**gmhX, a novel gene required for the incorporation of L-glycero-D-manno-heptose into lipooligosaccharide in Neisseria meningitidis**

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Lipooligosaccharide (LOS) is a critical virulence factor of Neisseria meningitidis. A Tn916 insertion mutant, designated 469, was found to exhibit a markedly truncated LOS of 2–9 kDa when compared by Tricine/SDS-PAGE to the parental LOS (4–6 kDa). Electrospray mass spectrometry analysis of 469 LOS revealed that it consisted of the deep rough, heptose-deficient structure, Kdo2-lipid A. Sequencing of chromosomal DNA flanking the Tn916 insertion in mutant 469 revealed that the transposon had inserted into an ORF predicted to encode a 187 aa protein with sequence homology to the histidinol-phosphate phosphatase domain of Escherichia coli HisB and to a family of genes of unknown function. The gene, designated gmhX, is part of a polycistronic operon (ice-2) containing two other genes, nlaB and orfC. nlaB encodes a lysophosphatidic-acid acyltransferase and orfC is predicted to encode an N-acetyltransferase. Specific polar and non-polar gmhX mutations in the parental strain, NMB, exhibited the truncated LOS structure of mutant 469, and repair of gmhX mutants by homologous recombination with the wild-type gmhX restored the LOS parental phenotype. GmhX mutants demonstrated increased sensitivity to polymyxin B. GmhX mutants and other Kdo2-lipid A mutants also demonstrated increased sensitivity to killing by normal human serum but were not as sensitive as inner-core mutants containing heptose. In the genomes of Helicobacter pylori and Synechocystis, gmhX homologues are associated with heptose biosynthesis genes; however, in N. meningitidis, gmhX was found in a location distinct from that of gmhA, rfaD, rfaE, aut and rfaC. GmhX is a novel enzyme required for the incorporation of L-glycero-o-manno-heptose into meningococcal LOS, and is a candidate for the 2-o-glycero-manno-heptose phosphatase of the heptose biosynthesis pathway.

**Keywords:** meningococcus, lipopolysaccharides, heptose biosynthesis

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**INTRODUCTION**

*Neisseria meningitidis* (the meningococcus) is a leading cause of bacterial meningitis and rapidly fatal sepsis throughout the world. It remains a serious public health threat, infecting hundreds of thousands annually in developing countries and causing epidemics worldwide (Robbins & Freeman, 1988; Tikhomirov et al., 1997). Lipooligosaccharide (LOS) is an important virulence factor in several steps of meningococcal disease, including nasopharyngeal colonization, survival in the bloodstream, septicemia and meningitis (Kahler & Stephens, 1998; Tunkel & Scheld, 1993).

Meningococcal LOS is structurally distinct from the lipopolysaccharide (LPS) of other Gram-negative
organisms in that, among other features, it lacks the repeating O-antigen side chains. Meningococcal LOS is composed of a conserved inner-core structure: a membrane-associated hexa-acylated lipid A moiety attached to two molecules of 3-deoxy-d-manno-2-octulosonic acid (Kdo) and two molecules of L-glycerod-manno-heptose (Hep). Attached to the Hep₂-Kdo₂ lipid A inner-core structure in LOS are oligosaccharide chains of varying length and composition. In _N. meningitidis_ and _Neisseria gonorrhoeae_ LOS, the oligosaccharides are composed of glucose, galactose, N-acetylglucosamine and N-acetylmuramic acid.

The biochemical and genetic aspects of LPS synthesis have been extensively studied in _Escherichia coli_ and _Salmonella_. The isolation and characterization of heptose-deficient inner-core mutants in _E. coli_ and _Salmonella_ have helped identify genes necessary for heptose transfer (rfaC, rfaE) (Chen & Coleman, 1993; Sirisena _et al_. 1992) and biosynthesis (gmhA, rfaE, rfaD) (Brooke & Valvano, 1996a, b; Draizek _et al_., 1995; Eidel & Osborn, 1974; Nichols _et al_., 1997; Sirisena _et al_., 1992, 1994). rfaC encodes heptosyltransferase I, which transfers the first heptose from the ADP-L-glycerod-manno-heptose donor molecule to the Kdo₂-lipid A substrate to create Hep₂-Kdo₂-lipid A, and rfaE encodes heptosyltransferase II, which transfers the second heptose from ADP-L-glycerod-manno-heptose to the Hep₂-Kdo₂-lipid A substrate to create the inner-core structure Hep₂-Kdo₂-lipid A (Chen & Coleman, 1993; Kadrmas & Raetz, 1998; Sirisena _et al_., 1992).

Over the last 5 years a number of studies, aided by data from meningococcal and gonococcal genome sequencing projects, have identified meningococcal and gonococcal homologues of _E. coli_ and _Salmonella_ LPS inner-core assembly and biosynthesis genes (Gotschlich, 1994; Jennings _et al_., 1993; Kahler _et al_., 1996a, b; Lee, F. K. N. _et al_., 1995; Zhou _et al_., 1994; for a review, see Kahler & Stephens, 1998). These studies have also helped to define unique features of meningococcal inner-core LOS, such as differences in lipid A phosphorylation and differences in the composition and attachment of acyl chains on lipid A (Kahler & Stephens, 1998). Nevertheless, issues regarding compartmentalization and modification of LOS and LPS inner-core structures remain unresolved and questions still remain about the genetics and biochemistry of heptose biosynthesis and incorporation in _E. coli_, _Salmonella_ (Valvano _et al_., 2000) and _N. meningitidis_.

