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Biochemical Analysis of Lpt3, a Protein Responsible for Phosphoethanolamine Addition to Lipooligosaccharide of Pathogenic *Neisseria*

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The inner core of neisserial lipooligosaccharide (LOS) contains heptose residues that can be decorated by phosphoethanolamine (PEA). PEA modification of heptose II (HepII) can occur at the 3, 6, or 7 position(s). We used a genomic DNA sequence of *lpt3*, derived from *Neisseria meningitidis* MC58, to search the genomic sequence of *N. gonorrhoeae* FA1090 and identified a homolog of *lpt3* in *N. gonorrhoeae*. A PCR amplicon containing *lpt3* was amplified from F62ΔLgtA, cloned, mutagenized, and inserted into the chromosome of *N. gonorrhoeae* strain F62ΔLgtA, producing strain F62ΔLgtAlpt3::Tn*5*. LOS isolated from this strain lost the ability to bind monoclonal antibody (MAb) 2-1-L8. Complementation of this mutation by genetic removal of the transposon insertion restored MAb 2-1-L8 binding. Mass spectrometry analysis of LOS isolated from the F62ΔLgtA indicated that this strain contained two PEA modifications on its LOS. F62ΔLgtAlpt3::Tn*5* lacked a PEA modification on its LOS, a finding consistent with the hypothesis that *lpt3* encodes a protein mediating PEA addition onto gonococcal LOS. The DNA encoding *lpt3* was cloned into an expression vector and Lpt3 was purified. Purified Lpt3 was able to mediate the addition of PEA to LOS isolated from F62ΔLgtAlpt3::Tn*5*.

Lipooligosaccharide (LOS) is an important neisserial virulence determinant. It consists of an oligosaccharide component attached to lipid A via 3-deoxy-D-manno-octulosonic acid (KDO). Unlike lipopolysaccharide produced by enteric organisms, the oligosaccharide component of LOS does not contain a repeating O antigen. The structure of a sufficient number of LOS molecules from neisserial strains has been determined to form a coherent yet incomplete picture as to the structural diversity of its LOS. The different LOS structures are generated by varying the composition of the OS attached to heptose I (HepI; α-chain variation), by varying the attachment of an OS or phosphoethanolamine (PEA) onto HepII (β-chain variation), or by adding a galactose onto the GlcNAc found on HepII (γ-chain extensions) (1, 8, 10, 15, 16, 45, 46).

Neisseria strains synthesize LOS by sequentially adding monosaccharides onto a basal unit. Genetic studies on LOS biosynthesis in strain F62 identified a gene cluster *lgtA-E* (10) that was responsible for the addition of most of the sugars found on the α chain. Biochemical and genetic analysis have confirmed the functions of each of these genes (3, 24, 38, 40) and their involvement in the synthesis of the α chain. Additional genes needed to synthesize the β and γ chain have also been identified (1, 18), and most of the biochemical properties of these gene products have been defined (41). The expressed LOS can be classified based on their ability to bind specific monoclonal antibodies (MAbs). The LOS structure recognized by MAb 2-1-L8 is shown in Fig. 1. This antibody binds the 3.6-kDa LOS resulting when LgtE is expressed, and when LgtA, LgtG, and LgtC are not produced (11). MAb 2-1-L8 loses its affinity for LOS if LgtA, LgtC, or LgtG is expressed (4, 6, 7, 33). The absence of PEA at 3-C of HepII (3-HepII) also results in the loss of MAb 2-1-L8 binding. This loss was demonstrated by an oligosaccharide with a L8 epitope composition $(Hex)_2(Hep)_2(HexNAc)_1(PEA)_1(KDO)_2$ failing to bind the MAb 2-1-L8 when PEA was absent from 3-HepII (11).

Both the gonococcus and meningococcus are able to decorate the 3, 6, or 7 positions of HepII with PEA (27–29). Genetic studies utilizing random transposon mutagenesis of *N. meningitidis* MC58 identified a gene, *lpt3*, that resulted in the expression of LOS devoid of PEA on HepII (22). Although the enzymatic activity of the *lpt3* gene product has not been verified, a transferase has been demonstrated in an *Escherichia coli* homolog that possessed the ability to mediate the addition of PEA to the 7-position of KDO (30). Also, the *pmrC* homolog in *Salmonella enterica* has been shown to mediate addition of PEA to lipid A (21). These findings suggest that Lpt3 possesses a corresponding biochemical function (20).

Previous reports, based on Southern hybridization data, suggested that *lpt3* was present in several strains of *N. meningitidis* but that a homolog was not present in *N. gonorrhoeae* FA1090 (22). However, using the FA1090 DNA sequence database (University of Oklahoma) we identified a homolog, NG1198, that has a 96% nucleotide identity to *N. meningitidis* MC58 *lpt-3*. In the present study, the role of *lpt3* was investigated. We

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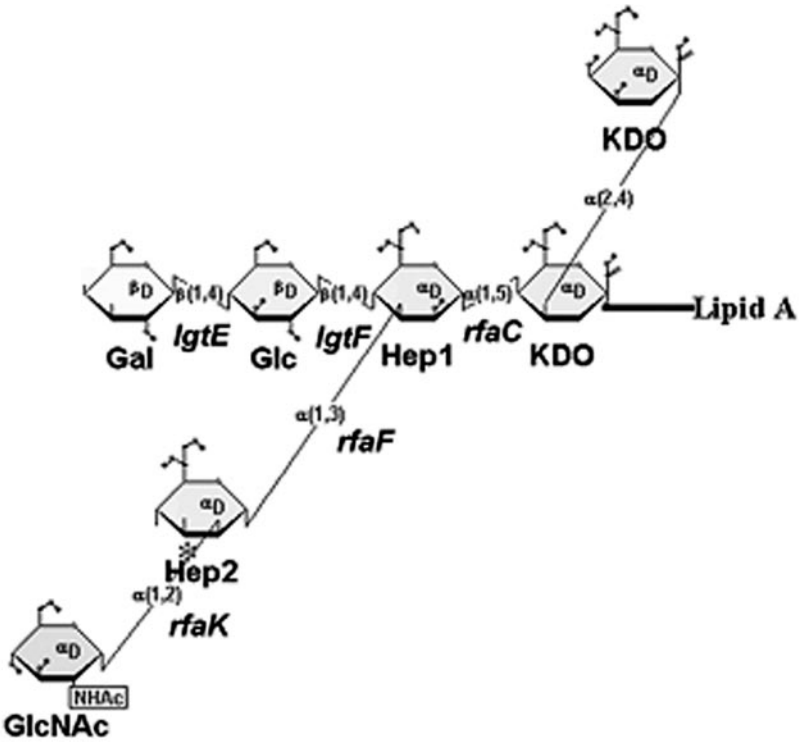


