Intracellular Survival of *Neisseria gonorrhoeae* in Male Urethral Epithelial Cells: Importance of a Hexaacyl Lipid A

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*Neisseria gonorrhoeae* is a strict human pathogen that invades and colonizes the urogenital tracts of males and females. Lipooligosaccharide (LOS) has been shown to play a role in gonococcal pathogenesis. The acyl transferase MsbB is involved in the biosynthesis of the lipid A portion of the LOS. In order to determine the role of an intact lipid A structure on the pathogenesis of *N. gonorrhoeae*, the msbB gene was cloned and sequenced, a deletion and insertion mutation was introduced into *N. gonorrhoeae* and the mutant strain was designated 1291A11K3. Mass spectrometric analyses of 1291A11K3 LOS determined that this mutation resulted in a pentaacyl rather than a hexaacyl lipid A structure. These analyses also demonstrated an increase in the phosphorylation of lipid A and an increase in length of the oligosaccharide of a minor species of the msbB LOS. The interactions of this mutant with male urethral epithelial cells (uec) were examined. Transmission and scanning electron microscopy studies indicated that the msbB mutants formed close associations with and were internalized by the uec at levels similar to those of the parent strain. Gentamicin survival assays performed with 1291A11K3 and 1291 bacteria demonstrated that there was no difference in the abilities of the two strains to adhere to uec; however, significantly fewer 1291A11K3 bacteria than parent strain bacteria were recovered from gentamicin-treated uec. These studies suggest that the lipid A modification in the *N. gonorrhoeae* msbB mutant may render it more susceptible to innate intracellular killing mechanisms when internalized by uec.

*Neisseria gonorrhoeae* is a strict human pathogen, which invades and colonizes the epithelia of the urogenital tracts of both males and females (2, 12, 18). The lipooligosaccharide (LOS) of *N. gonorrhoeae* has been shown to play a role in the pathogenesis of human infections (19, 40). The LOS is composed of three major components: the oligosaccharide chain extensions, the core region, and lipid A. The oligosaccharide extensions of the LOS contain determinants which resemble human glycosphingolipid antigens that play a role in molecular mimicry. Studies have also shown that the oligosaccharide region of the LOS can be involved in receptor-mediated interactions (17, 19, 37). Lipid A of *N. gonorrhoeae* is similar in structure to lipid A from other gram-negative bacteria (31, 44, 46).

htrB (alternatively *lpxL* or *waaM*) is involved in the biosynthesis of lipid A of lipopolysaccharides (LPSs) and LOSs. HtrB is one of the 2-keto-3-deoxyoctulosonic acid (Kdo)-dependent acyl transferases responsible for the addition of a secondary acyl substitution on the lipid A portion of LPS or LOS. The htrB gene has been well characterized for *Escherichia coli*, *Haemophilus influenzae*, and *Salmonella enterica* serovar Typhimurium (6, 36, 44).Mutations in this gene have been shown to have a number of effects on the organism. One such effect is temperature sensitivity. *E. coli*, *H. influenzae*, and *S. enterica* serovar Typhimurium *htrB* mutants have all been shown to be initially sensitive to temperatures above 32.5°C (28, 31, 44). A number of different genes are able to subsequently suppress temperature sensitivity (27, 29) (D. M. B. Post and M. A. Apicella, unpublished data). msbB (alternatively *lpxM* or *waaN*) has been identified as one of these suppressors (27).

Work done with *E. coli* has shown that MsbB is a late-functioning acyl transferase, which functions optimally after laurate incorporation by HtrB onto the *E. coli* KDO₂-lipid IV₅A structure (9). Therefore, secondary acyl substitutions of the lipid A structure are thought to occur in a sequential manner.

Chemical analyses of LPS and LOS isolated from the serovar Typhimurium and *H. influenzae* htrB mutants demonstrated that the lipid A portion of LPS or LOS was modified (31, 44). Additional studies showed that LPS or LOS purified from these *htrB* mutants was reduced in its toxicity compared to LPS or LOS isolated from the parent strains (26, 36). Animal model studies using *H. influenzae* and serovar Typhimurium *htrB* mutants demonstrated that these organisms were less virulent than the parent strains (11, 26).

