

Roles of 3-Deoxy-D-manno-2-Octulosonic Acid Transferase from *Moraxella catarrhalis* in Lipooligosaccharide Biosynthesis and Virulence

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Lipooligosaccharide (LOS), a major outer membrane component of *Moraxella catarrhalis*, is a possible virulence factor in the pathogenesis of human infections caused by the organism. However, information about the roles of the oligosaccharide chain from LOS in bacterial infection remains limited. Here, a *kdtA* gene encoding 3-deoxy-D-manno-2-octulosonic acid (Kdo) transferase, which is responsible for adding Kdo residues to the lipid A portion of the LOS, was identified by transposon mutagenesis and construction of an isogenic *kdtA* mutant in strain O35E. The resulting O35E*kdtA* mutant produced only lipid A without any core oligosaccharide, and it was viable. Physicochemical and biological analysis revealed that the mutant was susceptible to hydrophobic reagents and a hydrophilic glycopeptide and was sensitive to bactericidal activity of normal human serum. Importantly, the mutant showed decreased toxicity by the *Limulus* amoebocyte lysate assay, reduced adherence to human epithelial cells, and enhanced clearance in lungs and nasopharynx in a mouse aerosol challenge model. These data suggest that the oligosaccharide moiety of the LOS is important for the biological activity of the LOS and the virulence capability of the bacteria in vitro and in vivo. This study may bring new insights into novel vaccines or therapeutic interventions against *M. catarrhalis* infections.

Moraxella catarrhalis, a gram-negative diplococcus, is now considered to be an important human respiratory tract pathogen in children and adults (6, 29). In children, it causes otitis media, the most common childhood infection and the leading cause of conductive hearing loss, while in adults it exacerbates chronic obstructive pulmonary diseases, the fourth leading cause of death in the United States. Sporadic cases of conjunctivitis, meningitis, endocarditis, ophthalmia neonatorum, keratitis, urethritis, peritonitis, and septicemia have also been reported in individuals with reduced immune defense (36). In addition, the number of antibiotic-resistant strains of *M. catarrhalis* has significantly increased over the past decades (3, 23). Currently, the molecular pathogenesis of *M. catarrhalis* infection is not fully understood. Based on limited information about this organism and other respiratory tract pathogens, it is generally believed that it has several virulence factors involved in colonization, adherence, or complement resistance that enable the organism to evade the normal host defense and to establish the infection (47).

Previous studies suggested that complement resistance involving multifactorial processes might be an important mechanism of *M. catarrhalis* virulence. Several *M. catarrhalis* isogenic mutants of UspA2 (1, 30), Fur (15), CopB (22), and outer membrane protein (OMP) E (37) showed their susceptibility to the bactericidal activity of normal human serum when compared with their parental strains. This indicates that these bacterial components may play critical roles for the bac-

terial resistance to killing caused by normal human serum. In addition, many studies also revealed that bacterial adherence or colonization could be another important mechanism of *M. catarrhalis* virulence since surface outer membrane components such as UspA1, UspA2H (30), OMP CD (24), McaP (42), MID (14), and Hag (25) have been reported as adhesion molecules that bind to host cells.

Lipooligosaccharide (LOS), a major outer membrane component of *M. catarrhalis*, is also considered to be a possible virulence factor in the pathogenesis of the infections (9, 13). Unlike enteric bacterial lipopolysaccharide (LPS), *M. catarrhalis* LOS consists of an oligosaccharide core and lipid A without an O-antigen polysaccharide side chain (10). Structural studies have revealed that the oligosaccharides from three major serotypes have branched structures, with a common inner core composed of glucosyl residues and 3-deoxy-D-manno-2-octulosonic acid (Kdo) (26). The lipid A portion is similar to that of other gram-negative bacteria but with seven shorter fatty acyl chains (C₁₀ to C₁₂) (10, 33).

Despite these structural data, information regarding the biosynthesis of *M. catarrhalis* LOS is limited. This information is critical for elucidating the virulence functions of the LOS molecule. Previously, Zaleski et al. identified a UDP-glucose-4-epimerase and showed that a corresponding knockout mutant resulted in a truncated LOS structure lacking two terminal galactosyl residues (50). Recently, Luke et al. identified a *kdsA* gene coding for Kdo-8-phosphate synthase and found a *kdsA*-deficient mutant consisting only of lipid A in its LOS structure (32). Both mutant strains showed reduced resistance to complement killing caused by normal human serum. However, the

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description	Source or reference
Strains		
<i>E. coli</i> TOP10	Cloning strain	Invitrogen
<i>M. catarrhalis</i> O35E	Wild-type strain	46
<i>M. catarrhalis</i> O35E705	Strain with EZ::TN transposon insertion in <i>kdtA</i> gene	This study
<i>M. catarrhalis</i> O35EkdtA	<i>kdtA</i> knockout mutant strain	This study
Plasmids		
pCR2.1	TOPO TA cloning vector	Invitrogen
pBluescript II SK(+)	Cloning vector	Fermentas
pUC4k	Kanamycin resistance cassette	Amersham
pCRA	<i>kdtA</i> cloned into pCR2.1	This study
pSA	EcoRI-SalI <i>kdtA</i> fragment cloned into SK(+)	This study
pSAK	EcoRI-blunted kanamycin resistance cassette inserted into blunted HindIII site of pSA	This study
Primers		
31	5'-GAA ACC TGA GAG GTA ACT GCA GCC AGT T-3'	This study
32	5'-GTA TCA TTG CTC AAG ATG CCA GCT CTG C-3'	This study
33	5'-ATG GAC GGG CAA ATC AGG TGC TGT ATT G-3'	This study
34	5'-AGC CAT GAA GAT TTG GTG-3'	This study
35	5'-CTC GTC GAC TTG TCG CAC AAG CAA CGC-3' (SalI site underlined)	This study
36	5'-CTC GAA TTC CGT GAC AGT AAT GGT GAA-3' (EcoRI site underlined)	This study

interactive roles between the LOS and the host epithelial cells in vitro or in animal challenge models remain unstudied.

