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Inner Core Biosynthesis of Lipooligosaccharide (LOS) in *Neisseria meningitidis* Serogroup B: Identification and Role in LOS Assembly of the α1,2 *N*-Acetylglucosamine Transferase (RfaK)

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A lipooligosaccharide (LOS) mutant of *Neisseria meningitidis* serogroup B strain NMB (immunotype L3,7,9) was identified in a Tn*916* (*tetM*) mutant bank by loss of reactivity with monoclonal antibody 3F11, which recognizes the terminal Galβ1→4GlcNAc epitope in the lacto-*N*-neotetraose moiety of the wild-type LOS structure. The mutant, designated 559, was found to express a truncated LOS of 3.0 kDa. Southern and PCR analyses demonstrated that there was a single intact Tn*916* insertion (class I) in the mutant 559 chromosome. Linkage of the LOS phenotype and the Tn*916* insertion was confirmed by transformation of the wild-type parent. Nucleotide sequence analysis of the region surrounding the transposition site revealed a 1,065-bp open reading frame (ORF). A homology search of the GenBank/EMBL database revealed that the amino acid sequence of this ORF had 46.8% similarity and 21.2% identity with the α1,2 *N*-acetylglucosamine transferase (RfaK) from *Salmonella typhimurium*. Glycosyl composition and linkage analysis of the LOS produced by mutant 559 revealed that the lacto-*N*-neotetraose group which is attached to heptose I (HepI) and the *N*-acetylglucosamine and glucose residues that are attached to HepII in the inner core of the parental LOS were absent. These analyses also showed that the HepII residue in both the parent and the mutant LOS molecules was phosphorylated, presumably by a phosphoethanolamine substituent. The insertion of nonpolar and polar antibiotic resistance cartridges into the parental *rfaK* gene resulted in the expression of LOS with the same mobility as that produced by mutant 559. This result indicated that the inability to add the lacto-*N*-neotetraose group to the 559 LOS is not due to a polar effect on a gene(s) downstream of *rfaK*. Our data indicate that we have identified the meningococcal α1,2 *N*-acetylglucosamine transferase responsible for the addition of *N*-acetylglucosamine to HepII. We propose that the lack of α-chain extension from HepI in the LOS of mutant 559 may be due to structural constraints imposed by the incomplete biosynthesis of the LOS inner core.

Lipooligosaccharide (LOS) is a major virulence factor of the obligate human pathogen *Neisseria meningitidis* (54, 55). Levels of LOS correlate with the release of inflammatory cytokines (interleukin 1, interleukin 6, and tumor necrosis factor alpha) important in the pathogenesis of meningococcemia and meningococcal meningitis (2). Direct cytotoxic effects of meningococcal LOS have also been observed in endothelial cell tissue models infected with *N. meningitidis* (7). The LOS structures of *N. meningitidis* have been well characterized both immunologically (53) and biochemically (9, 17, 23, 31, 35, 57). Meningococcal LOS consists of a lipid A to which a conserved inner core of two heptoses and two 2-keto-3-deoxyoctulosonic acid (KDO) residues is attached. The sugar composition of the α-chain oligosaccharide attached to heptose I (HepI) of the inner core is variable (55) but in certain immunotypes is a perfect mimic of the sugars attached to the human sphingoglycolipid paragloboside (27). Unlike the lipopolysaccharide (LPS) expressed by members of the family *Enterobacteriaceae*, *Neisseria* LOS does not have O antigens (16).

Variation of the oligosaccharide composition of meningococcal LOS is postulated to modulate the immune response and virulence phenotype (46, 55), and LOS has recently been shown to act as a ligand during meningococcal attachment to cultured epithelial cells (36, 37). In addition, phase variation of LOS from immunotype L1,8,10 to L3,7,9 has been correlated with the conversion of asymptomatic carriage of meningococci in the nasopharynx to septic bacteremia in a mouse model (25), and similar observations have been noted for human populations (19). Therefore, there is intense interest in characterizing the genes involved in LOS biosynthesis and in the phase variation of these structures.

Synthesis of the meningococcal LOS structure requires enzymes which perform lipid A core synthesis, sugar biosynthesis, and sugar transfer. Several genes encoding the enzymes involved in LOS sugar biosynthesis and transfer have been found, including the UDP-galactose epimerase gene, *galE* (18, 24, 41); the phosphoglucomutase gene, *pgm* (42, 60); and *rfaD*, which epimerizes ADP-heptose to L-glycero-D-mannoheptose (5). In addition, a cluster of five sugar transferase genes (*lgtA* to *lgtE*) capable of synthesizing the lacto-*N*-neotetraose group has also been identified (10), and the heptosyl transferase

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genes *rfaC*, which attaches HepI to KDOI (59), and *rfaF*, which attaches HepI to HepII (43), have been described. This paper describes the characterization of an α1,2 *N*-acetylglucosamine transferase gene homolog (*rfaK*) and its role in LOS biosynthesis in *N. meningitidis* group B.

MATERIALS AND METHODS

Growth conditions for bacterial strains. Strain NMB (CDC8201085) was grown under aerobic conditions with 3.5% CO_2 at 37°C on GC agar (Difco) supplemented with 0.4% glucose and 0.68 mM $Fe(NO_3)_3$. Liquid cultures were vigorously aerated at 37°C in GC broth with the same supplements and 0.51 M sodium bicarbonate (32). Tn*916* transposition mutants of NMB (20, 50) were cultured in the presence of 5 μg of tetracycline (Sigma Chemical Co., St. Louis, Mo.) per ml. Meningococcal transformants containing the spectinomycin resistance gene (Ω cartridge) from pHP45 (38) were selected and maintained in the presence of 80 μg of spectinomycin (chloride salt; Sigma Chemical Co.) per ml. Similar transformants containing the kanamycin resistance gene *aphA-3* from pUC18K (30) were selected and maintained on brain heart infusion agar supplemented with 5% fetal calf serum (Difco) and 60 μg of kanamycin (sulfate salt; Sigma) per ml. The antibiotics and concentrations (in micrograms per milliliter) used to select *Escherichia coli* strains were as follows: ampicillin, 100; chloramphenicol, 50; kanamycin, 50; and spectinomycin, 100.