In this study, we investigated the role of an intact lipid A structure on the pathogenesis of *N. gonorrhoeae*. To perform these investigations, we created an msbB lipid A mutant of *N. gonorrhoeae* strain 1291. This mutation resulted in a pentaacyl rather than the hexaacyl lipid A structure found in the wild-type strain. The interactions of this mutant with male urethral epithelial cells (uec) were examined. These studies suggest that the lipid A modification in the *N. gonorrhoeae* msbB mutant may render it more susceptible to killing when internalized by the uec.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37, 32, or 30°C and supplemented with appropriate antibiotics. For growth curves, an overnight culture was used to inoculate
fresh medium. Cells were grown at 37°C with agitation, and readings were taken every 30 min. N. gonorrhoeae and Neisseria meningitidis organisms were grown on gonococcal agar (GCA) (Becton Dickinson, Sparks, Md.) supplemented with 1% IsoVitalex or on brain heart infusion (BHI) agar (Becton Dickinson) supplemented with 2.5% heat-inactivated fetal calf serum (FCS) at 37°C in 5% CO₂. Liquid cultures of N. gonorrhoeae were grown in BHI broth supplemented with 2.5% FCS or in gonococcal broth supplemented with 1% IsoVitalex at 37°C. Kanamycin-resistant N. gonorrhoeae isolates were grown on supplemented BHI agar plates or in supplemented BHI broth with 50 μg of kanamycin per ml.

**Recombinant DNA and transformation methods.** Restriction and modifying enzymes were purchased from New England Biolabs (Beverly, Mass.) and Promega (Madison, Wis.). Standard DNA recombinant protocols were performed as previously described (38). Transformation of E. coli with plasmid DNA was done using the CaCl₂ method (16). Transformation of N. gonorrhoeae was performed as previously described (43).

**Complementation of an E. coli htrB mutant.** An overnight culture of E. coli MLK217 cells was inoculated into 500 ml of fresh LB medium. Cells were grown at 30°C with vigorous agitation to an optical density at 600 nm (OD₆₀₀) of 0.5. The cells were chilled briefly on ice and centrifuged at 2,200 × g for 20 min at 2°C. The cell pellet was washed with ice-cold water and centrifuged at 2,200 × g for 20 min at 2°C. The cell pellet was washed a second time, centrifuged as described above, and resuspended in an equal volume of sterile 10% (vol/vol) glycerol-water (4). Thirty microliters of the cells was electroporated (4 kV, 330 μF, low-resistance ohms, fast charge rate) with 1 μl of the pNMBA11pUC19 DNA by using a Cell Porater (Invitrogen, Carlsbad, Calif.). Then, cells were incubated in 1 ml of SOC medium at 30°C for 90 min with agitation and plated on LB agar plates containing tetracycline (20 μg/ml) and ampicillin (100 μg/ml). Cells were grown in a 37°C incubator overnight. Colonies that were able to grow at 37°C were then tested using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses.

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

**Southern blot analyses.** Hybridization experiments were carried out according to the manufacturer’s protocols (Boehringer Mannheim Corp., Indianapolis, Ind.). All probes were labeled by using either PCR labeling or random labeling with digoxigenin-labeled deoxycytidine triphosphates (Boehringer Mannheim Corp.).

**DNA sequencing and analyses.** DNA sequencing reactions were performed by using dye terminator cycle sequencing chemistry with AmpliTaq DNA polymerase and FS enzyme (PE Applied Biosystems, Foster City, Calif.). The reactions were run on and analyzed with an Applied Biosystems model 373A stretch fluorescence automated sequencer at the University of Iowa DNA Facility. All primers were either commercially available or purchased from Genosys Corporation (Aldrich, Milwaukee, Wis.) or IDT Technologies (Corvalle, Iowa).

**Sequence analysis was performed by using Assembly LIGN, version 1.0 (Oxford Molecular Group Inc., Oxford, United Kingdom), MacVector (Oxford Molecular Group Inc.), and Wisconsin Package, version 10.0 (Genetics Computer Group, Madison, Wis.).

**Cloning and mutagenesis of msbB gene.** The E. coli htrB gene sequence was used to search the N. gonorrhoeae strain FA1090 sequence at the University of Oklahoma website. The sequence that showed the highest homology to the E. coli gene (N. gonorrhoeae sequence bp 160985 to 162427) was used for the design of PCR primers. PCR amplification of this region was performed with N. meningitidis strain NMB genomic DNA and the primers gchtrB3 5'-TTCGGCATCCACTCCCCTTTG-3' and gchtrB5 5'-TCGCGATCCTACCCCTTTCTG-3'. The 1,443-bp PCR product was cloned using the TA cloning vector pCR2.1 (Invitrogen), and this construct was designated pNMBA11. The XbaI and HindIII sites flanking the PCR product were used to clone the PCR fragment into XbaI- and HindIII-restricted pUC19 (New England Biolabs). This construct was ligated using T4 DNA ligase and subsequently transformed into E. coli DH5α cells (Invitrogen). This construct was designated pNMBA11pUC19 (Fig. 1).
1). Resuspended in phosphate-buffered saline (PBS) to an OD 650 of 0.9. Cells were

