

Modification of Lipooligosaccharide with Phosphoethanolamine by LptA in *Neisseria meningitidis* Enhances Meningococcal Adhesion to Human Endothelial and Epithelial Cells[∇]

Hideyuki Takahashi,^{1*} Russel W. Carlson,² Artur Muszynski,² Biswa Choudhury,² Kwang Sik Kim,³ David S. Stephens,^{4,5,6} and Haruo Watanabe¹

Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan¹; University of Georgia, Complex Carbohydrate Research Center, Athens, Georgia²; Division of Pediatric Infectious Diseases, Department of Pediatrics, School of Medicine, John Hopkins University, Baltimore, Maryland³; Division of Infectious Diseases, Department of Medicine,⁴ and Department of Microbiology and Immunology,⁵ Emory University School of Medicine, Atlanta, Georgia; and Department of Veterans Affairs Medical Center, Atlanta, Georgia⁶

Received 30 May 2008/Returned for modification 10 July 2008/Accepted 9 September 2008

The lipooligosaccharide (LOS) of *Neisseria meningitidis* can be decorated with phosphoethanolamine (PEA) at the 4' position of lipid A and at the O-3 and O-6 positions of the inner core of the heptose II residue. The biological role of PEA modification in *N. meningitidis* remains unclear. During the course of our studies to elucidate the pathogenicity of the ST-2032 (invasive) meningococcal clonal group, disruption of *lptA*, the gene that encodes the PEA transferase for 4' lipid A, led to a approximately 10-fold decrease in *N. meningitidis* adhesion to four kinds of human endothelial and epithelial cell lines at an multiplicity of infection of 5,000. Complementation of the *lptA* gene in a Δ *lptA* mutant restored wild-type adherence. By matrix-assisted laser desorption ionization–time-of-flight mass spectrometry analysis, PEA was lost from the lipid A of the Δ *lptA* mutant compared to that of the wild-type strain. The effect of LptA on meningococcal adhesion was independent of other adhesins such as pili, Opc, Opa, and PilC but was inhibited by the presence of capsule. These results indicate that modification of LOS with PEA by LptA enhances meningococcal adhesion to human endothelial and epithelial cells in unencapsulated *N. meningitidis*.

Neisseria meningitidis is a gram-negative diplococcal pathogen that colonizes the nasopharynx of humans as a unique host and may enter the bloodstream, where it can cause fulminant septicemia or induce acute meningitis after passing through the brain-blood barrier and gaining access to cerebrospinal fluid (CSF). Meningococcal factors such as Opa, Opc, pili, and PilC are known to participate in the adhesion to and invasion of human host cells (reviewed in reference 9). Non-protein factors such as capsule and lipooligosaccharide (LOS) also play important roles in meningococcal infections in humans (30, 34, 42, 49). However, many questions about meningococcal pathogenicity remain unsolved (26–28).

Meningococcal LOS, an important inducer of the host inflammatory response in meningococcal sepsis, is structurally distinct from lipopolysaccharide (LPS) of enteric gram-negative bacteria. Instead of the repeating O antigens of enteric LPS, meningococcal LOS consists of short variable oligosaccharide (OS) residues linked to two conserved core heptose molecules attached to lipid A via a ketodeoxyoctulonic acid molecule (Kdo I). The OS chains are the alpha chain branching from heptose I (Hep I) in a β (1,4) linkage and the beta and gamma chains branching from heptose II (Hep II) in α (1,3) and α (1,2) linkages, respectively (Fig. 1) (44). Lipid A triggers the Toll-like receptor 4/MD-2-mediated inflammatory response. Changes in the configuration or the conformational

structure of lipid A affect the biological response (31). Structural diversity of meningococcal LOS is due to variability of OS and inner core moieties and leads to immunological differences characterized as 12 distinct immunotypes (44). Diversity is due to the presence, absence or variability of expression of LOS biosynthesis-associated genes (3, 18, 20). Variation of the meningococcal LOS structure also mediates differences in host immune responses and bacterial virulence (32, 44).

Meningococcal LOS is modified with one or possibly multiple phosphoethanolamine (PEA) groups at the 4' lipid A moiety by a PEA transferase named LptA (PEA transferase specific for lipid A) (7). In addition to the modification at lipid A, the LOS may also be modified with PEA groups at Hep II by two kinds of other PEA transferases, named Lpt6 (50) and Lpt3 (25). Lpt6 and Lpt3 add a PEA group at the 6 or 3 position, respectively (50). A glucose residue can also be added at the 3 position by the *lgtG* gene product (α 1,3-galactosyl transferase) (25). The presence of *lpt6*, *lpt3*, and *lgtG* varies among meningococcal isolates (50). The LOS modification by LgtG is phase variable due to slippage of a homopolymeric tract of cytidines (18). The Hep II LOS modification with PEA is dependent on the presence of *lpt3* and/or *lpt6* in a strain, but the addition of the glucose residue at the 3 position is dominant if the *lgtG* and *lpt3* genes are both present in a strain (20). The modification by Lpt3, Lpt6, and LgtG changes the LOS inner core structure and influences the immunotype (3, 18). There is epidemiological evidence that specific kinds of immunotypes, such as L1,8 (33) and L3,7,9 (19), are correlated with meningococcal pathogenesis. Experimentally, an *N. meningitidis* strain with the L3,7,9 immunotype enhances colonization at

* Corresponding author. Mailing address: Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111. Fax: 81-3-5285-1163. E-mail: hideyuki@nih.go.jp.

[∇] Published ahead of print on 29 September 2008.

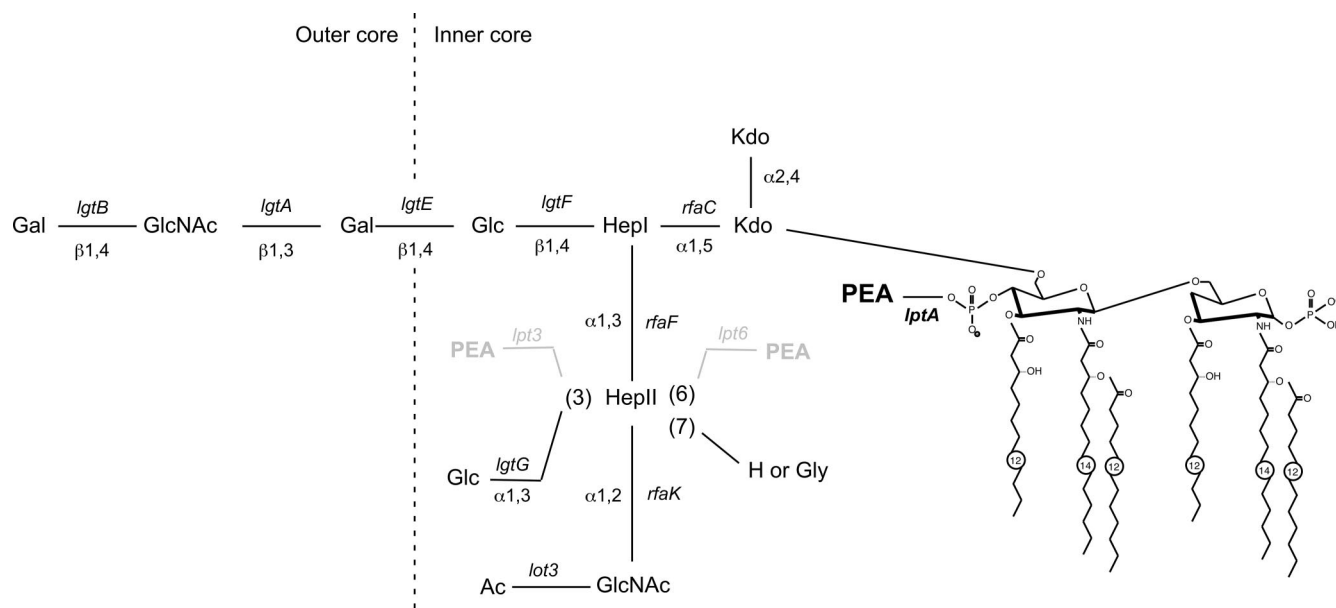


FIG. 1. Schematic representation of the LOS structure in strain HT1125 of *N. meningitidis*. The LOS structure of *N. meningitidis* HT1125 was determined by glycosyl composition, glycosyl linkage, nuclear magnetic resonance, and MALDI-TOF MS analyses of the intact LOS, de-O-acylated LOS, isolated lipid A, and isolated OSs from purified LOS (data not shown). The biosynthesis-associated genes are shown in italic at the respective positions. PEA groups at Hep II of the 3 and 6 positions, shown in gray, were absent in the HT1125 strain.

the nasal region in an infant mouse intranasal infection model (24), and an L8 *N. meningitidis* strain adheres to human umbilical vein endothelial cells (HUVEC) more efficiently than an L3 immunotype strain (48). However, the precise role of LOS structure in meningococcal adherence and invasion remains unclear. An encapsulated LOS-deficient mutant shows impaired adherence to human epithelial cells (2), while encapsulated *N. meningitidis* *pgm*, *rafK*, or *gmnB* mutants, producing a gradual truncation of the outer and inner cores of the LOS, showed enhanced adherence to Detroit 562 epithelial cells (29). An unencapsulated meningococcal strain enhanced the adherence to dendritic cells by the gradual truncation of meningococcal LOS with a mutation in the *lst*, *lgt*, *galE*, or *pgm* gene (22). However, on the whole, the relationship between adhesive ability and LOS structure, including the PEA modification, in *N. meningitidis* is still unclear (7).

We previously reported that a Japan-specific invasive clone, ST-2032, efficiently adheres to and invades human cultured endothelial and epithelial cell lines (36). During the course of our studies to elucidate the pathogenicity of the ST-2032 clone, we noted a relationship between LOS modifications and meningococcal adherence. In the present study, the role of PEA modification in meningococcal adherence to human cells was examined, and it was found that the PEA modification by LptA enhanced meningococcal adherence to human endothelial and epithelial cells.

MATERIALS AND METHODS

Bacterial growth conditions. *N. meningitidis* strains stored at -80°C were routinely grown on GC agar plates, consisting of GC medium agar (Becton-Dickinson) supplemented with 1% IsoVitalX enrichment (Becton-Dickinson), at 37°C in 5% CO_2 (37). For selecting kanamycin-resistant *N. meningitidis* strains, brain heart infusion agar (Becton-Dickinson) agar containing 3% defibrinated horse blood (Nihon Biotest, Japan) was used. *Escherichia coli* was grown on L

plates or in L broth liquid culture at 37°C . When required, antibiotics were added at the following concentrations: kanamycin at $150\ \mu\text{g/ml}$, erythromycin at $4\ \mu\text{g/ml}$, chloramphenicol at $5\ \mu\text{g/ml}$, and spectinomycin at $75\ \mu\text{g/ml}$ for *N. meningitidis*, and kanamycin at $50\ \mu\text{g/ml}$, erythromycin at $150\ \mu\text{g/ml}$, chloramphenicol at $10\ \mu\text{g/ml}$, ampicillin at $50\ \mu\text{g/ml}$, and spectinomycin at $75\ \mu\text{g/ml}$ for *E. coli*. All of the strains used in this study are listed in Table 1.

Production of anti-LptA rabbit antiserum. A 1.6-kb DNA fragment containing the coding region of the *lptA* gene was amplified with a set of primers (NMB1638-pET24a-5 and NMB1638-pET24a-2 [Table 2]) by PCR. The DNA fragment was digested with BamHI and XhoI and cloned into the same cutting sites of the expression vector pET24a (Novagen), resulting in pHT463. Plasmid pHT463 was transformed into *E. coli* strain BL21(DE3) (Novagen). The purification and raising of a polyclonal rabbit serum to LptA were performed as described previously (38).