FIG. 1. Structure of neisserial LOS. The structure presented in this figure represents the LOS molecules that can be synthesized by the gonococcus that is the epitope for MAb 2-1-L8 (modified with permission from Tong et al. [36]). LOS biosynthetic genes are italicized. PEA can variably added to multiple sites designated with an asterisk on the HepII residue.

provide biochemical evidence that this gene encodes a PEA transferase in the gonococcus.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains used in the present study are described in Table 1. *Neisseria* strains were grown in phosphate-buffered gonococcal medium (Difco) supplemented with 20 mM D-glucose and growth supplements either in broth with the addition of 0.042% $NaHCO_3$ or on agar at 37°C in a CO_2 incubator (43). *E. coli* strains were grown on Luria-Bertani medium (32). Kanamycin was used at 50 μg/ml, ampicillin was used at 60 μg/ml, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at 35 μg/ml.

Chemicals, reagents, and enzymes. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.). All chemicals used for the present study were reagent grade or better and were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. Tris-Tricine gels (16.5%) and running buffer were obtained from Bio-Rad Laboratories (Richmond, Calif.).

TABLE 1. List of bacterial strains and plasmids used

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
N. gonorrhoeae F62		P. Frederick Sparling
N. gonorrhoeae F62ΔLgtA	622-bp BspEI and AgeI *lgtE* deletion (MAb 2-1-L8+)	34
N. gonorrhoeae F62ΔLgtAlpt3::Tn*5*	Tn*5* insertion at bp 866 of *lpt3* (MAb 2-1-L8−)	This study
N. gonorrhoeae F62ΔLgtAΔlpt6lpt3::Tn*5*	Replacement of *lpt6* with Spec^r cassette in *N. gonorrhoeae* F62ΔLgtAlpt3::Tn*5*	This study
N. gonorrhoeae FA1090		William Shafer
E. coli DH5αMCR	Cloning host strain F$^-$ *mcrA* (*mrr-hsdRMS-mcrBC*)	BRL[a]
E. coli: M15	Expression strain for vector pQE30	QIAGEN
E. coli: BL21	Expression strain for vector pET15b	Novagen
Plasmids		
pUC19	Cloning vector, replicates in *E. coli* DH5αMCR	BRL[a]
pLPT3	F62ΔLgtAlpt3 cloned into NdeI and EcoRI of pUC19	This study
pLPT6	F62ΔLgtAlpt6 cloned into HindIII and EcoRI of pUC19	This study
pET15b	Expression vector, replicates in BL21	Novagen
pET15b::PEA	FA1090 *lpt3* cloned into NdeI and EcoRI of pET15b	This study
pQUE30	Expression vector, replicates in M15	QIAGEN
pQE30::PEA	FA1090 *lpt3* cloned into BamHI and PstI of pQUE30	This study

[a] Bethesda Research Laboratories (BRL) is now part of Invitrogen.

TABLE 2. List of primers used in this study

Primer	Sequence (5′ to 3′)[a]	Description
PTE-6	ATTCCAAGGATCCAAAAAATCCCTTTTCGTTCTCTTTCTGTATTC	Amplifies the 5′ end of *lpt3* with BamHI site
PTE-7	CGTACTGCAGATCTAAATTTGTCTTTATTGGTTTTCACGCTGCTTATT	Amplifies the 3′ end of *lpt3* with PstI site
PTE-4	ATTCCAACATATGAAAAAATCCCTTTTCGTTCTCTTTCTGTATTC	Amplifies the 5′ end of *lpt3* with NdeI site
PTE-5	CGTAGAATTCATCTAAATTTGTCTTTATTGGTTTTCACGCTGCTTATT	Amplifies the 3′ end of *lpt3* with EcoRI site
PEA-R	CCGGAATTCCGGAATCACCAATATTTCCGCCATGCAGTCGTG	Amplifies a sequence downstream of *lpt3* with EcoRI site
O6-PEAR	ATTTGAATTCGGCATTCCCGAATACG	Amplifies 3′ end of *lpt6* region with EcoRI site
O6-PEAF	CGAAGCTTTCCCAGCCGCTTTGG	Amplifies 5′ end of *lpt6* region with HindIII site
Lpt6 MutF	CCCCTGCAGTAGCTTTTTGCCGCACGGC	Mutation primer to replace *lpt6* with Spec^r cassette with PstI site
Lpt6 MutR	CCCCTGCAGCCCGCCCCACTCCTCAAA	Mutation primer to replace *lpt6* with Spec^r cassette with PstI site

[a] The underlined sequences represent the location of the restriction enzyme site.

The MAb 2-1-L8 was graciously provided by Wendell Zollinger (Walter Reed Army Institute of Research, Washington D.C.).

DNA isolation procedures. Chromosomal DNA was isolated by using Promega's Wizard Genomic DNA Purification Kit. Plasmid DNA was isolated by the Birnboim and Doly alkaline lysis method (2).

Transformation. Competent cells of *E. coli* DH5-MCR were prepared according to the method of Inoue et al. (14). Transformation of *E. coli* with pLPT3, as well as with the transposon-mutagenized pLPT3, was done according to the heat shock protocol (32). DNA transformation into *N. gonorrhoeae* was done by resuspending piliated cells in GCP broth containing 1× Kellogg's solution, 0.042% $NaHCO_2$, 10 mM $MgCl_2$, and 1 μg of the DNA of interest (35). Cells were incubated for about 3 h with shaking at 37°C. Cells were plated onto GCK plates containing kanamycin. The spot transformation consisted of suspending 10 isolated piliated colonies in 500 μl of GCP broth containing 10 mM $MgCl_2$. Serial dilutions of cells were spotted onto GCK plates, and 2 μg of DNA was mixed into each spot. Plates were allowed to dry prior to overnight incubation. Colonies were patch plated onto GCK and GCK plates containing kanamycin (12).

LOS purification and SDS-PAGE. Purified LOS was obtained from broth grown cells through the hot-phenol method, followed by lyophilization (42). Quick preparations of gonococcal LOS were prepared from plated cultures as described by Hitchcock and Brown and diluted 1:25 in lysing buffer (13). The suspension was boiled for 10 min immediately before loading. Approximately 0.1 μg of LOS was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 16.5% Tris-Tricine gel (from Bio-Rad) in Tris-Tricine running buffer according to the protocol suggested by the manufacturer. The gel was fixed overnight in 40% ethanol–5% acetic acid, and the LOS was visualized by silver staining (37).

