: PorA, ~42 kDa; PorB, ~35 kDa; LOS, ~5 kDa.

the MPs from the *msbB* mutants is dramatically reduced compared with their relative abundance in the parental strains. However, this reduction is significantly more pronounced in the encapsulated *msbB* mutant (NMBA11K3) than in the acapsular *msbB* mutant (NMBA11K3cap-). These data suggest that the amounts of lipid A, and hence LOS, expressed in the outer membranes of NMBA11K3 and NMBA11K3cap- are significantly reduced compared to the amounts expressed in the outer membranes of their parent strains. Additionally, the relative amounts of C16:0 in the MPs from the *msbB* mutants were higher than those in the MPs from the wild-type strains. Consistent with these findings, a previous study by Steeghs et al. also found that the relative amounts of short-chain fatty acids increased in a LOS-deficient *N. meningitidis* *lpxA* mutant (25).

DISCUSSION

Previous studies involving *htrB* mutants from *E. coli*, *H. influenzae*, and *S. enterica* serovar Typhimurium have demonstrated that they exhibit a number of phenotypes (10, 12, 16, 28). *E. coli*, *H. influenzae*, and *S. enterica* serovar Typhimurium *htrB* mutants were all shown to be initially sensitive to temperatures above 32°C (10, 12, 28). However, work from our laboratory and others has demonstrated that *N. gonorrhoeae* and *E. coli* *msbB* mutants are not temperature sensitive (18, 24). In agreement with these studies, both NMBA11K3 and NMBA11K3cap- were able to grow on solid medium at 37°C. Growth curves demonstrated that NMBA11K3cap- was able to grow at the same rate as NMB and NMBcap-. However, NMBA11K3 had a slower growth rate and was unable to reach the same OD, after 56 h of growth, as the other three strains. Since the *msbB* mutants were able to grow at 37°C and since NMBA11K3cap- was able to grow at the same rate as NMB and NMBcap-, it seems unlikely that the lag in the growth of NMBA11K3 is due to temperature sensitivity.

An additional characteristic of *htrB* and *msbB* mutants is modification of the LPS and LOS structures. Studies performed with *S. enterica* serovar Typhimurium and *H. influenzae*