Construction of meningococcal mutants. An *N. meningitidis* mutant with *lptA* deleted was constructed as follows. A 4-kb DNA fragment containing *lptA* was amplified with primers *lptA*-1 and *lptA*-2 (Table 2) from HT1125 chromosomal DNA and cloned in the SmaI site of pMW118 (Nippon Gene, Japan) to construct pHT432. A 1-kb SmaI-HincII spectinomycin resistance gene (*spe*) was inserted into the EcoRV and NruI sites of pHT432, which are located inside and outside the coding region of the *lptA* gene, respectively, to generate pHT433. Five hundred nanograms of linearized pHT433 was transformed into NIID280 and HT1125, and spectinomycin-resistant (Sp^r) clones were selected, resulting in *lptA* deletion mutants named HT1219 and HT1222, respectively.

The transgenic meningococcal mutant that contained a wild-type *lptA* gene ectopically complemented in the $\Delta lptA$ mutant at the *ggt* locus was constructed as follows. A 1.2-kb fragment containing a chloramphenicol resistance gene (*cat*) was inserted in a blunted SphI site of pHT432, resulting in pHT473. A SacI-HindII fragment containing *lptA*⁺-*cat* genes was inserted in a blunted BstXI site, which is located in the middle of the coding region of *ggt*, of pHT195 (38) to construct pHT507. The negative control was also constructed by inserting only a *cat* gene in the BstXI site and was named pHT508. Five hundred nanograms of linearized pHT507 was transformed into HT1222 and chloramphenicol-resistant (Cm^r) clones were selected, resulting in the ectopic complementation of the *lptA*⁺ gene at the *ggt* loci in the *lptA* deletion mutant of NIID280; this strain was named HT1277. The negative control mutant HT1278 was constructed by the same procedure by transforming HT1222 with linearized pHT508.

HT1002 ($\Delta\text{opc}::\text{cat}$) and HT1015 (*pilE::ermC*) were constructed by transforming linearized pHT10, which is a derivative of pHT148 (37) with the *ermC* gene

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant properties	Reference(s) or source
<i>N. meningitidis</i> strains		
Wild-type parent strains (ST, serogroup, immunotype)		
NIID280 (2032, untypeable, L5)	<i>lpt3 lpt6 lgtG⁺ lot3⁺</i>	36
H44/76 (32, B, L3)	<i>lpt3⁺ lpt6 lgtG lot3^a</i>	18, 36, 37
NIID311 (5, A, L2)	<i>lpt3 lpt6⁺ lgtG⁺ lot3⁺</i>	36
Mutants		
NIID280 derivatives		
HT1125	NIID280 Δ <i>siaB-D::kan</i>	36
HT1219	NIID280 Δ <i>lptA::spc</i>	This study
HT1222	NIID280 Δ <i>siaB-D::kan</i> Δ <i>lptA::spc</i>	This study
HT1277	NIID280 Δ <i>siaB-D::kan</i> Δ <i>lptA::spc</i> <i>ggt::lptA⁺-cat</i>	This study
HT1278	NIID280 Δ <i>siaB-D::kan</i> Δ <i>lptA::spc</i> <i>ggt::cat</i>	This study
HT1238	NIID280 Δ <i>siaB-D::kan</i> <i>pilE::ermC</i>	This study
HT1249	NIID280 Δ <i>siaB-D::kan</i> <i>pilE::ermC</i> Δ <i>lptA::spc</i>	This study
HT1178	NIID280 <i>pilC1::spc</i>	36
HT1182	NIID280 Δ <i>pilC2::ermC</i>	36
HT1186	NIID280 <i>pilC1::spc</i> Δ <i>pilC2::ermC</i>	36
H44/76 derivatives		
HT1034	H44/76 Δ <i>siaB-D::kan</i>	36
HT1378	H44/76 Δ <i>lptA::spc</i>	This study
HT1243	H44/76 <i>siaB-D::kan</i> Δ <i>lptA::spc</i>	This study
HT1235	H44/76 <i>siaB-D::kan</i> <i>pilE::ermC</i>	This study
HT1245	H44/76 <i>siaB-D::kan</i> Δ <i>lptA::spc</i> <i>pilE::ermC</i>	This study
HT1234	H44/76 <i>siaB-D::kan</i> Δ <i>opc::cat</i>	This study
HT1244	H44/76 <i>siaB-D::kan</i> Δ <i>lptA::spc</i> Δ <i>opc::cat</i>	This study
HT1236	H44/76 <i>siaB-D::kan</i> <i>pilE::ermC</i> Δ <i>opc::cat</i>	This study
HT1246	H44/76 <i>siaB-D::kan</i> Δ <i>lptA::spc</i> Δ <i>opc::cat</i> <i>pilE::ermC</i>	This study
HT1295	H44/76 <i>lptA⁺-FLAG-cat</i>	This study
HT1015	H44/76 <i>pilE::ermC</i>	This study
HT1002	H44/76 Δ <i>opc::cat</i>	This study
NIID311 derivatives		
HT1336	NIID311 Δ <i>sacAB::kan</i>	36
HT1380	NIID311 Δ <i>lptA::spc</i>	This study
HT1359	NIID311 <i>sacAB::kan</i> Δ <i>lptA::spc</i>	This study
<i>E. coli</i> strains		
JM109	<i>endA1 gyrA96 hsdR17(r_K⁻ m_K⁺) mcrB⁺ recA1 relA1 supE44 thi-1 Δ(lac-proAB) F⁻ [traD36 proAB lacI^qZΔM15]</i>	Takara Bio
BL21(DE3)	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dem</i> (DE3)	Novagen
Plasmids		
pET24a	Expression vector; Km ^r	Novagen
pHT463	Derivative of pET24a expressing <i>lptA</i> gene; Km ^r	This study
pMW118	Cloning vector; Ap ^r	Nippon Gene
pHT432	Derivative of pMW118 carrying an <i>lptA</i> gene; Ap ^r	This study
pHT433	Derivative of pHT432 carrying a Δ <i>lptA::Spc^r</i> gene; Ap ^r Spc ^r	This study
pHT473	Derivative of pHT432 carrying a <i>cat</i> gene at the SphI site; Ap ^r Cm ^r	This study
pTWV228	Cloning vector; Ap ^r	Takara Bio
pHT195	Derivative of pTWV228 carrying a <i>ggt</i> gene; Ap ^r	38
pHT507	Derivative of pHT195 carrying <i>lptA⁺-cat</i> genes at the BstXI site; Ap ^r Cm ^r	This study
pHT508	Derivative of pHT195 carrying a <i>cat</i> gene at the BstXI site; Ap ^r Cm ^r	This study
pGEM-T	PCR product cloning vector; Ap ^r	Promega
pHT515	Derivative of pGEM-T carrying a 1-kb downstream region of the <i>lptA</i> gene; Ap ^r	This study
pHT516	Derivative of pGEM-T carrying a 3' part of the <i>lptA</i> gene with a FLAG epitope; Ap ^r	This study
pHT518	Derivative of pHT516 carrying a 1-kb downstream region of the <i>lptA</i> gene; Ap ^r	This study
pHT519	Derivative of pHT518 carrying a <i>cat</i> gene at the HpaI site; Ap ^r Cm ^r	This study
pUC118	Cloning vector; Amp ^r	Takara Bio
pHT148	Derivative of pUC118 carrying a Δ <i>opc::ermC</i> gene; Ap ^r Erm ^r	37
pHT10	Derivative of pHT148 carrying a Δ <i>opc::cat</i> gene; Ap ^r Cm ^r	This study
pHT434	Derivative of pHT432 deleted of 0.7 kb of the NruI-SphI DNA fragment; Ap ^r Spc ^r	This study
pGSS33	Broad-host-range vector; Ap ^r Cm ^r Str ^r Tet ^r	37
pSTV28	Cloning vector; Cm ^r	Takara Bio
pHT261	Derivative of pGSS33 carrying a <i>cat</i> gene and multicloning site of pSTV28 at the SacI and Sall sites; Cm ^r	This study
pHT435	Derivative of pHT261 carrying an <i>lptA</i> gene; Cm ^r	This study

^a The genes were detected but not expressed by the frameshift (18, 20).

TABLE 2. Oligonucleotides used in this study

Primer	Target gene	Nucleotide sequence (5'→3') ^a
NMB1638-pET24a-5	<i>lptA</i>	CGggtaccTACGCCTCGTTTTTCCG CAACAATAAATCA
NMB1638-pET24a-2	<i>lptA</i>	CCGctcgagTGC CGCGGACGGCGGC AGGCTGCCAA
<i>lptA</i> -1	<i>lptA</i>	ACGCAGGGTTCAAGATGAGT TGAA
<i>lptA</i> -2	<i>lptA</i>	CCCAGCAAACCTGCCTATCC GAAA
<i>lptA</i> -13	<i>lptA</i>	GCCGATATGCCGTCTGAAGCC TTCGG
<i>lptA</i> -14	<i>lptA</i>	AActgcagCCGGATACCGCTACCA CAACTGGGG
<i>lptA</i> -15	<i>lptA</i>	GCCGCTGCTTGCCGCGCGCG GCGA
<i>lptA</i> -16-FLAG	<i>lptA</i>	GGGgttaacTTACTTGTCGTCATC <u>GTCTTGTAGTCGCGCGGGC</u> AGGCTGCCAATATATC

^a Lowercase indicates additional nucleotides used to create the restriction sites. The underlined nucleotides were originally added to attach the FLAG epitope (see Materials and Methods).

replaced with a *cat* gene, and linearized pHT72 (37) into H44/76 and selecting Cm^r and erythromycin-resistant (Erm^r) clones, respectively.

The Δ *lptA::spc* (HT1219), *pilE::ermC* (HT1015), or Δ *opc::cat* (HT1002) mutation was transferred to another genetic background by transforming an *N. meningitidis* strain with the purified chromosomal DNA and selecting Spc^r, Erm^r, or Cm^r clones, respectively (Table 1).

Construction of an *lptA*-FLAG epitope-tagged fusion *N. meningitidis* strain. An *N. meningitidis* strain with a FLAG epitope-tagged *lptA* gene was constructed as follows. A 1-kb DNA fragment of the downstream region of the *lptA* gene amplified by PCR with primers *lptA*-13 and *lptA*-14 (Table 2) was cloned into the pGEM-T vector (Promega) to construct pHT515. A 0.9-kb DNA fragment containing the 3' part of the *lptA* gene was amplified with primers *lptA*-15 and *lptA*-16-FLAG (Table 2) and cloned into the pGEM-T vector to construct pHT516. A 1-kb SpeI-PstI fragment of pHT515 was inserted in the same site of pHT516 to construct pHT518. A blunted 1.2-kb fragment containing a *cat* gene was inserted in an HpaI site of pHT518, resulting in pHT519. Five hundred nanograms of linearized pHT519 was transformed into the H44/76 *N. meningitidis* strain and Cm^r clones were selected, resulting in the *lptA*-FLAG epitope-tagged strain of H44/76, named HT1295 (see Fig. 4).

SDS-polyacrylamide gel electrophoresis and Western blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (36, 37).