Colony immunoblot screening procedure. The initial colony immunoblot screen was conducted with monoclonal antibody (MAb) 3F11, generously supplied by Michael Apicella (University of Iowa). This antibody recognizes the unsialylated terminal Galβ1→4GlcNAc epitope in the lacto-*N*-neotetraose moiety of LOS from both pathogenic and commensal *Neisseria* spp. (28, 57). Secondary colony immunoblots were performed using the group B capsular polysaccharide-specific MAb 5C1-3, kindly donated by Wendell Zollinger (Walter Reed Army Institute of Research). A Tn*916* mutant bank of NMB consisting of 1,500 mutants has been created and stored as frozen stocks at −70°C (50). These strains were revived in 1-ml GC broth cultures incubated at 37°C overnight in a benchtop rotary incubator. Fifty microliters of each broth culture, including the control strains NMB and M7 (50), was applied to prewetted nitrocellulose membranes (BA-S NC; Schleicher & Schuell) with a BioDot apparatus according to the manufacturer's instructions (Bio-Rad). After the samples were pulled onto the membrane by vacuum, the nitrocellulose membrane was removed from the BioDot apparatus and air dried for 1 h. The membrane was rinsed in 25 ml of Tris-Tween buffer (0.01 M Tris, 0.15 M NaCl [pH 8.0], 0.05% [vol/vol] Tween 20 [Sigma]) containing 3% (wt/vol) gelatin (Bio-Rad) for MAb 3F11 or 3% bovine serum albumin (Sigma) for MAb 5C1-3 and gently agitated on a rotating platform for 1 h at room temperature. After the blocking buffer was removed, the membrane was washed once in Tris-Tween buffer for 5 min and then treated with diluted primary antibody (1:100 for MAb 3F11 incubated overnight or 1:500 for MAb 5C1-3 incubated for 1 h). The membrane was then washed three times for 10 min each in Tris-Tween buffer. The membrane was then incubated for 1 h with goat anti-mouse immunoglobulin M-immunoglobulin G-alkaline phosphatase conjugate (Jackson Immunochemicals) diluted 1:5,000 and then rinsed three times for 10 min each in Tris-Tween buffer. The color development solution contained 35 μl of 4-nitroblue tetrazolium chloride (NBT) solution (Boehringer Mannheim) and 45 μl of 5-bromo-4-chloro-3-indolylphosphate (BCIP) solution (Boehringer Mannheim) in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$). The reaction was performed in the dark until the positive and negative controls were fully developed.

Tricine-SDS-PAGE analysis of LOS preparations. A mini Protean apparatus (Bio-Rad) was used for Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to the description of Schagger and von Jagow (44). Crude LOS preparations were prepared from whole-cell lysates of meningococcal growth suspended in distilled water. The protein concentrations of these preparations were approximated by the Bradford assay (Bio-Rad). Proteinase K digests consisted of 1 μg of protein in 2% SDS (total volume, 10 μl) to which 2 μl of 25-mg/ml proteinase K (Sigma) was added, and the mixture was incubated at 55°C for 30 min. A second aliquot of proteinase K was added and the digestion was repeated before the reaction was stopped with the addition of 25 μl of loading buffer (1 M Tris [pH 8.45], 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue). A 4-μl aliquot of each sample was boiled for 4 min before being loaded onto a 16% Tricine–SDS-PAGE gel. After electrophoresis, the gels were fixed in 40% ethanol–5% acetic acid overnight. The gels were silver stained according to the method of Hitchcock and Brown (14).

DNA preparation. Chromosomal DNA was purified by the method of Nath (33). Plasmid DNA was extracted by the method of Birnboim and Doly (1). The plasmids and genetic elements used in this study are described in Table 1. Restriction digests were performed with the universal potassium-glutamate buffer described by Hanisch and McClelland (11). Restriction enzymes were inactivated according to the manufacturer's instructions. Ligation conditions were as described by Maniatis et al. (29). PCR products were cloned with a TA cloning kit (Invitrogen). Klenow DNA polymerase was used to fill in 3′ protrusions from restriction digests as described by Maniatis et al. (29).

TABLE 1. Plasmids and genetic elements

Plasmid or primer	Description or sequence	Source or reference
Plasmids		
pCR2	Ap^r Km^r	Stratagene
pPR510	Cm^r	39
pUC18K	Km^r (*aphA-3*), Ap^r	30
pHSG298	Km^r	52
pHP45	Sp^r (Ω) Ap^r	38
pCK19	pCR2 containing the cloned internal fragment of *rfaK* PCR amplified with primers CK2 and CK15	This study
pCK20	pPR510 plus *Eco*RI insert from pCK19	This study
pCK21	pCK20 with nonpolar Km^r cassette inserted into the *Cla*I site of *rfaK*	This study
pCK22	pHSG298 plus *Eco*RI insert from pCK21	This study
pCK23	pCK20 with polar Ω cassette inserted into the *Cla*I site of *rfaK*	This study
pCK24	pHSG298 plus *Eco*RI insert from pCK23	This study
Primers		
CK2	5′-GATGCACAGACGCTTTTCCG-3′	This study
CK4	5′-GTCAACCACAGCTATTACACG-3′	This study
CK13	5′-CGCATAATGACAAACGGAATGCC-3′	This study
CK15	5′-TCATCGCTTTGGCGGTAAAACG-3′	This study
CM10	5′-CTCGAAAGCACATAGAATAAGGC-3′	49
CM13	5′-GACCTTGATAAAGTGTGATAAGTCC-3′	49
JS65	5′-CAAGGATCTGGATTTCGATCACG-3′	This study
JS66	5′-GGGCTTTACTAAGCTGATCCGG-3′	This study
KANC	5′-GTGGTATGACATTGCCTTCTGCG-3′	This study

Construction of nonpolar and polar cassettes. The region surrounding the central unique *Cla*I site in *rfaK* was amplified by PCR using the chromosome-specific primers CK2 and CK15 and was then cloned into the pCR2 vector to form pCK19 (Table 1). To allow for counterselection in subsequent manipulations, the insert from pCK19 was transferred into the Cm^r vector pPR510 (pCK20) by using the flanking *Eco*RI sites. The nonpolar *aphA-3* cassette was excised from pUC18K (30) with *Eco*RI and *Bam*HI, and the overhangs were filled with Klenow DNA polymerase. The polar Ω cassette was excised from pHP45 (38) by using *Sma*I. Both cartridges were cloned into the *Cla*I site of pCK20, which had been treated with Klenow DNA polymerase to produce blunt ends. The recombinant plasmids were extracted from transformants which had been selected by using the appropriate antibiotic combinations and partially restriction mapped to determine the orientation of the cassettes. The junctions of pCK21 (*rfaK*::*aphA-3*) were sequenced to verify the in-frame fusion of the ATG start codon downstream of *aphA-3* with the carboxy terminus of the *rfaK* open reading frame (ORF). Similarly, the orientation of the Ω cassette in pCK23 was determined by PCR (see Fig. 5). The flanking *Eco*RI sites were used to transfer both polar and nonpolar constructs from pCK21 and pCK23 into pHSG298 to form pCK22 (*rfaK*::*aphA-3*) and pCK24 (*rfaK*::Ω), respectively.

