The MisR/MisS Two-component Regulatory System Influences Inner Core Structure and Immunotype of Lipooligosaccharide in Neisseria meningitidis*

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Lipooligosaccharide (LOS) of Neisseria meningitidis is the major inflammatory mediator that contributes to meningococcal pathogenesis. Variable attachments to the HepII residue of the LOS inner core together with the α-chain heterogeneity result in immunologically distinct LOS structures, which may be selected for during human infection. Lpt-3, a phosphoethanolamine (PEA) transferase, and LgtG, a glucosyltransferase, mediate the substitution of PEA or glucose at the O-3 position of HepII in L3 or L2 LOS immunotypes, respectively. Inactivation of a two-component response regulator, encoded by NMB0595, in N. meningitidis strain NMB resulted in the loss of all PEA decorations on the LOS inner core expressed by the NMB0595 mutant. When compared with the parental strain NMB that predominately express L2 immunotype LOS and other minor LOS structures, the NMB0595 mutant expresses a pure population of HepII residues of the LOS inner core together with PEA substitutions at the O-3 position with glucose, but lacking other PEA decorations on the inner core. Quantitative real time PCR experiments showed increased transcription of lgtG in the NMB0595 mutant, and no significant change in lpt-3 transcription. Inactivation of lgtG resulted in LOS inner cores without glucose, but these structures, even though the lpt-3 transcription was unaffected, also lacked the O-3-linked PEA. Consistently, a double mutation of lgtG and misR in strain NMB yielded a LOS structure without PEA or Glc substitution of HepII. These data indicated a new pathway for the regulation of LOS inner core structure in N. meningitidis through an environmental sensing two-component regulatory system, named misR(NMB0595)/misS(NMB0594) for regulator and sensor of the meningococcal inner core structure.

Neisseria meningitidis, an obligate human pathogen, causes systemic meningococcal infection ranging from bacteremia, meningitis, and fulminant meningococcal septicemia (1). The morbidity and mortality of meningococcal disease are equated with the levels of circulating endotoxin or lipooligosaccharide (LOS), which is released from the meningococcal cell surface as blebs (2). Not only does the LOS structure mediate the host proinflammatory response, LOS also influences colonization and resistance to killing by serum bactericidal activity (3-5). Meningococcal LOS has been serologically classified into 12 immunotypes of which eight have been structurally characterized (for review, see Kahler and Stephens, Ref. 6). The PEA and/or sugar substitutions of the inner core HepII residue, terminal sialylation of the α chain (7), and O-acetylation of some glycosyl residues (8) define each immunotype. Whereas a given meningococcal strain may express a dominant LOS immunotype, structures of other immunotypes are present in minor amounts. How variability in meningococcal LOS structure is produced is of considerable biological importance.

The HepII residue of the meningococcal LOS inner core can be substituted with PEA at either the O-3 (L1, L3, L7, and L8 immunotypes) or O-6 position (L2, L4, and L6 immunotypes), whereas glucose (Glc) is found at the O-3 of HepII in two LOS immunotypes (L2 and L5 immunotypes) of N. meningitidis (Fig. 1). Lpt-3 (9), a PEA transferase, or LgtG (10), a glucosyltransferase, mediates the substitution of PEA or Glc at the O-3 position, respectively. Lpt-3 is a member of the YhhX/YhiW/YipP/YjdB family (9), and there are two other sequence homologues, NMB1638 (lpta) and NMB0415 (dca), present in the meningococcal genome (11). Both Lpta and Dca share 27% identity and 42% similarity to Lpt-3 at the amino acid level. Located within the division cell wall gene cluster, dca has been implicated in transformation efficiency of Neisseria gonorrhoeae and is phase variable (12). Lpta has recently been identified as the PEA transferase specific for the lipid A head groups (13). The gene encoding the O-6 PEA transferase, tentatively termed Lpt-6, has not been identified. LgtG is phase variable in meningococci and gonococci through a slipped-strand mispairing mechanism because of the presence of a poly(C) tract within the coding region (10, 14, 15) enabling antigenic variation of the LOS inner core in these two neisserial pathogens.