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transformed with E. coli was isolated from this transformation with the

armed with PCR and re-

aphA (17,000 g) and the fragment ions for diphosphoryl lipid A (952.0) and the

mass accuracy of 0.25% trypsin, 0.1% EDTA) for 4 min at room temperature, removal of the

samples (3:1) and mixed 1:1 with super DHB matrix solution (2,5-dihydroxybenzoic acid/5-methoxysalicylic acid [9:1, wt/wt] in a saturated solution in CHCl3/CH3OH [3:1, vol/vol]) (15). Approximately 100 laser shots were recorded for each sample. The spectra were then smoothed with a 19-point Savitsky-Golay function and mass calibrated with an external mass calibrant consisting of resin substrate trisdecapeptide, insulin chain B (oxidized), and bovine insulin (all from Sigma, St. Louis, Mo.). For external calibrations under these conditions, a mass accuracy of 0.1% was obtained. For comparison purposes, a two-point correction was made to the spectra of the O-deacylated LOS using the expected fragment ions for diphosphoryl lipid A (m/z 952.0) and the N. gonorrhoeae nonaccharide (m/z, 1,839.6). All masses measured under these MALDI-TOF conditions were average mass values.

ESI-MS/MS. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was run on an ABI 300 triple quadrupole mass spectrometer (PE-Sciex, Concord, Ontario, Canada) equipped with a Protona nanospray ion source (MDS Proteomics A/S, Odense, Denmark). Lipid A samples were dissolved in CH3OH/CHCl3/CH3OD (3:1) and mixed 1:1 with super DHB matrix solution (2,5-dihydroxybenzoic acid/5-methoxysalicylic acid [9:1, wt/wt]) in a saturated solution in CHCl3/CH3OH [3:1, vol/vol]) (15). Approximately 100 laser shots were recorded for each sample. The spectra were then smoothed with a 19-point Savitsky-Golay function and mass calibrated with an external mass calibrant consisting of resin substrate trisdecapeptide, insulin chain B (oxidized), and bovine insulin (all from Sigma, St. Louis, Mo.). For external calibrations under these conditions, a mass accuracy of 0.1% was obtained. For comparison purposes, a two-point correction was made to the spectra of the O-deacylated LOS using the expected fragment ions for diphosphoryl lipid A (m/z 952.0) and the N. gonorrhoeae nonaccharide (m/z, 1,839.6). All masses measured under these MALDI-TOF conditions were average mass values.

Bacterial assays. Ten milliliters of gonococcal broth with supplements was inoculated with bacteria to an OD600 of 0.03. These cultures were grown for 2 h at 37°C with agitation. Then, samples were diluted to a final concentration of 108 bacteria/ml in a phosphate-buffered salt solution consisting of 10 mM K2HPO4, 10 mM KH2PO4, 136 mM NaCl, 5 mM KCl, 1 mM CaCl2, 0.3 mM MgCl2, 1 mM MgSO4, 7 H2O, and 0.01% BSA, pH 7.0. The assay was performed as previously described by Zaleski et al., with some modifi-

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Growth of immortalized uuec. Human papillomavirus (HPV) was used to trans-

duce male uuec (H. A. Harvey, D. M. B. Post, and M. A. Apicella, unpublished data). HPV-transduced uuec were grown in prostate epithelial growth medium (PrEGM) (Clonetics, San Diego, Calif.) on 100-mm-diameter tissue culture- treated petri dishes (Corning Inc., Corning, N.Y). When cultures were 10 to 14 days old, they were passaged to 24-well tissue culture plates (Corning Inc., Corning, N.Y) for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Cells were lifted from 100-mm-diameter dishes by treatment with a trypsin solution (0.25% trypsin, 0.1% EDTA) for 4 min at room temperature, removal of the

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trypsin solution, and incubation of the cells at 37°C for 4 min. Then, the cells were suspended in 5% FCS/PrEGM, centrifuged for 2 min at 1,380 × g, and resuspended in the desired volume of PrEGM prior to seeding. When the uue were >90% confluent, gonococcal challenges were performed as described below.

**Assay for the invasion of male uue.** Invasion assays were performed as previously described with some modifications (25). Once uue were >90% confluent, cells were grown for at least 48 h prior to infection in antibiotic-free PrEGM. All challenges were performed using piliated colonies, based on colony morphology. Cells were grown for at least 48 h prior to infection in antibiotic-free PrEGM. All samples were viewed with microscopes located in the Central Microscopy Research Facility at the University of Iowa.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the mshB genes from *N. meningitidis* strain NMB and *N. gonorrhoeae* strain 1291 are available in the GenBank database under accession numbers AF428103 and AY057903, respectively.