In this study, we identified *kdtA* gene in *M. catarrhalis* and constructed an isogenic *kdtA* mutant. Based on studies from *Escherichia coli* and other enteric bacteria, the *kdtA* gene encodes Kdo transferase, which catalyzes the sequential addition of two Kdo sugars onto a lipid IV_A moiety, a key precursor of lipid A. Prior to its incorporation, Kdo is synthesized and activated to CMP-Kdo by the enzymes encoded by *kdsA* and *kdsB* genes (40). Analysis of the physicochemical and biological functions of the *kdtA* mutant was performed to study functions of *kdtA* gene and its resulting LOS in vitro and in vivo.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Bacterial strains, plasmids, and primers are described in Table 1. *M. catarrhalis* strains were cultured on chocolate agar plates (Remel, Lenexa, KS) or brain heart infusion (BHI) (Difco, Detroit, MI) agar plates at 37°C in 5% CO₂. Mutant strains were selected on BHI agar supplemented with kanamycin at 20 µg/ml. Growth rates of wild type and mutant were measured as follows. An overnight culture was inoculated in 10 ml of BHI media (adjusted optical density at 600 nm [OD₆₀₀] = 0.05) and shaken at 37°C with 250 rpm. The bacterial cultures were monitored spectrophotometrically at OD₆₀₀. *E. coli* was grown on Luria-Bertani agar plates or broth with antibiotic supplementation. The antibiotic concentrations used for *E. coli* were as follows: kanamycin, 30 µg/ml; ampicillin, 50 µg/ml.

General DNA methods. DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase I Klenow fragment, and *Taq* DNA polymerase were purchased from Fermentas (Hanover, MD). Preparation of plasmid and purification of PCR products and DNA fragments were performed using kits manufactured by QIAGEN (Santa Clarita, CA). Bacterial chromosomal DNA was isolated using a genomic DNA purification kit (Promega, Madison, WI). DNA nucleotide sequences were obtained via 3070xl DNA analyzer (Applied Biosystems, Foster City, CA) and analyzed with DNASTAR software (DNASTAR Inc., Madison, WI).

Transposon mutagenesis and identification of *kdtA* gene. A 20-ml portion of culture (OD₆₀₀ = 0.5) of strain O35E was harvested by centrifugation and washed three times with 10% (vol/vol) glycerol in distilled water. The final cell pellet was suspended in 150 µl glycerol solution. A 20-µl portion of this cell suspension was mixed with 1 µl of EZ::TN <KAN-2> Tnp Transposome (Epicentre, Madison, WI), transferred into a microelectroporation chamber, and electroporated using a field strength of 2.2 kilovolts over a 1-mm distance for 4

ms (Micropulser, Bio-Rad, Hercules, CA). After the electroporation, the cell suspension was added to 1 ml of BHI broth, shaken at 250 rpm at 37°C for 3 h, and plated on BHI agar plates containing kanamycin. After 24 h incubation, the resulting kanamycin-resistant colonies were screened by colony blot assay and further identified by whole cell enzyme-linked immunosorbent assay (28) for loss of binding reactivity to a rabbit anti-LOS antibody. The transformants without anti-LOS antibody binding activity were subsequently evaluated by examining the LOS profiles from proteinase-K-treated whole-cell lysates (45) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining analysis (43). The clones with no detectable LOS were selected for isolation of chromosomal DNA and nucleotide sequence analysis. A first pair of sequence primers was provided by the EZ::TN <KAN-2> Tnp Transposome kit; other primers were designed according to the results of nucleotide sequence analysis (Table 1; Fig. 1, primers 31, 32, and 33). The *kdtA* homologue from strain O35E was identified by BLAST searches at GenBank of the National Center for Biotechnology Information.

Cloning of *kdtA* homologue and construction of the knockout *kdtA* mutant. The putative *kdtA* homologue was amplified from chromosomal DNA of strain O35E using primers 35 and 36 (Table 1; Fig. 1). This PCR product was cloned into pCR2.1 using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) to obtain pCRA. The insertion was released by EcoRI-SalI digestion and then subcloned into an EcoRI-SalI site of SK(+) to form pSA. A kanamycin resistance cassette

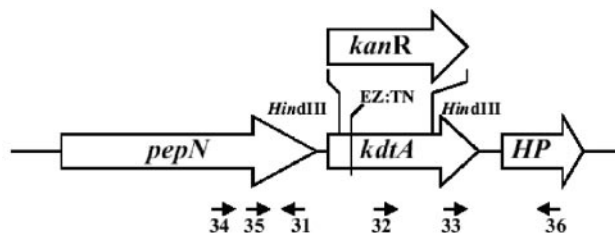


FIG. 1. Genetic organization of *kdtA* locus in *M. catarrhalis* O35E genome. *pepN*, aminopeptidase N; *HP*, hypothetical protein. Large arrows represent the direction of transcription; the site of the transposon insertion identified in O35E705 is denoted as EN:TN, and the location of deletion replaced by the kanamycin resistance gene (*kanR*) is between two HindIII cleavage sites. The sites of primers used are indicated as small arrows (primers 31, 32, 33, 34, 35, and 36 are described in Table 1).

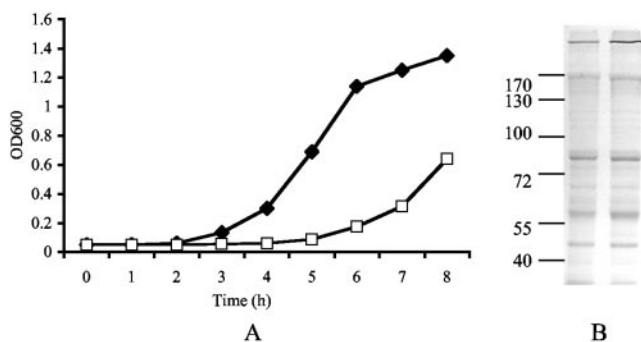


FIG. 2. (A) Growth curves of *M. catarrhalis* wild-type strain O35E (◆) and mutant O35EkdtA (□) in BHI broth at 37°C. (B) The corresponding OMP profile of *M. catarrhalis* wild-type O35E (left lane) and mutant O35EkdtA (right lane) were shown by SDS-PAGE and Coomassie brilliant blue staining. Molecular size standards are shown in kilodaltons.