Immunological methods. After SDS-PAGE, LOSs were electrotransferred onto Immobilon-P membrane (Millipore Corp.) in a Tris-Tricine-methanol buffer (10 mM Tris [pH 8.3], 10 mM Tricine, 0.01% SDS, 20% methanol) at a constant voltage of 100 V for 1 h according to the protocol provided by Bio-Rad Corp. After air drying for 1 h, the membrane was processed in buffer (20 mM Tris, 150 mM NaCl, 2% milk powder) to block all nonspecific binding sites and then screened for reactivity with MAb 2-1-L8. Bound MAb was detected by reacting the Immobilon-P membrane with horseradish peroxidase-labeled goat anti-mouse immunoglobulin G, and testing for the presence of bound horseradish peroxidase by using the following development conditions (50 mM Tris [pH 8.0], 0.006% H_2O_2, 0.08% 4-chloro-1-naphthol). When colony blot analysis was performed, overnight colonies were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) and processed as described above.

PCR. Primers used in the present study are listed in Table 2. The PCR was performed by using the Expand Long Template PCR kit (Roche) according to the manufacturer's specifications. Primers were purchased from Integrated DNA technologies (Coralville, Iowa). PCRs were resolved on 1% agarose gels containing 500 μg of ethidium bromide in Tris-borate EDTA running buffer/ml (32). Sequencing of PCR products was performed by Macrogen, Inc. (Seoul, South Korea).

Strain construction. Bacterial strains and plasmids used in the present study are listed in Table 1. *N. gonorrhoeae lpt3* was cloned by PCR amplification of the *lpt3* region with primers PTE-4 and PEA-R. The amplicon was digested with EcoRI and NdeI and cloned into the EcoRI and NdeI sites of pUC19, giving pLPT3. This plasmid was used as the target DNA in a transposon mutagenesis reaction (using a kit purchased from Epicenter), giving pLPT3::Tn*5* (9, 31). The location of the transposon insertion was determined by restriction mapping, and a construct was chosen that had the transposon inserted near the beginning of the coding region. This insertionally inactivated construct was introduced into F62ΔLgtA, giving rise to F62ΔLgtAΔlpt3::Tn*5*. The exact site of transposon insertion was determined by DNA sequence analysis to be at bp 866 of the coding region. For biochemical analysis, the *N. gonorrhoeae* FA1090 chromosome was used as a template for PCR amplification of *lpt3* with primers PTE-4 and PTE-5. The product was cloned into the EcoRI and NdeI sites of expression vector pET15b, giving pET15b::PEA. Amplification with primers PTE-6 and PTE-7 yielded a product used for cloning into vector pQE30 to create pQE30::PEA.

N. gonorrhoeae lpt6 was cloned by PCR amplification of *lpt6* (NGO 2071) and its flanking regions with primers O6-PEAR and O6-PEAF. The amplicon was digested with EcoRI and HindIII and cloned into the EcoRI and HindIII sites of pUC19, giving pLPT6. The clone was sequenced to confirm the presence of lpt6 on the amplicon. The *lpt6* gene was replaced with a Spec^r cassette by using pLPT6 as a template in a PCR with mutation primers Lpt6 MutF and Lpt6 MutR. The amplicon was digested with PstI, the site into which the Spec^r cassette was inserted. This construct was introduced into F62ΔLgtAΔlpt3::Tn*5*, giving rise to F62ΔLgtAΔlpt6Δlpt3::Tn*5*.

Isolation and purification of Lpt3. Recombinant DNAs were transformed into strain M15 (vector pQE30) or BL21 (vector pET15b). Inoculated cultures were grown at 37°C until the optical density at 600 nm reached between 0.4 and 0.6. IPTG (isopropyl-β-D-thiogalactopyranoside) inducer was added and cultures were incubated at 37°C for 4.5 h. The cultures were sonicated, and proteins were purified on Ni-agarose columns according to the manufacturer's specifications (QIAGEN, Valencia, CA) except that after loading the materials, the column was washed with sonication buffer (10 volumes of column), followed by washing buffer for 16 h. Proteins were eluted from the column with increasing concentrations of imidazole (twice with 1 ml at 5 mM, twice with 1 ml at 10 mM, and twice with 1 ml at 25 mM). All buffers contained 1 mM phenylmethylsulfonyl fluoride.

Enzymatic reaction. Reactions contained 3 μl of 10-fold-concentrated buffer (500 mM Tris-HCl [pH 8.8], 50 mM β-mercaptoethanol, 1% Triton X-100), 3 μl of 100 mM $MgCl_2$, 2 μl of 1-palmytiol-2-oleyol-phosphatidylethanolamine (to a final concentration of 1 mM), and either 3 μl of LOS (1 mg/ml) or cell suspension to a final volume of 16 μl. Prior to initiating the reaction, phosphatidylethanolamine was dissolved a 1:4 mixture of methanol and chloroform and stored at −80°C. To begin the reaction, the phosphatidylethanolamine solution was added to an Eppendorf tube and dried in a CO_2 incubator. The other components were added into the reaction tube and mixed until the PEA donor was suspended.