Transformation procedures. Meningococci were transformed with DNA preparations by the technique of Janik et al. (15). Competent *E. coli* JM109 was prepared by the method described by Chung et al. (3).

Southern analysis. Appropriate restriction digests of chromosomal and plasmid DNA were loaded onto 0.5% agarose gels (Type V; Sigma) and electrophoresed in Tris-acetate EDTA buffer (29). The DNA fragments were transferred to a nylon membrane (Magnagraph; Micron Separations Inc.) by the method of Southern (48). Chromosome-specific PCR products were labelled with digoxigenin-^{11}dUTP by using the random primed method and a nonradioactive DNA labelling kit (Genius 2 kit; Boehringer Mannheim) for use as probes. Prehybridization, hybridization, and chemiluminescent detection procedures were performed as described elsewhere (1a).

Sequencing procedures. Single-specific-primer PCR (47) was used to chromosome walk in both the 3′ and 5′ directions from the insertion site of Tn*916* by using primers specific to the transposon. Chromosome-specific primers were then designed from this information to continue the process in both directions and to generate overlapping chromosome-specific PCR fragments for confirmation of the primary sequences. PCR products were generated with an AmpliTaq kit (Perkin-Elmer) and were purified by using QIAquick Spin columns (Quiagen) to remove excess nucleotides and AmpliTaq polymerase. The products were sequenced with an AmpliCycle sequencing kit (Perkin-Elmer).

Preparation of purified LOS. Purified LOS was prepared from 5 g (dry weight) of bacteria by a modified version of the procedure described by Galanos et al. (8).

Strains NMB and 559 were used to inoculate 8 liters of GC broth which was incubated for 18 h in a shaking incubator (New Brunswick). The cells were harvested and air dried in a SpeedVac (Savant). The LOS extraction solvent consisted of 90% phenol-chloroform-petroleum ether (bp, 40 to 60°C) (2:5:8). The dried bacteria were thoroughly suspended in 50 ml of LOS extraction solvent and then subjected to 4 min of homogenization with an Ultra Turmax Tissumizer (Janke and Tunkel). This mixture was stirred for 2 h and then centrifuged at 10,500 × *g* for 15 min at 4°C (J2M1 Beckman centrifuge). The supernatant was retrieved, and the extraction of the pellet was repeated as described above. The two supernatants were pooled, and the final pellet was discarded. The chloroform and petroleum ether were removed in 1 h by rotary evaporation with a model RE-III rotary evaporator (Buchel) with a water bath temperature of 40°C. The residual phenol phase was chilled on ice, and the LOS was precipitated by the addition of 6 volumes of cold diethyl ether-acetone (1:5). The white precipitate was collected as a pellet after centrifugation at 5,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the pellet was washed three times with 80% phenol and twice with diethyl ether. The pellet was dried in a SpeedVac (Savant) overnight, then resuspended in distilled water, and aliquoted into Eppendorf tubes. These samples were dried as described above and stored at 4°C. This procedure generally yielded 60 mg of LOS from 5 g (dry weight) of bacteria. The purity of these LOS samples was determined by SDS-PAGE (44) with visualization by silver staining (14).

Preparation of LOS OSs. The LOS preparations (10 mg each) from *N. meningitidis* 559 and NMB *rfaK*::Ω were hydrolyzed in 1% acetic acid (5 ml) for 2 h at 100°C. The hydrolysates were centrifuged at 5,000 × *g* for 20 min, and the supernatants were removed. The pellets were washed once with 3 ml of water and centrifuged again. The wash water was added to the supernatant, and the remaining lipid A was extracted with diethyl ether (three times, 5-ml volumes each time). The aqueous phase containing the oligosaccharides (OSs) was lyophilized. The lyophilized OSs were dissolved in 0.5 ml of water, filtered with Microfilterfuge tubes containing 0.45-μm-pore-size Nylon-66 membrane filters, applied to a Bio-Gel P-4 column (70 by 1.6 cm), and eluted with water containing 1% 1-butanol. Fractions were assayed for carbohydrate by phenol-sulfuric acid assay. Fractions representing the OS peaks were pooled and lyophilized.

O deacylation of LOS. The LOS sample of *N. meningitidis* mutant 559 was O deacylated according to the procedure of Helander et al. (13). Approximately 8 mg of LOS was incubated with 1 ml of anhydrous hydrazine for 20 min at 37°C. The solution was cooled to −20°C, and 5 ml of chilled acetone was added dropwise to precipitate the O-deacylated LOS. The samples were then centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was removed, and the pellet was washed again with cold acetone and centrifuged. The precipitated O-deacylated LOS was then resuspended in 1 ml of water and lyophilized.

Preparation of LOS-HF. The LOS and OS samples (2 mg) were placed in 1.5-ml polypropylene tubes. The samples were treated with cold aqueous 48% hydrogen fluoride (HF) (100 μl) and kept for 24 h at 4°C. The HF was removed by a flushing under a stream of air followed by the addition of diethyl ether (300 μl) and drying with a stream of air. This step was repeated three times. The dry pellet was dissolved in water and lyophilized.

Glycosyl composition analyses. The LOSs and OSs were hydrolyzed in 2 M trifluoroacetic acid (0.5 ml) in a closed vial at 120°C for 3 h. The glycosyl residues were converted to their alditol acetates (58) and analyzed by gas-liquid chromatography (GLC) and by combined GLC-mass spectrometry (GLC-MS).

Glycosyl linkage analyses. Glycosyl linkages were determined by the preparation and GLC-MS analysis of partially methylated alditol acetates (PMAAs) (58). Methylation of the LOS and OS samples was accomplished by the method of Ciucanu and Kerek (4). The permethylated products were further purified by reversed-phase chromatography on a Sep-Pak C_{18} cartridge (56). Hydrolysis of the methylated LOS or OS samples was performed with 2 M trifluoroacetic acid (120°C, 3 h), and the PMAA derivatives were prepared as described previously (58). During PMAA preparations, NaB^2H_4 was used to reduce the partially methylated glycosyl residues to their alditols.