(1,282 bp) obtained from pUC4k with EcoRI digestion was subsequently inserted into the *kdtA* gene using the HindIII site to form pSAK.

After verification by sequence analysis, the mutagenic construct (containing an insertion of the kanamycin resistance cassette within a deletion of the *kdtA* coding region) was amplified by PCR using primers 35 and 36. The PCR-generated DNA product was purified and used for electroporation. After 24 h incubation, the resulting kanamycin-resistant colonies were selected for PCR analysis of chromosomal DNA using primers 34 and 36. The inactivated *kdtA* mutant was verified by sequence analysis and named as O35EkdtA. OMPs of both mutant and wild-type strains were prepared by the Zwittergent extraction method (5) and examined on SDS-PAGE gels with 7.5% separating gel.

Kdo assay. Outer membrane vesicles (OMV) were prepared by the EDTA-heat-induced method (38). Kdo was determined as described previously (48) by using 80 µg OMV preparation of wild-type or mutant strain as samples and Kdo ammonium salt (Sigma, St. Louis, MO) as a standard.

LOS determination. A crude LOS extraction was performed from both wild-type strain O35E and the mutant O35EkdtA using the proteinase-K-treated whole-cell lysate method (45). The resulting extracts from each bacterial suspension (1.9 µg of protein concentration) were resolved by 15% SDS-PAGE and visualized by silver staining (43). The OMV preparation was also applied since there was no detectable LOS in O35EkdtA whole-cell lysates. Western blot using a rabbit anti-LOS antibody was performed (28). For the composition analysis, 30 to 35 g of wet cells from O35E and O35EkdtA were prepared for LOS purification by phenol-water extraction (21) and then the phenol-chloroform-petroleum ether (PCP) method (17), due to the lack of LOS extracts from O35EkdtA. The glycosyl compositions of O35E LOS purified by phenol-water extraction and O35EkdtA LOS purified by PCP were determined by gas chromatography-mass spectrometry (GC-MS) analysis of trimethylsilyl methylglycosides. Also fatty acid methyl esters (49) were performed on an HP-5890 GC interfaced to a mass selective detector 5970 MSD using a Supelco DB1 fused silica capillary column (30 by 0.25 mm inside diameter; J & W Scientific, Folsom, California). The fatty acyl components of samples were detected by MS analysis. The sample was dissolved in 3:1 chloroform-methanol mixture at 1 µg/µl concentration and mixed with equal volume of 0.5 M 2,5-dihydroxy benzoic acid (matrix) and spotted with 1 µl on a 100-well stainless steel matrix-associated laser desorption ionization (MALDI) plate. The mass spectra were collected on a MALDI-time of flight (TOF) instrument (Applied Biosystems) in reflectron mode using 337-nm N₂ laser in negative mode.

Negative staining of bacteria. Bacteria were suspended on a slide in a small amount of water (28). Formvar-coated 200 mesh grids (Electron Microscopy Sciences, Fort Washington, PA) were floated on the bacterial suspension for 1 min, blotted dry, and then floated on a mixture of equal volumes of 2% ammonium acetate (Mallinckrodt Chemical, Inc., Paris, KY) and 2% ammonium molybdate (Sigma). The grids were viewed with a JEOL-1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Limulus amoebocyte lysate assay. The chromogenic *Limulus* amoebocyte lysate assay for endotoxin activity was performed using the QCL-1000 kit (Bio-Whittaker Inc., Walkersville, MD). Overnight cultures from chocolate agar plates were suspended in BHI broth to OD₆₀₀ of 0.1, and serial dilutions of these stocks were used as samples.

Susceptibility determination. The sensitivity of strains to a panel of hydrophobic agents or a hydrophilic glycopeptide was performed using standard disk diffusion assays (39). Bacteria were cultured in BHI to an OD₆₀₀ of 0.2, and 100-µl portions of the bacterial suspension were spread onto chocolate agar plates. Antibiotic disks or sterile blank paper disks (6 mm; Becton Dickinson, Cockeysville, MD) saturated with the various agents were plated on the lawn in triplicate at 37°C for 18 h. Sensitivity was assessed by measuring the diameter of the zone of growth inhibition in two axes, and the mean value was calculated.

Bactericidal assay. A complement-sufficient normal human serum was prepared and pooled from 8 healthy adult donors. A 200-µl bactericidal assay was performed in a 96-well plate (32). Normal human serum was diluted to 0.5, 2.5, 5.0, 12.5, and 25% in pH 7.4 Dulbecco's phosphate-buffered saline containing 0.05% (wt/vol) gelatin (DPBSG). Bacteria (10 µl of 10⁶ CFU) were inoculated into 190-µl reaction wells containing the diluted normal human serum, 25% of heat-inactivated normal human serum, or DPBSG alone and incubated at 37°C for 30 min. Serial dilutions (1:10) of each well were plated onto chocolate agar plates. The resulting colonies were counted after 24 h of incubation.

Adherence assay. Chang (conjunctival; CCL20.2), HeLa (cervix; CCL-2), and A549 (lung; CCL-185) human epithelial lines were cultured in Eagle's minimal essential medium (American Type Culture Collection, Manassas, VA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum in an atmosphere of 5% CO₂ at 37°C. A quantitative adherence assay was performed on a 24-well tissue culture plate (Corning Incorporated, Corning, NY) (1). Adherence was expressed as the percentage of bacteria attached to the human cells relative to the original bacteria added to the well. The data represent the average of 3 independent assays.