**RESULTS**

**Homology analysis.** The National Center for Biotechnology Information website was used to obtain the amino acid sequences of HtrB from *H. influenzae* and MsbB from *E. coli*. The MsB protein sequences of *E. coli*, *N. gonorrhoeae*, and *N. meningitidis* and the HtrB protein sequence from *H. influenzae* were compared with alignment programs from Genetics Computer Group. This alignment showed that these proteins had two regions that were highly conserved among all of the organisms. These segments were designated conserved regions one and two. A BLAST search was performed using these conserved regions, and the only proteins that were found to show high homology with them were lipid A acyl transferases from a number of bacteria (Fig. 2A and B). The two conserved regions showed high homology among a number of bacteria. These results suggest that these regions are possible active sites for these enzymes.

**Complementation of an *E. coli* htrB mutant with pNMB11 pUC19.** Complementation of an *E. coli* htrB mutant, MLK217, was performed. The complemented strain was designated MLK217A11. Growth curves (Fig. 3A) demonstrated that MLK217 was unable to grow at 37°C, consistent with previous reports of htrB mutants (28, 31). Additionally, these studies showed that the MLK217A11 bacteria were able to grow al-

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**FIG. 2.** Homology analyses of predicted amino acid sequences of HtrB and MsbB from various bacteria. Residues with homology are shaded in black, and residues with similarity are shaded in grey. The consensus sequence is shown at the bottom of each panel. (A) Alignment of conserved region one (*N. meningitidis* strain NMB MsbB amino acids 186 to 203). (B) Alignment of conserved region two (*N. meningitidis* strain NMB MsbB amino acids 272 to 290). N.gono1291 and N.gono2, *N. gonorrhoeae* MsbB (Genbank accession no. AY057903); N.mening and N.mening2, *N. meningitidis* MsbB (GenBank accession no. AF428103); S. typhimurium and S. typhimurium HtrB (GenBank accession no. AAC43515); H.ducreyi and H.ducreyi HtrB, *Haemophilus ducreyi* (GenBank accession no. AAF34642); H.influenzae and H.influenzae strain Rd HtrB (GenBank accession no. AAC23173); H.pylori and H.pylori HtrB, *Helicobacter pylori* (GenBank accession no. AAP34642); H.influenzae strain Rd MsbB (GenBank accession no. AAC21868); H.ducreyi and H.ducreyi HtrB, *Haemophilus ducreyi* (GenBank accession no. AAF34642); H.influenzae MsbB, H. influenzae strain Rd MsbB (GenBank accession no. AAC21868); V.cholerae and V.cholerae2, *Vibrio cholerae* strain N16961 lauroyl transferase (HtrB) (GenBank accession no. AAF3389); H.ducreyi MsbB, H. ducreyi MsbB (GenBank accession no. AAF33777); E.coliLpxP, *E. coli* strain K-12 LpxP (GenBank accession no. AA66658); X.fastidiosa, *Xylella fastidiosa* lauroyl transferase (GenBank accession no. AAF32917); H.pylori2, *Helicobacter pylori* strain 26695 IpBp (GenBank accession no. AAD07343).
MLK2 and MLK217A11 stained black, whereas the LPS isolated from MLK217 stained brown. The brown staining pattern was consistent with previous reports of \textit{htrB} mutants (31, 39). These results demonstrated that the pNMBA11pUC19 was able to complement the \textit{E. coli} \textit{htrB} mutant.

\textbf{Mutagenesis of the \textit{msbB} gene.} A deletion of the \textit{msbB} gene was made in the pNMBA11pUC19 construct using the restriction enzymes \textit{BclI} and \textit{BssHII}. These restriction enzyme reactions removed 138 nucleotides from the \textit{msbB} gene. A kanamycin resistance gene, \textit{aphA}-3, was ligated into the site of deletion. This construct was transformed into \textit{E. coli} DH5\textalpha cells. Restriction enzyme analysis and PCRs were used to confirm that the proper construct had been made. This construct was designated pNMBA11K3.

Since the \textit{N. meningitidis} \textit{msbB} gene was used to construct the \textit{msbB} mutation in \textit{N. gonorrhoeae}, the putative \textit{msbB} gene sequence from \textit{N. gonorrhoeae} strain 1291 was compared with the putative \textit{msbB} gene sequence from the \textit{N. meningitidis} strain NMB using the Fasta program from Genetics Computer Group. This comparison showed a divergence of 54 nucleotides (94\% homology). Analysis of the predicted protein sequences showed a difference in 15 amino acids (94.8\% homology). Transformation of pNMBA11K3 into \textit{N. gonorrhoeae} strain 1291 was performed as previously described. Selection for transformants was done on gonococcal medium base plates containing kanamycin. Transformants were able to grow at 37\°C. PCR and Southern blot analyses demonstrated that the proper mutation had been incorporated into the \textit{N. gonorrhoeae} strain 1291 genomic DNA (data not shown). This transformant was subsequently designated 1291A11K3.