Transmission electron microscopy. *N. meningitidis*, grown on GC agar plates overnight, was scraped from the top of colony and dispensed in 1 drop of HEPEs-minimal essential medium. A 400-mesh Formvar-coated copper grid (Electron Microscopy Sciences) was inversely put on the bacterial suspension and left for 1 min. The grid was then fixed with 1 drop of 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 min, followed by washing in 2 drops of sterile H₂O for a few seconds. The fixed grid was stained with 1 drop of 1% sodium phosphotungstate (pH 6.0) for 40 s. After removal of the residual solution, the grids was completely dried and stored until observation. Electron microscopy was performed with a Hitachi H-7500 transmission electron microscope.

Preparation of cellular protein fractions. Cellular fractionation was performed as described previously (38). In brief, bacterial cells in suspension in ice-cold Dulbecco's phosphate-buffered saline (PBS) were disrupted by sonication and centrifuged at 1,000 × *g* for 5 min to remove undisturbed cells. The supernatant was transferred to a new tube and centrifuged again at 15,000 × *g* for 10 min. Proteins in the supernatant fraction (cytoplasmic and periplasmic proteins) were precipitated with trichloroacetic acid, and the resultant pellet was dissolved in 20 μl of 2 M Tris base and an equal volume of 2× SDS buffer. The pellet fraction (membrane fractions) was further fractionated by suspension in 20 μl of 4% Triton X-100 in Dulbecco's PBS and, after incubation at 37°C for 30 min, centrifuged at 15,000 × *g* for 10 min. The Triton-soluble supernatant (inner membrane-enriched fraction) was mixed with an equal volume of 2× SDS buffer,

and the Triton-insoluble pellet (outer membrane-enriched fraction) was dissolved in 40 μl of SDS buffer.

Tissue culture. Human brain microvascular endothelial cells (HBMEC), HUVEC (Toyobo, Japan), and epithelial cells (larynx carcinoma cell line Hep-2 and lung carcinoma cell line A549) were cultivated as described previously (36). A549 cells were also cultivated in serum-free medium (XTen GO; Thermo Trace) supplemented with 1 mg/ml of streptomycin and penicillin for experiments performed under serum-free condition (see Fig. 9C).

Determination of bacteria associated with and internalized in host cells. Infection assays using tissue culture were performed as described previously (36, 45). Endothelial or epithelial cells were seeded on gelatin-coated (for endothelial cells) or noncoated (for epithelial cells) 96-well tissue culture plates (Iwaki) (10⁴ cells) and incubated at 37°C with 5% CO₂ for 1 day. At 2 h prior to the bacterial infection, the culture medium was replaced with assay medium (AM), which was MCDB131 supplemented with 10% fetal bovine serum, 90 μg ml⁻¹ of heparin, and 3 mM glutamine unless otherwise indicated. In a standard experiment, the bacterial suspension was prepared in AM at an optical density at 600 nm (OD₆₀₀) of 0.5, which corresponded to approximately 5 × 10⁸ CFU ml⁻¹. If a reduced multiplicity of infection (MOI) was indicated, the bacterial suspension was prepared by further dilution. One hundred microliters of the bacterial suspension was inoculated onto host cell monolayers in duplicate for each assay and incubated at 37°C with 5% CO₂ for 4 h. To determine bacterial adherence, the monolayers were washed with AM four times to remove nonadherent bacteria. Adherent bacteria were released by addition of PBS (Nissui, Japan) containing 2% saponin, and CFU were determined on GC agar plates with appropriate dilution to count cell-adherent bacteria. To determine internalized bacteria, AM containing 150 μg ml⁻¹ of gentamicin was added and further incubated for 1 h to kill all extracellular bacteria. It was confirmed that 5 × 10⁷ extracellular meningococci were almost completely (>99.999%) killed under these experimental conditions (data not shown). Internalized bacteria that were not killed by the gentamicin treatment were determined by addition of PBS containing 2% saponin and plating on GC agar after appropriate dilutions to count the bacterial numbers as CFU. Results are expressed as means ± standard deviations, and bacterial numbers were statistically compared using a two-tailed Student *t* test.

Observation of infected cells by Giemsa staining. HBMEC were seeded on one well of two-well Lab-Tek II chamber slides (Nalge Nunc International) (10⁵ cells) and incubated at 37°C with 5% CO₂ for 1 day. One milliliter of bacterial suspension containing 5 × 10⁸ bacteria was inoculated onto cell monolayers and incubated at 37°C with 5% CO₂ for 4 h. The monolayer was washed four times with AM, fixed with absolute methanol for 1 min, and stained with Giemsa staining solution (Wako, Japan). After the stained cells were washed with H₂O, the bacterial adhesion was observed under oil immersion.

Purification of meningococcal LOS and isolation of the LOS OSs and lipid A. LOS samples were prepared from *N. meningitidis* strains grown on 120 GC agar plates. The bacteria were scraped and put into a 200-ml glass flask. Fifty milliliters of fresh extraction solvent (6.7 ml of 90% phenol, 16.7 ml of chloroform, and 26.7 ml of petroleum ether) was added to the flask, and the bacteria were homogenized with a PT1300D homogenizer (Polytron) five times for 30 s each with 30-s intervals in an ice bath at 15,000 rpm. The bacterial suspension was stirred for 2 h at room temperature and then centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was transferred to a new flask, and the resultant pellet was again extracted with a new 50 ml of fresh extraction solvent by the same procedure. The total 100 ml of supernatant obtained by this procedure was evaporated in a water bath at 40°C for 3 min; the evaporation condenser was cooled at 4°C and vacuumed at 670 hPa, resulting in approximately 14 ml of solvent. The solvent was precipitated by adding 14 ml of diethyl ether and 70 ml of acetone and centrifuged at 10,000 × *g* at 4°C for 15 min. The precipitated LOS was dried for overnight under reduced pressure. The dried LOS was dissolved in 5 ml of H₂O and stored at -70°C before use. Samples were then subjected to mild acid hydrolysis in 1% (vol/vol) aqueous acetic acid for 1.5 h at 100°C with constant stirring. The lipid A fractions were separated from OS by centrifugation at 3,500 × *g* for 15 min at 4°C, suspended in water, and centrifuged again; this water washing was repeated one more time. The lipid A sediment fractions were saved for analysis. The original and wash supernatants were combined and lyophilized for each LOS. They contained the released OSs. The OSs were further purified by gel filtration chromatography using a Bio-Gel P-4 (Bio-Rad) column (120 by 1 cm) and eluted with deionized water. The eluting fractions were recorded with a Shimadzu refractive index detector (RID-10A).

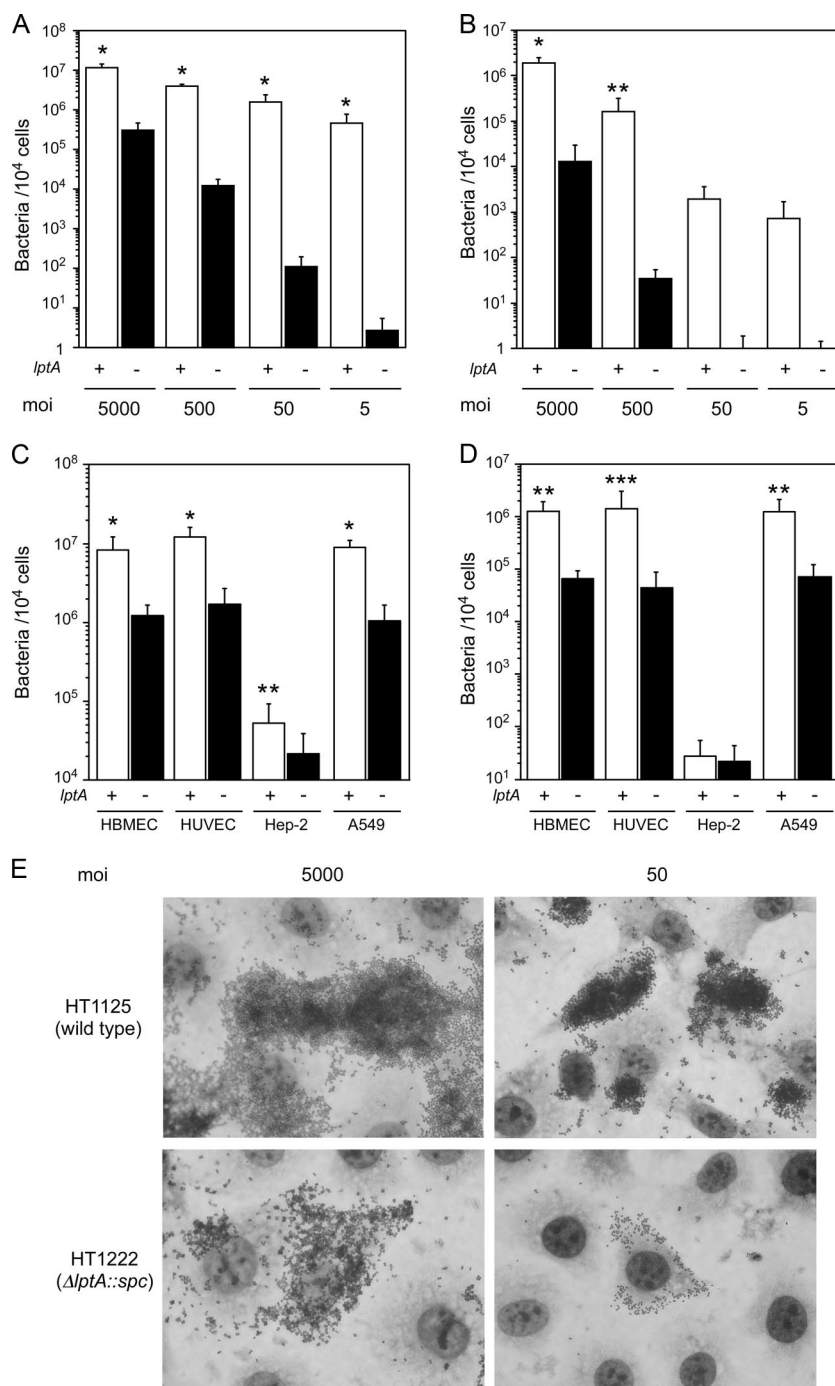


FIG. 2. Adherence and internalization of the $\Delta lptA$ *N. meningitidis* mutant to human endothelial and epithelial cells. (A and B) Adhesion (A) and internalization (B) of *N. meningitidis* to HBMEC at MOIs of 5,000, 500, 50, and 5. (C and D) Adhesion (C) and internalization (D) of *N. meningitidis* to human endothelial cells (HBMEC and HUVEC) and human epithelial cells (HEp-2 and A549) at an MOI of 5,000. Bacterial numbers are represented as CFU. Internalized bacteria were determined as the gentamicin-resistant bacterial number. Mean CFU of adherent bacteria (A and C) or gentamicin-resistant bacteria (B and D) per 10^4 cells in at least three experiments are shown, and error bars represent the standard errors of the means. Open and filled bars indicate the numbers of wild-type (HT1125) and $\Delta lptA$ (HT1222) *N. meningitidis* cells, respectively. *, $P < 0.001$; **, $P < 0.05$; ***, $P = 0.06$ (compared to the $\Delta lptA$ strain). (E) Meningococcal adhesion to HBMEC was observed by light microscopy with Giemsa staining. Infection with wild-type (HT1125) and $\Delta lptA$ (HT1222) *N. meningitidis* strains at MOIs of 5,000 and 50 is shown.

MS analysis of lipid A fractions. The lipid A fraction was analyzed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) on an Applied Biosystems 4700 proteomics analyzer. Lipid A samples were dissolved in chloroform-methanol (3:1, vol/vol) and mixed with 0.5 M 2,4,6-trihydroxyacetophenone matrix in methanol. The lipid A analyses were performed in a negative-reflectron mode.