Chromatographic and MS techniques. GLC and GLC-MS analyses were performed by using fused silica capillary columns (length, 30 m; inner diameter, 0.32 mm) with helium as the carrier. A DB-1 column (J&W Scientific) was used for aminoglycosyl derivatives, and an SP2330 column (Supelco, Bellefonte, Pa.) was used for the neutral glycosyl derivatives. Fast atom bombardment mass spectrometry (FAB-MS) analysis was performed in the positive mode with a JEOL (Tokyo, Japan) SX/SX 102A tandem four-sector mass spectrometer, which was operated at 10 kV accelerating potential. Ions were produced by FAB with xenon, using a JEOL FAB gun operated at 6 kV in a conventional FAB ion source. The spectra acquired are averaged profile data of three scans as recorded by a JEOL complement data system. These spectra were acquired from *m*/*z*s of 200 to 2,000 at a rate that would scan from an *m*/*z* of 0 to 2,500 in 1 min. A filtering rate of 100 Hz and an approximate resolution of 1,000 were used in acquiring these spectra. The samples were dissolved in dimethyl sulfoxide, and 1-μl aliquots were mixed with an equal volume of the FAB matrix, thioglycerol, on the probe tip.

Nucleotide sequence accession number. The DNA sequence of the *rfaK* gene from *N. meningitidis* appears in the EMBL and GenBank nucleotide sequence databases under accession no. U35713. The GAP sequence comparison program of the Genetics Computer Group software package version 7.3.1 UNIX was used in the amino acid alignment studies.

RESULTS

Isolation and initial characterization of mutant 559. Parental NMB LOS is shown in Fig. 1 and contains a lacto-*N*-neotetraose structure (Galβ1→4GlcNAcβ1→3Galβ1→4Glc) attached to HepI of the inner core (24). Under normal growth conditions, this LOS structure is ~50% sialylated at the terminal galactose residue (24), resulting in two major LOS structures with different mobilities (4.5 and 4.8 kDa) when separated by Tricine-SDS-PAGE (Fig. 2). The capsule-deficient mutant, M7, is unable to sialylate LOS (e.g., absent 4.8-kDa band in Fig. 2) since the Tn*916* insertion interrupts *synX*, the first gene in the group B capsule biosynthesis operon required for the synthesis of CMP-*N*-acetylneuraminic acid (51). Since MAb 3F11 recognizes the unsialylated terminal galactose residue in the lacto-*N*-neotetraose structure (Galβ1→4GlcNAc epitope [6]), parental NMB is MAb 3F11 positive, while mutant M7 is very positive in immunoblot screens. Tn*916* mutations which result in the removal of the terminal galactose or increased shielding by sialylation would have a MAb 3F11-negative phenotype.

Initial colony immunoblots of the Tn*916* mutant bank of NMB probed with MAb 3F11 revealed 14 nonreactive mutants. A second immunoblot in which the cell densities for each strain and controls were equilibrated was performed to confirm the initial result. Mutants identified by this approach were also screened with immunoblots for the expression of group B capsule by using MAb 5C1-3 (50) to exclude mutants defective in *N*-acetylneuraminic acid biosynthesis. The Tn*916* mutant strain 559 (MAb 3F11 negative, MAb 5C1-3 positive) was identified by this approach and selected for further study. Tricine-SDS-PAGE analysis of the LOS prepared from this strain (Fig. 2) revealed a truncated structure which migrated more rapidly than the LOSs from two previously defined Tn*916* LOS mutants, SS3 (24, 49) and R6 (49, 60).

To confirm linkage of the 559 mutant phenotype to the Tn*916* insertion, chromosomal DNA was extracted from mutant 559 and used to transform wild-type NMB. More than 500 tetracycline-resistant back-transformants and wild-type NMB were screened with MAb 3F11. Approximately 95% of the back-transformants were MAb 3F11 negative, whereas all wild-type NMB colonies were MAb 3F11 positive.

Analysis of the Tn*916* insertion in 559. Restriction digests of 559 chromosomal DNA were probed with digoxigenin-labelled, *Sau*3A1-digested pAM120, the original carrier plasmid of Tn*916* (20). The hybridization patterns indicated that Tn*916* was intact and in a single copy in the 559 chromosome (data not shown). The integrity of Tn*916* was confirmed by single-specific-primer PCR using primers homologous to the ends of the left (CM10) and right (CM13) flanking arms of the transposon (Fig. 3). A comparison of this sequence with the insertion site in wild-type NMB revealed a 5-bp carryover from pAM120 (Fig. 3), characteristic of Tn*916* transposition events.

Identification of the *rfaK* gene in *N. meningitidis*. The region surrounding the Tn*916* insertion in 559 was sequenced and analyzed for ORFs. The Tn*916* insertion site was 6 bp downstream of the initiation codon of a 1,065-bp ORF. A homology search of the GenBank/EMBL database showed that the ORF had significant amino acid identity with the proposed α1,2 *N*-acetylglucosamine transferase from *Salmonella typhimurium* (26). An alignment revealed that these proteins were 21.1% identical (46.8% similarity) (Fig. 4). Therefore, the NMB ORF was designated *rfaK*.

Introduction of polar and nonpolar insertions into *rfaK* in *N. meningitidis*. The hypothesis that the inability to extend the α-chain from HepI in 559 LOS was due to a polar effect of the