In this study, we have identified and characterized a novel gene, _gmhX_, which, based on genetic and biochemical evidence, is required for the incorporation of heptose into meningococcal LOS and appears to be involved in heptose biosynthesis. We demonstrate that _gmhX_ is encoded in a polycistronic operon (ice-2; LOS inner-core extension, locus 2), which also contains a gene involved in meningococcal phospholipid biosynthesis. While _gmhX_ is required for heptose incorporation, it is distinct from the kinase and ADP-heptose synthetase domains encoded by _rfaE_, which in _N. meningitidis_ are found as two separate genes (_rfaE_ and _aut_), and from the ADP-heptose isomerase (_rfaD_). Homologues of _gmhX_ are found associated with heptose biosynthesis operons in other species.

**METHODS**

**Media and growth conditions.** Strains of _N. meningitidis_ (Table 1) were grown on GC base agar or broth (Difco) at 37 °C in the presence of 3-5% CO₂ under aerobic conditions. Strains of _E. coli_ (Table 1) were grown in LB base agar or broth (Gibco-BRL) at 37 °C under the same conditions. Antibiotics were used at a concentration of 5 μg ml⁻¹ for tetracycline, 50 μg ml⁻¹ for kanamycin, 60 μg ml⁻¹ for spectinomycin and 100 μg ml⁻¹ for ampicillin.

**Creation of a Tn916 mutant library and screening for mutations in LOS.** Strain NMB [B:2b-P1.2,5-L2] was originally isolated from a patient with meningitis in Pennsylvania in 1982. A Tn916 mutant library in _N. meningitidis_ strain NMB was created and screened as described previously (Kahler _et al_., 1996a; Stephens _et al_., 1994; Swartley _et al_., 1993).

**LOS isolation and SDS-PAGE.** LOS was examined by proteinase K digestion and Tricine/SDS-PAGE on a mini Protein apparatus (Bio-Rad) (Lesse _et al_., 1990; Schagger & von Jagow, 1987).

**Composition and linkage analysis of LOS.** Purified LOS was prepared from _N. meningitidis_ strains by a modified version of the procedure described by Galanos (Galanos _et al_., 1969; Kahler _et al_., 1996a, b), and analysed by electrospray mass spectrometry (ES-MS) as previously described (Kahler _et al_., 1996a, b).

**DNA preparation and transformation procedures.** Chromosomal DNA was prepared from NMB by the method of Nath (1990). Plasmid DNA was extracted from _E. coli_ as described by Engbrecht & Brent (1996). NMB and _E. coli_ were transformed with DNA as described by Chung _et al_. (1989) and Janik _et al_. (1976). Restriction digests and ligation reactions were performed under conditions specified by the manufacturer (New England Biolabs).

**Single specific primer-PCR cycle sequencing.** This was used to amplify chromosomal DNA flanking the transposon insertion in 469 as described by Shyamala & Ames (1989). The amplified products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced with the AmpliCycle Sequencing Kit (Perkin-Elmer) or by automated sequencing at the Emory Core DNA Sequencing Facility. Nucleotide and amino acid sequence analysis was performed using either the GCG Wisconsin Package version 8.1 (Genetics Computer Group) or the DNASTAR sequence analysis package.

**Southern blotting techniques.** Chromosomal DNA was prepared from NMB as described by Nath (1990) and digested with _SspI_, _ClaI_ or _EcoRI_ under conditions specified by the manufacturer (New England Biolabs). The DNA fragments were separated on a 0.7% agarose gel, run at 30 V for 6 h, transferred to a nylon membrane (Micron Separation), hybridized with a _gmhX_-specific probe, and developed using the Genius chemiluminescence system (Boehringer Mannheim).

**Construction of polar and non-polar _gmhX_ mutants.** An internal fragment of _gmhX_ containing a unique _SspI_ restriction site was amplified from NMB and cloned in pCR2 (Invitrogen). To create a polar mutation in _gmhX_, an Ω_SspI restriction site was amplified from NMB and cloned in pCR2 (Invitrogen). To create a polar mutation in _gmhX_, an Ω_SspI
Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description/LOS structure</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Neisserial strains</td>
<td>B:2B:P1.2.5::L2</td>
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</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>lgIF (CMK2)</td>
<td>Hep(3GlcNAc)PEA-Kdo₃-lipid A</td>
<td>Kahler et al. (1998)</td>
</tr>
<tr>
<td>pgm (R6)</td>
<td>Hep(3GlcNAc,Glc)PEA-Kdo₂-lipid A</td>
<td>Kahler et al. (1998)</td>
</tr>
<tr>
<td>rfaK (CMK1)</td>
<td>Hep₂PEA-Kdo₂-lipid A</td>
<td>Kahler et al. (1996a)</td>
</tr>
<tr>
<td>469</td>
<td>NMB containing orfC gmbX::Tn916</td>
<td>This study</td>
</tr>
<tr>
<td>GS152.7</td>
<td>NMBgmbX::ΩSp⁸</td>
<td>This study</td>
</tr>
<tr>
<td>GS158</td>
<td>NMBgmbX::aphA-3</td>
<td>This study</td>
</tr>
<tr>
<td>GS159</td>
<td>NMBrfaC::lacZ–ermC</td>
<td>This study</td>
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<tr>
<td>GS160</td>
<td>GS158rfaC::lacZ–ermC</td>
<td>This study</td>
</tr>
<tr>
<td>CMK3</td>
<td>NMBrfaD::ΩSp⁸</td>
<td>This study</td>
</tr>
<tr>
<td>GS161</td>
<td>NMBaut::aphA-3</td>
<td>This study</td>
</tr>
<tr>
<td>M7</td>
<td>NMBsymA::Tn916</td>
<td>Kahler et al. (1998)</td>
</tr>
<tr>
<td>GS156</td>
<td>NMBsnaB::ΩSp⁸</td>
<td>Shih et al. (1999)</td>
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<tr>
<td>E. coli strains</td>
<td>W3110 parent</td>
<td>Polissi &amp; Georgopoulos (1996)</td>
</tr>
<tr>
<td>DH5α</td>
<td></td>
<td>Engebrecht &amp; Brent (1996)</td>
</tr>
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<td>MLK3</td>
<td>W3110htrB::Tn10</td>
<td>Polissi &amp; Georgopoulos (1996)</td>
</tr>
<tr>
<td>MLK33</td>
<td></td>
<td>Polissi &amp; Georgopoulos (1996)</td>
</tr>
</tbody>
</table>