Construction of the meningococcal *lptA* gene on a multicopy plasmid and introduction into *N. meningitidis*. To isolate the DNA region containing *lptA*, pHT432 was digested with *Nru*I and *Sal*I, followed by blunting and self-ligation to obtain pHT434. *Eco*RI-*Sph*I 2.4-kb DNA fragments of pHT434 were cloned into the same sites of pHT261, which was a derivative of the IncQ broad-host-range vector pGSS33 (37) (Table 1), resulting in pHT435. Intro-

duction of the plasmids into *N. meningitidis* was performed as described previously (37).

RESULTS

Disruption of *lptA* in *N. meningitidis* reduces meningococcal adherence to HBMEC at any MOI. To study the biological role of LptA in *N. meningitidis*, a $\Delta lptA$ mutation was introduced into the ST-2032 strain, and adherence and internalization were examined by in vitro infection assays using HBMEC at MOIs of 5,000, 500, 50, and 5 (Fig. 2A). The growth rate of the $\Delta lptA$ mutant (HT1222) in AM (see Materials and Methods) was not different from that of the wild-type strain HT1125 (data not shown). At an MOI of 5,000, the $\Delta lptA$ mutant HT1222 adhered to and invaded HBMEC approximately 10 times less efficiently than the wild-type strain HT1125 (Fig. 2A and B). The inefficiency of adhesive and invasive activities of the $\Delta lptA$ mutant was more apparent in proportion to the reduction of the MOI (Fig. 2A and B). These results suggested that the mutation in the *lptA* gene influenced the meningococcal adhesion to HBMEC even at an MOI of 5,000. Thereafter, to maximize the number of internalized bacteria and to exclude the overestimation of adhesion defectiveness of the $\Delta lptA$ mutant compared to the wild-type strain, bacteria associated with and internalized in host cells were determined at an MOI of 5,000 unless otherwise indicated.

Disruption of *lptA* in *N. meningitidis* reduces meningococcal adherence to human endothelial and epithelial cell lines. The defectiveness of adhesion of the $\Delta lptA$ mutant was also examined with the other endothelial cell line (HUVEC) and two kinds of epithelial cell lines (A549 and HEp-2). The HT1222 strain adhered to all of the human cell lines approximately 10 times less efficiently than the HT1125 strain (Fig. 2C). The number of internalized HT1222 bacteria also decreased to approximately 1/10 of that of HT1125 (Fig. 2D). All these results suggested that the *lptA* gene affected the adhesion of *N. meningitidis* to human cells.

Confirmation of the adhesion defect of the $\Delta lptA$ mutant by microscopy. The adhesion of the $\Delta lptA$ *N. meningitidis* mutant to HBMEC was further examined by light microscopy. At an MOI of 5,000, the $\Delta lptA$ mutant HT1222 adhered less to HBMEC than the wild-type strain HT1125 (Fig. 2E, left panels). At an MOI of 50, decreased adherence of the $\Delta lptA$ mutant was also observed (Fig. 2E, right panels). These data confirmed that disruption of the *lptA* gene in *N. meningitidis* reduced meningococcal adhesion to human cells.

Complementation with the wild-type *lptA* gene in the $\Delta lptA$ mutant restores meningococcal adhesion to HBMEC. To confirm the effect of $\Delta lptA$ *N. meningitidis*, the wild-type *lptA* gene was ectopically complemented in *ggt* of the $\Delta lptA$ mutant. The insertion at the *ggt* locus does not affect adhesive activity (35). The LptA protein was sufficiently expressed in the *lptA*⁺-complemented $\Delta lptA$ mutant HT1277 but not in the isogenic strain HT1278, which was not complemented (Fig. 3A). The adhesive activity of strain HT1277 was completely recovered to the level of the wild-type strain HT1125, but recovery was not observed in the HT1278 strain (Fig. 3B). The same results were obtained for meningococcal bacterial internalization (Fig. 3C). These results suggested that the *lptA* gene product affects the adhesion of *N. meningitidis* to human cells.

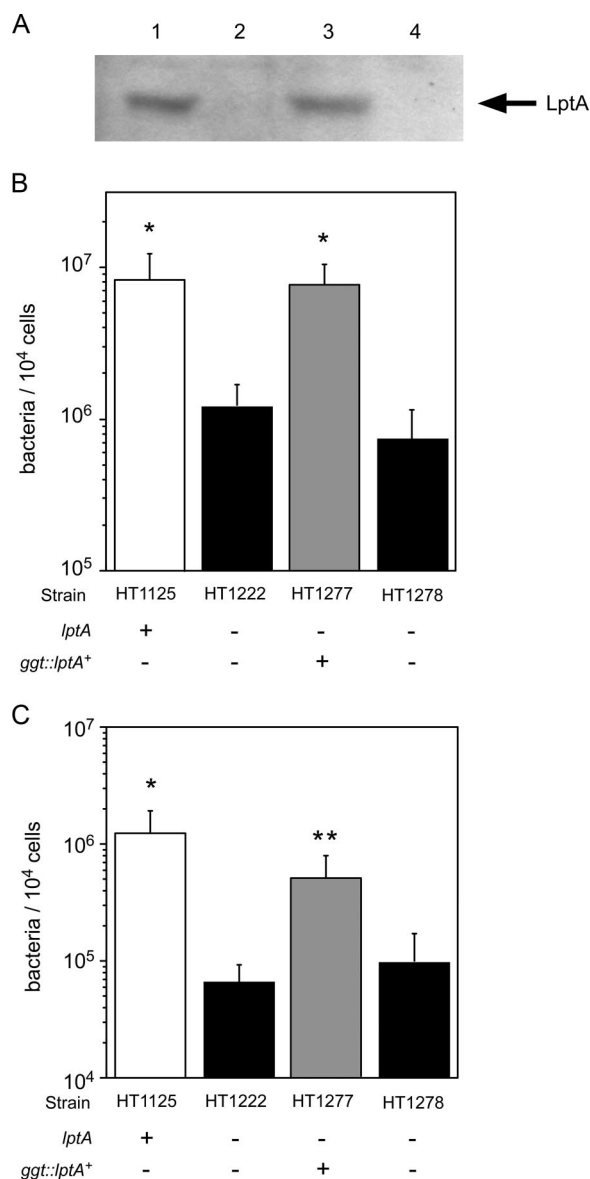


FIG. 3. Effect of complementation of the *lptA*⁺ gene in $\Delta lptA$ *N. meningitidis* on bacterial adhesion to HBMEC. (A) Western blotting for LptA protein. Bacterial extracts equivalent to an OD₆₀₀ of 0.05 were analyzed. Lane 1, HT1125 (wild type); lane 2, HT1222 ($\Delta lptA$); lane 3, HT1277 ($\Delta lptA$ *ggt::lptA*⁺-*cat*); lane 4, HT1278 ($\Delta lptA$ *ggt::cat*). (B and C) Adhesion (B) and internalization (C) of the $\Delta lptA$ *N. meningitidis* mutant in which the *lptA*⁺ gene was ectopically complemented. The internalized bacteria were determined as the number of gentamicin-resistant bacteria. Mean CFU of adherent bacteria (B) or gentamicin-resistant bacteria (C) per 10⁴ cells of HBMEC in at least three experiments are shown, and error bars represent the standard errors of the means. *, $P < 0.01$; **, $P < 0.05$ (compared to the strain not complemented with the *lptA*⁺ gene).

LptA does not directly act as an adhesin molecule. The LptA protein might act as a direct adhesion molecule on the meningococcal surface. In fact, a signal peptide sequence was found in the deduced amino acid sequence of the LptA protein (data not shown). To assess the location of meningococcal LptA in *N. meningitidis*, the cellular proteins were fractionated and analyzed by Western blotting with an anti-meningococcal

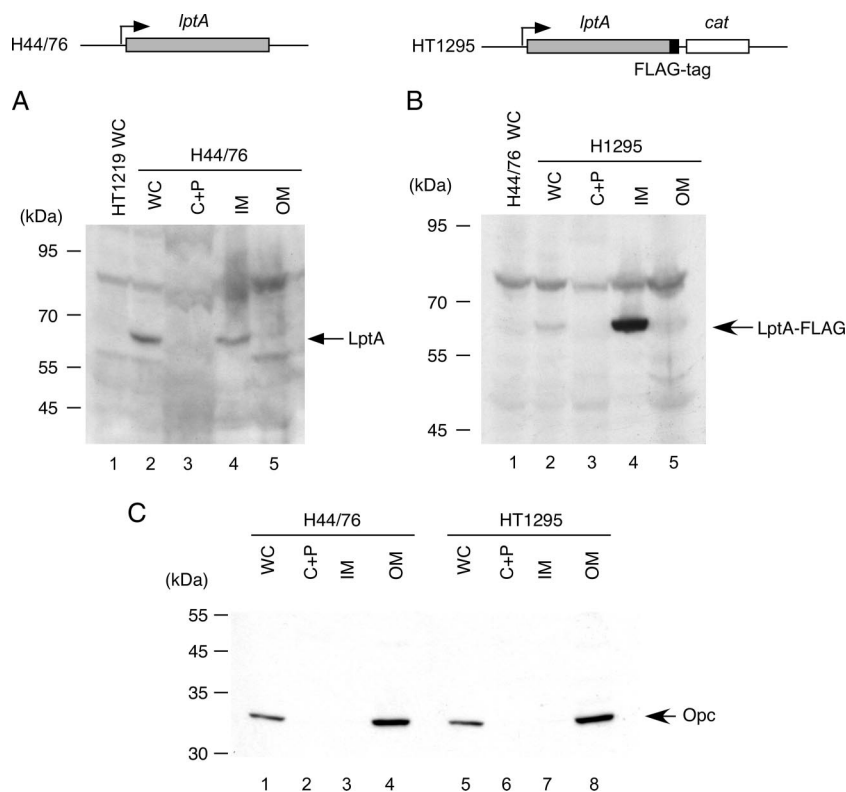


FIG. 4. Localization of the LptA protein in *N. meningitidis*. Whole-cell lysates (WC) and the cytoplasmic and periplasmic fraction (C+P), the inner-membrane-enriched fraction (IM), and the outer-membrane-enriched fraction (OM) prepared by sonication from *N. meningitidis* strain H44/76 (A and C) or HT1295 (B and C) were separated and analyzed by Western blotting. (A and B) Five microliters of each sample was analyzed by Western blotting with anti-LptA rabbit antiserum (A) or with anti-FLAG monoclonal antibody (mAb) (B). (C) One microliter of each sample was analyzed to detect the Opc protein by Western blotting with monoclonal antibody B306 (1) as an outer membrane protein marker.

LptA rabbit antiserum (Fig. 4A). The typical outer membrane protein in *N. meningitidis*, the Opc protein, was not detected in the cytosolic fraction (Fig. 4C, lanes 2 and 6), indicating that separation of the total membrane fraction from the soluble (cytosolic and periplasmic) fraction was successful. Furthermore, since the Opc protein was detected only in the outer membrane fraction isolated from the total membrane fraction with 4% Triton X-100 (Fig. 4C, lanes 4 and 8), it was confirmed that the inner and outer membranes were also successfully separated from the total membrane fraction in this study. The LptA protein was detected in the inner membrane fraction (Fig. 4A, lane 4) but not in the outer membrane fraction (Fig. 4A, lane 5). To confirm this result, we further examined the LptA cellular localization by using the LptA protein fused to a FLAG tag at the C terminus (see Materials and Methods). The LptA-FLAG protein was also detected only in the inner membrane fraction (Fig. 4B, lanes 4 and 5). These data suggested that the LptA protein was not exposed on the meningococcal surface, excluding the possibility that the LptA protein itself could function as an adhesin molecule in *N. meningitidis* (see Discussion).