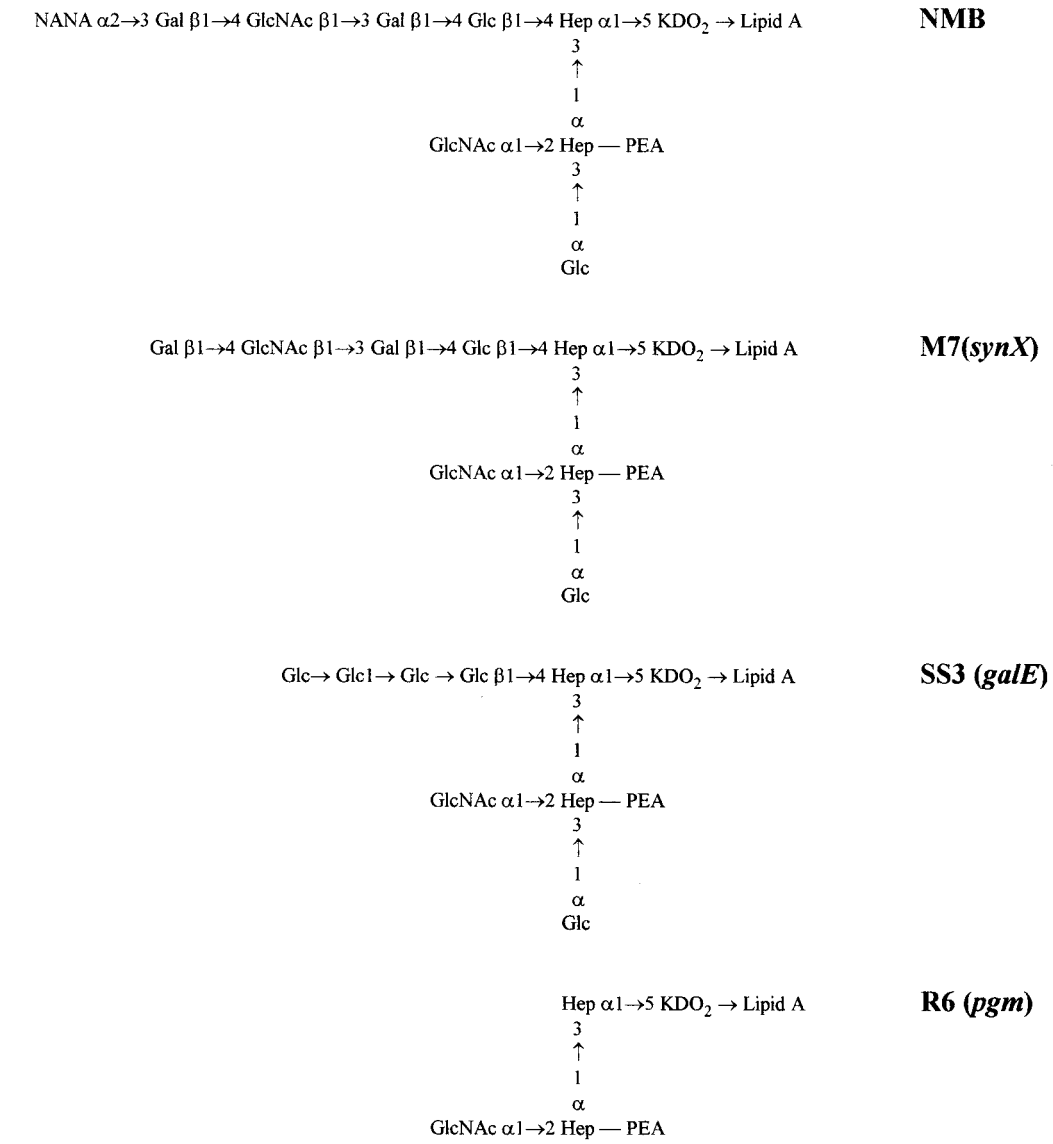

FIG. 1. Biochemical structures of the parental *N. meningitidis* serogroup B strain (NMB) and Tn*916* LOS mutants M7 (51), SS3 (24), and R6 (60). Abbreviations: Gal, galactose; Glu, glucose; GlcNAc, *N*-acetylglucosamine; and NANA, *N*-acetylneuraminic acid.

Tn*916* insertion in *rfaK* on potential downstream genes involved in LOS biosynthesis was examined. Polar and nonpolar antibiotic cassettes were cloned into an internal *Cla*I site of *rfaK* and then reintroduced into the wild-type chromosome via transformation and homologous recombination. Both pCK22 (*rfaK*::*aphA-3*) and pCK24 (*rfaK*::Ω) (Materials and Methods; Fig. 5) were used to transform parent strain NMB. Colony blots of 200 to 500 transformants from each transformation were probed with MAbs 3F11 and 5C1-3. All NMB*rfaK*::*aphA-3* and NMB*rfaK*::Ω transformants were MAb 3F11 negative and MAb 5C1-3 positive. Colony PCR was performed on six randomly selected individual colonies from each transformation to confirm the site of insertion. In all cases, the *aphA-3* and Ω cassettes had recombined into the wild-type chromosomal copy of *rfaK*. A comparison of the LOS patterns of NMB*rfaK*::*aphaA-3* and NMB*rfaK*::Ω with mutant 559 showed no differences in mobility when analyzed by Tricine-SDS-PAGE (Fig. 6). These results suggest that the 559 LOS phenotype is due solely to the inactivation of *rfaK*.

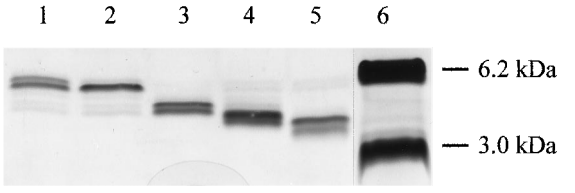

FIG. 2. Comparison of the relative mobilities of LOSs from the parent strain, NMB, and mutant 559 with those of LOS mutants M7 (51), SS3 (24), and R6 (60) after separation by Tricine-SDS-PAGE. LOS preparation and gel electrophoresis conditions were as described in Materials and Methods. Lane 1, NMB (wild type); lane 2, M7; lane 3, SS3; lane 4, R6; lane 5, 559; and lane 6, prestained low-molecular-mass markers (Gibco BRL) (with sizes shown on the right).

Structural analysis of the truncated LOS expressed by mutant 559. The glycosyl compositions of LOSs and OSs from the parent NMB and both the 559 and the NMB*rfaK*::Ω mutants