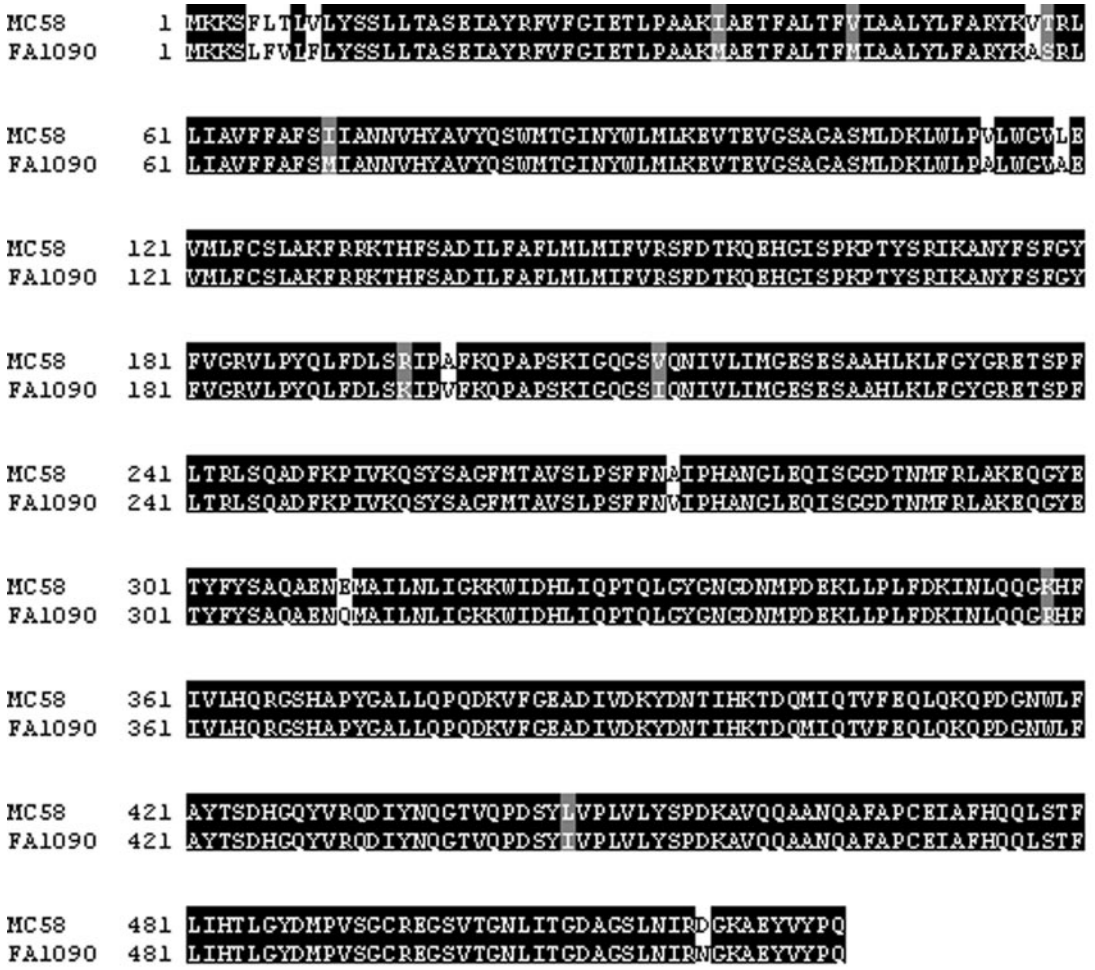


FIG. 2. Lpt3 amino acid alignment. *N. meningitidis* MC58 Lpt3 is aligned against *N. gonorrhoeae* FA1090 Lpt3. Shaded regions indicate sites of conservation. Gray boxes indicate where minor amino acid differences exist, and white areas show sites of more significant variation.

Lastly, 1 to 2 μg of enzyme was added, and the reaction was carried out for 90 min at 30°C.

MS analysis. Purified LOS from F62ΔLgtA, F62ΔLgtAlpt3::Tn*5*, the mutant complement, F62ΔLgtAΔlpt6lpt3::Tn*5* and were O-deacylated prior to matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) (16). Anhydrous hydrazine (200 μl) was added to 0.5 mg of LOS and kept at 37°C with periodic vortexing for 20 min. O-deacylated LOS was precipitated with −20°C acetone and centrifuged at 12,000 × g for 20 min. The pellet was washed with cold acetone and resuspended in H_2O at 2 μg/μl. Samples were desalted with cation-exchange beads (Dowex 50X) and combined with 100 mM 2,5-dihydrobenzoic acid concentrated in MeOH. Negative ion MALDI-MS was performed in linear mode with delayed extraction on a Voyager Elite TOF instrument equipped with a 337-nm nitrogen laser (PerSeptive Biosystems, Framingham, MA). Analyses were performed with a 150-ns time delay and a grid voltage of 92 to 94% of full acceleration voltage, 20 kV, and external calibration.

RESULTS

Identification of *N. gonorrhoeae* FA1090 *lpt3* homolog. A search of the *N. gonorrhoeae* FA1090 chromosome at the University of Oklahoma yielded a FA1090 homolog of *N. meningitidis* MC58 *lpt3*. An alignment of the two Lpt3 proteins is shown in Fig. 2. A search of FA1090 Lpt3 conserved domains at the NCBI conserved domain website yielded results consistent transferase ability. The region between residues 211 and 497 was 90.6% aligned with pfam00884, a sulfatase. Three other proteins' domains aligned with Lpt3 over a broader range. Lpt3 residues 16 to 519 aligned 94.8% with COG 2194, from a predicted membrane-associated metal-dependent hydrolase. COG 1368 from a phosphoglycerol transferase of the alkaline phosphatase superfamily aligned 71.7% between Lpt3 residues 49 to 464. A hydrolase of the alkaline phosphatase superfamily contained COG 3083 which aligned 50.5% with Lpt3. These results indicate that *lpt3* should encode a membrane-associated transferase.

Construction of *lpt3* mutant in *N. gonorrhoeae* F62Δ*LgtA*. In order to study the activity of the *lpt3* gene product, *N. gonorrhoeae* F62ΔLgtA was chosen as a starting point because F62ΔLgtA contains a deletion of the *lgtA* gene, resulting in the expression of a single LOS component that binds the MAb 2-1-L8 (see Fig. 3). Using primers PTE-4 and PEA-R, a PCR was performed with the F62ΔLgtA chromosome as a template. The DNA sequence of the resulting 1.8-kb amplicon was determined. The region encoding *lpt3* showed 100% nucleotide identity to *N. gonorrhoeae* FA1090. The flanking regions contained only a few base changes in noncoding regions (data not shown). This fragment was cleaved with restriction enzymes EcoRI and NdeI and cloned into pUC19 at

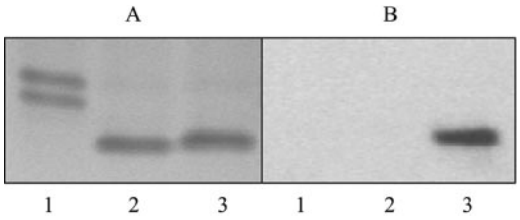


FIG. 3. Reactivity of F62ΔLgtAlpt3::Tn*5* with MAb 2-1-L8. (A) Silver-stained LOS electrophoresed through a 16.5% Tris-Tricine polyacrylamide gel. Lanes: 1, LOS isolated from F62; 2, LOS isolated from F62ΔLgtAlpt3::Tn*5*; 3, LOS isolated from F62ΔLgtA. (B) Western blot with MAb 2-1-L8 on a gel identical to the one shown in panel A.