Structural analyses of meningococcal LOS. To examine the structural change in the meningococcal LOSs due to the Δ *lptA* mutation, the lipid A moieties of the LOSs from *N. meningitidis* strains HT1125 (wild type) and HT1222 (Δ *lptA*) were analyzed by MALDI-TOF MS (Fig. 5). The ions observed in the lipid A

of HT1125 were not different from those of HT1222, except for the ions of PEA-containing molecules (Fig. 1 and 5 and Table 3). No other structural changes in the OS moieties were observed by gas chromatography-MS, MALDI-TOF MS, or nuclear magnetic resonance analyses (Fig. 1 and data not shown). These results indicated that only the PEA group at the 4' position of lipid A was lost in the LOS of the Δ *lptA* *N. meningitidis* mutant, which is consistent with a previous report (7), and suggested that the loss of the PEA group led to the decrease in adhesion of the Δ *lptA* *N. meningitidis* mutant to human cells.

Influence of pili, Opc, Opa, and PilC on the adhesion defect of the Δ *lptA* *N. meningitidis* mutant. We sought to determine whether the reduction of the adhesive activity in the Δ *lptA* mutant was dependent on or associated with changes in known adhesive molecules such as pili, Opc, Opa, and PilC. In the NIID280 (ST-2032) genetic background, *pilE::ermC* (HT1238) and Δ *lptA::spc* (HT1222) mutations reduced the adhesive activity to approximately 1/100 and 1/10 of the level in the wild-type strain (HT1125), respectively (Fig. 6A). The adhesive ability of the *pilE* Δ *lptA* double mutant (HT1249) was decreased to approximately 1/10,000 of the level in the wild-type strain (Fig. 6A). In an H44/76 (ST-32) genetic background, *pilE* (HT1235) and Δ *lptA* (HT1243) mutations also reduced the adhesive activity to approximately 1/100 and 1/10 of the level in the wild-type strain (HT1034), respectively (Fig. 6A).

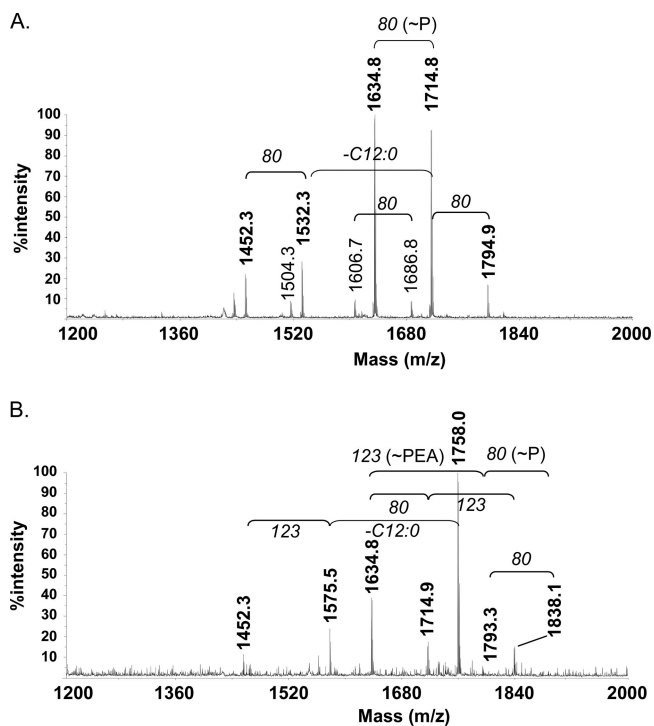


FIG. 5. MALDI-TOF spectra of lipid A purified from strains HT1222 ($\Delta lptA::spc$) (A) and HT1125 (wild type) (B). The masses of the abundant fragments are indicated at the tops of the corresponding peaks. The ions of molecules containing a PEA group in HT1125 lipid A ($m/z = 1838, 1758, \text{ and } 1575$) (Table 3) are not detected in those of HT1222 lipid A.

The cooperative reduction of the adhesive ability was also observed in the *pilE* $\Delta lptA$ double mutant (HT1245) (Fig. 6A). The cooperative reduction of adhesive ability caused by the *pilE* and $\Delta lptA$ mutations was also observed in a $\Delta opc::cat$ H44/76 genetic background (HT1234, HT1244, HT1236, and HT1246) (Fig. 6A). The results genetically suggested that the reduction of adhesive activity in the $\Delta lptA$ mutant was independent of pili regardless of the presence of Opc. We examined the effect of the $\Delta lptA$ mutation on PilC, Opc, Opa, and pilin production by Western blotting. The expression of Opc appeared to be slightly reduced in the $\Delta lptA$ mutant, while the amounts of pili and PilC proteins were not significantly different in wild-type and $\Delta lptA$ *N. meningitidis* strains (Fig. 6B, lanes 3 and 4). The *opc* gene was absent in the NIID280 strain (Fig. 6B, lanes 1 and 2) (36), and PilC and Opa proteins were faintly detected in the H44/76 strains (Fig. 6B, lanes 3 and 4) (36). It was further confirmed by transmission electron microscopy that ~6 to 12 diplococcus and pilus bundles were found in both HT1125 (wild type) and HT1222 ($\Delta lptA$) in the NIID280 (ST-2032) genetic background, while ~0 to 4 pili per diplococcus and a few pilus bundles were expressed in both the wild-type HT1034 and $\Delta lptA$ HT1243 strains in the H44/76 (ST-32) genetic background (Fig. 6C). The reduction in adhesion produced by the $\Delta lptA$ mutation was observed even in an *opc*-negative strain (ST-2032) and in an *Opa*-negative strain expressing little PilC (H44/76) (Fig. 6A and B). All the results suggested that the Opc, Opa, and PilC proteins and pili appear

not to be directly related to the reduction of adhesive activity in the $\Delta lptA$ mutant (see Discussion).

Influence of capsule on LptA-mediated meningococcal adhesion. We further examined whether the LOS modification by LptA and the effect on adhesion was affected by the presence of capsule, because the strains examined so far were all derived from capsule-negative strains (Table 1). As shown in Fig. 7, the $\Delta lptA$ mutation reduced the adhesive activities of both the NIID311 and H44/76 unencapsulated *N. meningitidis* strains ($P < 0.001$ for HT1336 versus HT1359 and for HT1034 versus HT1243) (Fig. 7). However, the *lptA* mutation did not affect the reduced adhesive ability in an encapsulated *N. meningitidis* NIID311 genetic background ($P = 0.78$, NIID311 versus HT1380) (Fig. 7), while the $\Delta lptA$ mutation slightly reduced the adhesive activity in the H44/76 genetic background ($P < 0.05$, H44/76 versus HT1378). Adhesion of encapsulated meningococcal strains was less affected by the $\Delta lptA$ mutation than that of unencapsulated strains with the same NIID311 and H44/76 genetic backgrounds. This suggested that LptA-mediated adhesion may be masked when meningococci are encapsulated (see Discussion).

Influence of a multicopy *lptA* gene in a wild-type strain. Increased expression or copy number of the *lptA* gene in wild-type *N. meningitidis* could lead to the enhancement of the number of PEAs in the LOS molecule as observed for some meningococcal strains (7). This could result in increased adhesive activity of *N. meningitidis*. To examine this possibility, the *lptA* gene was introduced on a plasmid into the wild-type *N. meningitidis* strain HT1125, and adhesion was examined using HBMEC. While LptA protein expression increased in HT1125 harboring the pHT435 plasmid (HT1125/pHT435) (Fig. 8B, lane 4), the adhesion was not significantly different in the HT1125/pHT435 transformant compared to the HT1125/pHT261 transformant (Fig. 8A). MALDI-TOF MS analysis of lipid A preparations from these *N. meningitidis* transformants revealed that the ions of the PEA-containing molecules in the LOS from HT1125/pHT435 ($m/z = 1836$ and 1756) (Fig. 8C, lower panel) did not change compared to those in the LOS from HT1125/pHT261 (Fig. 8C, upper panel). The results sug-

TABLE 3. MALDI-TOF MS analysis (negative mode) of lipid A fractions of LOSs from *N. meningitidis* HT1222 and HT1125 and proposed compositions for observed ions^a

Strain	Observed [M - H] ⁻ ion ^b	Proposed composition
HT1222 ($\Delta lptA::spc$)	1794.9	$P_3\text{GlcN}_2\text{C}_{12:0}2_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$
	1714.8	$P_2\text{GlcN}_2\text{C}_{12:0}2_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$
	1634.8	$P_1\text{GlcN}_2\text{C}_{12:0}2_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$
	1532.3	$P_2\text{GlcN}_2\text{C}_{12:0}1_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$
	1452.3	$P_1\text{GlcN}_2\text{C}_{12:0}1_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$
	HT1125 (wild type)	1838.1
1758.0		$P_1\text{PEAGlcN}_2\text{C}_{12:0}2_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$
1793.3		$P_3\text{GlcN}_2\text{C}_{12:0}2_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$
1714.9		$P_2\text{GlcN}_2\text{C}_{12:0}2_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$
1634.8		$P_1\text{GlcN}_2\text{C}_{12:0}2_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$
1575.5		$P_1\text{PEAGlcN}_2\text{C}_{12:0}1_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$
1452.3		$P_1\text{GlcN}_2\text{C}_{12:0}1_3\text{OHC}_{14:0}2_3\text{OH}(\text{C}_{12:0})_2$

^a See also Fig. 5 and 8.

^b The ions for PEA-containing molecules are shown in bold.