Two-component regulatory systems prevalent in prokaryotes (16) may coordinate expression of virulence determinants in bacterial pathogens. These signal transduction systems gener-

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# The abbreviations used are: LOS, lipooligosaccharide; PEA, phosphoethanolamine; Glc, glucose; LPS, lipopolysaccharide; OS, oligosaccharide; Hep, heptose; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; RT, reverse transcriptase; HF, hydrogen fluoride.
Two-component Regulatory System Affects LOS Inner Core

Construction of Mutations in misR, lgtG, and lptA

misR—A unique HincII site was incorporated into misR by splice-overlap PCR. Two internal fragments of misR were amplified using the primer pairs 14325 (5'-GGCAATGCTTTTCGAGCCATCTC-3')/14322 (5'-GGTCTTGGCAGGAGCTTTCGC-3') and 19143 (5'-GCAGCGATTTAATCTGGATA-3')/14324 (5'-GCCATTGACGGGCTCAAGGTTTCG-3'). Equal molar amounts of these products were mixed together and used as a template for PCR amplification using 14325 (5'-GGTATAGTGACGGTAGCCCTGTCGAC-3') and 14320 (5'-GACAAACAGGTAGCCCGA-AAGGTTTCG). The internal fragment of misR containing the unique HincII site was cloned into the HincII site of pHSG5576 to form pJKD5357. The ermC marker was liberated from pARmC'G (23) by BamHI and NotI. The fragment was subsequently polished using T4 DNA polymerase (New England Biolab) and ligated into the HincII site of pJKD5357 to form pJKD5358. The tetM cassette from Tsb916 was amplified with primers 14326 (5'-GGCATATATCCACGTTCCTACAC-3') and tetM2 (5'-GAAAAAGATATCAGCCTGTCG-3'), which contain EcoRV sites. The PCR product was purified, treated with T4 DNA polymerase, and ligated into the HincII site of pUC18 to form pUC8tetM. This plasmid was digested with EcoRV and ligated to HincII-digested pJKD5357. Chloramphenicol- and tetracycline-resistant colonies were examined and the resultant construct, pJKD5359, containing misR::tetM was isolated. The plasmid was purified and used to transform N. meningitidis strain NMB using the plate transformation method (Kahler et al., Ref. 6). Transformants were selected for resistance to erythromycin or tetracycline and correct transformants, named NbMbisR and NbMbisRT, were saved and further characterized. The inactivation of misR was confirmed by colony PCR using primers 14325 (5'-GGTATAGTGACGGTAGCCCTGTCGAC-3') and 14659 (5'-TTGACAA-ACAGGTAGCCCAACG-3') and Southern blots.

lgtG—An internal fragment of lgtG was amplified using lgtG1 (5'-GACACAACAACTTACACACAGG-3') and lgtG2 (5'-GCTTATGACGGTACCTACAGC-3') primers. The PCR product was polished using T4 DNA polymerase and ligated into the unique HincII site of pHSG5298 to create pCK48. The apka-3 cassette was liberated from pUC18K (24) using EcoRI and BamHI followed by treatment with T4 DNA polymerase. The polished apka-3 cassette was ligated into the unique BssHI site of the cloned lgtG fragment to produce pCK49. The purified plasmid was used to transform strain NMB and the transformants were selected by resistance to kanamycin.

lptA—A PCR product was generated using primer pair NMB1638-1 (5'-AATGGTCATCACGGCCATG-3')/NMB1638-2 (5'-GTGTGGCCTCCTGTCGAC-3') primers. The PCR product was amplified using lptA primer and ligated into the unique HincII site of pHSG5576 to form pCK48. The apka-3 cassette was liberated from pUC18K (24) using EcoRI and BamHI followed by treatment with T4 DNA polymerase. The polished apka-3 cassette was ligated into the unique BssHI site of the cloned lgtG fragment to produce pCK49. The purified plasmid was used to transform strain NMB and the transformants were selected by resistance to kanamycin.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Growth Conditions

Meningococcal strains were grown with 3.5% CO2 at 37 °C unless specified otherwise. GC base agar (Difco), supplemented with 0.4% glucose and 0.68 mM Fe(NO3)3, or GC broth with same the supplements and 0.043% NaHCO3 was used. BHI medium (37 g/liter brain heart infusion) with 1.25% fetal bovine serum was used when kanamycin selection was required. Antibiotic concentrations (µg/mL) used for E. coli strains were ampicillin, 100; kanamycin, 50; spectinomycin, 100; and erythromycin, 300; and for N. meningitidis were kanamycin, 50; erythromycin, 3; spectinomycin, 60; and tetracycline, 5. E. coli strain DH5α cultured on Luria Bertani (LB) medium was used for cloning and propagation of plasmids. Meningococci were transformed by the procedure of Janik et al. (22). E. coli strains were transformed by electroporation with GenePulser (Bio-Rad) according to the manufacturer’s protocol.