Construction of rfaC::lacZ–ermC reporter in NMB and integration into the genome of N. meningitidis. A unique BamHI restriction site was created in rfaC based on the method described by Hughes & Andrews (1996). Primers RN91 (5'-CCGTTTGGCATTTACGTTAACGGC-3') and RN12 (5'-TTACAAACGGATCCACCGTG-3') were used to PCR-amplify an internal 5' section of rfaC from NMB, and RN11 (5'-ATTGACGCGTTAGAGGCCCGT-3') and RN14 (5'-GTCCTGCAATCTGGTCCGGCAG-3') were used to PCR-amplify an internal 3' section of rfaC from NMB. Complementary primers RN11 and RN12 are mutagenic primers that introduce a unique BamHI site in the rfaC amplification product. Equivalent amounts of the amplification products were combined and used as a template in a second round of PCR amplification with nested primers RN10 (5'-GAGGTGGATCGTAAGACGCC-3') and RN13 (5'-CCCAAGGGGATAAAAAGTCC-3'). The resulting 900 bp amplification product containing rfaC with a unique BamHI site was then cloned in pGEM-T (Promega) to create pRN1013. A BamHI fragment from pAErnmC (encoding promoterless β-galactosidase and erythromycin resistance) was inserted into the unique internal BamHI site in rfaC in pRN1013 to create pRN1013#. The rfaC::lacZ–ermC construct was amplified from pRN1013# and used to transform NMB and GS158 to create GS159 and GS160 reporter strains, respectively.

Quantification of β-galactosidase activity in rfaC reporter strains. β-Galactosidase activity of the GS159 and GS160 reporter strains was measured by a whole cell ELISA protocol adapted from Takahashi et al. (1992). Briefly, Maxisorp microtitre plates (Nunc) were incubated overnight at 4 °C with mouse anti-β-galactosidase mAb (Promega) in 0.1 M carbonate buffer and blocked with 10% calf serum in TBST (0.01 M Tris base, 0.015 M NaCl, with 0.1% Tween 20, pH 8.0). A standardized bacterial cell suspension (2.5×10⁴ c.f.u. ml⁻¹) was lysed by repeated freeze-thawing; 50 µl of each lysate was then added to the wells and incubated at 37 °C for 1 h. Rabbit anti-β-galactosidase mAb (ICN) in TBST was added to the wells and incubated at 37 °C for 1 h, followed by incubation with goat anti-rabbit IgG conjugated to peroxidase (ICN) in TBST at room temperature for 1 h. The O-phenylenediamine dihydrochloride substrate (Sigma) in citrate/phosphate buffer (0.05 M citric acid, 0.1 M Na₂HPO₄, with 0.72% H₂O₂) was added, incubated for 30 min at room temperature and stopped with 2.5 M H₂SO₄. Absorbance at 490 nm was measured on a microtitre plate reader (Bio-Tek).

Complementation of htrB mutant. To test whether the gmbX gene product exhibits MsbA activity and suppresses the temperature-sensitive phenotype of an htrB mutant, E. coli htrB::Tn10 mutant MLK3 (Polissi & Georgopoulos, 1996) was transformed with pGEM-TgmbX and grown at 30 °C overnight on LB plates with the appropriate selection. Twenty-four colonies of each transformant were patched onto duplicate plates containing the appropriate selective antibiotic and incubated at 30 or 42 °C, respectively.

4'-Phosphatase assay. Whole-membrane extracts of NMB and GS158 were analysed for 4'-phosphatase activity with the kind assistance of Shib Basu and C. R. H. Raetz. Membrane extracts were incubated with [4'-32P]P(Kdo)₄-IVₐ as described by Price et al. (1995); the products were then separated by TLC and analysed using a phosphimager.

Polymyxin sensitivity assays. 10⁻¹ and 10⁻⁵ c.f.u. of each meningococcal strain or mutant were resuspended in GC broth and spotted in duplicate on GC agar plates containing serial twofold dilutions of the agent to be tested. The concentration ranges for polymyxin B were 15.5–500 µg ml⁻¹.
The plates were incubated overnight at 37 °C and the MIC was determined as described by Shafer et al. (1984).