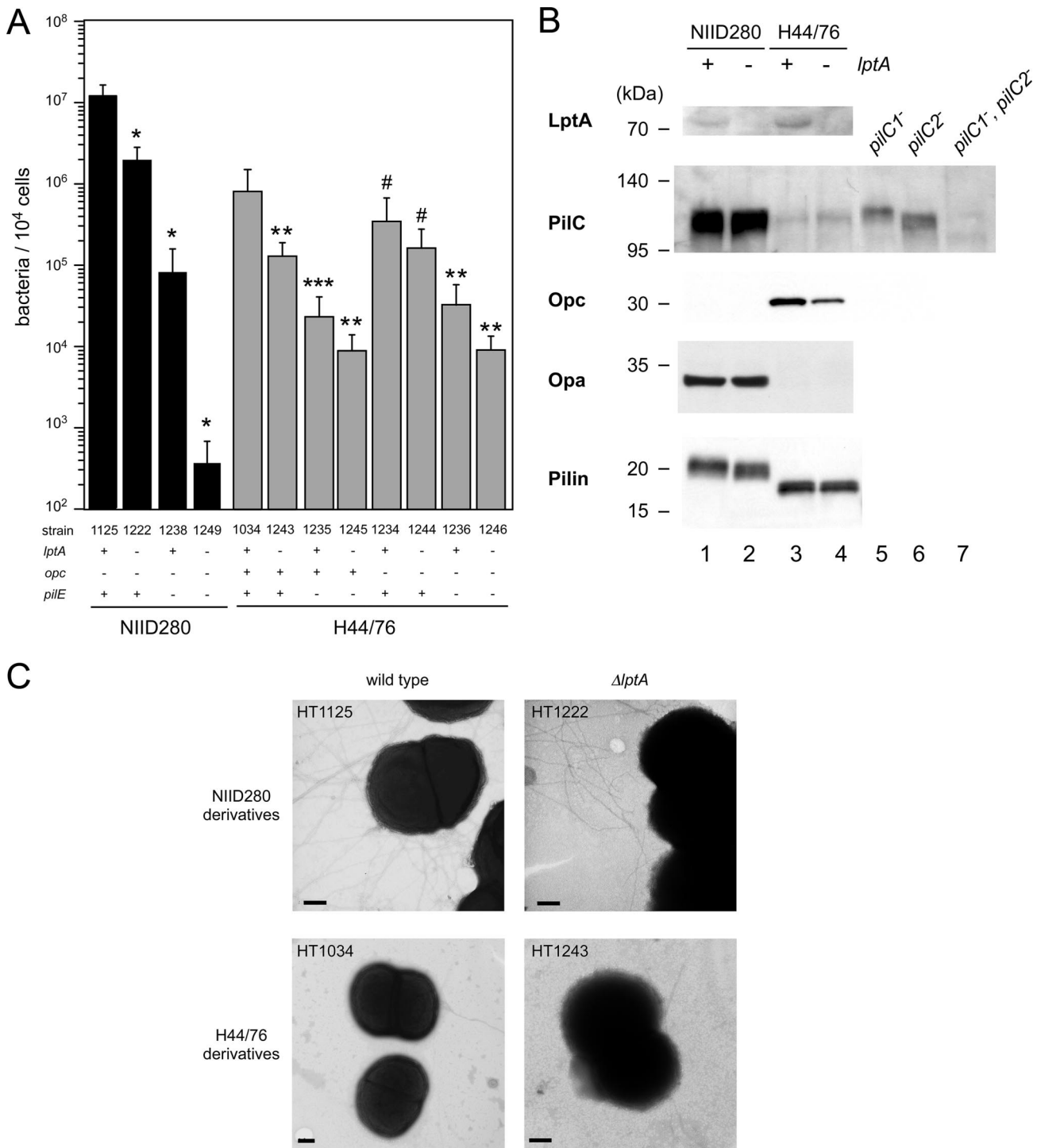


FIG. 6. (A) Adhesion of *pilE::ermC* and *Δopc::cat* mutants with NIID280 (ST-2032) and H44/76 (ST-32) genetic backgrounds to HBMEC. Mean CFU of adherent bacteria per 10⁴ cells of HBMEC in at least three experiments are shown, and error bars represent the standard errors of the means. *, *P* < 0.01; **, *P* < 0.05; ***, *P* < 0.06; and #, *P* > 0.1 (compared to the isogenic wild-type strain). (B) Western blotting for LptA, PilC, Opc, Opa, and pilin proteins. Bacterial extracts equivalent to an OD₆₀₀ of 0.05 (for LptA and PilC), 0.01 (for Opc and Opa), or 0.0025 (for pilin) were analyzed by Western blotting. Lane 1, HT1125 (ST-2032 wild type); lane 2, HT1222 (ST-2032 *ΔlptA*); lane 3, HT1034 (ST-32 wild type); lane 4, HT1243 (ST-32 *ΔlptA*). The *pilC1* mutant (HT1178, lane 5), the *pilC2* mutant (HT1182, lane 6), and the double mutant (HT1186, lane 7) were used only as controls for anti-PilC rabbit serum. (C) Transmission electron micrographs showing piliation of meningococcal strain HT1125 and the *ΔlptA* mutant HT1222 and of meningococcal strain HT1034 and the *ΔlptA* mutant HT1243. Bars, 200 nm.

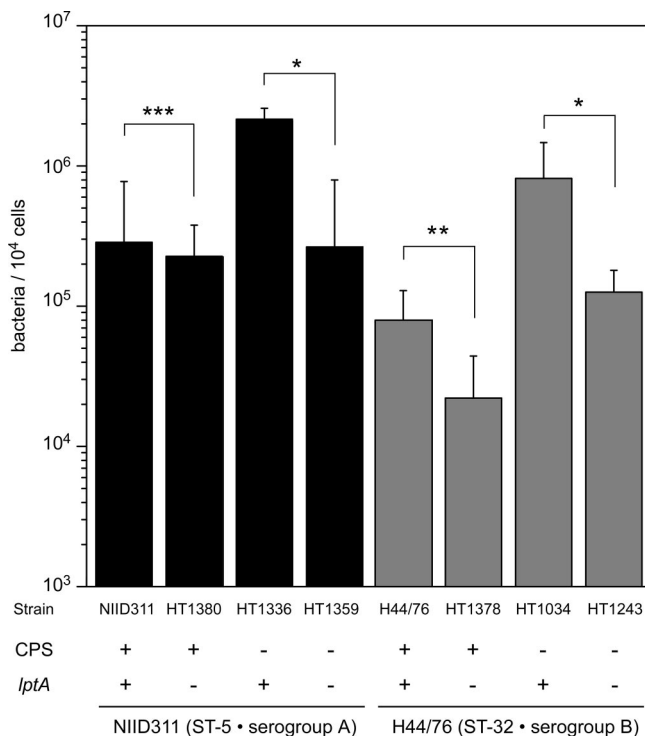


FIG. 7. The enhancement of bacterial adhesion to HBMEC with LOS modification by LptA is not prominent in encapsulated *N. meningitidis*. NIID311 (ST-5) and H44/76 (ST-32) genetic backgrounds were studied. Mean CFU of adherent bacteria per 10^4 cells of HBMEC in at least three experiments are shown, and error bars represent the standard errors of the means. *, $P < 0.001$; **, $P < 0.05$; ***, $P = 0.78$ (compared to the isogenic $\Delta lptA$ strain). CPS, capsular polysaccharide.

gested that the overexpression of LptA in wild-type *N. meningitidis* did not affect the PEA of meningococcal LOS and adhesion to human cells.

Influence of the presence of purified PEA-LOS and bovine serum albumin on meningococcal infection. To obtain some clues about the possibility that LOS modified with PEA (PEA-LOS) could act as an adhesin molecule, the meningococcal adhesion to HBMEC was examined in the presence of the LOS purified from wild-type *N. meningitidis* strain HT1125 during the infection assay. The meningococcal adhesion was not inhibited by as much as $100 \mu\text{g}$ of purified PEA-LOS, and the inhibitory effect was not also observed even when HBMEC were preincubated with the purified PEA-LOS for 2 h prior to the bacterial infection (Fig. 9A). These results suggested that PEA-LOS could not directly act as an adhesin molecule (see Discussion). We also examined the effect of serum on the LptA-mediated meningococcal adhesion. Under infection conditions without serum (serum-free AM), it was still observed that the $\Delta lptA$ mutant HT1222 adhered to HBMEC approximately 10 times less efficiently than the wild-type strain HT1125 (Fig. 9B). To completely exclude the presence of serum or serum factors, bacterial adhesion was also examined with serum-free AM and A549 epithelial cells grown in serum-free medium (see Materials and Methods). Approximately 10 times less efficient adherence by the $\Delta lptA$ mutant was also observed under the completely serum-free conditions (Fig.

9C). These results suggested that LptA-mediated meningococcal adhesion was not related to serum (see Discussion).

DISCUSSION

The modification of the 4' position of the lipid A moiety of LOS (or LPS) with PEA is known to confer resistance to cationic antimicrobial peptides such as polymyxin B on *N. meningitidis* (41), *Salmonella enterica* (23), and *E. coli* (21). However, other biological roles of the modification have not been elucidated. In the present study, we demonstrated that the modification of LOS by LptA can enhance meningococcal adhesion to human cells.

The LptA-mediated effect on meningococcal adhesion seemed to be masked by capsular polysaccharides, since the modification of LOS by LptA did not significantly affect meningococcal adhesion to HBMEC in encapsulated *N. meningitidis* (Fig. 7). The meningococcal capsule confers serum resistance (14, 49) and decreases meningococcal adhesion to and invasion of the cultured human cells in in vitro experiments (Fig. 7) (47). Although *N. meningitidis* clinical isolates from blood or CSF are encapsulated (5), *N. meningitidis* can undergo frequent phase variation (16) to unencapsulated variants, which may be selected at the surface of the human nasopharynx. Unencapsulated meningococci efficiently enter the epithelial and endothelial cells and subsequently disseminate into blood or CSF after the reversion to the encapsulated state (15, 42, 51). LptA-mediated meningococcal adhesion may play an important role in the unencapsulated state of *N. meningitidis* in the natural reservoir of the human nasopharynx.

The exact mechanism of LptA-mediated meningococcal adhesion is interesting but at present unknown. It seems unlikely that LOS modification by LptA affects meningococcal adhesion by altering the overall charge of the LOS molecule; disruption of *lpt3* or *lpt6*, which encode proteins adding PEA to the inner core heptose II, in immunotype L2 (*lpt6*⁺) or L3 (*lpt3*⁺) *N. meningitidis*, respectively, did not affect the meningococcal adhesion to HBMEC (data not shown). Another possibility is that the PEA-LOS itself acts as an adhesin molecule. It is known that gonococcal LOS directly mediates interactions with host cells; *N. gonorrhoeae* adheres to primary human cervical epithelial cells by the interaction between gonococcal lipid A and a human C3 acceptor molecule (CR3) (10–12), and gonococcal LOS binds to an asialoglycoprotein receptor on HepG2 epithelial cells (30) and on human sperm (17). However, it is less likely that *N. meningitidis* PEA-LOS interacts with human cells via the CR3, since the LptA-mediated meningococcal adhesion with A549 epithelial cells, which do not produce any CRs themselves (8), could be observed under completely serum-free conditions (Fig. 9C). We also tried to examine the direct interaction between purified PEA-LOS and HBMEC by immunostaining and flow cytometry with Alexa Fluor 488-labeled PEA-LOS (39), but we could not detect adherence (data not shown). In addition, meningococcal adhesion to HBMEC was not inhibited by the addition of the PEA-LOS (Fig. 9A). However, meningococcal attachment to human cells is a multistep process involving initial pilus-mediated adherence followed by close adherence with Opc and Opa. PEA-LOS may be involved in this close adherence through an unknown mechanism.