\textbf{Characterization of the LOS by SDS-PAGE and Western blot analyses.} Silver staining showed that the 1291A11K3 LOS migrated through the gel slightly faster than the 1291 LOS and that the 1291A11K3 LOS stained brown instead of black (Fig. 4A). This staining pattern was consistent with previous reports of LPS and LOS isolated from \textit{htrB} mutants of \textit{E. coli} and \textit{H. influenzae} (31, 39). It has been previously demonstrated that MAb 6B4 binds to \textit{N. gonorrhoeae} LOS through the terminal N-acetyl-lactosamine (3). Western blot analysis was performed using LOS isolated from \textit{N. gonorrhoeae} strains 1291 and 1291A11K3 and the MAb 6B4. This blot showed that this antibody reacted with both the 1291 LOS and the 1291 A11K3 LOS (Fig. 4B). These results indicated that the oligosaccharide portion of the 1291A11K3 LOS maintained the terminal N-acetyl-lactosamine. This blot also showed a higher-molecular-weight band in addition to the expected LOS band.
To compare the intact lipid A structures from the *N. gonorrhoeae* 1291 and 1291A11K3 strains, the LOS samples were subjected to mild acid hydrolysis to cleave the lipid A from the oligosaccharide moiety. Lipid A fractions were analyzed by MALDI mass spectrometry in the negative-ion mode. As seen in Fig. 6A, the 1291 lipid A preparation gave a major deprotonated molecular ion at \((M - H)^- = 1,633.2\), corresponding to a hexaacyl, monophosphoryl structure (46). In addition to this major component, a diphosphoryl species at \((M - H)^- = 1,713.2\) and a monophosphorylated species bearing an additional PEA group at \((M - H)^- = 1,756.3\) are also present. Prompt fragments seen in the spectrum at \(m/z = 1,450.9\) and \(1,434.4\) arise from losses of lauric acid \((-182\ D)\) and 3-hydroxylauric acid \((-198\ Da)\), respectively.

In the MALDI spectrum of the 1291A11K3 lipid A, the major component gave a molecular ion at \((M - H)^- = 1,450.7\), which is shifted to lower mass by 182.5 Da. This mass difference is consistent with the loss a single lauric acid residue \((182.3\ Da)\) from the 1291 monophosphorylated lipid A structure. In addition to this species, the spectrum of the 1291A11K3 lipid A also contains peaks for a diphosphoryl species at \((M - H)^- = 1,530.9\) and a monophosphorylated species bearing an additional PEA at \((M - H)^- = 1,573.8\). Prompt fragments for the losses of lauric acid \((m/z = 1,268.5)\) and 3-hydroxylauric acid \((m/z = 1,252.3)\) are also present. The fact that mild acid hydrolysis of the 1291A11K3 LOS also gave mainly monophosphorylated lipid A suggests that the additional PEA moiety seen in the O-deacylated LOS (Fig. 5B) is linked primarily to the reducing terminal phosphate.

Strong acid hydrolysis of the lipid A samples was conducted to release all of the N-linked and O-linked fatty acids from the diglucosamine backbone. Following derivatization, the fatty acids were analyzed by GC-MS (Table 3). From the 1291 lipid A sample, lauric acid, 3-hydroxylauric acid, and 3-hydroxymyristic acid were detected in a 1.2:1.2:1.0 ratio. The 1291A11K3 lipid A sample contained lauric acid, 3-hydroxy lauric acid, and 3-hydroxymyristic acid in a 0.5:1.3:1.0 ratio. Consistent with the

Mass spectrometric analyses of *N. gonorrhoeae* 1291A11K3 LOS. To determine the effect of the msbB mutation on the *N. gonorrhoeae* 1291 LOS structure, LOSs from strains 1291 and 1291A11K3 were O-deacylated to remove O-linked fatty acids from the lipid A moiety. When analyzed by MALDI mass spectrometry in the negative-ion mode, the 1291 O-deacylated LOS (Fig. 5A) gave a major deprotonated molecular ion, \((M - H)^- = m/z = 2,792.9\), consistent with the previously published structure of the wild-type *N. gonorrhoeae* strain 1291 LOS (22). In the O-deacylated LOS mixture from the 1291A11K3 mutant, this species is also predominant but there is additional PEA heterogeneity in the sample. When present, the extra PEA group exists on the lipid A moiety (see inset, bottom), primarily on the reducing terminal phosphate (see text). Peaks marked with asterisks are \((M - H-H_2O)^-\) species.
MALDI data described above, these results suggest that the lipid A of the 1291A11K3 mutant has one less lauric acid residue than the lipid A from the parental 1291 strain.