Serum bactericidal assay. Serum bactericidal assays with 10, 25 and 50% normal human serum (NHS) were performed on meningococcal strains as previously described (Kahler et al., 1998). In this study, bactericidal assays were performed at 5, 15 and 30 min at 37 °C.

RT-PCR. RT-PCR experiments were performed as previously described (Kahler et al., 1996b). Histidine auxotrophy was examined as previously described (Erwin & Stephens, 1995).

RESULTS

Isolation of a Tn916 mutant that expresses a Kdo2-lipid A truncated LOS structure

To identify genes involved in LOS biosynthesis, Tn916 insertion mutants in the serogroup B meningococcal strain NMB were screened for loss of reactivity with mAb 3F11, which recognizes the unsialylated terminal galactose of the neisserial LOS α-chain oligosaccharide (Apicella et al., 1981) (Fig. 1). A class II Tn916 mutant, designated 469, was identified by this method and found to be 100% linked to the Tn916 insertion by back transformation. Mutant 469 exhibited a markedly truncated LOS structure of 2–9 kDa when compared by Tricine-SDS-PAGE to the parental LOS of 4–6 kDa (Fig. 2a). Composition and linkage analysis of 469 LOS indicated that the mutant LOS structure consisted of two Kdo sugars and a hexa-acylated lipid A LOS structure with variable phosphorylation (phosphate or phosphoethanolamine) at 1′ and 4′ positions of the lipid A (Fig. 2b). The truncated LOS structure of mutant 469 is identical to the parent NMB LOS inner core except for greater variability of phosphorylation of the lipid A head groups, a feature that was also observed for other inner-core mutants (Kahler et al., 1996a, b).

DNA sequence analysis of the region disrupted by insertion of Tn916 in mutant 469

Sequencing of chromosomal DNA flanking the Tn916 insertion in mutant 469 revealed that a truncated Tn916 (class II insertion) had inserted into a region with three ORFs (orfC, gmbX, nlaB). Insertion of the transposon resulted in an 805 bp chromosomal deletion that completely removed orfC, partially deleted gmbX, and potentially had a polar effect on nlaB expression (Fig. 3). The first gene in this cluster, orfC, was predicted to encode a 190 aa protein containing a hexapeptide motif common to many acetyltransferases and acyltransferases (Dicker & Seetharam, 1992; Vuorio et al., 1994). The predicted orfC gene product shared greatest homology (33–7% identity, 56–4% similarity over 101 aa) with vioB, a gene in the E. coli O7 LPS biosynthesis cluster which is proposed to encode an N-acetyltransferase for dTDP-4-acetamido-4,6-dideoxyglucose synthesis (GenBank accession number AAD44155).

Downstream of orfC and partially deleted in mutant 469 was gmbX, an ORF predicted to encode a 187 aa protein that contained an ATP-binding motif common to the ATP-binding cassette (ABC) family of bacterial transporters (Walker et al., 1982) and shared homology with

Fig. 1. Chemical structure of the L2 immunotype LOS expressed by N. meningitidis strain NMB as described by Rahman et al. (1998). The mAb 3F11 recognizes the unsialylated terminal group Gal-β-(1→4)-GlcNac (Apicella et al., 1981). R = H or phosphate; 70% of NMB LOS lipid A molecules lack this 4′-phosphate.
the N-terminal histidinol-phosphate phosphatase domain of *E. coli* HisB (28.4% identity, 51.8% similarity over 141 aa). BLAST searches of the GenBank database with the predicted *gmhX* gene product revealed that, in addition to homology with the N-terminal region of *E. coli* HisB, significant homology at the protein sequence level was also shown to YaeD, a predicted protein of unknown function in *Haemophilus influenzae*. *E. coli* (29.7% identity, 54.4% similarity over 182 aa and 31.6% identity, 49.5% similarity over 190 aa, respectively) and a variety of other species. The meningococcal *gmhX* gene product also revealed homology (28% identity, 34% similarity over 184 aa) to *Streptomyces lincolnensis* LmbK, which is involved in synthesis of lincomycin, an antibiotic composed of a peptide and a carbohydrate derivative (Peschke et al., 1995).

The third gene in this cluster, *nlaB* (242 aa), shared homology (27% identity, 46% similarity over 242 aa) with *E. coli* lysophosphatidic-acid acyltransferases gene, *plsC* (Coleman, 1992). We have shown that *nlaB* encodes a meningococcal enzyme with *in vivo* and *in vitro* lysophosphatidic-acid acyltransferase activity (Shih et al., 1999).

Since the Tn916 mutant in mutant 469 was demonstrated to have a polar effect on *nlaB* expression, we presumed that *gmbX* and *nlaB* were transcriptionally linked and that the promoter for *nlaB* must lie upstream of *gmbX*. RT-PCR of the *orfC–nlaB* region demonstrated that *orfC*, *gmbX* and *nlaB* were co-transcribed in an operon (Fig. 4). Because these genes constitute the second operon involved in LOS inner-core biosynthesis identified in our laboratory, we named the operon *ice-2* (LOS inner-core extension, locus 2).