Pulmonary and nasopharyngeal clearance patterns in animal model. Female BALB/c mice (6 to 8 weeks of age) were obtained from Taconic Farms Inc. (Germantown, NY). The mice were housed in an animal facility in accordance with National Institutes of Health guidelines under animal study protocol 1158-04. Bacterial aerosol challenges were carried out in mice using the same OD₅₄₀ value of wild-type strain O35E (1.24 × 10⁹ CFU/ml) or mutant O35EkdtA (7.8 × 10⁸ CFU/ml) in 10 ml DPBSG (27). The number of bacteria present in the lungs and nasal washes was measured at various time points postchallenge. The minimum detectable numbers of viable bacteria were 100 CFU per lung and 4 CFU per nasal washing. Clearance of *M. catarrhalis* was expressed as the percentage of bacterial CFU at each time point compared with the number deposited at time zero. Due to the lower inoculum of the mutant strain, the challenge was repeated with similar doses of 6.2 × 10⁸ or 6.0 × 10⁸ (CFU/ml) for O35E or O35EkdtA to confirm if the clearance rate is strain dependent rather than inoculum dependent.

Statistical analysis. The adherence and clearance percentage was analyzed by chi-square test.

Nucleotide sequence accession number. The nucleotide sequence of *kdtA* gene in *M. catarrhalis* strain O35E has been deposited at GenBank under accession number AY854633.

RESULTS

Identification and cloning of *M. catarrhalis* O35E *kdtA*. *M. catarrhalis* mutants were constructed by transposon mutagenesis. The resulting kanamycin-resistant colonies were screened for loss of reactivity to a specific anti-LOS antibody. One clone (O35E705) with no detectable LOS band in the silver staining gel after SDS-PAGE was selected. The chromosomal DNA of O35E705 was purified and subjected to nucleotide sequence analysis which showed that the EZ:TN transposon was inserted in a 279-bp position of a DNA fragment within a single open reading frame of 1,308 bp (Fig. 1). BLAST searches at GenBank with the deduced polypeptide sequence revealed 35% or 34% identity and 52% or 51% similarity when compared with the *kdtA* amino acid sequence of *E. coli* or *Neisseria meningitidis*. Unlike the Kdo biosynthetic operon (*pyrG-kdsA-eno*) in *M. catarrhalis* (32), though the predicted *pepN* coding for an aminopeptidase N is in close proximity to the *kdtA* (Fig. 1), it is not related to LOS biosynthesis.

Construction of knockout *kdtA* mutant. To confirm the function of the predicted *kdtA* gene, a *kdtA* knockout mutant was

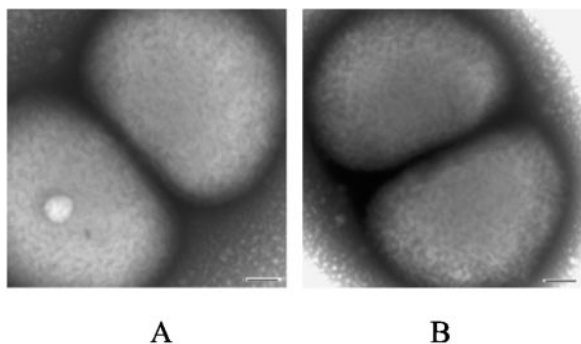


FIG. 3. Electron photomicrographs of negative staining of *M. catarrhalis* mutant O35EkdtA (A) and wild-type strain O35E (B). Scale bars, 100 nm.

constructed by allelic exchange with an insertion of a kanamycin resistance cassette into the HindIII site of the *kdtA* gene with a 956-bp deletion (Fig. 1). Primers 34 and 36 were used to amplify the *kdtA* gene from kanamycin-resistant colonies. The sequence analysis of PCR products confirmed that the kanamycin resistance cassette was inserted into *kdtA* of O35E chromosomal DNA at the predicted position. The clone was named as O35EkdtA.

Morphology and growth rate of *kdtA* mutant. O35EkdtA formed smaller colonies on chocolate agar plates when compared with the wild-type strain. The growth rate of O35EkdtA in BHI broth was significantly slower than that of wild-type strain at logarithmic phase (Fig. 2A). Comparative analysis of OMP by SDS-PAGE indicated that both wild-type and mutant strains showed similar OMP profiles (Fig. 2B). Electron microscopy further revealed that the shape and size of the O35EkdtA mutant (Fig. 3A) is similar to those of the wild-type strain (Fig. 3B).

Determination of Kdo and LOS contents in *kdtA* mutant. The OMV preparations were evaluated for the presence of Kdo, a specific sugar component of LOS. The concentration of Kdo in the OMV from the wild-type strain was 73 ng/80 µg of OMV, while that of the mutant was below detectable levels (<5 ng/80 µg of OMV). To detect any LOS-derived oligosaccharide component in the *kdtA* mutant, the LOS content of

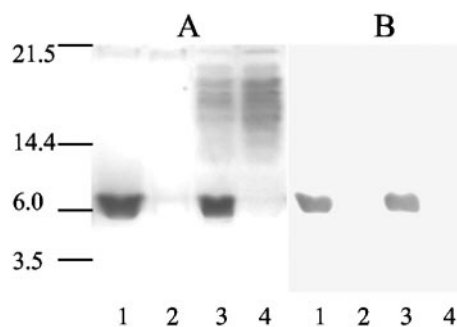


FIG. 4. LOS patterns of SDS-PAGE followed by silver staining (A) or Western blot (B) of *M. catarrhalis* wild-type strain O35E (lanes 1 and 3) and mutant O35E *kdtA* (lanes 2 and 4). Lanes 1 and 2 represent extracts from proteinase K-treated whole-cell lysates from 1.9 µg of each bacterial suspension, and lanes 3 and 4 represent 0.8 µg of each OMV. A rabbit anti-LOS antibody was used at 1:50 dilution (B).