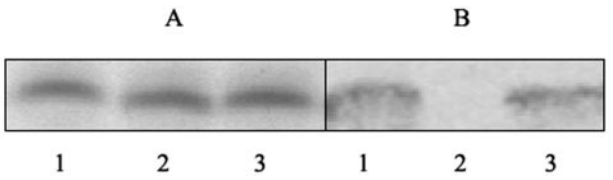


FIG. 4. Complementation analysis of F62ΔLgtAlpt3::Tn*5*. (A) Silver-stained LOS electrophoresed through a 16.5% Tris-Tricene polyacrylamide gel. Lanes: 1, LOS isolated from F62ΔLgtA; 2, LOS isolated from F62ΔLgtAlpt3::Tn*5*; 3, LOS isolated from the *lpt3* complemented F62ΔLgtAlpt3::Tn*5*. (B) Western blot with MAb 2-1-L8 on a gel identical to the one shown in panel A.

those respective sites. The resulting plasmid, pLPT3, was used as a target of transposon mutagenesis.

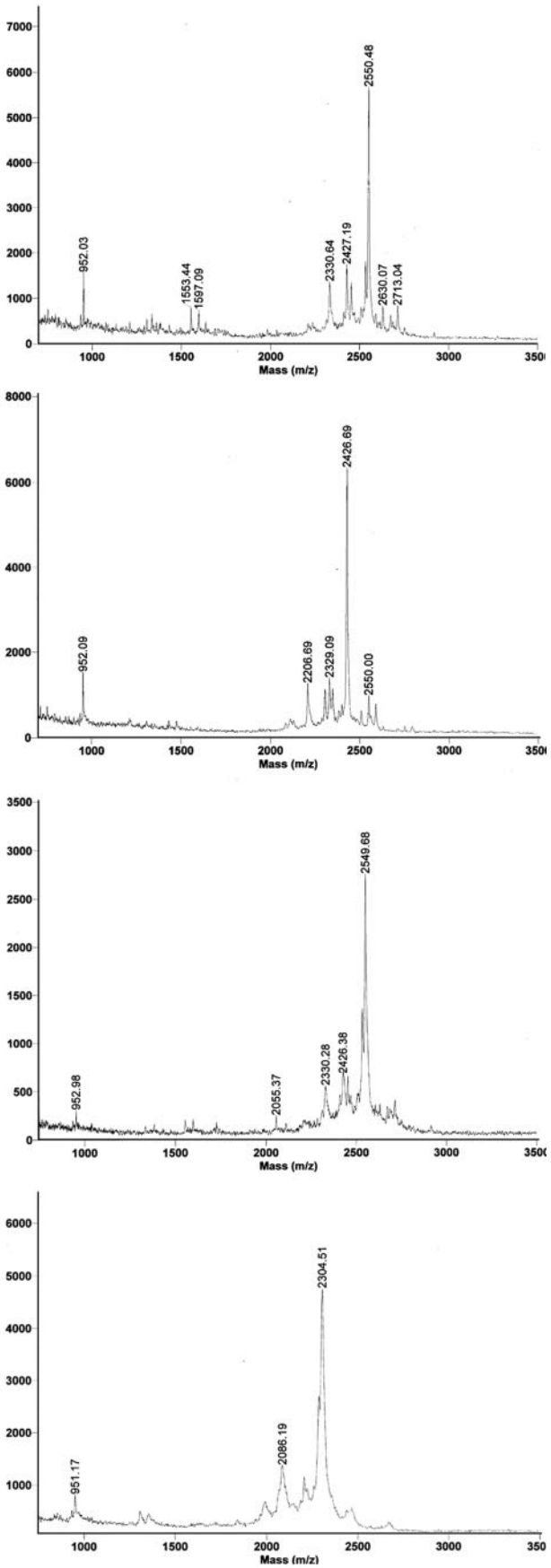
Transposon-inserted pLPT3 was used to transform F62ΔLgtA, and kanamycin-resistant transformants were identified. These colonies were screened for loss of reactivity with MAb 2-1-L8. PCRs were performed on individual MAb 2-1-L8 unreactive transformants using PTE-4 and PTE-5, and the amplicons all had the predicted increase in size, corresponding to the transposon insertion into *lpt3*. DNA sequence analysis of the amplicons confirmed that each contained a transposon insertion into *lpt3*. One of these mutants, F62ΔLgtAlpt3::Tn*5* was selected for further study. The data presented in Fig. 3 indicates that the SDS-PAGE profile of the mutant strain produced a LOS that migrated slightly faster than F62ΔLgtA LOS. Western blot analysis of an identical gel, using MAb 2-1-L8, indicated that this component no longer bound the MAb. Because the antibody requires PEA addition at 3-HepII, this mutant presumably contained an insertion that inactivated *lpt3* and prevented this specific decoration.

Complementation analysis of *lpt3* mutant. To prove that the loss of MAb 2-1-L8 affinity for F62ΔLgtAlpt3::Tn*5* LOS was caused by the inactivation of *lpt3*, complementation analysis was performed. The plasmid pLPT3, containing wild-type gonococcal *lpt3*, was used as a source of DNA to transform F62ΔLgtAlpt3::Tn*5* cells; kanamycin-sensitive transformants were identified for further study. PCR analysis with primers PTE-4 and PTE-5 revealed that all kanamycin-sensitive transformants lost the transposon insertion and contained an amplicon of the same size as the original parent. All kanamycin-sensitive transformants reacquired the ability to bind MAb 2-1-L8. One of these transformants was selected for further study. LOS was isolated from this complemented mutant and its SDS-PAGE profile compared to that of F62ΔLgtA and F62ΔLgtAlpt3::Tn*5*. The data indicated (Fig. 4) that the complemented mutant and the parent showed identical LOS migration patterns. Western blot analysis of a duplicate gel indicate that both the parent and the complemented mutant LOS bound the antibody, whereas the *lpt3* mutant LOS did not. These results indicate that the insertional inactivation of *lpt3* was responsible for the inability of mutant LOS to bind MAb 2-1-L8.