Chromosomal DNA Isolation and Southern Blots

Meningococcal chromosomal DNA was prepared according to the method of Nath (26). The Genius 2 DNA labeling and detection system (Bio-Rad, Molecular Biochemicals) was used to perform DNA hybridization. The digoxigenin-labeled probe for detecting misR was generated by the random primed labeling reaction with an internal coding sequence of misR as template, obtained with PCR amplification using primers YT45 (5'-GGTATAGTGACGGTAGCCCTGTCGAC-3') and YT46 (5'-GGCAATGCTTTGCGAGGCATCTC-3'). The purified DNA was digested with HincII or EcoRV and resolved on a 0.7% agarose gel. DNA was transferred to a nylon membrane using a Turboblotter apparatus (Schleicher & Schuell). Hybridization and development of the Southern blots were performed following the manufacturer’s recommended protocol.

Polymyxin B Assays

Resistance to polymyxin B was ascertained by growth on a series of GC agar plates containing doubling amounts of polymyxin B (8 to 1,025 µg/mL). Three single colonies from mutant and wild type strains were patched on the plates from the highest to the lowest concentration. The plates were incubated for 18 h and then scored for growth of each patch. This assay was repeated three separate times for consistency. In addition, broth cultures of mid-late exponential phase were diluted to equal cell density, and aliquots of 2 µl of cell suspension were spotted...
Two-component Regulatory System Affects LOS Inner Core

RESULTS

Generation and Characterization of NMB0595 Mutant—The genome of strain MC58 was compared with the receiver domain sequence of the two-component regulatory systems and one open reading frame, NMB0595, was identified to encode a homologue of the response regulator family. NMB0595 was predicted to encode a 225-amino acid protein with a C-terminal winged helix-turn-helix DNA binding domain homologous to the OmpR subfamily of response regulators (30). The critical amino acid residues forming the active site of the response regulator are present at the respective conserved location in NMB0595 (Asp-9, Asp-10, Asp-52, Thr-79, and Lys-101). Immediately downstream of the response regulator was a homologue of the histidine kinase, NMB0594 (Fig. 2A). Sequence analysis of NMB0594 predicted that it contains a N-terminal transmembrane domain and a large periplasmic loop. Two large proteins flank this two-component regulatory system: NMB0596, a putative integral membrane protein and NMB0593, an 808-amino acid protein with a putative acetyltransferase domain (PF00583, Pfam data base) at the C terminus.

Various two-component regulatory systems in several bacteria mediate LPS structural changes and result in enhancement of resistance to cationic antimicrobial peptides when activated (18, 19, 31–36). Johnson et al. (15) inactivated the response regulator gene encoded by NMB0595 in an uncharacterized serogroup C meningococcal isolate and showed that this mutant exhibited sensing properties to defensins. To examine whether this phenotype correlated with a change in the structure of lipooligosaccharide in meningococci, the response regulator gene was mutated in our laboratory isolate, strain NMB, in which the LOS biosynthesis pathway has been comprehensively examined in conjunction with detailed structural analyses of LOS structures produced by mutations within this pathway (5, 27, 37, 38).

Two mutations in NMB0595 were constructed with either an erythromycin or tetracycline antibiotic resistance cassette inserted into misR. The plasmid constructs containing these cassettes were transformed into strain NMB and the correct transformants, named NMBmisR and NMBmisRT, in which the cassettes had recombined into the genome were confirmed by colony PCR and Southern blots (Fig. 2, B and C, and data not shown) and further characterized. The polarity of these cassettes on the genome of misR, misS (NMB0594) and NMB0593, was determined by quantitative real time PCR. The transcription of these two genes was first normalized to that of lpxA, encoding the UDP-N-acetylglucosamine acetylttransferase involved in lipid A biosynthesis (39), and the -fold of changes in relative expression values for the...
Two-component Regulatory System Affects LOS Inner Core

Mutant strains were determined by comparison to the expression value obtained for the wild type parent strain NMB. Based upon two biological replicates for NMBmisR, NMBmisRT and NMB, we determined that the expression of NMB0594 decreased by less than 2-fold in the NMBmisR mutant (0.654 ± 0.37), whereas it decreased by 13-fold in the NMBmisRT mutant (0.075 ± 0.03). This result clearly demonstrated that genes encoding the response regulator and the histidine kinase of this two-component signal transduction system are transcriptionally coupled. In addition, the tetM cassette in the NMBmisRT mutant was polar on the expression of NMB0594, whereas the ermC cassette of the NMBmisR mutant was most likely non-polar. Using similar analysis, the expression of NMB0593 was shown to be transcribed independently of the misRS operon (data not shown).