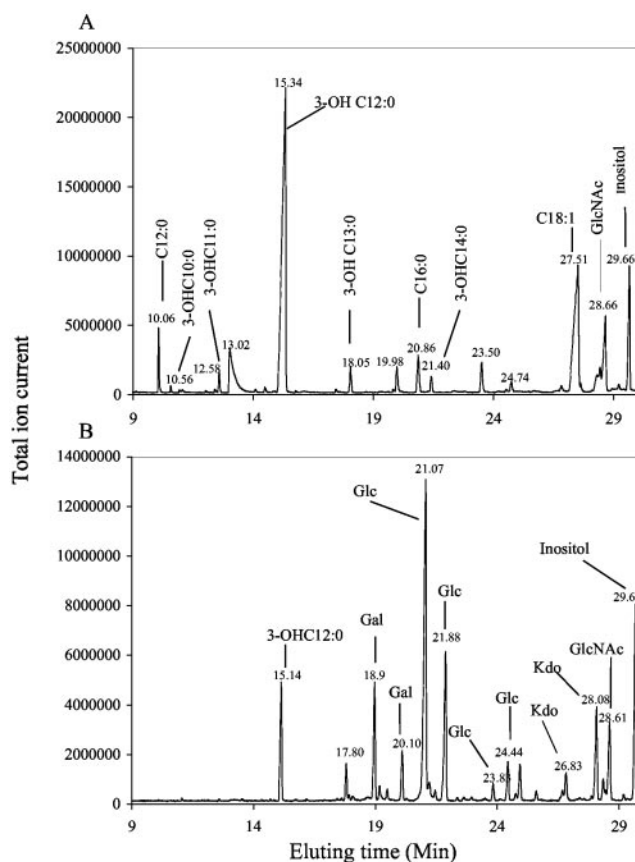


FIG. 5. Total ion current spectrum from GC-MS analysis of glycosyl compositions with 100 µg of PCP extract material from *M. catarrhalis* mutant O35EkdtA (A) or phenol-water extract from wild-type strain O35E (B). The components of the O35EkdtA (A) were identified as indicated and show the presence of lipid A fatty acids (3-OHC_{12:0}, 3-OHC_{10:0}, etc.) and *N*-acetylglucosamine (GlcNAc), as well as the fatty acids C_{16:0} and C_{18:1}, due to the presence of phospholipids (A). The components of the O35E (B) showed the sugar constituents (Kdo, Gal [galactose], Glc [glucose], and GlcNAc) and lipid A fatty acids (3-OHC_{12:0}). Inositol was added as internal standard.

both proteinase-K-treated cell lysates and OMVs from O35E and O35EkdtA was examined by SDS-PAGE analysis. Silver staining for carbohydrate (sensitivity up to 10 ng for LOS) after SDS-PAGE revealed that LOS was detected in O35E but not in O35EkdtA (Fig. 4A). The LOS band was confirmed by Western blot analysis; here a specific anti-LOS antibody detected LOS in the O35E but not in the O35EkdtA (Fig. 4B).

Structural analysis. GC-MS composition analysis of the PCP-extracted “LOS” preparation from the O35EkdtA mutant showed that the only glycosyl residue present was *N*-acetylglucosamine, together with fatty acyl residues that are typical of the lipid A (Fig. 5A). The presence of *N*-acetylglucosamine is presumably due to the diglucosamine backbone of the lipid A. In contrast, LOS preparation from the wild-type strain showed a number of glycosyl components typical of the core oligosaccharide, including Kdo (Fig. 5B). The MALDI-TOF MS analysis of the O35EkdtA PCP extract confirmed that the extract contained a variety of lipid A structures reflecting variations in molecular species. These are due to differences in phosphate and phosphoethanolamine (PEA) groups as well as some het-