MS analysis of LOS. To confirm that the loss of a PEA residue from F62ΔLgtAlpt3::Tn*5* LOS caused the loss of MAb 2-1-L8 reactivity, MS analysis was performed. LOS was purified according to the method of Westphal and Jann (42) from F62ΔLgtA, F62ΔLgtAlpt3::Tn*5*, F62ΔLgtAΔ lpt6lpt3::Tn*5*, and the complemented mutant. The resulting spectra are shown in Fig. 5. Table 3 contains the proposed compositions for the LOS molecules that correspond to peaks on the spectra. As shown in the spectra in panel A, the most abundant ion for F62ΔLgtA is at *m/z* 2,550.48. This value is consistent with the composition $(Hex)_2(HexNAc)_1(Hep)_2(PEA)_2(KDO)_2$ and a lipoidal moiety (952 Da). The presence of minor peaks in the spectra allowed us to obtain further structural validation. The peak representing the *m/z* 2,427.19 ion differs from the major peak by the mass of PEA (123.1 Da). The *m/z* 2330.64 ion corresponds to the loss of KDO (220.2 Da) compared to the major ion. This molecule occurs as the result of the lability of the ketosidic linkage resulting from hydrazine mediated O-deacetylation (15, 16, 23). Compared to the most abundant ion, the *m/z* 2,630.07 ion differs by the gain of HexNAc (203.2 Da) and the loss of KDO and PEA. This addition is probably mediated by the aberrant action of LgtD. A mass difference consistent with the gain of a Hex (162.2 Da) is seen in the *m/z* 2,713.04 molecular ion compared to the *m/z* 2,550.48 ion. The presence of this peak is best explained by the activity of *lgtC*, which can phase vary to produce a functional glycosyl transferase in this strain, albeit at a low frequency.

Comparison of the F62ΔLgtA and F62ΔLgtAlpt3::Tn*5* LOS MALDI-MS spectra showed compositional differences in the LOS produced by these two strains. The spectra in panel B shows that the major ion of the mutant LOS is at *m/z* 2,426.69, corresponding to a loss of PEA compared to the most abundant peak in the parental spectrum. Ions of *m/z* 2,329.09 and 2,206.69 are consistent with the loss of KDO from ions *m/z* 2,426.69 and 2,550.00, respectively. Both the parent and mutant spectra contain a peak at *m/z* 952 representing the lipoidal moiety. F62ΔLgtA does not decorate its lipoidal moiety with appreciable PEA residues, as shown by the absence of peaks at 1,032, 1,155, 1,075, and 1,278, which would correspond to the addition of one or two phosphate groups and one or two PEA groups, respectively, to the lipoidal moiety. Therefore, loss of PEA groups in the family of PEA transferase mutants created from this strain must be from the oligosaccharide component of the LOS.

LOS from the complemented *lpt3* mutant LOS was also analyzed by MS. The spectra in Fig. 5C indicates that there was a recovery of wild-type LOS. This is best viewed through the change in ratios of ions containing PEA decoration in comparison to the mutant spectra. The major peak is at *m/z* 2,550.48, which is characteristic of $(Hex)_2(HexNAc)_1(Hep)_2(PEA)_2(KDO)_2$ and the lipoidal moiety. Again, like the wild-



type spectra, a minor ion of *m/z* 2,427.19 is seen which differs from the major ion by a PEA residue. Other similarities to the wild-type spectra are three ions corresponding to the loss of KDO, a gain of HexNAc with a simultaneous loss of KDO and PEA, and the gain of a Hex residue. These results indicate that inactivation of Lpt3 is responsible for the loss of PEA shown in the mutant spectra.

Lpt3 mutant LOS contained a small amount of an ion at *m/z* 2,550.00 corresponding to $(Hex)_2(HexNAc)_1(Hep)_2(PEA)_2(KDO)_2$lipoidal moiety. The presence of this ion is best explained by the existence of a subset of LOS molecules that contain PEA on carbons 6 and 7 of Hep II, since the *lpt3* mutant LOS failed to bind MAb 2-1-L8. Recently, *lpt6* was identified in the meningococcus and is speculated to be a transferase that mediates the addition of PEA to 6-HepII (44). To determine whether this transferase is responsible for the presence of the *m/z* 2,550.00 ion expressed by the *lpt3* mutant LOS, *lpt6* was deleted from the F62ΔLgtAlpt3::Tn*5* chromosome. The deletion of *lpt6* completely oblates the presence of the *m/z* 2,550 ion. These data indicate that F62ΔLgtAΔlpt6lpt3::Tn*5* LOS is completely devoid of PEA decoration. These data indicate that Lpt6 is responsible for the PEA modifications in the *lpt3* mutant.

Isolation and purification of Lpt3. In order to demonstrate that the *lpt3* gene product was a PEA transferase, Lpt3 was isolated and purified. Two PCRs were performed with FA1090 chromosomal DNA as a template: one using primers PTE-6 and PTE-7 and the other using primers PTE-4 and PTE-5. Each product yielded a 1.5-kb fragment that was confirmed by nucleotide sequencing to contain *lpt3*. Each amplicon was cloned, respectively, into expression vectors pQE30 and pET15b, creating pQE30::PEA and pET15b::PEA. These plasmid DNAs were transformed into *E. coli* M15 (for vector pQE30) or BL21 (for vector pET15b). Expression in these vectors creates a fusion protein in which a six-residue His tag is added to Lpt3. As shown in Fig. 6, the protein fractions taken immediately after the end of induction of BL21 cells containing pET15b::PEA showed the presence of a protein of the appropriate molecular mass (54 kDa). After purification on a nickel column, even in the presence of the protease inhibitor phenylmethylsulfonyl fluoride, a degradation product of a lower molecular mass was always observed.

Determination of Lpt3 activity. The activity of Lpt3 was determined by using whole cells of F62ΔLgtAlpt3::Tn*5*. LOS was isolated from the F62ΔLgtAlpt3::Tn*5*, with or without enzymatic treatment and subjected to SDS-PAGE and Western blot analysis. As shown in Fig. 7, silver staining of the gel revealed that, as expected, the F62ΔLgtAlpt3::Tn*5* cells that underwent the reaction with or without the enzyme yielded a slightly different migration pattern. LOS that underwent reaction with Lpt3 migrated at a reduced rate compared to the

FIG. 5. MALDI-MS Profiles of F62ΔLgtA and F62ΔLgtAlpt3::Tn*5* LOS. (A, B, C, and D) Spectra of O-deacylated purified LOS. (A) MS profile of F62ΔlgtA LOS. (B) Spectrum produced by F62ΔLgtAlpt3::Tn*5*. (C) Spectrum produced by the complemented mutant. (D) F62ΔLgtA Δlpt6lpt3::Tn*5* LOS profile. The masses of the abundant fragments are indicated at the top of the corresponding peaks.