Considering that the modulation of LOS can expose or in-

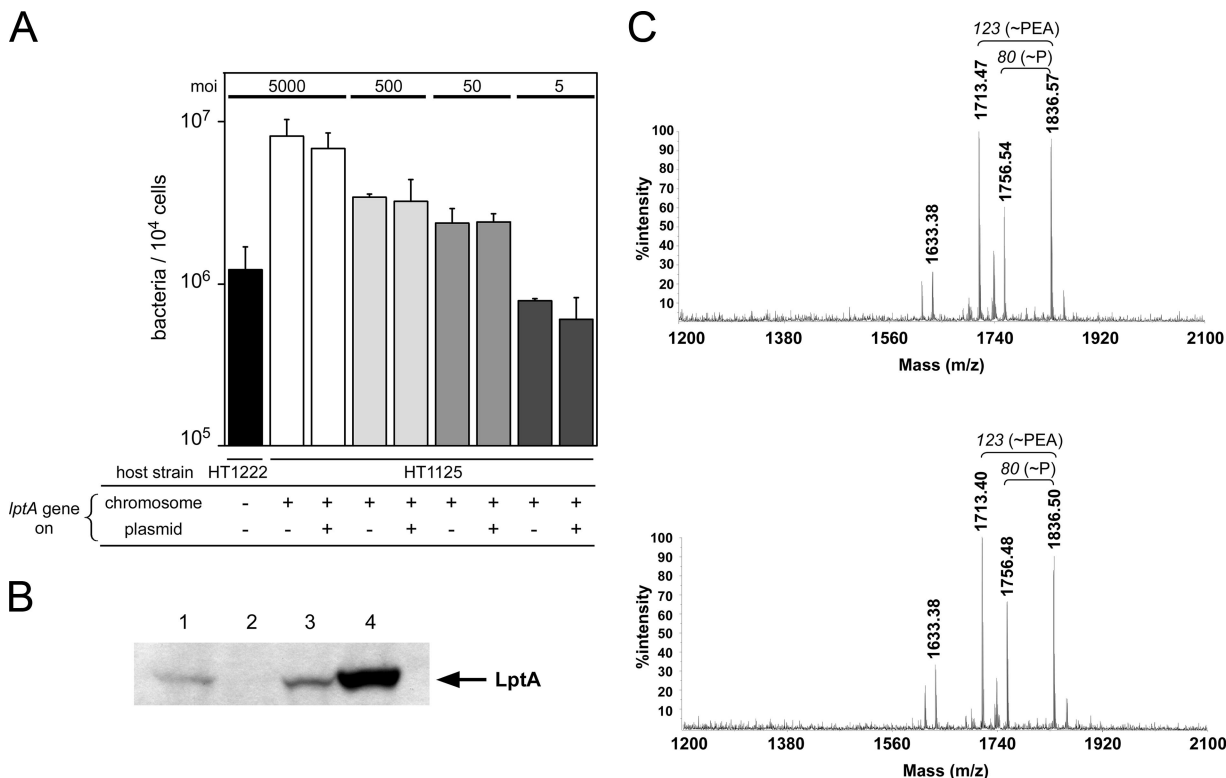


FIG. 8. Introduction of the *lptA* gene on a multicopy plasmid into wild-type *N. meningitidis* does not enhance LOS modification with the PEA group or bacterial adhesion to HBMEC. (A) Adhesion of HT1125 harboring a plasmid containing the *lptA* gene (pHT435) or a vector plasmid (pHT261) to HBMEC. The bacterial adhesion was examined at MOIs of 5,000, 500, 50, and 5. Mean CFU of adherent bacteria per monolayer of HBMEC in at least three experiments are shown, and error bars represent the standard errors of the means. (B) Western blotting for LptA protein. Bacterial extracts equivalent to an OD₆₀₀ of 0.05 were analyzed by Western blotting. Lane 1, HT1125 (ST-2032 wild type); lane 2, HT1222 (ST-2032 Δ *lptA*); lane 3, HT1125 harboring pHT261 (vector); lane 4, HT1125 harboring pHT435 (*lptA*⁺ on a plasmid). (C) MALDI-TOF spectra of lipid A purified from HT1125 harboring pHT261 (upper panel) and from HT1125 harboring pHT435 (lower panel). The masses of the major fragments are indicated at the tops of the corresponding peaks. The fragments and peaks of these ions were nearly identical in the two *N. meningitidis* transformants.

duce conformational changes of certain proteins in *N. gonorrhoeae* (13) and affect Opc-mediated meningococcal interactions with HUVEC (46), LOS modification with PEA by LptA may also induce configurational or conformational changes on the meningococcal surface, which could result in the exposure of a specific adhesin molecule in *N. meningitidis*. Since the reduction of adhesive activity by the Δ *lptA* mutation was observed in all *N. meningitidis* strains examined in this study (Fig. 2, 6, 7, and 8) and in ST-11 and ST-1011 strains (data not shown), the PEA-LOS-mediated adherence mechanism is generally present among *N. meningitidis* strains. We showed in this study that meningococcal adhesion enhanced by the LptA was independent of pili and Opc and probably of Opa and PilC (Fig. 6), which was consistent with a previous report by Unkmeir et al. that meningococcal adhesion to HBMEC is not mediated by Opc and human fibronectin (43). Further, it is unlikely that meningococcal adhesion enhanced by LptA is mediated by recently reported new meningococcal adhesins such as NadA (4) and MspA (40), because NadA facilitates adherence to epithelial cells but not to endothelial cells (4) and both proteins are not expressed in all *N. meningitidis* strains (6, 40). Enhancement of meningococcal adhesion by LptA appears to be mediated by unidentified adhesin molecules, which

was also an implication of our previous study (36). This concept is consistent with the observation that LptA-mediated adhesion to HBMEC and Opc-mediated meningococcal adhesion to HUVEC are masked by capsule (Fig. 7) (47). However, further experiments are required to elucidate the precise mechanism.

Since growth conditions affect the lipid A phosphorylation pattern in *N. meningitidis* (7), PEA levels on the LOS may also vary among *N. meningitidis* strains that show different adhesive activities (36). However, the LptA protein was found equally among all *N. meningitidis* strains examined (data not shown) (7). The amount of PEA-LOS did not increase even with the overexpression of the *lptA* gene in wild-type *N. meningitidis* (Fig. 8) and in the noninvasive ST-2046 strain (data not shown) (36). Considering all these results, the PEA level on lipid A of the LOS might be constant in *N. meningitidis*.

We found that the LOS modification by LptA enhanced the adhesion of *N. meningitidis* to human epithelial or endothelial cells. Since homologues of *lptA* are found in other neisserial genomes (*N. gonorrhoeae*, *N. lactamica*, and *N. elongata*) (unpublished data), as well in those of other bacteria (23), enhancement of adhesion by LptA may be observed in other

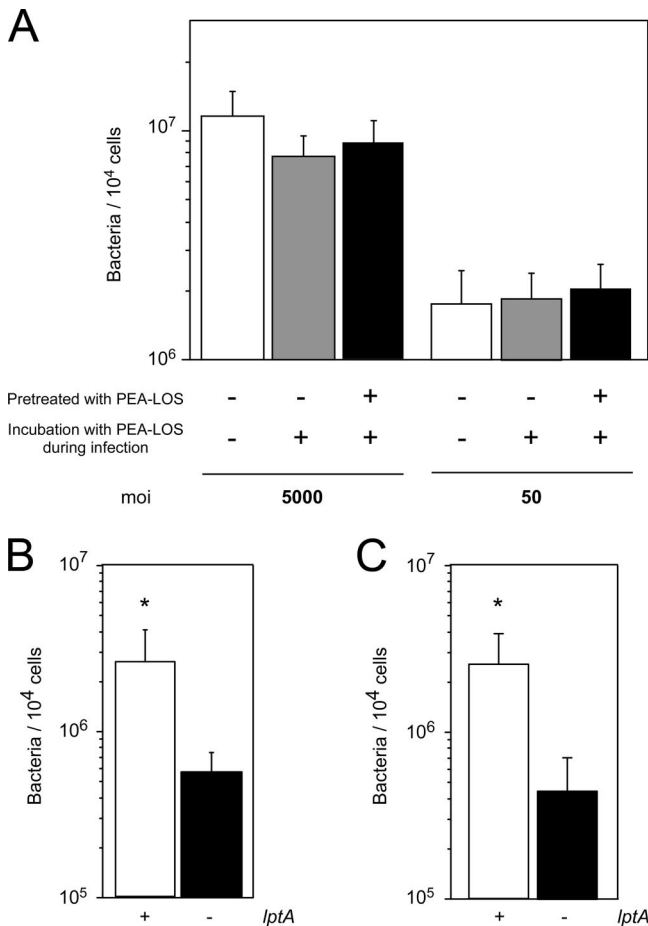


FIG. 9. Influence of the presence of purified PEA-LOS and bovine serum albumin on LptA-mediated meningococcal adherence to human cultured cells. (A) Adhesion of *N. meningitidis* to HBMEC in the presence of 100 μ g of PEA-LOS (purified from wild-type *N. meningitidis* strain HT1125) during infection at MOIs of 5,000 and 50. Gray and open bars indicate the bacterial number with or without PEA-LOS, respectively. Solid bars indicate the number of bacteria adhering to HBMEC pretreated with 100 μ g of PEA-LOS for 2 h prior to the bacterial infection. (B and C) Adhesion of *N. meningitidis* to HBMEC (B) and to A549 cells grown in serum-free medium (C) in serum-free AM during the infection assay. Opened and filled bars indicate the numbers of wild-type (HT1125) and Δ lptA (HT1222) *N. meningitidis* bacteria, respectively, represented as CFU. Mean CFU of adherent bacteria per 10⁴ cells in at least three experiments are shown, and error bars represent the standard errors of the means. *, $P < 0.001$ compared to the Δ lptA strain.

bacteria. The general ability and the mechanism of enhancement of bacterial adhesion by LptA will be further examined.

ACKNOWLEDGMENTS

We thank Makoto Ohnishi for helpful discussion.

This work was supported by grants from the Ministry of Health, Welfare and Labor of Japan. H.T. was also supported by a grant from the Ministry of Education, Science and Culture of Japan (grant no. 16790265), D.S.S. and R.W.C. were supported by NIH grant R01 AI-33517. R.W.C. was partly supported by the U.S. Department of Energy-funded (DE-FG09-93ER-20097) Center for Plant and Microbial Complex Carbohydrates.