**Location of the *ice-2* operon in the meningococcal and gonococcal genomes (Fig. 3)**

Searches of the two meningococcal genome databases (MC58, http://www.tigr.org; Z2491, http://www.sanger.ac.uk) and *N. gonorrhoeae* FA1090 genome sequence database (http://www.genome.ou.edu/gono.html) with the genetic sequence of the *ice-2* operon revealed that the operon was located 1.8 kb downstream of *lgtG*, a recently characterized glucosyltransferase which forms the α-(1→3) bond between glucose and heptose II on the β-chain of gonococcal LOS (Banerjee et al., 1998). PCR amplification of NMB chromosomal DNA with primers specific for *lgtG* and *nlaB* confirmed that *lgtG* was upstream of the *ice-2* operon in strain NMB. A divergently transcribed ORF with homology (72% identity, 74% similarity) to the *H. influenzae* hypothetical protein HI0275 is located between *gmbX* of *ice-2* and *lgtG* in FA1090, Z2491 and NMB genomes, but not in the serogroup B MC58 genome. The presence of this ORF was confirmed by sequencing of the *lgtG–ice-2* intergenic region in strain NMB. *lgtG* was absent in the serogroup A *N. meningitidis* Z2491 genome sequence database. *orfC* was absent in the FA1090 genome sequence database and is located downstream of *nlaB* in the MC58 genome. These data are summarized in Fig. 3.

**Construction and analysis of the meningococcal *gmhX* mutant**

To determine which gene(s) were responsible for the LOS phenotype of mutant 469, specific mutations were constructed in *orfC*, *gmbX* or *nlaB* and the mutations were introduced into the parent strain NMB by homologous recombination. LOS prepared from NMB*gmhX::Ωsp* and NMB*gmhX::aphA* non-polar mutant GS158 demonstrated the truncated LOS phenotype of mutant 469 (Fig. 2a), whereas GS156 (NMB *nlaB::Ωsp* ) exhibited the parental LOS phenotype (data not shown). Composition and linkage analysis of the LOS from *gmhX* mutants GS152.7 and GS158 confirmed that both mutants exhibited a Kdo<sub>2</sub>-lipid A LOS structure with variable phosphorylation at the 1′ and 4′ positions of lipid A, similar to mutant 469. To confirm that the
mutant LOS phenotype in the GS158 mutant was caused by insertion of a non-polar cassette into gmbX and not due to a second site orfC mutation in the GS158 background, the nucleotide sequences of orfC in NMB and GS158 were examined and found to be identical. Although the predicted gmbX gene product shared homology with the N-terminal histidinol-phosphate phosphatase domain of E. coli HisB (Chiariotti et al., 1986) and its homologues in H. influenzae (YaeD, accession number P46452) and Salmonella typhimurium (HisB, P10368), neither mutant 469 nor meningococcal gmbX mutants GS152.7 and GS158 demonstrated histidine auxotrophy.

Southern hybridization of the NMB chromosome with a gmbX-specific probe confirmed that gmbX was present as a single copy in the NMB chromosome. To demonstrate that the gmbX mutation in GS158 was responsible for the LOS inner-core defect, we restored the gmbX LOS mutant to the parental LOS phenotype by homologous recombination with a wild-type copy of gmbX. Non-polar gmbX mutant GS158 was transformed with a 4 kb PCR product amplified from GS156 (Shih et al., 1999), encompassing wild-type copies of orfC and gmbX, in addition to nlaB::ΩSpR. Spectinomycin-resistant, kanamycin-sensitive transformants were isolated and tested for mAb 3F11 reactivity. All transformants tested had a restored mAb LOS phenotype (3F11+). We concluded that the gmbX mutation was responsible for the truncated LOS mutant phenotype in mutant 469.

**Phenotype of gmbX mutants**

Similar to other deep rough mutants (Wilkinson et al., 1972), gmbX mutants demonstrated decreased growth rates at 30 °C and 37 °C based on optical density measured at 550 nm (data not shown). Outer-membrane profiles in mutant 469 appeared to be identical to parent strain NMB. The gmbX mutants demonstrated increased sensitivity to the antimicrobial agent polymyxin B. The MICs to polymyxin B were 250 µg ml⁻¹ for parent strain NMB and 62.5 µg ml⁻¹ for gmbX mutants.

We have previously shown that, compared to the serum-resistant encapsulated parent strain NMB, encapsulated mutant 469 was more sensitive to killing by 25 and 50% NHS (P < 0.002) (Kahler et al., 1998). However, mutant 469 was less sensitive to killing in 25% NHS at 30 min (data not shown for the 5 and 15 min time points) when compared to encapsulated serum-sensitive LOS mutants.
containing heptose (Table 2) (CMK2, \( P < 0.001 \); CMK1, \( P < 0.001 \)). The serum sensitivity of mutant 469 was not due to a polar effect on \( nlaB \) or the deletion of \( orfC \), since specific \( nlaB \) mutants were resistant to killing by NHS, and polar and non-polar \( gmhX \) mutants were not significantly different from 469. Mutations in other genes (e.g. \( gmhX \), \( rfaD \), \( aut \)) which resulted in the expression of Kdo\(_2\)-lipid A LOS structures demonstrated similar survival in NHS.