TABLE 3. Compositions and masses of LOS from MALDI-MS

Strain	LOS composition[a]	M_r	
		Experimental	Calculated
F62ΔLgtA	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_2(KDO)_2$ lipoidal moiety	2,550.48	2,551.27
	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_1(KDO)_2$ lipoidal moiety	2,427.19	2,428.22
	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_2(KDO)_1$ lipoidal moiety	2,330.64	2,331.28
	$(Hex)_2(HexNAc)_2(Hep)_2(PEA)_1(KDO)_1$ lipoidal moiety	2,630.07	2,631.41
	$(Hex)_3(HexNAc)_1(Hep)_2(PEA)_2(KDO)_2$ lipoidal moiety	2,713.04	2,713.83
F62ΔLgtA1pt3::Tn*5*	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_1(KDO)_2$ lipoidal moiety	2,426.69	2,428.22
	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_2(KDO)_1$ lipoidal moiety	2,329.09	2,331.09
	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_1(KDO)_1$ lipoidal moiety	2,206.69	2,208.04
	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_2(KDO)_2$ lipoidal moiety	2,550.00	2,551.27
F62ΔLgtA1pt3::Tn*5* pLPT3 complement	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_2(KDO)_2$ lipoidal moiety	2,549.68	2,551.27
	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_1(KDO)_2$ lipoidal moiety	2,426.38	2,428.22
	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_2(KDO)_1$ lipoidal moiety	2,330.28	2,331.28
	$(Hex)_2(HexNAc)_2(Hep)_2(PEA)_1(KDO)_1$ lipoidal moiety	2,629.99	2,631.41
	$(Hex)_3(HexNAc)_1(Hep)_2(PEA)_2(KDO)_2$ lipoidal moiety	2,712.96	2,713.83
F62ΔLgtAΔ1pt61pt3::Tn*5*	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_0(KDO)_2$ lipoidal moiety	2,304.51	2,305.17
	$(Hex)_2(HexNAc)_1(Hep)_2(PEA)_0(KDO)_1$ lipoidal moiety	2,086.19	2,084.99

[a] Structures given are the predicted structure. The calculated M_r is derived from this structure.

mutant LOS. The difference is difficult to see because the gel contained overloaded LOS, which was required for Western blotting. The Western blot of an identical gel showed a difference in MAb 2-1-L8 reactivity. Only F62ΔLgtAlpt3::Tn*5* cells that underwent the reaction with the Lpt3 enzyme regained the ability to bind MAb 2-1-L8. This suggests that PEA had been transferred to the 3-HepII on the gonococcal LOS.

Because LOS was contained in whole cells in the biochemical experiment described above, it is possible that some additional components contained in these cells were needed for the successful LOS modification. As a direct measure of Lpt3 modification of LOS, LOS from F62ΔLgtAlpt3::Tn*5* was isolated and purified, and an enzymatic reaction was performed. As shown in Fig. 8, purified mutant LOS incubated with phosphatidylethanolamine and the purified Lpt3 enzyme produced a strong positive reaction with MAb 2-1-L8, when the reaction mix was directly spotted onto a nitrocellulose filter. When the reaction was performed either without PEA or without Lpt3, no MAb 2-1-L8 reactivity was seen. As an additional control, we reacted lysate from sonicated *E. coli* BL21 cells with LOS from F62ΔLgtAlpt3::Tn*5*. We were never able to demonstrate any MAb 2-1-L8 reactivity (data not shown). This control eliminates the possibility that gonococcal Lpt3 has been contaminated with a PEA transferase from the cloning host. Attempts to demonstrate this reactivity by SDS-PAGE analysis failed to produce a satisfactory gel. This is most likely due to the fact that we needed to add large quantities of LOS (3 μg) to get a readily visible signal when the modified LOS was blotted directly onto nitrocellulose, and this quantity of LOS grossly overloads and SDS-PAGE gel. However, the ability of purified Lpt3 to add PEA to purified LOS provides direct evidence that *lpt3* encodes a functional PEA transferase that adds PEA onto 3-HepII of gonococcal LOS.

DISCUSSION

In this study, we used a bioinformatic approach to identify a *lpt3* homolog in *N. gonorrhoeae*. We constructed a mutation in the identified DNA sequence by in vitro transposon mutagenesis, introduced the mutated DNA sequence into the gonococcus, and analyzed transformants for their ability to bind MAb

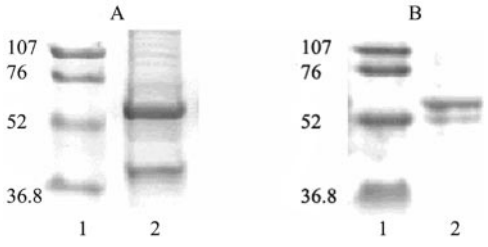


FIG. 6. SDS-PAGE profile of proteins isolated from various *E. coli* strains after induction with IPTG. *E. coli* strains were grown to mid-log phase, and Lpt3 expression was induced by the addition of IPTG. (A) Supernatants of *E. coli* BL21 containing pET15b::PEA were analyzed on an SDS–6.5% PAGE gel. Lane 1 shows the molecular weight standard, and lane 2 shows the extracted supernatants. (B) Lpt3 protein purified on a nickel column. Lane 1 shows the molecular weight standard, and lane 2 shows the purified Lpt3. A degradation product was obtained that did not show enzymatic activity in subsequent steps (data not shown). The molecular mass standard is given in kilodaltons.

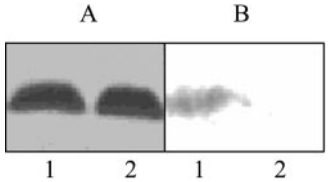


FIG. 7. Enzymatic Lpt3 activity on whole F62ΔLgtAlpt3::Tn5 cells. Reactions contained 10 μl of mutant cell suspension, 500 mM Tris-HCl buffer (pH 8.8), 2 μl of phosphatidylethanolamine, 3 μl of 100 mM $MgCl_2$, 2 μl of Lpt3 enzyme, and water to a final volume of 30 μl. The reactions were carried out for 90 min at 30 C. A total of 10 μl of the reaction sample was withdrawn, and samples were analyzed on a 15% Tris-Tricine gel. (A) Silver-stained gel of LOS resulting from the enzymatic reactions. Lane 1 contains F62ΔLgtAlpt3::Tn*5* LOS from a reaction containing Lpt3. Lane 2 shows the LOS resulting from the same reaction, but in the absence of Lpt3. (B) Western blot with MAb 2-1-L8 of a gel identical to the gel shown in panel A.