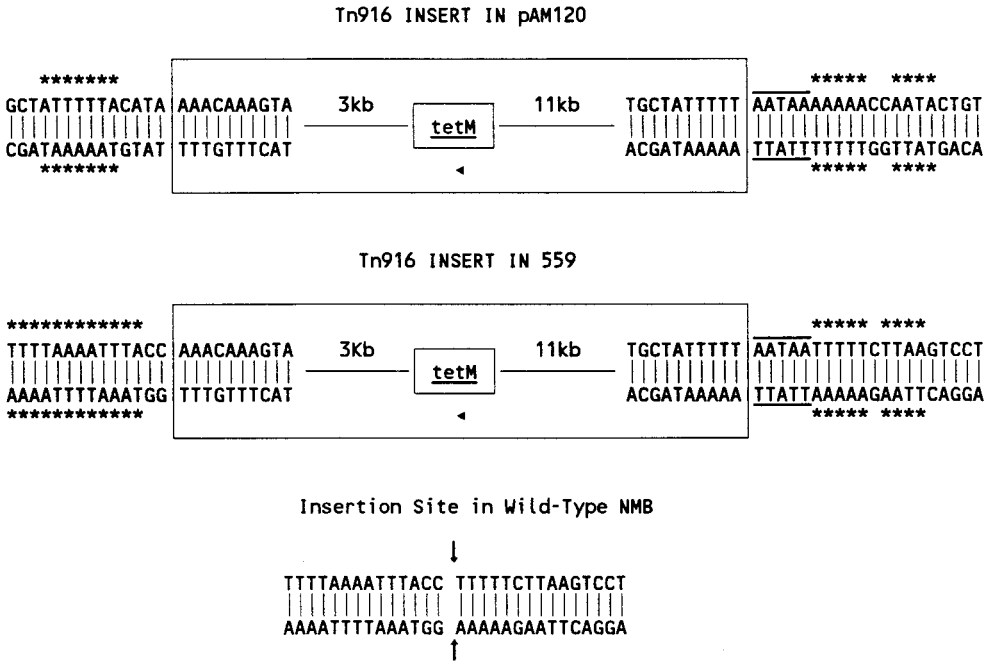

FIG. 3. Sequence analysis of the insertion site of Tn*916* in mutant 559. The sequences of the left and right junctions of Tn*916* are shown. AT-rich regions (stars), the 5-bp carryover from pAM120 (underlines), and the direction of transcription through the *tetM* gene in Tn*916* (arrowheads) are indicated.

are shown in Table 2. The glycosyl components of the NMB LOS and OS were consistent with the previously reported results for the LOS from this strain (60). Analysis of the 559 LOS by PAGE revealed that a small amount of parental NMB LOS, indicated by the presence of relatively small amounts of glucose, galactose, and glucosamine, was present in this preparation. Since the linkage of the 559 phenotype with the Tn*916* insertion was 95%, the presence of NMB LOS was probably due to the 5% unlinked colonies. NMB LOS glycosyl residues were completely absent from the NMB*rfaK*::Ω OS preparation, which contained only heptose, indicating that this strain does not synthesize any parental NMB LOS. These composition

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NMB RFAK      1   MEKEFRILNIVSAKIWGGG..EQYVYDVSKALGLRGCTM......FTA 40
SALTY RFAK    1 MIKKIIFTVTPIFSIPPRGAAAVETWIYQVAKRLSIPNAIACIKNAGYPE 50
NMB RFAK     41 VNKNNELMHRRFSEVSSVFTTRLHTLNGLFSLCALTRFIRENRISHLMIH 90
SALTY RFAK   51 YNKINDNCDIHYIGFSKVYKRLFQKWTRLDPLPYSQRIL..NIRDKVTTQ 98
NMB RFAK     91 TGKIAALSILLKKLTGVRLIFVKHNVVANKTDFYHRLIQKNTDRFICVSR 140
SALTY RFAK   99 EDSVIVIHNSMKLYRQIRERNPNAKLVMHMHNAFEPELPDNDAKIIVPSQ 148
NMB RFAK    141 LVYDVQTADNPFKEKYRIIHNGIDTDRFPPSQEKPDSRFFTVA....... 183
SALTY RFAK  149 FLKAFYEERLP.AAAVSIVPNGFCAETYKRNPQDNLRQQLNIAEDATVLL 197
NMB RFAK    184 YAGRISPEKGLENLIEACVILHRKYPQIRLKLAGH.......GHPDYMCR 226
SALTY RFAK  198 YAGRISPDKGILLLLQAFKQLRTLRSNIKLVVVGDPYASRKGEKAEYQKK 247
NMB RFAK    227 LKRDVSASGAEPFVSFEGFTENIASFYRQSDVVVLPSLVPEAFGLSLCEA 276
SALTY RFAK  248 VLDAAKEIGTDCIMAGGQSPDQMHNFYHIADLVIVPSQVEEAFCMVAVEA 297
NMB RFAK    277 MYCRTAVISNTLGAQKEIVEHHQSGI.LLDRLTPESLADEIERLVLNPEA 325
SALTY RFAK  298 MAAGKAVLASKKGGISEFVLDGITGYHLAEPMSSDSIINDINRALADKE. 346
NMB RFAK    326 KNALATAAHQCVAARFTINHTADKLLDAI...... 354
SALTY RFAK  347 RHQIAEKAKSLVFSKYSWENVAQRFEEQMKNWFDK 381
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FIG. 4. Amino acid sequence alignment of RfaK proteins from *N. meningitidis* and *S. typhimurium* (SALTY). Identity (lines) and conserved amino acid replacements (colons) between the aligned amino acid sequences are indicated.

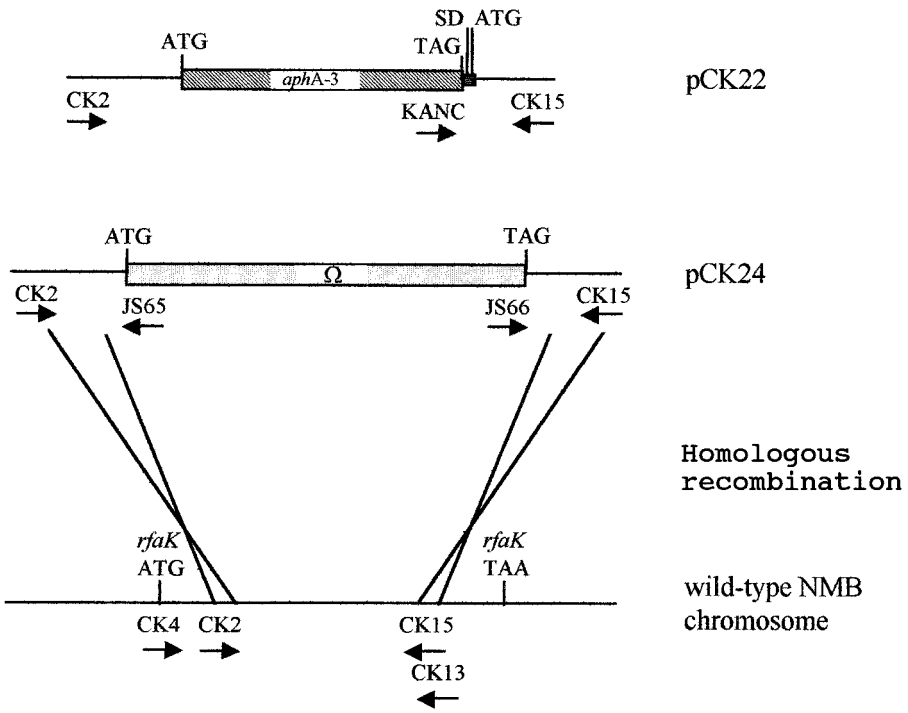
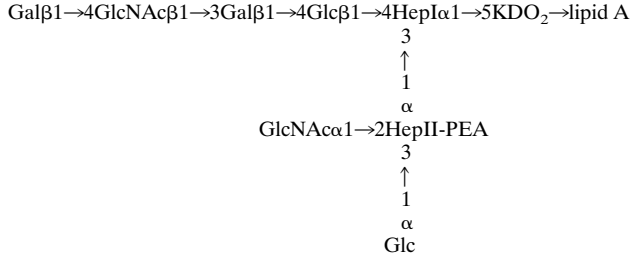