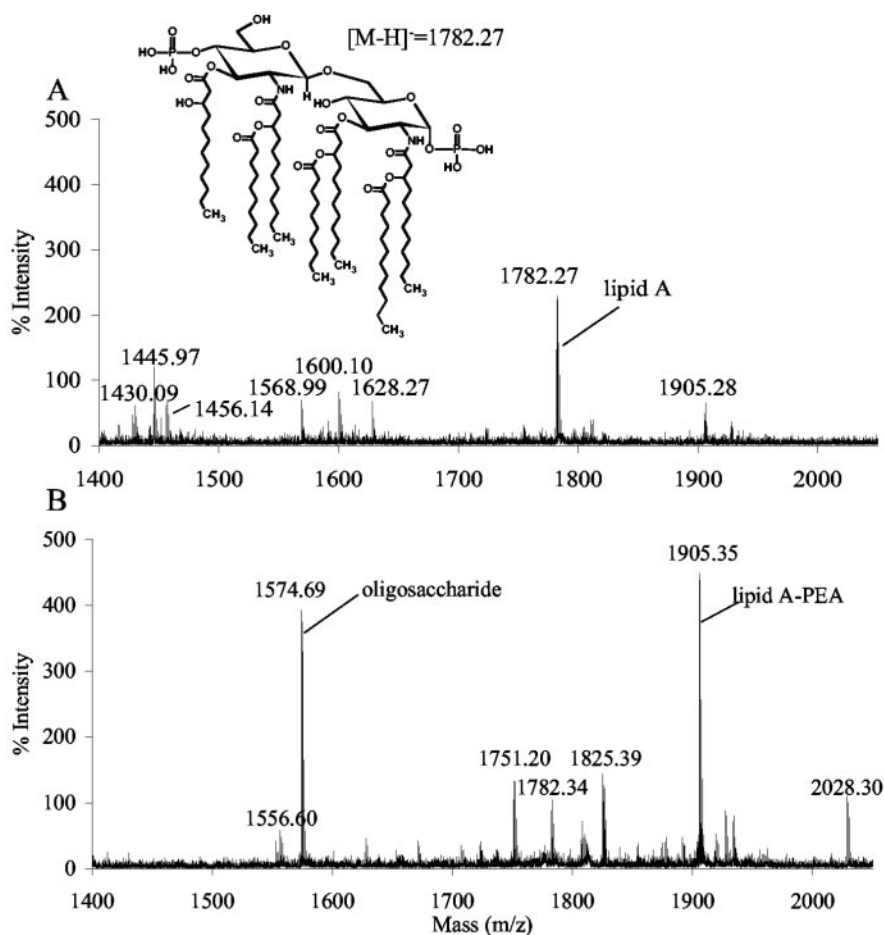


FIG. 6. MALDI-TOF mass spectral analysis of lipid A compositions with 1 μg of PCP extract material from *M. catarrhalis* mutant O35EkdA (A) or phenol-water extract from wild-type strain O35E (B). This analysis was done in the negative mode, and all ions are represented as deprotonated $[\text{M-H}]^-$ ions. The proposed lipid A composition for each ion is given in Table 2, and major ions with m/z values of 1,574.6 and 1,556.6 are due to the LOS oligosaccharide and its anhydro form, respectively. The upper portion of the figure shows the structure of the major species at 1,782.27.

erogeneity due to variations in fatty acid constituents (Fig. 6A; Table 2). A prominent species of the O35EkdA extract was the bis-phosphorylated lipid A, which is devoid of PEA groups (m/z 1,782). However, MALDI-TOF MS analysis of the paren-

tal lipid A isolated from the LOS by mild acid hydrolysis showed a predominant lipid A species with an additional PEA group (m/z 1,905 = m/z 1,782 + 123) (Fig. 6B; Table 2). These data indicate that the LOS from O35EkdA consists of intact

TABLE 2. Predicted lipid A compositions of LOS preparation from O35E and O35EkdA mutant based on MALDI-TOF MS analysis

Source	Ion m/z		Predicted lipid A composition ^a	Relative intensity ^b (%)
	Observed	Calculated		
O35E	2,028.30	2,029.19	$\text{P}_2\text{-PEA}_2\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_2}\text{-C}_{12:0_1}$	24
	1,905.35	1,906.18	$\text{P}_2\text{-PEA}_1\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_2}\text{-C}_{12:0_1}$	100
	1,825.39	1,826.18	$\text{P}_1\text{-PEA}_1\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_2}\text{-C}_{12:0_1}$	32
	1,782.34	1,783.17	$\text{P}_2\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_2}\text{-C}_{12:0_1}$	23
	1,751.20	1,752.04	$\text{P}_2\text{-PEA}_1\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_1}\text{-C}_{12:0_1}$	29
	O35EkdA	1,905.28	1,906.18	$\text{P}_2\text{-PEA}_1\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_2}\text{-C}_{12:0_1}$
1,782.27		1,783.17	$\text{P}_2\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_2}\text{-C}_{12:0_1}$	100
1,628.27		1,629.03	$\text{P}_2\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_1}\text{-C}_{12:0_1}$	29
1,600.10		1,601.00	$\text{P}_2\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_2}$	35
1,568.99		1,569.87	$\text{P}_2\text{-PEA}_1\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_1}$	29
1,456.14		1,456.89	$\text{P}_2\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{12:0_1}$ (minus H_2O)	30
1,445.97		1,446.86	$\text{P}_2\text{-GlcNAc}_2\text{-3-OHC}_{12:0_4}\text{-C}_{10:0_1}$	52
1,430.09		1,430.87	$\text{P}_2\text{-GlcNAc}_2\text{-3-OHC}_{12:0_3}\text{-C}_{10:0_1}\text{-C}_{12:0_1}$	27

^a P, phosphate moieties; GlcNAc, *N*-acetylglucosamine; 3-OHC_{12:0}, 3-hydroxydodecanoyl acyl chain; C_{12:0}, dodecanoyl acyl chain; C_{10:0}, decanoyl acyl chain.

^b The highest value of each strain represents 100% and others compared with the highest one.

TABLE 3. Susceptibilities of *M. catarrhalis* wild-type strain O35E and mutant O35EkdtA to a panel of hydrophobic agents or a hydrophilic glycopeptide

Compound (amt or concn)	Zone of growth inhibition for strains (mm) ^a	
	O35E	O35EkdtA
Azithromycin (15 µg)	29.0 ± 0.5	35.4 ± 0.5
Fusidic acid (10 mg/ml)	29.0 ± 0.9	32.5 ± 0.5
Novobiocin (5 µg)	13.8 ± 0.3	16.1 ± 0.3
Polymycin B (300 iu)	11.5 ± 0.5	15.5 ± 0.0
Rifampin (5 µg)	22.1 ± 0.3	29.0 ± 0.5
Vancomycin (5 µg)	<6.0 ^b	8.2 ± 0.3
Deoxycholate (100 mg/ml)	22.0 ± 0.9	26.8 ± 0.3
Triton X-100 (5% [wt/vol])	15.5 ± 0.5	24.5 ± 0.5
Tween 20 (5% [vol/vol])	11.5 ± 0.5	14.2 ± 0.3

^a Sensitivity was assessed by measuring the diameter of the zone of growth inhibition in two axes, and the mean value was calculated. Values are the means ± standard deviations. The data represent the averages of three separate experiments.

^b Stands for no inhibition for bacterial growth.

lipid A structures similar to O35E but with no Kdo or other oligosaccharide components.

Susceptibility of *kdtA* mutant. To determine if inactivation of the *kdtA* gene had an effect on the permeability of the outer membrane of the mutant strain, a broad range of hydrophobic agents and a hydrophilic glycopeptide were used to test the mutant's susceptibility. As shown in Table 3, O35EkdtA was more susceptible to most hydrophobic antibiotics, reagents, and a hydrophilic glycopeptide, vancomycin, than the wild-type strain.