![Fig. 4. RT-PCR of the \( orfC-nlaB \) region in meningococcal strain NMB, demonstrating that \( orfC \), \( gmhX \) and \( nlaB \) constitute an operon. + and — designate the presence or absence of reverse transcriptase, respectively. cDNA was prepared from RNA from strain NMB and amplified with primers GS9 (5'-GCGCG-GTTGTGTCGTCATCG-3') and GS34 (5'-TACCGATTTATGGTGTGGAG-3'). Chromosomal DNA (Chrom) amplified from strain NMB with primers GS9 and GS34 served as a positive control. Lane M contains DNA size markers (1 kb ladder).](image)

**Studies of the role of the \( gmhX \) gene product in the incorporation of heptose into meningococcal LOS**

Four hypotheses were considered to explain the role of \( gmhX \) in the incorporation of heptose into meningococcal LOS: (1) an effect on transcription of the heptosyltransferase I gene \( rfaC \); (2) modification of the Kdo\(_2\)-lipid A substrate; (3) transport of the Kdo\(_2\)-lipid A substrate across the inner membrane; and (4) enzymic activity in the heptose biosynthesis pathway.

To study the possibility that inactivation of \( gmhX \) affected heptose transfer to the Kdo\(_2\)-lipid A by affecting transcription of the heptosyltransferase I gene \( rfaC \), an \( rfaC::lacZ-ermC \) reporter construct was inserted into the wild-type background (GS159) and the non-polar \( gmhX \) mutant (GS160). No differences in \( \beta \)-galactosidase activity were observed in GS159 and GS160 (data not shown), suggesting that the \( gmhX \) gene product did not affect \( rfaC \) transcription, and by inference, RfaC activity.

In *Rhizobium leguminosarum*, the 4'-phosphate substitution on lipid A is removed by a phosphatase before further additions (e.g. heptose) to the structure can be made. Therefore, we investigated whether \( gmhX \) could encode a phosphatase involved in Kdo\(_2\)-lipid A assembly. As noted, analysis of the lipid A portion of 469 LOS by ES-MS demonstrated variable phosphorylation of the 4' position of lipid A. We tested whether whole-membrane extracts of NMB and GS158 contained lipid A 4'-phosphatase activity (Price *et al.*, 1995). No release of 4'-phosphate from the [4',32P][Kdo]_2-IV_A substrate was observed in either parent strain NMB or the \( gmhX \) mutant GS158.

Recently, \( msbA \), first identified as a multicopy suppressor of \( htrB \) mutants defective in LPS synthesis, was proposed to transport nascent core–lipid A molecules

**Table 2. Sensitivity of \( gmhX \) and other inner-core meningococcal mutants to NHS**

<table>
<thead>
<tr>
<th>Meningococcal strain</th>
<th>Description/LOS structure</th>
<th>Serum resistancea</th>
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<tr>
<td></td>
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<td>10%</td>
</tr>
<tr>
<td></td>
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<td>25%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50%</td>
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<tr>
<td>NMB</td>
<td>B:2B::P1.2.5:1.2/Lacto-N-neotetraose-Hep(_2)GlcNAC PEA-Kdo(_2)-lipid A</td>
<td>4.42 ± 0.04</td>
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<tr>
<td>CMK2</td>
<td>NMBgtF::aphA-3 Hep(_2)GlcNAC PEA Kdo(_2)-lipid A</td>
<td>3.97 ± 0.10</td>
</tr>
<tr>
<td>CMK1</td>
<td>NMBrafA::aphA-3 Hep(_2)PEAKdo(_2)-lipid A</td>
<td>3.97 ± 0.07</td>
</tr>
<tr>
<td>469</td>
<td>NMBorfC gmhX nlaB::Tn916/Kdo(_2)-lipid A</td>
<td>4.73 ± 0.02</td>
</tr>
<tr>
<td>GS152.7</td>
<td>NMBgmbX::ΩSp(_1)//Kdo(_2)-lipid A</td>
<td>3.64 ± 0.15</td>
</tr>
<tr>
<td>GS158</td>
<td>NMBgmbX::aphA-3/Kdo(_2)-lipid A</td>
<td>3.61 ± 0.09</td>
</tr>
<tr>
<td>GS159</td>
<td>NMBrafC::lacZ-ermC/Kdo(_2)-lipid A</td>
<td>3.53 ± 0.82</td>
</tr>
<tr>
<td>CMK3</td>
<td>NMBrafD::ΩSp(_1)//Kdo(_2)-lipid A</td>
<td>3.33 ± 0.16</td>
</tr>
<tr>
<td>GS161</td>
<td>NMBaut::aphA-3/Kdo(_2)-lipid A</td>
<td>3.39 ± 0.25</td>
</tr>
<tr>
<td>GS156</td>
<td>NMBnlaB::aphA-3, L2/LOS</td>
<td>ND</td>
</tr>
<tr>
<td>M7</td>
<td>NMBsynA::Tn916</td>
<td>0</td>
</tr>
</tbody>
</table>

\( a \) Resistance to killing by NHS [10, 25 and 50% (v/v) concentrations] over 30 min compared to time 0 value (−40 log units). Data are Log\(_{10}\) survival values ± SEM from 2–15 separate experiments per variable, with quadruplicate determinations of each variable per experiment. ND, Not done.
across the inner membrane (Polissi & Georgopoulos, 1996; Zhou et al., 1998). Because the \textit{msbA} gene product contains Walker box motifs and is predicted to be an ABC transporter involved in LPS biosynthesis, we postulated that \textit{gmhX} could play a similar role in LOS biosynthesis and tested the ability of \textit{gmhX} to complement an \textit{E. coli} \textit{btrB} mutant. None of the \textit{btrB} mutants transformed with a multicopy plasmid expressing the \textit{gmhX} gene product restored the temperature-sensitive phenotype of the \textit{btrB} mutant. In addition, searches of the meningococcal and gonococcal sequence databases revealed a homologue of \textit{msbA} that was distinct from \textit{gmhX}.