FIG. 5. Schematic diagram of the construction of polar (pCK24) and nonpolar (pCK22) mutations in *rfaK* of *N. meningitidis*. pCK22 and pCK24 were constructed as detailed in Materials and Methods. Primers used to confirm these constructs (arrows) are indicated. These plasmids were introduced into naturally competent NMB by transformation. Since the carrier plasmid, pHSG298, is unable to replicate in NMB, the resistance markers were rescued by homologous recombination into the chromosome via the flanking *rfaK* DNA. Fifty transformants from each transformation were tested for kanamycin resistance and found to be sensitive to this marker. Insertions were confirmed by PCR of NMB*rfaK*::*aphA-3* with CK4-CK13 and NMB*rfaK*::Ω with CK4-JS65 and CK13-JS66. These PCR fragments were sequenced to confirm that there were no deletions or rearrangements in the regions flanking the inserts.

results show that the OSs from 559 and NMB*rfaK*::Ω are composed of only L-*glycero*-D-*manno*-heptose in addition to KDO.

Methylation analysis of the NMB LOS gave a molar ratio of terminal glucose to terminal galactose to 4-linked glucose to 3-linked galactose to 3,4-linked heptose to terminal-glucosamine to 4-linked glucosamine of 1.1:1.0:1.2:0.9:0.8:0.6:0.7. The procedure and columns used did not allow the detection of phosphorylated glycosyl residues. However, methylation analysis after 48% aqueous HF treatment, which removes phosphate and ethanolamine phosphate substituents, resulted in an additional 2,3-linked heptosyl residue. This result indicates that this additional heptosyl residue (i.e., HepII) contains a phosphate or an ethanolamine phosphate substituent. It has been previously reported that a terminal *N*-acetylglucosamine is attached to the HepII residue at O-2 (9, 17, 35, 60) and that a terminal glucose is attached at O-3 (17). Therefore, the above results suggest that the terminal glucosyl residue is linked at the O-3 position of this HepII residue. These data, in conjunction with those previously reported for neisserial LOS (9, 17, 24, 35), suggest the following structure of NMB LOS:



Methylation analysis of the LOS and OS from mutant 559

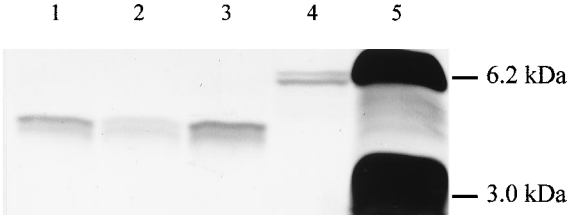


FIG. 6. Comparison of the LOS patterns of the polar and nonpolar *rfaK* mutations in NMB with that of the original 559 mutant. Results of Tricine-SDS-PAGE of LOSs prepared from mutant 559 (lane 1), NMB*rfaK*::*aphA-3* (lane 2), NMB*rfaK*::Ω (lane 3), and NMB (parent strain) (lane 4) are shown. Lane 5, prestained molecular mass markers (sizes are shown on the right).

TABLE 2. Glycosyl composition analyses of *N. meningitidis* wild-type (NMB) and mutant (559 and NMB*rfaK*::Ω) LOSs and OSs[a]

Residue	Composition (relative mol%)					
	NMB		559		NMB*rfak*::Ω	
	LOS	OS	LOS	OS	LOS	OS
Glucose	29	34	4.3	9.7	ND[b]	ND
Galactose	31	38	5.1	12	ND	ND
L,D-Heptose	20	14	44	67	73	100
GlcN[c]	20	14	47	12	27	ND

[a] The procedure used does not detect KDO.
[b] ND, not detected.
[c] GlcN, the total glucosamine and *N*-acetylglucosamine content in intact LOS or the *N*-acetylglucosamine content in OS.

gave only one PMAA derivative, 1,3,5-tri-*O*-acetyl-2,4,6,7-tetra-*O*-methylhepitol, indicating the presence of a 3-linked heptosyl residue (i.e., HepI). Methylation analysis after aqueous 48% HF treatment showed the presence of an additional terminal heptosyl residue; the terminal heptose/3-linked heptose ratio was 1.0:1.2. Therefore, in this LOS, the HepII residue is terminally linked to O-3 of HepI and the HepII residue contains a phosphate and/or an ethanolamine phosphate substituent(s), since it is detected only after 48% HF treatment. These results were supported by FAB-MS analysis (in the positive mode) of the O-deacylated 559 LOS. Three major ions were observed, with *m*/*z*s of 1,985.9, 2,108.8, and 2,231.1. These values are consistent with lactonized $[M+Na]^+$ ions of O-deacylated molecules with the following compositions: $Hep_2P_1PEA_1KDO_2$lipid A, $Hep_2P_1PEA_2KDO_2$lipid A, and $Hep_2P_1PEA_3KDO_2$lipid A (where PEA is phosphoethanolamine). The exact locations of the phosphate and ethanolamine phosphate groups are not known. The PEA substituents may be on the HepII residues and/or on the lipid A glucosamine residues, i.e., at the 1′ and/or 4′ positions (23). A complete structural analysis of this LOS, as well as of the parent NMB LOS, is in progress to determine the locations of these substituents.

DISCUSSION

Tn*916* mutagenesis of *N. meningitidis* in conjunction with Western immunoblot analysis has been a very successful strategy for identifying mutants with alterations in LOS biosynthesis (24, 49, 60) and capsule biosynthesis (50, 51). The wild-type strain, NMB, which was used in these studies has an L3,7,9 LOS immunotype, although biochemical and physical analyses have determined that the predominant LOS structure is L2 (24, 40a). The L2 and the L3 structures have identical α-chains and differ primarily by the attachment of glucose to O-3 of HepII in L2 (9) versus a PEA to O-3 of HepII in L3 (17, 35). Currently, there are no MAbs that readily distinguish this region (60a), and therefore L2 and L3 structures appear immunologically similar when standard serotyping techniques are used.