To establish which of the two possible lauric acids was missing in the 1291A11K3 mutant strain, the monophosphorylated lipid A samples were analyzed by ESI-MS/MS in the negative-ion mode. The major molecular ions for the 1291 (m/z 1,632.9) and 1291A11K3 (m/z 1,450.7) lipid A species were selected for collision-induced dissociation. As seen in Fig. 7, the lipid A samples gave similar fragmentation patterns, with the fragment series for 1291A11K3 shifted to lower masses by 182 Da. Most of the fragments arise from single and multiple losses of O-linked fatty acids from the parent ions, as indicated on the spectra and the structures shown (Fig. 7, insets). However, the low-mass fragments in the spectra of the 1291 (m/z 691.2 and 709.2) and 1291A11K3 (m/z 509.0 and 527.0) samples would appear to arise from two-bond cleavages: the loss of the 3-hydroxylauric acid residue on the distal glucosamine, as a free acid or a ketene, and a cross-ring cleavage of the reducing terminal glucosamine (indicated as an e-type cleavage in Fig. 7). In the nomenclature of Costello and Vath (10), this e-type cross-ring cleavage is designated a0,4A2-type fragment. As this cross-ring cleavage releases all of the substituent-bearing positions from the reducing terminal glucosamine, the low-mass fragment ions thus contain only the lipids on the distal glucosamine. In this case, the fact that the fragments for the 1291A11K3 lipid A are 182 Da lower in mass than the fragments for the 1291 lipid A suggests that in strain 1291A11K3, a lauric acid is missing from the distal glucosamine of the lipid A structure.

**Bactericidal activity of PNHS against N. gonorrhoeae strains 24-1, 1291, and 1291A11K3.** To determine if the msbB mutation affects serum susceptibility, three N. gonorrhoeae strains, 24-1, 1291, and 1291A11K3, were tested in triplicate in a serum bactericidal assay. Strain 24-1 was used as a serum-sensitive control. All three strains were able to grow in the presence of heat-inactivated normal human serum (data not shown). Strain 24-1 was highly serum sensitive in the presence of PNHS, while the serum sensitivity of strain 1291A11K3 was unchanged from that of the parent strain 1291 (data not shown). These studies indicated that modifications of the lipid A structure had no effect on susceptibility to killing by PNHS. DeMaria et al. reported similar findings with an htrB mutant of H. influenzae (11).
TEM and SEM analyses of uec infected with either 1291 or 1291A11K3. TEM studies showed cells infected with 1291A11K3 and 1291 and showed bacteria on the surfaces of the uec beginning to be internalized into the cells (Fig. 8A and B). These studies also showed both 1291 and 1291A11K3 bacteria entering uec and being internalized within vacuoles. In Fig. 8A and B, the tight association between the plasma membrane and the gonococci, indicative of a clathrin-dependent receptor-mediated endocytic process, is seen. SEM demonstrated a close association between the uec’s plasma membrane and both 1291A11K3 and 1291 bacteria (Fig. 8C and D). The close association between the plasma membrane and the bacteria suggests internalization by clathrin-independent receptor-mediated endocytosis. Additionally, the frequency at which the 1291A11K3 mutant was found within uec, on analysis of multiple fields using TEM, suggested that it was internalized at a rate similar to that of the parent strain.

Invasion assays performed with 1291 and 1291A11K3. In order to study the effects of the mshB mutation on gonococcal invasion of and adherence to uec, standard gentamicin survival invasion assays were performed. The results from the experiment shown in Fig. 9 are representative of what was consistently seen in six separate experiments. These results showed that there was no significant difference in the abilities of strains 1291 and 1291A11K3 to adhere to uec ($P = 0.6947$). However, there was a significant difference between the numbers of 1291 and 1291A11K3 bacteria recovered from the gentamicin-treated uec ($P = 0.0130$). These data, combined with the electron microscopic analyses, suggest that strain 1291A11K3 enters cells in a fashion similar to that of strain 1291 but may be more susceptible to innate intracellular killing mechanisms found within urethral epithelial cells.

### Table 3. Fatty acid analysis of the lipid A samples

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative % in:</th>
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<tbody>
<tr>
<td></td>
<td>1291 wild type</td>
</tr>
<tr>
<td>Lauric acid ($C_{12:0}$)</td>
<td>29.8</td>
</tr>
<tr>
<td>Palmitic acid ($C_{16:0}$)</td>
<td>1.6</td>
</tr>
<tr>
<td>Stearic acid ($C_{18:0}$)</td>
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<td>3-OH-lauric acid ($3-OH-C_{12:0}$)</td>
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</tr>
<tr>
<td>3-OH-myristic acid ($3-OH-C_{14:0}$)</td>
<td>25.7</td>
</tr>
<tr>
<td>Other components$^b$</td>
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$^a$ These fatty acids were detected in the lipid A preparations but were not observed in our analyses of intact lipid A species by mass spectrometric methods.