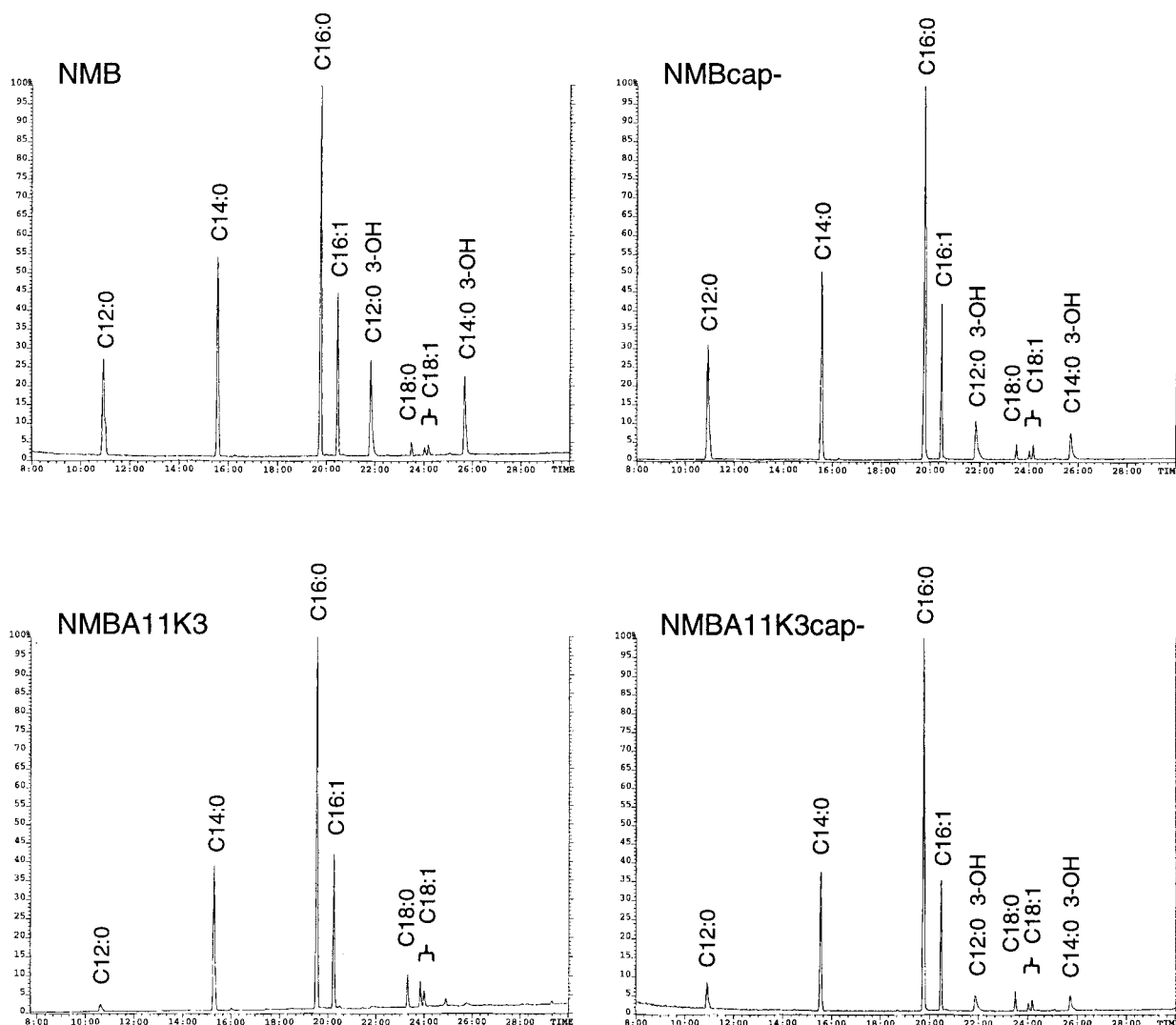


FIG. 6. GC/MS chromatograms of fatty acid methyl esters derived from membrane preparations of the designated *N. meningitidis* strains. Relevant peaks are labeled in each chromatogram. The y axes show the relative peak intensities of the summed ion abundances.

htrB mutants demonstrated that the lipid A structures of both were modified (12, 28). The lipid A from the *H. influenzae htrB* mutant was determined to be approximately 90% tetraacyl and 10% pentaacyl instead of the normal hexaacyl structure. Additionally, studies of an *N. gonorrhoeae msbB* mutant demonstrated that the lipid A was pentaacyl instead of hexaacyl (18). In the study presented here, we were able to isolate and begin to characterize LOS from NMBA11K3cap-. The increase in migration rate and the change in the staining pattern of the NMBA11K3cap- LOS were consistent with data that have been previously reported for *msbB* and *htrB* mutants (12, 18, 23). Additionally, the binding of MAb 6B4, which recognizes the terminal two sugars of the oligosaccharide, to NMBA11K3cap- LOS indicates that the oligosaccharide region of the LOS is intact. Similar to findings with the *N. gonorrhoeae msbB* mutant (18), MS analysis of the NMBA11K3cap- lipid A demonstrated that it is missing one lauric acid (C12:0) substitution and thus may have a pentaacyl rather than a hexaacyl structure. These

results are consistent with a previous study of an *N. meningitidis* strain H44/76 *msbB* mutant (33).

We were unable to isolate LOS from NMBA11K3 despite using a variety of standard methods. Immuno-SEM using MAb 6B4 demonstrated a lack of surface labeling of NMBA11K3, while NMB, NMBcap-, and NMBA11K3cap- all showed surface binding of the antibody. Immuno-TEM micrographs showed that, unlike what was found for NMB, NMBcap-, and NMBA11K3cap-, there was no MAb 6B4 binding to the outer membrane of NMBA11K3. However, binding of MAb 6B4 was visible in the cytoplasm of NMBA11K3. Since we could not isolate LOS from this mutant, we speculate that this labeling represents MAb 6B4 binding to the Gal β 1-4GlcNAc of the oligosaccharide chain extensions still attached to its carrier, undecaprenol phosphate. MAb 6B4 has been previously shown to bind to lacto-*N*-neotetraose-ceramide in human erythrocytes (14). That study, by Mandrell et al., demonstrated that MAb 6B4 is able to recognize its epitope on a lipid carrier

TABLE 2. Fatty acid analysis of outer membranes from designated strains^a

Fatty acid	Relative abundance (%) in:			
	NMB	NMBA11K3	NMBcap-	NMBA11K3cap-
C12:0	42.1	3.2	43.2	10.0
C14:0	63.4	42.5	51.3	40.8
C16:0	100.0	100.0	100.0	100.0
C16:1 ⁹	40.9	36.9	34.4	34.8
C18:0	3.1	7.5	3.1	4.6
C18:1 ⁹ trans	1.9	5.9	1.7	1.7
C18:1 ⁹ cis	2.9	3.9	3.3	2.7
C12:0 3-OH	37.4	1.5	18.0	8.4
C14:0 3-OH	30.1	1.3	10.8	6.5