Biological activity of *kdtA* mutant. To investigate the effect on bacterial toxicity or virulence due to loss of functional *kdtA* gene, the *kdtA* mutant was tested for LOS-associated biological activity. In a *Limulus* amebocyte lysate assay, whole-cell suspensions (OD₆₀₀ = 0.1) gave 3.7×10^3 endotoxin units (EU)/ml for O35E and 6×10^2 EU/ml for the O35EkdtA mutant, a sixfold reduction. In a bactericidal assay with normal human serum, strain O35E survived at 25% normal human serum, which was the highest concentration used. However, 50% of the mutant cells died at 2.5% normal human serum and 95% of the mutant cells died at 25% normal human serum (Fig. 7). The results indicated that the mutant strain showed reduced resistance to the complement killing activity of the normal human serum.

To test the adherence of the *kdtA* mutant to human epithelial cells, Chang, HeLa, and A549 cell lines were used (Table 4). Lack of expression of the *kdtA* gene in the mutant caused an approximately 50% reduction in adherence to Chang or HeLa epithelial cells, and no reduction for A549 epithelial cells.

To investigate the effect on survival of the *kdtA* mutant in a murine respiratory tract clearance model, mice were challenged with wild-type or mutant strain by aerosolization (Table 5). Mutant O35EkdtA present in the lungs showed a significantly accelerated clearance rate compared to the wild type at 3 h (86.0% versus 68.2%, $P < 0.01$) or 6 h (97.2% versus 84.0%, $P < 0.01$). Mutant O35EkdtA present in the nasopharynx showed a similar pattern at 3 h (92.3% versus 70.2%, $P < 0.01$) or 6 h (99.3% versus 90.1%, $P < 0.05$).

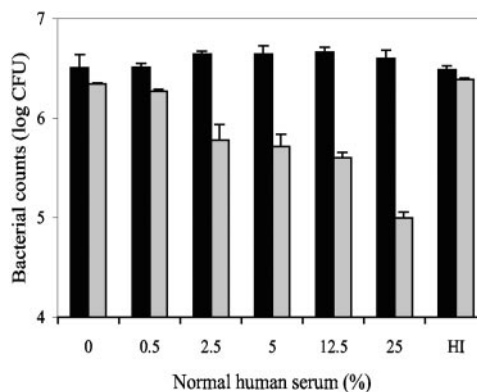


FIG. 7. Bactericidal activity of normal human serum against *M. catarrhalis* wild-type strain O35E (black bar) and mutant O35E *kdtA* (gray bar). "HI" represents the group of 25% heat-inactivated normal human serum. The data represent the averages of three independent assays.

Due to the lower inoculum of the mutant strain, the challenge was repeated with similar doses of 6.2×10^8 or 6.0×10^8 (CFU/ml) for O35E or O35EkdtA. The numbers of O35E and O35EkdtA present in lungs at 0 h were 32,854 (26,099 to 41,356) and 22,238 (16,276 to 30,386), respectively. Similar to the first challenge, the mutant O35EkdtA present in the lungs showed an accelerated clearance rate compared to the wild type at 3 h (85.6% versus 67.9%, $P < 0.01$) or at 6 h (94.5% versus 83.8%, $P < 0.05$) (data not shown). Meanwhile, the numbers of O35E and O35EkdtA present in nasopharynx at 0 h were 9,646 (6,865 to 13,556) and 7,916 (5,598 to 11,193), respectively. The mutant O35EkdtA present in the nasopharynx also showed an accelerated clearance rate compared to the wild type at 3 h (85.6% versus 61.4%, $P < 0.01$) (data not shown).

DISCUSSION

A *kdtA* gene from *M. catarrhalis* was identified by using random transposon mutagenesis and further confirmed by the construction and characterization of a *kdtA* knockout mutant. As expected, the mutant O35EkdtA did not produce a detectable LOS band by silver staining or Western blot from whole-cell lysates or OMVs. No Kdo component was detected in OMV prepared from O35EkdtA. These observations were supported by GC-MS and MS analysis, which showed that the LOS extracted from the O35EkdtA consisted of only the bisphosphorylated lipid A with seven fatty acyl chains. These data

TABLE 4. Adherence of *M. catarrhalis* wild-type strain O35E and mutant O35EkdtA to human epithelia

Strain	Adherence ^a (%)		
	Chang cells	HeLa cells	A549 cells
O35E	44.0 ± 6.6	42.6 ± 7.6	25.9 ± 11.2
O35EkdtA	20.6 ± 6.4*	19.0 ± 6.3*	25.1 ± 2.6

^a Adherence of *M. catarrhalis* organisms to human epithelia is expressed as mean (± standard deviation) percentage of bacteria attached to the human cells, based on the number of original bacteria that were added to the well. *, compared with O35E ($P < 0.01$, using chi-square test).

TABLE 5. Time course of bacterial recovery in mouse respiratory tract after an aerosol challenge with *M. catarrhalis* wild-type strain O35E and mutant O35EkdTA

Group ^a (sample source)	Bacterial recovery ^b (CFU/mouse) (SD range) and reduction ^c (%) at:							
	0 h		3 h		6 h		24 h	
	Recovery		Recovery	Reduction	Recovery	Reduction	Recovery	Reduction
O35E (lung)	112,489 (77,097–164,129)		35,782 (23,721–53,977)	68.2*	18,480 (11,764–29,031)	84.0*	102 (50–211)	ND ^d
O35EkdTA (lung)	63,400 (54,963–73,130)		8,979 (7,861–10,256)	86.0**	1,771 (787–3,986)	97.2**	<100	ND
O35E (nasal washes)	15,661 (12,596–19,470)		4,662 (2,776–7,829)	70.2*	1,541 (1,104–2,149)	90.1***	26 (11–61)	ND
O35EkdTA (nasal washes)	14,614 (9,976–21,410)		1,123 (659–1,912)	92.3**	101 (56–181)	99.3****	4 (1–12)	ND

^a Mice were challenged by aerosol with 10 ml of 1.24×10^9 CFU/ml O35E or 7.8×10^8 CFU/ml O35EkdTA.

^b Lung and nasal washes were collected for bacterial counts, and each time point represents a geometric mean bacterial CFU of six mice.