$^b$ This category includes minor amounts of $C_{12:1}$ and $C_{14:1}$ fatty acids and a few unidentified components.

![FIG. 7. Negative-ion electrospray MS/MS spectra of the monophosphorylated lipid A species from N. gonorrhoeae strain 1291 (left) and N. gonorrhoeae strain 1291A11K3 (right). The parent (M − H)$^-$ ions selected for collision-induced dissociation are indicated with arrows. Fragment ions are labeled on the spectra and indicated on the structures using a letter code. O-linked fatty acids are lost as free acids (a, b, c, and d cleavages) or ketenes (a', b', c', and d' cleavages). Fragments of type e and f are cross-ring cleavages.](image-url)
HtrB and MsbB are Kdo-dependent acyl transferases involved in the biosynthesis of LPS and LOS (6, 9, 36). These transferases are thought to act in a sequential manner, with HtrB acting first and MsbB acting second (9). The mass spectrometric analysis of lipid A isolated from mutants supports this: lipid A from an H. influenzae htrB mutant was determined to be 90% tetraacyl and 10% pentaacyl (31), whereas lipid A from an E. coli msbB mutant was determined to be pentaacyl (41). This suggests that HtrB is able to be fully functional in the absence of a functional MsbB acyl transferase. Mutants in htrB isolated from E. coli, H. influenzae, and serovar Typhimurium have all been shown to be initially sensitive to temperature (28, 31, 44); however, the msbB mutant isolated from E. coli was not temperature sensitive (41). It has been proposed that if the lipid A is at least pentaacyl, it is able to grow at higher temperatures (9).

Discerning whether the gene identified in these studies is an htrB or msbB homologue is difficult due to the high similarity of these acyl transferases at both the amino acid and DNA levels. The gene identified in these studies was able to complement an E. coli htrB mutant. However, previous studies have shown that both htrB and msbB can complement an htrB mutant (27, 28, 31). We may, however, be able to draw some conclusions based on the phenotypes of the mutant. Previous studies of an msbB mutant demonstrated that these bacteria were not temperature sensitive (41). The mutant, 1291A11K3, developed in these studies grew normally at 37°C, both in liquid broth and on solid medium (data not shown). Additionally, mass spectrometric analyses revealed that the lipid A isolated from 1291A11K3 was pentaacyl. Previous studies have suggested that the addition of the secondary acyl substitutions occurs in a sequential manner, with HtrB acting first and MsbB acting second (9). Therefore, the lipid A of an htrB mutant would be expected to be primarily tetraacyl, and the lipid A from an msbB mutant would be expected to be pentaacyl. Therefore, it seems likely that the gene identified in these studies is an msbB homologue. In addition, a recent study by van der Ley et al. (49) demonstrated that mutations in two htrB/msbB homologues in N. meningitidis resulted in lipid A
structures that were pentaacyl and tetraacyl. Their studies suggest that *Neisseria* bacteria may assemble their lipid A in a sequential manner, and therefore it seems likely that the gene identified in these studies acts in a fashion similar to that of the *msbB* gene from *E. coli*.

To establish the acylation pattern of the pentaacyl lipid A in 1291A11K3, we analyzed the monophosphoryl lipid A molecules from 1291 and 1291A11K3 using tandem mass spectrometry. In monophosphorylated lipid A molecules, the single phosphate group exists on the distal glucosamine and thus creates asymmetry in the structures. Furthermore, in negative-ion MS analysis, charge is expected to be retained on the lone phosphate group, facilitating interpretation of fragmentation pathways. The low-mass fragments observed in these studies were attributed to two-bond cleavages comprised of the loss of a single fatty acid moiety and a diagnostic cross-ring cleavage. The cross-ring cleavages established that the distal nonreducing terminal glucosamine of the pentaacyl 1291A11K3 lipid A sample is missing a lauric acid compared to the 1291 hexaacyl lipid A structure. In ESI-MS/MS investigations of the lipid A molecules from *Enterobacter agglomerans*, *Salmonella minnesota*, *S. minnesota E. agglomerans*, *Shigella flexneri*, low-mass fragment ions corresponding to those seen in the 1291 lipid A spectrum (m/z 691 and 709) were also observed. However, in those species, the fragment ions were assigned as nonreducing terminal acylium ions (5, 8). As acylium ions of that composition could not arise from the *N. gonorrhoeae* 1291 lipid A structure, we assigned the fragments differently. Comparison of the lipid A structures, however, suggests that in fact our assignments could also fit for *E. agglomerans*, *S. minnesota*, and *S. flexneri*, since the lipid A molecules from those species have the same N-linked acyloxyacyl fatty acids on their distal glucosamines as *N. gonorrhoeae* 1291. Consequently, the shift of those fragment ions to lower mass in the 1291A11K3 mutant provides strong evidence that a lone 3-hydroxymyristyl moiety exists in amide linkage on the distal glucosamine of the lipid A.