FIG. 8. Enzymatic Lpt3 activity on purified LOS. Purified LOS from F62ΔLgtAlpt3::Tn*5* was reacted with purified Lpt3. Reactions contained 3 μl of mutant LOS, 500 mM Tris-HCl buffer (pH 8.8), 2 μl of phosphatidylethanolamine, 3 μl of 100 mM $MgCl_2$, 2 μl of Lpt3 enzyme, and water to a final volume of 30 μl. The reactions were carried out for 90 min at 30 C. A total of 10 μl of each reaction was spotted onto the nitrocellulose filter. Lane 1 represents the above reaction, lane 2 shows the complete reaction without Lpt3, and lane 3 shows the complete reaction without phosphatidylethanolamine.

2-1-L8. Failure of mutant LOS to bind MAb 2-1-L*8* in Western blots and MS analyses of this mutant LOS confirmed that the inactivation of gonococcal *lpt3* resulted in the production of LOS that was deficient in PEA modification at 3-HepII. Genetic complementation by restoring the parental DNA sequence into transposon-mutagenized strains restored the parental LOS phenotype, verifying that the loss of reactivity was due to the insertion of the transposon into *lpt3*. Although it is possible that the loss of MAb binding could have been due to a polar effect of the transposon insertion effecting the expression of downstream genes, we have discounted this possibility because DNA sequence analysis of both *N. gonorrhoeae* F62 and FA 1090 strains revealed that the downstream gene (*hrpA* homolog) has significant homology with DNA helicases and that the intergenic region between *lpt3* and the *hrpA* homolog is homologous with promoter elements. In addition, purified Lpt3 was able to mediate the proposed biochemical function of Lpt3.

We devised a cloning/purification procedure that allowed us to purify Lpt3. This protein proved difficult to purify to homogeneity and rapidly degraded in solution. Amino acid sequence analysis of this protein suggests that it is intimately associated with the bacterial membrane. Our biochemical tests with the purified protein clearly indicate that Lpt3 is a PEA transferase because it modified 3-HepII of *lpt3* mutant LOS. However, it does not function well in solution. The low activity and the high degree of instability of the purified protein prevented us from performing a more detailed biochemical analysis of Lpt3.

Both *N. gonorrhoeae* and *N. meningitidis* are capable of expressing LOS molecules that are decorated with PEA at positions 3, 6, and 7 of HepII. The exact locations of the addition(s) are biologically significant because neisserial LOS has been shown to be a target of the immune system. For example, antibodies recognizing the B5 epitope, PEA bound to 3-HepII, were found in patients (25). Also, the position of PEA addition has been shown to be important in the role LOS plays as an acceptor for complement. PEA present at the 6-HepII residue was shown to form amide linkages with C4b better than corresponding LOS structures with PEA added to the 3 position. As a result, meningococcal strains containing PEA at 6-HepII are more susceptible to the bactericidal effects of serum than strains with the 3-position modification (29). It has been suggested that this difference in C4b interaction depending on the PEA modification site may account for that fact that more than 70% of meningococcal strains isolated from humans contain PEA at 3-HepII (26) and why, during an outbreak of meningococcal meningitis, 97% of disease-causing isolates carried the same modification (17).

A closer examination of the MALDI-MS results provides insight into the action of PEA transferases. The F62ΔLgtAlpt3::Tn*5* LOS spectrum contained peaks at *m*/*z* 2,426.69 and 2,550.00, indicating that these ions, respectively, contained one and two PEA residues, even though *lpt3* had been inactivated. The Western blot with MAb 2-1-L*8* in the present study shows that *lpt3* inactivation results in the loss of PEA decoration at 3-HepII. However, the *lpt3* mutant produced small amounts of LOS decorated with PEA at sites other than 3-HepII. The production of LOS devoid of PEA by F62ΔLgtAΔlpt6lpt3::Tn*5* indicates that Lpt6 is responsible for the additional decorations at sites other than 3-HepII in the *lpt3* mutant. The genetic and biochemical basis for the addition onto position 7 is unknown. One possibility is that PEA at 6-HepII of LOS may spontaneously migrate to the 7-HepII position. In mutants that cannot add PEA onto this position, no addition would be possible at position 7. A second possibility is that the protein needed to mediate this addition requires the presence of PEA modified on 6-HepII in order to serve as an acceptor. Another possibility is that Lpt6 may be slightly promiscuous at the site of decoration. The gene *lpt6* has been identified in the meningococcus and is thought to encode a PEA transferase that decorates 6-HepII (44). Because the donor and substrate are similar in the PEA modification processes of the positions 3, 6, and 7 of HepII, it is possible that Lpt6 could act on 3-HepII, 6-HepII, or 7-HepII with different efficiencies.

Previous studies have shown that different neisserial strains have various ratios of PEA modification at HepII that do not correspond to the presence or the absence of *lpt3* and *lpt6*. For example, even though *N. meningitidis* strains BZ147 and 2220Y both possess *lpt3* and *lpt6*, BZ147 LOS predominantly contains 3-HepII PEA modification, and 2220Y LOS is simultaneously modified at the 3 and 6 positions of HepII with PEA (5, 29, 44). Also, it has been noted that meningococcal strain NMB contains a *lpt3* homolog but only expresses a LOS with PEA decorated at 3-HepII when a truncated mutant, NMB*lgtK*, is created that expresses the major LOS form of Hep2Kdo2-lipid A. Rather, majority of NMB LOS displays an L2 immunotype with glucose at 3-HepII and PEA at 6-HepII (19).

Because the presence of *lpt3* does not directly correlate to PEA modification at 3-HepII, as in the example described above, regulation of modification must occur. One process that affects the ability of PEA to add to 3-HepII is the activity of LgtG. It is known that Lpt3 competes with LgtG for the 3-HepII (1). However, this competition between enzymes has complicating factors. First, it has recently been shown that environmental stimulation through the MisS/MisR system alters the level of LgtG activity (39). When the system is not functional LgtG is upregulated and a higher percentage of LOS molecules are modified with glucose at 3-HepII (19). However, it is important to note that when an NMB *lgtG* mutant was created the resulting LOS still failed to contain PEA at 3-HepII although the strain possesses *lpt3*. These data suggest that PEA modification at HepII also reflects the functionality of the Lpt3 enzyme. Lpt3 encoded by strain NMB may have a decreased affinity for either the donor or the substrate compared to FA1090 Lpt3. DNA sequence analysis of *lpt3* isolated from these three strains indicate that they contain

minor DNA sequence variations (E. T. O'Connor, unpublished observations). These minor sequence variations may alter the biochemical properties of this enzyme enough to produce readily measurable phenotypic differences. Further evaluation of enzymes from neisserial strains known to have various ratios of PEA modification of HepII is needed to fully understand the modification process of HepII of neisserial LOS.

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