Two Tn*916* LOS mutants of strain NMB, SS3 (*galE*) (24) and R6 (*pgm*) (60), defective in α-chain synthesis have previously been reported (Fig. 1). The LOS mutant 559 was identified by the inability to bind MAb 3F11, which recognizes an epitope in the terminal galactose of the lacto-*N*-neotetraose moiety of the α-chain of LOS. The mobility of the LOS from mutant 559 in Tricine-SDS-PAGE was more rapid than those of the LOSs of the two previously characterized mutants, SS3 and R6 (Fig. 2). Glycosyl composition, linkage, and FAB-MS analyses showed that the 559 LOS consisted of a $Hep_2P_1PEA_n$ KDO_2lipid A structure in which the number of PEA groups varied from one to three. This structure was deficient in both the addition of the α-chain sugars to HepI and the attachment of *N*-acetylglucosamine and glucose to HepII. The 559 LOS also differed in the amount of PEA substitution in comparison with the reported (24, 60) structures for the wild-type NMB and SS3 LOS structures. The latter two LOS molecules contain a single PEA group attached to HepII, whereas the 559 LOS contained up to three PEA groups as well as an additional phosphate substituent. At least one of these PEA groups or phosphate substituents is attached to HepII, since the PMAA of this residue cannot be observed until after dephosphorylation with 48% aqueous HF. However, it is also possible that PEA or P substitutions occur at other locations as well; e.g., PEA groups have been reported at the 1′ and 4′ positions of the lipid A from L3 LOS of *N. meningitidis* (23). A complete structural analysis of 559 and NMB LOSs will determine the exact location and number of these substituents.

As noted above, the analysis of the LOS of mutant 559 indicates that the O-3 glucose attached to HepII is absent and that one to three PEA groups are present (at least one attached to HepII). Since our studies have found that the parent NMB LOS is L2, we predict that one of the PEA groups is at O-6 or -7 of HepII, characteristic of the L2 structure. We suggest that the absence of the glucose at O-3 HepII in 559 has occurred because LOS inner core synthesis is incomplete, that is, *N*-acetylglucosamine must be added to the inner core before glucose can be added. PEA substitution of LOS is a specifically regulated pathway which has been implicated in the polymyxin resistance phenotypes found in *S. typhimurium* (12) and *E. coli* (34). Raetz (40) and Schnaitman and Klena (45) have also suggested that attachment and removal of PEA to LPS is involved in the transportation and maturation of LPS in the outer membrane. Therefore, we postulate that the increased PEA substitution of 559 LOS could be required to stabilize the truncated structure.

Sequence analysis of the region surrounding the Tn*916* insertion site revealed that the transposon had interrupted a 1,065-bp ORF. The amino acid sequence deduced from this ORF contained 21.2% identity and 46.8% similarity with the protein encoded by the α1,2 *N*-acetylglucosamine transferase gene (*rfaK*) from *S. typhimurium* LT2 (22, 26). We propose that the neisserial ORF be designated *rfaK*, since previously identified transferases involved in inner core LOS biosynthesis in *Neisseria* spp. have also retained the *rfa* designations from *E. coli* (43, 45, 59). This name designation clearly associates the neisserial inner core LOS biosynthesis genes with each other and distinguishes this ORF from the proposed *N*-acetylglucosamine transferase gene (*lgtA*) found in the *lgt* cluster involved in the biosynthesis of the α-chain (10). The product of the *S. typhimurium rfaK* gene is proposed to attach *N*-acetylglucosamine via an α1→2 linkage to the terminal glucose of the LPS structure. The structural analysis of NMB LOS suggests that RfaK attaches *N*-acetylglucosamine in the same way to HepII. Interestingly, neither the neisserial nor the *S. typhimurium rfaK* gene had any homology with the proposed *N*-acetylglucosamine transferase gene (*rfaK*) from *E. coli*, which is predicted to encode the enzyme that adds *N*-acetylglucosamine to an inner core heptose via an α1,7 linkage (45). Interruptions of the two *rfaK* homologs produce similar effects in both *E. coli* and *S. typhimurium* LPSs (i.e., a core deficient in *N*-acetylglucosamine and the abolition of O antigen addition to a truncated LPS core). However, *trans* complementation of *rfaK* mutants in *E. coli* and *S. typhimurium* by heterologous homologs has been difficult to demonstrate. For example, Klena et al. (21) found that the products of both the *E. coli rfaK* and *rfaL* genes were required to complement the *S. typhimurium rfaK* mutant. Therefore, it is unlikely that the neisserial RfaK, which has linkage and substrate specificities different from those of both of these enzymes, will complement the *rfaK* mutations in *E. coli* and *S. typhimurium*. Studies are under way to address this question.

While the identification of the interrupted ORF as an *rfaK* homolog may adequately explain the loss of the *N*-acetylglucosamine attached to HepII in the mutant 559 LOS structure, it does not account for the lack of the α-chain extension from HepI and the absence of glucose attached to HepII. One hypothesis explaining the multiple changes in the 559 LOS structure predicts that the interruption of *rfaK* has polar effects on a downstream gene(s) involved in α-chain extension from HepI. To test this hypothesis, polar and nonpolar antibiotic resistance cassettes were introduced into the chromosomal

copy of *rfaK* by transformation and homologous recombination. Analysis of the LOSs produced by the polar and nonpolar knockout mutants (NMB*rfaK*::Ω and NMB*rfaK*::*aphA-3*, respectively) were indistinguishable by Tricine-SDS-PAGE from that expressed by 559. These results suggest that the 559 phenotype is caused by the interruption of *rfaK* and does not involve other genes. A comparison of the LOS of mutant R6 (*pgm*), which expresses the LOS core structure $GlcNAcHep_2$ $PEAKDO_2$lipid A (60), and the LOS of mutant 559 suggests that the addition of *N*-acetylglucosamine to the inner core precedes the addition of the OSs to the heptose residues. Although the α-chain and the glucose residue at HepI and HepII are absent in R6 LOS because of the biosynthetic block, *N*-acetylglucosamine remains added to the inner core. Therefore, we propose that α-chain addition to HepI is probably constrained in mutant 559 by a requirement for a complete LOS inner core.

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