Modification of the acylation pattern of lipid A was not the only change that was found in the LOS of 1291A11K3. Changes in the phosphorylation pattern as well as the addition of higher-molecular-weight species of LOS were also seen. Previous studies by Lee et al. (31) showed similar modifications in the LOS of an *H. influenzae* htrB mutant. The high-molecular-weight species detected in the 1291A11K3 LOS appear to correspond to species that have been previously observed in the LOS from *N. gonorrhoeae* MS11mkC, a variant of *N. gonorrhoeae* strain MS1 isolated from men with gonorrhea (23). It is unclear if the bacteria use these modifications of their LOS to compensate for the changes in lipid A, or if the changes in the phosphorylation pattern and oligosaccharide portion of the LOS are directly regulated by HtrB and MsbB. Since HtrB and MsbB have both been shown to be acyl transferases, it seems unlikely that they have a direct role in the modifications of the oligosaccharide chain length or the amount of PEA present on the LOS (6, 9, 36). However, further studies are needed to try and determine more clearly the roles that *htrB* and *msbB* play in these modifications of the LOS.

Recent studies have focused on the factors involved in the entry of *N. gonorrhoeae* into eukaryotic cells. A number of bacterial and host factors have been identified. Piliation of the gonococcus, the presence of the Opa protein, and an intact LOS appear to be some of the prerequisite factors necessary for gonococcal invasion (20, 35). Additionally, studies involving the neisserial IgA1 protease have suggested that it may play a role in intracellular survival of the gonococcus (33). Our laboratory has been studying the role of gonococcal LOS in genital epithelial cell invasion. Our laboratory has shown that with male uec this process involves the asialoglycoprotein receptor (ASGP-R) (17). The ASGP-R is expressed on the surface of male uec and recognizes ligands with terminal galactose (17, 19, 37). The internalization of this complex has been shown to be a clathrin-dependent receptor-mediated endocytic process (17). Studies of both male urethral exudates and primary uec infected with *N. gonorrhoeae* have suggested that receptor-mediated endocytosis may be responsible for the internalization of a high percentage of these bacteria into male uec (2, 17, 18).

The invasion assays performed in this study suggested that...
there was no difference in the abilities of the 1291A11K3 bacteria and of the parent strain bacteria to adhere to uie. Additionally, the microscopy data clearly showed that both strains of bacteria were internalized by the uie and both were able to form close associations with the uie’s plasma membrane at similar rates. This association was indicative of entry by means of a receptor-mediated endocytosis mechanism. Therefore, the mutation in the msbB gene did not seem to have any apparent effects on the ability of this strain to be internalized by the uie.

The invasion assay data also showed that significantly fewer 1291A11K3 bacteria than parent strain bacteria were recovered from gentamicin-treated uie. At least two possibilities exist for explaining these results. First, the 1291A11K3 bacteria may invade the uie at a lower rate than the wild-type bacteria. Second, the 1291A11K3 organisms are more susceptible to intracellular killing mechanisms once they have been internalized by the uie. Since the bacteria seem to be internalized at similar rates the second explanation seems likely. After the male uie internalize the bacteria, the gonococci must be able to survive intracellularly. It is not known if the bacteria remain within a vacuole after internalization, and if so it is unclear how they are able to avoid endosomal degradation. Some studies have suggested that the neisserial IgA1 protease may be involved in intracellular survival of N. gonorrhoeae (33). IgA1 protease has been shown to cleave the host cell lysosome-associate membrane protein (LAMP-1) (33). LAMP-1 is thought to be involved in the maturation of late endosomes and lysosomes (33). The degradation of this glycoprotein is thought to contribute to the ability of the gonococcus to survive intracellularly (33). However, male volunteer studies demonstrated that a gonococcal IgA1 protease mutant was not compromised in its ability, compared to the parent strain, to cause urethritis (21).

Our studies presented here demonstrate that the mutation in the msbB gene in N. gonorrhoeae affects how these organisms interact with male uie. The modifications in the LOS may make the msbB organisms more susceptible to intracellular killing. Currently, the mechanisms by which epithelial cells are able to kill intracellular bacteria are not well understood. Further study of these killing mechanisms should enable future studies to determine more directly what role the LOS, and more specifically lipid A, plays in the survival of gonococci within male uie.

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