To confirm whether inactivation of the NMB0595/0594 two-component system in strain NMB presented a similar sensitivity profile to cationic peptides as described by Johnson et al. (15), we tested NMBmisR for sensitivity to polymyxin B, an acylated cyclic cationic antimicrobial peptide. Susceptibility was examined on GC agar plates containing increasing amounts of polymyxin B from 8 to 1,025 μg/ml. The parent meningococcal strain NMB is highly resistant to polymyxin B with a minimal inhibitory concentration of 32 μg/ml, whereas the NMBmisR had a minimal inhibitory concentration of 2 μg/ml, a 16-fold decrease in resistance to polymyxin. Therefore, the inactivation of NMB0595 encoding the putative response regulator in two separate isolates of meningococci resulted in similar sensitivity profiles to cationic peptides.

Structural Studies of Oligosaccharides Released from LOSs of NMBmisR, NMB0594, and NMB0594 Mutants—LPS (LOS) structural modifications have been correlated with polymyxin B susceptibility in other bacteria, most notably Salmonella typhimurium (40), thus the LOS structure of the non-polar NMBmisR mutant was studied in detail. The glycosyl compositions are shown in Table I. The NMBmisR mutant OS was similar to that of the wild type parent, except that the mutant OS was increased in the level of heptose. Because heptose residues containing phosphate substituents, e.g. PEA groups, are not observed during the glycosyl composition analysis, the results suggested that the NMBmisR mutant LOS structure was decreased in the level of heptose phosphorylation. Sequential chemical treatments were performed on the OS samples of the mutant and parent to further determine the glycosyl linkages and heptose PEA localization. The samples were methylated, treated with aqueous HF to remove any phosphate substituents, ethylated at the positions of phosphate removal, converted to partially methylated/ethylated alditol acetates, and analyzed by GC-MS (Table II).

### Table I

| Sugar | NMB | NMBmisR | NMBlgtG | NMBlgtG/misR |
|-------|-----|---------|---------|--------------|
| Glc   | 29  | 27      | 18      | 16           |
| Gal   | 27  | 27      | 35      | 32           |
| GlcNAc| 29  | 22      | 29      | 27           |
| 1,2-Hep| 15  | 24      | 18      | 25           |
| Kdo   | +   | +       | +       | +            |

Relative molar percentage.

* The values are from our previously published work (37) and are included for comparison.

Previous work indicated that NMB produced primarily an L2 immunotype structure (76% of the LOS) in which HepII is substituted at O-3 with Glc and at O-6 with PEA (structure I, Fig. 4), and a secondary structure (15% of the LOS) that lacked Glc substitution at O-3 (37’). Whereas the data indicated that this secondary structure that lacked Glc contained a PEA substituent on HepII, its location was not chemically determined.
Two-component Regulatory System Affects LOS Inner Core

The methylation/ethylation analysis of NMB LOS (Table II), again, confirmed that L2 is the predominant LOS structure present in strain NMB. Because the NMBmisR LOS was completely glucosylated at HepII O-3, it was possible that the lack of PEA at O-3 in NMBmisR LOS was because of an increased level of LgtG activity. To further examine the effect of misR on PEA substitution, lgtG mutants of NMB and NMBmisR were prepared and were named NMBlgtG and NMBlgtGmisR, respectively. The absence of lgtG would prevent glucosylation of HepII O-3, and, therefore, would leave this position available for the addition of PEA provided misR does not regulate PEA addition in some other manner.

The LOS of the NMBlgtG mutant showed the expected reduction in Glc (Table I) because of the absence of the terminal α-Glc attached to O-3 of HepII. In addition, neither terminal Glc nor 2,3-linked HepII was observed in the LOS of the NMBlgtG mutant by GC-MS analysis of partially methylated/ethylated alditol acetates (Table II), confirming the removal of the glucosyltransferase activity by the lgtG mutation. The location of the PEA groups in the NMBlgtG mutant was studied in detail with the purpose of providing a full structural comparison in this genetic background. A significant portion of the OS prepared from the NMBlgtG mutant contained 2-linked HepII that was ethylated at O-6, indicating that this OS contained a PEA group at O-6 of HepII. Surprisingly, the LOS of NMBlgtG prepared and were named NMBlgtG and NMBlgtGmisR, respectively. The absence of lgtG would prevent glucosylation of HepII O-3, and, therefore, would leave this position available for the addition of PEA provided misR does not regulate PEA addition in some other manner.

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Composition analysis of the double mutant, NMBlgtGmisR, OS (Table I) showed that Hep was increased over that of the NMBlgtG mutant OS suggesting a decrease in PEA addition to the HepII residue. Glycosyl linkage studies indicated that the OS from the double NMBlgtG/misR mutant contained only 2-linked HepII with no detectable ethylation showing that the misR mutation in the NMBlgtG mutant background (Table II), as with the misR mutation in the parent strain NMB, resulted in the absence of PEA substitution of the OS portion of the LOS. In summary, these glycosyl linkages were consistent with the conclusion that the OSs from both the NMBmisR and NMBlgtGmisR mutant LOSs lack detectable HepII PEA groups.