REFERENCES

- Achtman, M., B. Kusecek, G. Morelli, K. Eickmann, J. F. Wang, B. Crowe, R. A. Wall, M. Hassan-King, P. S. Moore, and W. Zollinger. 1992. A comparison of the variable antigens expressed by clone IV-1 and subgroup III of *Neisseria meningitidis* serogroup A. *J. Infect. Dis.* **165**:53-68.
- Albiger, B., L. Johansson, and A. B. Jonsson. 2003. Lipooligosaccharide-deficient *Neisseria meningitidis* shows altered pilus-associated characteristics. *Infect. Immun.* **71**:155-162.
- Berrington, A. W., Y. C. Tan, Y. Srikhanta, B. Kuipers, P. van der Ley, I. R. Peak, and M. P. Jennings. 2002. Phase variation in meningococcal lipooligosaccharide biosynthesis genes. *FEMS Immunol. Med. Microbiol.* **34**:267-275.
- Capocchi, B., J. Adu-Bobie, F. Di Marcello, L. Ciocchi, V. Masignani, A. Taddei, R. Rappuoli, M. Pizza, and B. Arico. 2005. *Neisseria meningitidis* NadA is a new invasins which promotes bacterial adhesion to and penetration into human epithelial cells. *Mol. Microbiol.* **55**:687-698.
- Cartwright, K. A., J. M. Stuart, D. M. Jones, and N. D. Noah. 1987. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. *Epidemiol. Infect.* **99**:591-601.
- Comanducci, M., S. Bambini, D. A. Caugant, M. Mora, B. Brunelli, B. Capocchi, L. Ciocchi, R. Rappuoli, and M. Pizza. 2004. NadA diversity and carriage in *Neisseria meningitidis*. *Infect. Immun.* **72**:4217-4223.
- Cox, A. D., J. C. Wright, J. Li, D. W. Hood, E. R. Moxon, and J. C. Richards. 2003. Phosphorylation of the lipid A region of meningococcal lipopolysaccharide: identification of a family of transferases that add phosphoethanolamine to lipopolysaccharide. *J. Bacteriol.* **185**:3270-3277.
- de Astorza, B., G. Cortes, C. Crespi, C. Saus, J. M. Rojo, and S. Alberti. 2004. C3 promotes clearance of *Klebsiella pneumoniae* by A549 epithelial cells. *Infect. Immun.* **72**:1767-1774.
- Dehio, C., S. D. Gray-Owen, and T. F. Meyer. 1998. The role of neisserial Opa proteins in interactions with host cells. *Trends Microbiol.* **6**:489-495.
- Edwards, J. L., and M. A. Apicella. 2002. The role of lipooligosaccharide in *Neisseria gonorrhoeae* pathogenesis of cervical epithelia: lipid A serves as a C3 acceptor molecule. *Cell. Microbiol.* **4**:585-598.
- Edwards, J. L., E. J. Brown, K. A. Ault, and M. A. Apicella. 2001. The role of complement receptor 3 (CR3) in *Neisseria gonorrhoeae* infection of human cervical epithelia. *Cell. Microbiol.* **3**:611-622.
- Edwards, J. L., E. J. Brown, S. Uk-Nham, J. G. Cannon, M. S. Blake, and M. A. Apicella. 2002. A co-operative interaction between *Neisseria gonorrhoeae* and complement receptor 3 mediates infection of primary cervical epithelial cells. *Cell. Microbiol.* **4**:571-584.
- Elkins, C., N. H. Carbonetti, V. A. Varela, D. Stirewalt, D. G. Klapper, and P. F. Sparling. 1992. Antibodies to N-terminal peptides of gonococcal porin are bactericidal when gonococcal lipopolysaccharide is not sialylated. *Mol. Microbiol.* **6**:2617-2628.
- Hammerschmidt, S., C. Birkholz, U. Zahringer, B. D. Robertson, J. van Putten, O. Ebeling, and M. Frosch. 1994. Contribution of genes from the capsule gene complex (*cps*) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis*. *Mol. Microbiol.* **11**:885-896.
- Hammerschmidt, S., R. Hilse, J. P. van Putten, R. Gerardy-Schahn, A. Unkmeir, and M. Frosch. 1996. Modulation of cell surface sialic acid expression in *Neisseria meningitidis* via a transposable genetic element. *EMBO J.* **15**:192-198.
- Hammerschmidt, S., A. Muller, H. Sillmann, M. Muhlenhoff, R. Borrow, A. Fox, J. van Putten, W. D. Zollinger, R. Gerardy-Schahn, and M. Frosch. 1996. Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (*stiaD*): correlation with bacterial invasion and the outbreak of meningococcal disease. *Mol. Microbiol.* **20**:1211-1220.
- Harvey, H. A., N. Porat, C. A. Campbell, M. Jennings, B. W. Gibson, N. J. Phillips, M. A. Apicella, and M. S. Blake. 2000. Gonococcal lipooligosaccharide is a ligand for the asialoglycoprotein receptor on human sperm. *Mol. Microbiol.* **36**:1059-1070.
- Jennings, M. P., Y. N. Srikhanta, E. R. Moxon, M. Kramer, J. T. Poolman, B. Kuipers, and P. van der Ley. 1999. The genetic basis of the phase variation repertoire of lipopolysaccharide immunotypes in *Neisseria meningitidis*. *Microbiology* **145**:3013-3021.
- Jones, D. M., R. Borrow, A. J. Fox, S. Gray, K. A. Cartwright, and J. T. Poolman. 1992. The lipooligosaccharide immunotype as a virulence determinant in *Neisseria meningitidis*. *Microb. Pathog.* **13**:219-224.
- Kahler, C. M., S. Lyons-Schindler, B. Choudhury, J. Glushka, R. W. Carlson, and D. S. Stephens. 2006. O-Acetylation of the terminal N-acetylglucosamine of the lipooligosaccharide inner core in *Neisseria meningitidis*. Influence on inner core structure and assembly. *J. Biol. Chem.* **281**:19939-19948.
- Kim, S. H., W. Jia, V. R. Parreira, R. E. Bishop, and C. L. Gyles. 2006. Phosphoethanolamine substitution in the lipid A of *Escherichia coli* O157:H7 and its association with PmrC. *Microbiology* **152**:657-666.
- Kurzai, O., C. Schmitt, H. Claus, U. Vogel, M. Frosch, and A. Kolb-Maurer. 2005. Carbohydrate composition of meningococcal lipopolysaccharide modulates the interaction of *Neisseria meningitidis* with human dendritic cells. *Cell. Microbiol.* **7**:1319-1334.

23. Lee, H., F. F. Hsu, J. Turk, and E. A. Groisman. 2004. The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. *J. Bacteriol.* **186**:4124–4133.
24. Mackinnon, F. G., R. Borrow, A. R. Gorringe, A. J. Fox, D. M. Jones, and A. Robinson. 1993. Demonstration of lipooligosaccharide immunotype and capsule as virulence factors for *Neisseria meningitidis* using an infant mouse intranasal infection model. *Microb. Pathog.* **15**:359–366.
25. Mackinnon, F. G., A. D. Cox, J. S. Plested, C. M. Tang, K. Makepeace, P. A. Coull, J. C. Wright, R. Chalmers, D. W. Hood, J. C. Richards, and E. R. Moxon. 2002. Identification of a gene (*lpt-3*) required for the addition of phosphoethanolamine to the lipopolysaccharide inner core of *Neisseria meningitidis* and its role in mediating susceptibility to bactericidal killing and opsonophagocytosis. *Mol. Microbiol.* **43**:931–943.
26. Nassif, X. 1999. Interaction mechanisms of encapsulated meningococci with eucaryotic cells: what does this tell us about the crossing of the blood-brain barrier by *Neisseria meningitidis*? *Curr. Opin. Microbiol.* **2**:71–77.
27. Nassif, X., S. Bourdoulous, E. Eugene, and P. O. Couraud. 2002. How do extracellular pathogens cross the blood-brain barrier? *Trends Microbiol.* **10**:227–232.
28. Nassif, X., C. Pujol, P. Morand, and E. Eugene. 1999. Interactions of pathogenic *Neisseria* with host cells. Is it possible to assemble the puzzle? *Mol. Microbiol.* **32**:1124–1132.
29. Plant, L., J. Sundqvist, S. Zughaier, L. Lovkvist, D. S. Stephens, and A. B. Jonsson. 2006. Lipooligosaccharide structure contributes to multiple steps in the virulence of *Neisseria meningitidis*. *Infect. Immun.* **74**:1360–1367.
30. Porat, N., M. A. Apicella, and M. S. Blake. 1995. *Neisseria gonorrhoeae* utilizes and enhances the biosynthesis of the asialoglycoprotein receptor expressed on the surface of the hepatic HepG2 cell line. *Infect. Immun.* **63**:1498–1506.
31. Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zahringer, U. Seydel, F. Di Padova, et al. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J.* **8**:217–225.
32. Schneider, H., J. M. Griffiss, R. E. Mandrell, and G. A. Jarvis. 1985. Elaboration of a 3.6-kilodalton lipooligosaccharide, antibody against which is absent from human sera, is associated with serum resistance of *Neisseria gonorrhoeae*. *Infect. Immun.* **50**:672–677.
33. Scholten, R. J., B. Kuipers, H. A. Valkenburg, J. Dankert, W. D. Zollinger, and J. T. Poolman. 1994. Lipo-oligosaccharide immunotyping of *Neisseria meningitidis* by a whole-cell ELISA with monoclonal antibodies. *J. Med. Microbiol.* **41**:236–243.
34. Song, W., L. Ma, R. Chen, and D. C. Stein. 2000. Role of lipooligosaccharide in Opa-independent invasion of *Neisseria gonorrhoeae* into human epithelial cells. *J. Exp. Med.* **191**:949–960.
35. Takahashi, H., K. Hirose, and H. Watanabe. 2004. Necessity of meningococcal γ -glutamyl aminopeptidase for the *Neisseria meningitidis* growth in rat cerebrospinal fluid (CSF) and CSF mimicking medium. *J. Bacteriol.* **186**:244–247.
36. Takahashi, H., K. S. Kim, and H. Watanabe. 2008. Differential in vitro infectious abilities of two common Japan-specific sequence-type (ST) clones of disease-associated ST-2032 and carrier-associated ST-2046 *Neisseria meningitidis* strains in human endothelial and epithelial cell lines. *FEMS Immunol. Med. Microbiol.* **52**:36–46.
37. Takahashi, H., and H. Watanabe. 2002. A broad-host-range vector of incompatibility group Q can work as a plasmid vector in *Neisseria meningitidis*: a new genetical tool. *Microbiology* **148**:229–236.
38. Takahashi, H., and H. Watanabe. 2004. Post-translational processing of *Neisseria meningitidis* γ -glutamyl aminopeptidase and its association with inner membrane facing to the cytoplasmic space. *FEMS Microbiol. Lett.* **234**:27–35.
39. Triantafyllou, K., M. Triantafyllou, and N. Fernandez. 2000. Lipopolysaccharide (LPS) labeled with Alexa 488 hydrazide as a novel probe for LPS binding studies. *Cytometry* **41**:316–320.
40. Turner, D. P., A. G. Marietou, L. Johnston, K. K. Ho, A. J. Rogers, K. G. Wooldridge, and D. A. Ala'Aldeen. 2006. Characterization of MspA, an immunogenic autotransporter protein that mediates adhesion to epithelial and endothelial cells in *Neisseria meningitidis*. *Infect. Immun.* **74**:2957–2964.
41. Tzeng, Y. L., K. D. Ambrose, S. Zughaier, X. Zhou, Y. K. Miller, W. M. Shafer, and D. S. Stephens. 2005. Cationic antimicrobial peptide resistance in *Neisseria meningitidis*. *J. Bacteriol.* **187**:5387–5396.
42. Tzeng, Y. L., and D. S. Stephens. 2000. Epidemiology and pathogenesis of *Neisseria meningitidis*. *Microbes Infect.* **2**:687–700.
43. Unkmeir, A., K. Latsch, G. Dietrich, E. Wintermeyer, B. Schinke, S. Schwender, K. S. Kim, M. Eigenthaler, and M. Frosch. 2002. Fibronectin mediates Opc-dependent internalization of *Neisseria meningitidis* in human brain microvascular endothelial cells. *Mol. Microbiol.* **46**:933–946.
44. Verheul, A. F., H. Snippe, and J. T. Poolman. 1993. Meningococcal lipopolysaccharides: virulence factor and potential vaccine component. *Microbiol. Rev.* **57**:34–49.
45. Virji, M., H. Kayhty, D. J. Ferguson, C. Alexandrescu, J. E. Heckels, and E. R. Moxon. 1991. The role of pili in the interactions of pathogenic *Neisseria* with cultured human endothelial cells. *Mol. Microbiol.* **5**:1831–1841.
46. Virji, M., K. Makepeace, D. J. Ferguson, M. Achtman, and E. R. Moxon. 1993. Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. *Mol. Microbiol.* **10**:499–510.
47. Virji, M., K. Makepeace, D. J. Ferguson, M. Achtman, J. Sarkari, and E. R. Moxon. 1992. Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by *Neisseria meningitidis*. *Mol. Microbiol.* **6**:2785–2795.
48. Virji, M., K. Makepeace, I. R. Peak, D. J. Ferguson, M. P. Jennings, and E. R. Moxon. 1995. Opc- and pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides. *Mol. Microbiol.* **18**:741–754.
49. Vogel, U., and M. Frosch. 1999. Mechanisms of neisserial serum resistance. *Mol. Microbiol.* **32**:1133–1139.
50. Wright, J. C., D. W. Hood, G. A. Randle, K. Makepeace, A. D. Cox, J. Li, R. Chalmers, J. C. Richards, and E. R. Moxon. 2004. *lpt6*, a gene required for addition of phosphoethanolamine to inner-core lipopolysaccharide of *Neisseria meningitidis* and *Haemophilus influenzae*. *J. Bacteriol.* **186**:6970–6982.
51. Yazdankhah, S. P., and D. A. Caugant. 2004. *Neisseria meningitidis*: an overview of the carriage state. *J. Med. Microbiol.* **53**:821–832.