^a Samples were normalized to the C16:0 component.

other than lipid A. Recent work in our laboratory with a meningococcal *bacA* mutant suggests that *N. meningitidis* LOS is assembled similarly to LPS (D. Post, A. Zaleski, E. Johansen, B. Gibson, and M. Apicella, unpublished data). Based on these studies and the current model for LPS assembly (36), it appears that the oligosaccharide chain extensions are assembled on the carrier lipid undecaprenol phosphate. Then, the oligosaccharide chain extensions and the lipid A-core region are transported to the periplasm by separate mechanisms, where they are subsequently ligated and transported to the outer membrane. The failure to isolate the lipid A-core region alone suggests that there is a defect in the assembly of the lipid A-core complex in NMBA11K3. Interestingly, recent findings by Tzeng et al. demonstrated that two *N. meningitidis* strain NMB mutants, defective in Kdo biosynthesis and Kdo transfer to the lipid A, expressed an LOS that consisted only of lipid A (31, 32). These results demonstrate that the lipid A can be assembled and transported to the bacterial surface in the absence of Kdo.

MS analyses of outer membranes isolated from NMBA11K3 demonstrated that the amounts of the lipid A-specific C12:0 3-OH and C14:0 3-OH present in these samples were 25- and 23-fold, respectively, less than the amount present in the MP from NMB. These data taken together with the microscopy data suggest that the amount of LOS expressed in the outer membranes of NMBA11K3 is significantly reduced compared to the amount expressed in the outer membranes of its parent strain. MP from NMBA11K3cap- showed 2- and 1.7-fold decreases in the amounts of C12:0 3-OH and C14:0 3-OH, respectively, present in these samples compared to the amounts present in MP from NMBcap-. Additionally, the relative amounts of C16:0 in the MPs from the *msbB* mutants were higher than those in the MPs from their respective wild-type strains. These data suggest that both NMBA11K3 and NMBA11K3cap- are defective in their abilities to assemble their LOS and that the presence of the capsular polysaccharide makes the defect in LOS assembly and subsequent transport more pronounced. These results also suggest that NMBA11K3 may place other lipids, most likely C16:0, the lipid that anchors the capsule to the outer membrane, in their outer membranes to compensate for the loss of the lipid A portion of the LOS. These mutants may preferentially express the lipidated capsule on their surfaces when the lipid A is altered. However, if the capsule components are absent in the *msbB* mutant, the me-

ningococcus may be able to incorporate the modified LOS structure into the outer membrane. Interestingly, Steeghs et al. determined that the presence of the capsular polysaccharide is essential for the viability of the LOS-deficient *N. meningitidis* *lpxA* mutant H44/76(pHBK30) (25), further suggesting that the C16:0 lipid may have a role in stabilizing the outer membranes of LOS-depleted mutants.

Western blot analyses demonstrated that the levels of porin expressed in the outer membrane of NMBA11K3 were also altered. Since porin is known to closely associate with LOS (4), it was not surprising to see a decrease in the levels of PorA and PorB in the outer membranes. SDS-PAGE analyses of MP from NMBA11K3 demonstrated that a number of other proteins expressed in the outer membrane were present at levels similar to those in the NMB MP. An explanation for the decrease in the levels of porin and LOS could be that they are transported to the outer membrane as a complex. The modification in the lipid A structure may have decreased the efficiency of LOS assembly; therefore, the transport and subsequent surface expression of the whole complex are altered.

A decrease in LOS and LPS expression on the bacterial surface has not been previously reported for any *htrB* or *msbB* mutant. Previous studies of an *N. meningitidis* *msbB* mutant by van der Ley et al. did not report any changes in the amount of LOS or porin expressed on the bacterial surface (33). One explanation for this difference may be the use of different strains of *N. meningitidis* in our respective studies.

Further study is required to more clearly determine the components involved in the maintenance of the outer membrane structure of NMBA11K3. This strain may prove to be an important tool for further elucidating the mechanisms of LOS assembly and transport in pathogenic *Neisseria*. Additionally, since NMBA11K3 is not greatly impaired in its growth rate and since the surface expression of LOS is markedly reduced, this strain may prove useful in the future development of meningococcal vaccines.

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