The lack of PEA substitution on the OSs from the NMBmisR and NMBlgtGmisR mutants was further confirmed by MALDI-TOF MS analysis. The results are shown in Fig. 3. Two molecular ions at m/z 1676 and 1658 were observed in OSs from the NMBmisR mutant LOS. These ions were consistent with molecules of the following composition: m/z 1676 = Galα2-Glcα2-GlcNac2-Hepβ2-Kdo (normal) and m/z 1658 = Galα2-Glcα2-GlcNac2-Hepβ2-Kdo (anhydro). Therefore, the NMBmisR mutant expressed only a L2-like structure lacking all PEA groups (Fig. 4, structure III), but differed from the wild type NMB L2 structure that contains a PEA substitution at O-6 of HepII (structure I) (37). The effect of the misR mutation in the lgtG background resulted in the absence of both a Glc residue and a PEA substituent producing a single OS with an m/z = 1514 (1496, anhydro) consistent with structure IV in Fig. 4. In comparison, the NMBlgtG mutant showed two OSs, one lacking both the Glc and the PEA consistent with the structure of the NMBlgtGmisR mutant; i.e. structure IV, and a second species with m/z = 1637 (1619, anhydro) that lacks a Glc residue but contains PEA, equivalent to an L4 immunotype (structure V, Fig. 4). This mixture of structures produced by NMBlgtG was consistent with the methylation/ethylation results described above showing a 1.0:1.3 ratio of 2-linked HepII:2-linked Hep (6-ethyl) residues. In summary, these results show that mutation of misR results in the inability to add PEA substituents to the HepII residue of the LOS inner core, even when lgtG is inactivated.

Structural Studies of Lipid A Released from LOS—As just described the NMBmisR mutant lacks PEA substituents on the LOS inner core and has increased susceptibility to polymyxin B. Because lipid A modification with PEA has been implicated in modulating polymyxin B susceptibility in several bacteria (19, 33, 44), it was of interest to determine whether PEA substitution of lipid A was affected by the misR mutation. Lipid A was isolated from intact LOS by a mild acid hydrolysis procedure with 1% SDS, 20 mM NaOAc, pH 4.5, at 100 °C for 1 h. This procedure, which is milder than the 1% HOAc hydrolysis protocol, is reported not to remove the glycosilylated linked phosphate from lipid A, if present (29). In addition to lipid A from the NMBmisR mutant, lipid A from the parent strain NMB and from NMBlptA were also analyzed. The latter lipid A is reported to lack PEA substituents because of a mutation in lpta, encoding the PEA transferase of the lipid A head groups (13) and, thus, serves as a PEA-deficient control of the lipid A structural analysis. The MALDI-TOF MS spectra of lipid A from NMB, NMBmisR, and NMBlptA are shown in Fig. 5. The NMBmisR lipid A preparation contained the same lipid A species as those found in the parent NMB preparation, including those with PEA groups. As predicted, lipid A from NMBlptA clearly lacked any of the PEA-containing ions. Thus the NMBmisR mutation, while preventing the addition of PEA to the inner core heptose of the oligosaccharide, does not prevent PEA addition to lipid A. Furthermore, the presence of PEA on the NMBmisR lipid A indicated that a defect in PEA biosynthesis is not the cause for the disappearance of PEA decorations on HepII of NMBmisR LOS inner core.

Quantitative Transcriptional Analysis—The HepII structural changes in the NMBmisR mutant indicated that the

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### Table II

| Glycosyl linkage | NMB | NMBmisR | NMBlgtG | NMBlgtGmisR |
|------------------|-----|---------|---------|-------------|
| T-Glc            | 14  | 12      | ND      | ND          |
| T-Gal            | 13  | 14      | 17      | 16          |
| 3-Gal            | 13  | 15      | 15      | 17          |
| 4-Glc            | 14  | 14      | 17      | 17          |
| T-GlcNac         | 11  | 12      | 12      | 13          |
| 4-GlcNac         | 11  | 10      | 12      | 12          |
| 3,4-Hep          | 12  | 12      | 13      | 13          |
| 2,3-Hep          | 1   | 11      | ND*     | ND          |
| 2-Hep            | ND  | ND      | 6       | 12          |
| 2-Hep(6/7-ethyl) | ND  | ND      | 8       | ND          |
| 2,3-Hep(6/7-ethyl)| 11 | ND      | ND      | ND          |

* ND, none detected.