The \textit{gmhX} homologues in \textit{Helicobacter pylori} (Rv0114 (CAIA7308)) and \textit{Streptomyces coelicolor} genomes were found with clusters of heptose biosynthesis genes (e.g. \textit{gmhA}, \textit{rfaE}, \textit{rfaD}). In \textit{Synechocystis} and \textit{Mycobacterium tuberculosis}, \textit{gmhX} was adjacent to \textit{gmhA} homologues. Because the heptose biosynthetic pathway has not been fully characterized, we considered whether the \textit{gmhX} gene product was a functional homologue of \textit{rfaD}, \textit{rfaE} or \textit{aut}. The meningococcal homologues of \textit{rfaD} and \textit{aut} were inactivated by insertional mutagenesis (Table 1) and the LOS phenotypes of these mutants determined by Tricine/SDS-PAGE analysis. In the parental background (\textit{gmhX} intact), meningococcal \textit{rfaD} and \textit{aut} isogenic mutants both exhibited heptose-deficient truncated LOS structures similar to that observed for the meningococcal \textit{gmhX} mutants, demonstrating that \textit{rfaD} and \textit{aut} were required for heptose incorporation into LOS and were distinct in function from \textit{gmhX}.

DISCUSSION

We have identified a new meningococcal gene, designated \textit{gmhX} (\textit{glycero-manno-heptose gene X}), which is necessary for the addition of \textit{L-glycero-d-manno-heptose} to \textit{Kdo}_{2}-lipid A of the meningococcal LOS inner core. Specific polar and non-polar meningococcal \textit{gmhX} mutants exhibit a heptose-deficient but otherwise wild-type meningococcal \textit{Kdo}_{2}-lipid A LOS structure. Repair of \textit{gmhX} mutants by homologous recombination with the wild-type \textit{gmhX} restored the LOS parental phenotype. \textit{GmhX}-specific mutants have increased sensitivity to polymyxin B and to killing by normal human serum. The \textit{gmhX} gene product does not appear to regulate heptosyltransferase I (\textit{RfaC}), act as a \textit{msbA} homologue (i.e. transport of nascent LPS) or modify lipid A. Further, \textit{gmhX} homologues are associated with putative heptose biosynthetic genes in other genomes and \textit{gmhX} is distinct from \textit{gmhA}, \textit{rfaD}, \textit{rfaE} and \textit{aut}.

Only two genes involved in bacterial heptose biosynthesis have been fully characterized (Fig. 5). \textit{gmhB} (formerly called \textit{lpC}) encodes the phosphoheptose isomerase, which converts sedoheptulose 7-phosphate into \textit{d-glycero-d-manno-heptose} 7-phosphate (Brooke & Valzano, 1996b). The second characterized gene, \textit{rfaD} (recently designated \textit{gmhD}), encodes the ADP-\textit{L-glycero-d-manno-heptose} epimerase, which converts ADP-\textit{d-glycero-d-manno-heptose} to ADP-\texti{L-glycero-d-manno-heptose} (Coleman, 1983; Pegues et al., 1990), the substrate for the heptosyltransferase reaction (Chen & Coleman, 1993; Kadrmaz & Raetz, 1998; Sirisena et al., 1992). Based on evidence for the initial substrate (sedoheptulose 7-phosphate) and end product (ADP-\textit{L-glycero-d-manno-heptose}), a putative pathway for heptose biosynthesis was proposed to include a mutase step for conversion of the \textit{d-glycero-d-manno-heptose} 7-phosphate to a \textit{d-glycero-d-manno-heptose} 1-phosphate and an ADP-heptose synthetase step for formation of the activated heptose sugar ADP-\textit{d-glycero-d-manno-heptose} (Eidels & Osborn, 1974) (Fig. 5a). ADP-heptose synthetase, rather than ADP-heptose synthase, more accurately describes the enzymatic reaction at this step and we propose the adoption of this nomenclature.