^c Compared with time point zero. * versus **, significant difference in bacterial reduction between O35E and O35EkdTA at $P < 0.01$. *** versus ****, significant difference at $P < 0.05$. The chi-square test was used for all comparisons.

^d ND, bacterial recoveries in some mice were under detectable level.

indicate that inactivation of the *kdtA* gene in *M. catarrhalis* results in viable bacteria with only an intact lipid A structure, similar to a known *kdtA* mutant in *N. meningitidis* (45) and a *kdsA* mutant in *M. catarrhalis* (32).

In the lipid A biosynthesis pathway of *E. coli* and other enteric bacteria, the late acyltransferases require the presence of the Kdo disaccharide in their substrate for activity since lipid IV_A is the major component that accumulates in all mutants with defects in Kdo (4, 7, 8). In our study, the lipid A precursor of *M. catarrhalis* is completely acylated prior to addition of Kdo. This phenomenon has been only observed in *Pseudomonas aeruginosa* (19, 34) and *N. meningitidis* (45). In addition, our data suggest that late acyltransferases of *M. catarrhalis* incorporate one or two secondary fatty acyl chains to each glucosamine unit with an asymmetrical pattern, while the acyltransferases of *P. aeruginosa* or *N. meningitidis* add one secondary fatty acyl chain in a symmetrical pattern.

The *M. catarrhalis kdtA* mutant showed complete or incomplete septum separation in binary cells with similar shapes and sizes as the wild type, as determined by electron microscopy. However the growth rate of the *kdtA* mutant was dramatically reduced in comparison to the wild type. In *N. meningitidis*, the *kdtA* mutant also showed a reduced growth rate, but with incomplete septum separation in tetrads. This is believed due to a defect in murein hydrolase activity or other cell division enzymatic activities (45). In addition, our *kdtA* mutant was susceptible to hydrophobic compounds as well as a hydrophilic glycopeptide that is normally excluded by the intact bacterial outer membrane, and also sensitive to the bactericidal activity of human normal serum. It is not clear if these changes are caused by a direct loss of the oligosaccharide moiety of the LOS on the outer membrane, or an indirect effect on surface display of the membrane molecules, such as proteins. It has been shown that UspA2 expression is involved in serum resistance by *M. catarrhalis* (1, 30) and the UspA2 tightly associated with the LOS (11). The lack of oligosaccharide moiety of the LOS may affect surface display of UspA2 and cause sensitive phenotype of the *kdtA* mutant. These data suggest that the oligosaccharide moiety of the LOS molecule is an important component in maintaining complement resistance of the bacteria to human normal serum and the integrity of the bacterial outer membrane.

Although lipid A has long been recognized as the active

moiety for endotoxic activity (16), variations in the saccharide content of LOS/LPS have been reported as structural determinants of macrophage activation by different LOS/LPS. For example, the polysaccharide portion covalently bound to lipid A played a principal role in *Salmonella* LPS-induced activation (35). Kdo₂ linked to meningococcal lipid A was structurally required for maximal activation of the macrophages (51). In the *Limulus* amoebocyte lysate assay, our *kdtA* mutant showed a sixfold reduction of endotoxin activity compared to that of the wild type. The data imply that oligosaccharides in *M. catarrhalis* LOS may play an important role in preserving its biological activity.

Bacterial adherence to the surface of epithelial cells plays a critical role in colonization and is believed to be the first step in the pathogenesis of microbial infections. It has been reported that several LOSs from respiratory tract bacteria are associated with bacterial adherence (2, 18, 20, 41, 44). Fitzgerald et al. showed that the bacterial adherence of *M. catarrhalis* might be mediated by carbohydrate moieties (12). Our previous study revealed that a specific anti-LOS monoclonal antibody significantly inhibits the adherence of *M. catarrhalis* to human epithelial cells (28). In this report, attachment to human epithelial cells by the *kdtA* mutant showed more than a 50% reduction in attachment to Chang or cervix epithelial cells while there was no reduction in attachment to lung epithelial cells. These data imply that the mutant has variable affinity to different types of epithelial cells and that LOS in *M. catarrhalis* is involved in adherence to certain epithelial cells, which may be associated with *M. catarrhalis* pathogenesis in specific organs. Besides the LOS, several OMPs have shown as adhesion molecules. Among them, UspA1 appeared spontaneous phase variation in vitro resulting in significant changes in binding to host cells (31). To rule out a possibility that the reduced adherence of the *kdtA* mutant was caused by such phase variation, the expression of *uspA1* was examined. It was reported that expression of the UspA1 was correlated with both adherence ability in vitro and the number of guanine (G) residues in a homopolymeric [poly (G)] tract located upstream of the *uspA1* open reading frame (31). High expression of UspA1 (10 Gs) would have higher binding capability while low expression of UspA1 (9 Gs) resulted in lower binding capability. When nucleotide sequence analysis of the *uspA1* was performed, both mutant and wild type strains showed 9 G residues in their

uspA1 poly(G) tracts (data not shown), suggesting that both strains retained a lower expression level of UspA1 protein, and the expression of UspA1 in *kdtA* mutant did not undergo phase variation spontaneously. In addition, the *kdtA* mutant showed enhanced clearance from the mouse nasopharynx and lung after an aerosol challenge. The involvement of LOS in the ability of *M. catarrhalis* to survive in vivo raises the possibility that LOS might be essential for the virulence of the organism in human respiratory tract.

In summary, we identified a *M. catarrhalis kdtA* homologue by a transposon mutagenesis approach and constructed an isogenic *kdtA* mutant that produces truncated LOS with only intact lipid A structure. The resulting mutant was sensitive to the bactericidal activity of human normal serum, and presented reduced endotoxin activity, reduced bacterial attachment in vitro, and/or increased bacterial clearance in vivo. This indicates that LOS is an important virulence factor in the pathogenesis of *M. catarrhalis* infections. The finding may lead to implications for the development of vaccines and to designs of therapeutic intervention against *M. catarrhalis* infections.

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