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MisR/MisS two-component regulatory system might modulate the transcription of genes involved in assembly of the HepII structure including lgtG and lpt-3. The expression of lgtG and the three genes, lpt-3, lptA, and dca, in the lpt-3 gene family was studied by quantitative real time PCR (Table III). Total RNAs were extracted from strains NMB and NMBmisR, the transcriptional profiles of these four genes was quantified relative to rpsE, encoding a constantly highly expressed ribosomal protein. No significant change was seen in lpt-3 and dca transcription, and the expression of lptA was down-regulated ∼2-fold. However, the expression of lgtG was up-regulated nearly 6-fold in the NMBmisR mutant. Similar data were obtained for the expression of lgtG in NMBmisR and NMBlgtG mutants when lpxA was used as the normalizing reference (5.1 ± 2.55 and 5.1 ± 2.36, respectively). Therefore, the complete substitution of Glc at the O-3 of HepII in the NMBmisR mutant is likely to be because of this increase in lgtG expression. However, even though lpt-3 transcription is unaffected in NMBmisR (Table III), and NMBlgtG (data not shown), their LOSs did not contain any detectable PEA on O-3 of HepII. The modest reduction in lptA expression in NMBmisR did not affect PEA substitution on the head groups of lipid A.

**DISCUSSION**

Two-component regulatory systems are prevalent in many prokaryotes, and are involved in processes important for bacterial pathogenesis. *N. meningitidis* is a pathogen that inhabits the human nasopharynx but can rapidly disseminate throughout the body during invasive infection. This organism contains only four pairs of predicted two-component regulatory systems, which presumably senses the restricted human environment encountered. Although sequence analysis strongly indicates that these proteins are members of the two-component regulatory systems, currently no biochemical evidence is available to demonstrate their functions. A major virulence factor of *N. meningitidis* is endotoxin or LOS and structural changes in LOS are important in meningococcal pathogenesis. We found that inactivation of one of the meningococcal two-component regulatory systems, NMB0595/NMB0594, designated misR/misS, increased sensitivity to cationic antimicrobial peptides and that this phenotype correlated to the loss of PEA substitutions of the LOS core HepII.

In this study, the LOSs of NMBmisR, NMBlgtG/misR, and NMBlgtG mutants were structurally characterized and compared with the LOS structure from the serogroup B meningococcal strain NMB. Meningococcal strain NMB expresses a mixed population of LOS structures ∼76% of which is the L2 immunotype (Fig. 1), which has the HepII O-3 and O-6 substituted with Glc and PEA, respectively (37), and a further 15% of LOS in which Glc is not present at O-3 of HepII (37). We found that the NMB lgtG mutant LOS did not contain a PEA at O-3 of HepII. The NMB lgtG mutant produced an LOS with two oligosaccharides; one which lacked both the HepII Glc residue and the PEA group (structure IV, Fig. 4), and a second OS that lacked the Glc residue but contained a PEA group at O-6 of HepII.
The structure of NMB octaose attached to HepI with Glc and GlcNAc attached to meningococcal LOS were absent (9). Similarly, the inner core composition of structure III is identical to that of the L5 immunotype (47); whereas structure IV has only been reported in a lpt-3 mutant of strain MC58 (9). A truncated LOS molecule with an inner core identical to that of structure IV has also been shown recently to be one of the structures synthesized by a phosphoglucomutase (pgm) mutant of strain NMB (48).

The lack of PEA groups in the LOSs from the NMBmisR mutants could indicate that the misR/S two-component regulatory system alters the biosynthetic pathway for phosphatidylethanolamine that is believed to be the substrate for the PEA transfer reaction (49). However, the persistence of PEA modifications of lipid A in the NMBmisR mutant indicates that the substrate is available. A second explanation for the observed phenotype is down-regulation of both lpt-3 and the putative lpt-6. Although we have no structural evidence that strain NMB expresses an L3 immunotype LOS, it does contain an intact lpt-3 gene and the NMBfraK (38) and NMBpgm (47) mutants were determined to express a LOS structure containing O3-PEA-substituted HepII, further supporting the active function of Lpt-3 in the NMB strain. However, lpt-3 transcription is unchanged in the misR mutant suggesting that transcriptional regulation of the O-3 transferase is not the cause of the misR LOS phenotype. The transcription of the putative lpt-6 could not be tested, as this gene is currently unidentified.

A third possibility is that the increased expression of lgtG results in complete glucosylation of the HepII O-3 position, thereby preventing the addition of PEA to HepII O-3. However, the continued absence of O-3 PEA groups in the LOSs of the NMBlgtGmisR and NMBlgtG mutants, as well as the absence of the O-6 PEA group in the NMBmisR mutant LOS does not support this explanation. A fourth possible explanation would be that NMB contains PEA hydrolases that are up-regulated in meningococci. Such PEA hydrolase activity has yet to be demonstrated in meningococci.