A third gene, named \textit{rfaE}, was implicated in heptose biosynthesis on the basis of complementation of the deep rough LPS mutant \textit{Salmonella} mutant SL1102 (Wilkinson et al., 1972) at the genetic (Lee, N.-G. et al., 1995; Levin & Stein, 1996; Valzano, 1999) and biochemical level (Sirisena et al., 1992). Functional homologues of \textit{rfaE} have been identified in \textit{N. gonorrhoeae} (Levin & Stein, 1996), \textit{H. influenzae} (Lee, N.-G. et al., 1995) and \textit{E. coli} (Valzano, 1999; Valzano et al., 2000). It was proposed that \textit{rfaE} encoded a bifunctional enzyme that could catalyse both the mutase and ADP-heptose synthetase steps in heptose biosynthesis (Raetz, 1996; Schmitman & Klena, 1993). Indeed, recent characterisation of the \textit{E. coli} \textit{rfaE} gene product demonstrated two distinct functional domains that were fused in some species but separate in others (Valzano, 1999; Valzano et al., 2000). However, the \textit{RfaE} N-terminal domain (amino acids 1–318) exhibits structural features common to kinases. The \textit{RfaE} C-terminal domain (amino acids 344–477) shares homology with a putative cytidylyltransferase (\textit{Aut}) from \textit{Ralstonia eutropha}. In the \textit{N. gonorrhoeae} FA1090 and \textit{N. meningitidis} Z2491 genome sequence databases, two widely dispersed and distinct ORFs, designated \textit{rfaE} and \textit{aut}, are predicted to encode proteins with homology to the \textit{N} terminus and the \textit{C} terminus of \textit{E. coli} \textit{RfaE}, respectively. In this study we demonstrate that the \textit{aut} homologue of \textit{N. meningitidis} is required for heptose incorporation into meningococcal LOS. Thus the \textit{N}-terminal and \textit{C}-terminal domains of \textit{RfaE} are encoded by two distinct genes in \textit{N. meningitidis} and \textit{N. gonorrhoeae}.

Based on the predicted enzyme activities of \textit{RfaE} and \textit{Aut}, two additional models for heptose biosynthesis have recently been proposed (Valzano, 1999; Valzano et al., 2000). One model predicts that the intermediate steps involve dephosphorylation of \textit{d-glycero-d-manno-heptose} 7-phosphate to create \textit{d-glycero-d-manno-heptose}, which is subsequently phosphorylated to produce \textit{d-glycero-d-manno-heptose} 1-phosphate (Fig. 5b). An alternative model proposes that an additional phosphate on \textit{d-glycero-d-manno-heptose} 1-phosphate is added to create \textit{d-glycero-d-manno-heptose} 1,7-diphosphate, which is then converted to ADP-\textit{d-glycero-d-manno-heptose} (Fig. 5c).
In this study, we describe the identification of a novel gene, \( \text{gmhX} \), which plays a role in heptose assembly, yet is distinct from heptose biosynthesis genes characterized to date. Meningococcal mutants defective in \( \text{gmhX} \), \( \text{rfaE} \), \( \text{aut} \) or \( \text{rfaD} \) each produced a truncated, heptose-deficient Kdo\(_{2}\)-lipid A LOS structure. Although \( \text{gmhX} \) homologues are present in several other species, the effect of this gene on LPS or LOS structure has not been documented previously. Based on the predicted ATP-binding and phosphatase properties of \( \text{GmhX} \), the predicted protein is a candidate for the enzyme that removes the 7-phosphate from \( \text{ADP-L-heptose} \) or from ADP-\( \text{heptose} \). The phosphatase removing 7-phosphate from subsequent substrates. \( \text{GmhX} \) and \( \text{Aut} \) could constitute a bifunctional enzyme with synthetase and phosphatase activity to create \( \text{ADP-L-heptose} \). In summary, we have identified a novel gene required for heptose biosynthesis in \( \text{N. meningitidis} \). The juxtaposition and co-transcription of LOS and phospholipid genes in the \( \text{ice-2} \) operon in \( \text{N. meningitidis} \) may also be important. The previously described \( \text{ice-1} \) operon encodes two enzymes involved in the addition of glucose and N-acetylglucosamine, respectively, to Hep\(_{2}\)-Kdo\(_{2}\)-lipid A LOS inner core (Kahler et al., 1996b). Upstream of these LOS glycosyltransferases in gonococcal strain FA1090 are homologues of \( \text{fabF} \) and \( \text{fabG} \), genes that encode fatty acid biosynthetic enzymes in E. coli (C. M. Kahler and others, unpublished data). Other meningococcal operons also contain genes associated with LOS assembly adjacent to genes associated with phospholipid assembly. For example, a \( \text{fabG} \) homologue is located downstream of the \( \text{IgtA–E} \) genes necessary for LOS biosynthesis (Kahler & Stephens, 1998) and the \( \text{lpxD–fabZ–} \) \( \text{lpxA} \) gene cluster contains the genes for lipid A (\( \text{lpxD, lpxA} \)) and fatty acid (\( \text{fabZ} \)) biosynthesis (Steeghs et al., 1997). Since LOS constitutes a large proportion of the outer membrane in meningococci, the coordinated regulation of membrane phospholipid genes with genes involved in LOS assembly would facilitate synchronized synthesis of these critical outer-membrane components and efficient adaptation to various environmental changes.

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In summary, we have identified a novel gene required for the incorporation of \( \text{L-glycero-d-manno-heptose} \) into the LOS of \( \text{N. meningitidis} \). This gene is distinct from other heptose biosynthesis genes. It is a candidate for the phosphatase removing 7-phosphate from ADP-\( \text{D-glycero-manno-heptose} \) or \( \text{D-glycero-manno-heptose} \) of the heptose biosynthesis pathway. Our finding supports the hypothesis that the heptose biosynthesis pathway in...
N. meningitidis and other species is more complex than originally proposed. Furthermore, mutations in gmbX and other genes that result in Kdo₂-lipid A structures demonstrate increased sensitivity to NHS. Finally, genes encoding LOS and phospholipid assembly in N. meningitidis appear to be coordinately regulated, which may facilitate adaptation to environmental changes.

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REFERENCES


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