Down-regulation or disruption of the MisR/MisS two-component system with the loss of HepII PEA substituents and with the concomitant elevated levels of LgtG glucosyltransferase would increase the ratio of immunotype structure III over the O-3 and/or O-6 PEA containing immunotype structures (e.g. L3 or L4). Conversely, the activation of MisR/S by inducing environmental signals would presumably increase the level of HepII PEA substitutions and suppress expression of the LgtG glucosyltransferase leading to lower levels of L2 immunotypes and an increase in other HepII-PEA containing immunotype structures. The coordinated regulation of HepII PEA substitutions and the LgtG glucosyltransferase would facilitate rapid switching between the immunotypes in response to environmental signal(s). Interestingly, switching of LOS immunotype expression was noted in a study of epidemiologically related meningococcal case and carrier isolates associated with an outbreak of an ET-5 serogroup B strain of *N. meningitidis*; 97% of case isolates expressed the L3, -7, and -9 immunotype, whereas the LOS immunotypes of the identical carrier strains were much more heterogeneous (50). Thus, the L3, -7, and -9 immunotypes appear to be selected during meningococcal invasion (50). Recently, Ram *et al.* (51) reported that the O-3 and O-6 PEA substituents of inner core HepII are the target sites for complement component C4b. These findings further empha-

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**Fig. 4. LOS structures expressed by meningococcal strains studied.** Structure I (L2) is the predominant immunotype expressed in wild type strain NMB but other structures such as structure II can be minor variants. Strain NMBmisR expresses exclusively structure III. The NMBlgtG strain expresses both structures IV and V (an L4 immunotype), whereas only structure IV is found in NMBlgtGmisR.

HepII (structure V in Fig. 4, an L4 immunotype). Mackinnon *et al.* (9) proposed a model whereby LOS inner core variation of meningococcal LOS was governed by the phase variability of lgtG expression in combination (10) with the presence or absence of an intact lpt-3 gene and that LgtG and Lpt-3 compete for the O-3 position of HepII. An L2 to L3 immunotype conversion frequency of $10^{-3}$, observed by immunoblot analyses, was reported in a serogroup B strain H44/76 (45) and was attributed to lgtG phase variation. Our structural data suggests that phase variation of lgtG in strain NMB genetic background results in the expression of L4 immunotype LOS (structure V) and structure IV LOS; however, this switching frequency has not been determined in strain NMB (46).

The NMBmisR OS structure consisted of the typical lactone-octetraose attached to HepI with Glc and GlcNAc attached to HepII at O-3 and O-2, respectively. The structure of NMBmisR differs from the parental L2 structure in that all PEA decorations on the inner core heptose (HepII) of the oligosaccharide of meningococcal LOS were absent (structure III in Fig. 4). Similarly, the misR mutation in an lgtG background resulted in a single LOS, which lacked glucose and all PEA modifications (structure IV in Fig. 4). As a nonpolar mutation of misR causes the loss of PEA inner core substitution in both the wild type and the lgtG genetic backgrounds, this PEA-related phenotype is not likely to be because of other nonspecific second site mutations that were selected for during transformation. Furthermore, the erythromycin cassette used to generate misR mutation was shown to be nonpolar, indicating that the LOS phenotype is not caused by inactivation of downstream genes. Meningococcal LOS structures III and IV have not been reported in any meningococcal isolates and may represent immunotypes for which structures have not currently been characterized. Interestingly, although the α chain structure is different, the inner core composition of structure III is identical to that of the L5 immunotype (47); whereas structure IV has only been reported in a lpt-3 mutant of strain MC58 (9). A truncated LOS molecule with an inner core identical to that of structure IV has also been shown recently to be one of the structures synthesized by a phosphoglucomutase (pgm) mutant of strain NMB (48).
size that modulating the LOS inner core structure is important in meningococcal pathogenesis.

The defect in the novel meningococcal misR/S two-component regulatory system resulted in the absence of PEA modification to the LOS inner core HepII residue, but with no changes in the PEA content of the lipid A head groups. The lack of PEA groups on HepII coincided with a reduction in polymyxin resistance of *N. meningitidis*. Phosphoryl groups linked to the lipid A head groups have been implicated in the formation of a stable outer membrane network enabling adjacent LPS molecules to be cross-linked via divalent cations (49, 52). The addition of aminoarabinose to the 4' phosphate, which presumably reduces electrostatic interaction between polymyxin and LPS, correlates with an increased resistance to polymyxin (18, 19, 32, 33). Our data indicates that a reduction of electrostatic interaction caused by the removal of PEA phos-
phorylation of the LOS inner core would result in a decrease in polymyxin resistance. Analogous to our finding, a decrease in resistance to polymyxin B was shown in the *uaaP* mutant of *E. coli* in which the heptose I kinase was inactivated resulting in elimination of the phosphoryl group of HepI and consequently the site of PEA substitution. In addition, the resistance offered by the aminooarabinose modifications of lipid A cannot rescue the increased polymyxin B sensitivity resulting from loss of inner core phosphorylation in the *uaaP* mutant (53). These observations are in agreement with the importance of polymyxin B-LPS interactions through interaction of a hydrophobic nature shown by calorimetric and surface plasma resonance studies (54, 55). Unlike other enteric bacteria that alter substitution of the lipid A head groups to modulate antimicrobial peptide resistance, meningococci may alter antimicrobial peptide susceptibility through modification of the inner core PEA phosphorylation.

Johnson et al. (15) reported that a serogroup C *NMB0595* mutant resembled the *phoP* mutant of *S. typhimurium*. The mutant was unable to grow in the presence of 1 mM magnesium and exhibited a longer lag phase yet displayed a similar growth rate as the wild type parent when grown in the presence of 10 mM MgCl₂ (15). However, the serogroup B *NMB0595* mutant characterized in our study did not show MgCl₂-dependent growth phenotype (data not shown). It is not clear whether this is a strain-dependent difference. The serogroup C *NMB0595* mutant also lacked other phenotypes specific for *phoP*; the *NMB0595* mutant was not sensitive to acidic pH (a typical *phoP* phenotype) (56) but showed increased sensitivity to alkaline pH (characteristics of *cpxR* regulatory mutants) (57) and high osmolality (an *ompR* phenotype) (58). Genes under the control of *NMB0595* were not identified in the study of Johnson et al. (15). Furthermore, the expression of *mgtA*, which encodes the magnesium uptake system and which is one of the most affected genes in the *S. typhimurium* *phoP* mutant (59), was not affected by the *NMB0595* mutation in meningococci (data not shown). A number of publications (18, 32, 35, 36, 60–62) have reported that both PhoP/PhoQ and PmrA/PmrB two-component regulatory systems modulate the resistance to antimicrobial peptides, such as defensins and polymyxin, through lipid A structural modification. Although Johnson et al. (15) have reported a defensin sensitivity of the serogroup C *NMB0595* mutant, no correlation between LOS structural change and antimicrobial peptide susceptibility was demonstrated. We have found that, unlike the *phoP* mutant of *S. typhimurium*, the meningococcal *NMB0595* mutation did not influence lipid A structure. These observations indicate that *NMB0595* is not a *phoP* functional homologue.

In *sito* analysis of the *NMB0595* (MisS) periplasmic domain also places this gene in a novel histidine kinase category. Many histidine kinases including CpxA and PhoQ contain two transmembrane domains flanking a large periplasmic loop. The periplasmic domain is usually considered to be the signal-sensing domain. Consequently, true functional homologues often share conserved periplasmic sequences to respond of the same inducing signal. In *E. coli*, transcription of *cpxR/A* is inhibited by CpxP, a periplasmic protein under the positive control of phos-

Table III

| Gene  | Experiment 1* | Ratio | Experiment 2 | Ratio |
|-------|---------------|-------|---------------|-------|
|       | WT | misR | misR/WT | Normalized | WT | misR | misR/WT | Normalized |
| *rpsE* | 3.32 x 10⁶ | 1.95 x 10⁵ | 0.59 | 1.0 | 2.69 x 10⁶ | 2.33 x 10⁵ | 0.87 | 1.0 |
| *lgtG* | 3.56 x 10³ | 1.65 x 10² | 4.64 | 7.9 | 2.16 x 10⁴ | 9.31 x 10³ | 4.31 | 5.0 |
| *lptA* | 1.49 x 10⁴ | 3.65 x 10³ | 0.25 | 0.4 | 1.82 x 10⁴ | 6.85 x 10³ | 0.37 | 0.4 |
| *lpt3* | 4.27 x 10³ | 3.09 x 10² | 0.75 | 1.2 | 2.39 x 10³ | 2.96 x 10² | 1.24 | 1.4 |
| *dca* | 1.88 x 10⁴ | 9.25 x 10³ | 0.49 | 0.8 | 2.37 x 10⁴ | 1.24 x 10³ | 0.52 | 0.6 |

*Average copy numbers, n = 4.*

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The MisR/MisS Two-component Regulatory System Influences Inner Core Structure and Immunotype of Lipooligosaccharide in *Neisseria meningitidis*

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