Published in final edited form as:

FEBS J. 2008 October ; 275(20): 5201–5214. doi:10.1111/j.1742-4658.2008.06651.x.

Identification of Two Late Acyltransferase Genes Responsible for Lipid A Biosynthesis in *Moraxella catarrhalis*

Song Gao^{1,3}, Daxin Peng^{1,3}, Wenhong Zhang¹, Artur Muszyński², Russell W. Carlson², and Xin-Xing Gu¹

¹ Vaccine Research Section, National Institute on Deafness and Other Communication Disorders, Rockville, Maryland 20850, USA

² Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602

Abstract

Lipid A is a biological component of the lipooligosaccharide (LOS) of a human pathogen, *Moraxella catarrhalis*. No other acyltransferases except UDP-GlcNAc acyltransferase responsible for lipid A biosynthesis in *M. catarrhalis* have been elucidated. By informatics, two late acyltransferase genes *lpxX* and *lpxL* responsible for lipid A biosynthesis were identified, and knockout mutants of each gene in *M. catarrhalis* strain O35E was constructed and named as O35ElpxX and O35ElpxL. Structural analysis of lipid A from the parental strain and derived mutants showed that O35ElpxX lacked two decanoic acids (C10:0) while O35ElpxL lacked one dodecanoic (lauric) acid (C12:0), suggesting that *lpxX* gene encoded decanoyl transferase and *lpxL* gene encoded dodecanoyl transferase. Phenotypic analysis revealed that both mutants were similar to the parental strain in their toxicity *in vitro*. However, the O35ElpxX was sensitive to bactericidal activity of normal human serum and hydrophobic reagents. It had a reduced growth rate in broth and an accelerated bacterial clearance at 3 h ($p < 0.01$) or 6 h ($p < 0.05$) after an aerosol challenge in a murine model of bacterial pulmonary clearance. Meanwhile, the O35ElpxL presented alike patterns as the parental strain except it was slightly sensitive to the hydrophobic reagents. These results indicate that these two genes, particularly *lpxX*, encoding late acyltransferases responsible for incorporation of the acyloxyacyl linked secondary acyl chains into the lipid A are important for biological activities of *M. catarrhalis*.

Keywords

Moraxella catarrhalis; late acyltransferase; lipooligosaccharide; *lpxX*; *lpxL*

Introduction

Moraxella catarrhalis is the third most common isolate following *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* as the causative agent of otitis media in infants and young children [1–3]. In developed countries, more than 80% of children under the age of 3 years will be diagnosed at least once with otitis media, and *M. catarrhalis* is responsible for 15 to 25% of all of these cases [4,5]. In adults with chronic obstructive pulmonary disease (COPD), which is the fourth leading cause of death in the United States, this organism is known to be the second cause of exacerbations of lower respiratory tract infections [6,7]. Approximately 20 million cases of such exacerbations are reported each

Correspondence: Xin-Xing Gu, National Institute on Deafness and Other Communication Disorders, 5 Research Court, Rockville, Maryland 20850, USA. Tel.: 301-402-2456; Fax: 301-435-4040; E-mail: guxx@nidcd.nih.gov.

³Current address: School of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu 225009, China.

year in the United States, up to 35% of them resulting from *M. catarrhalis* infections [8]. In immunocompromised hosts, *M. catarrhalis* causes a variety of severe infections including septicemia and meningitis. Clinical and epidemiological studies revealed high carriage rates in young children and suggested that a high rate of colonization was associated with an increased risk of the development of *M. catarrhalis*-mediated diseases [3]. Currently, the molecular pathogenesis of *M. catarrhalis* infection is not fully understood.

As a Gram-negative bacterium without capsular polysaccharides, *M. catarrhalis* is surrounded by an outer membrane consisting of lipooligosaccharide (LOS), outer membrane proteins and pili outside phospholipids [3]. LOS is a major outer membrane component of *M. catarrhalis* with three major LOS serotypes, A, B and C [9–12]. Quite a few studies have demonstrated that LOS is an important virulence factor for many respiratory pathogens, such as *Neisseria meningitidis* and *Haemophilus influenzae* [13–15]. Studies have also implicated that *M. catarrhalis* LOS is important in the pathogenesis of *M. catarrhalis* infection [16–19]. In contrast to the LOS or LPS molecules from most Gram-negative bacteria, *M. catarrhalis* LOS consists of only an oligosaccharide (OS) core and lipid A [9]. The inner core OS is attached to 3-deoxy-D-manno-octulosonic acid (Kdo) through a glucosyl residue instead of a heptosyl residue [10,20] while the lipid A portion consists of seven shorter fatty acid residues (decanoyl or dodecanoyl, C10:0 or C12:0) [10,11].

Recently, several genes associated with LOS biosynthesis of *M. catarrhalis*, especially for the core OS moiety, were reported. Zaleski et al. identified a *galE* gene encoding UDP-glucose-4-epimerase in *M. catarrhalis* and showed inactivation of the gene resulting in an LOS lacking two terminal galactosyl residues [21]. Luke et al. showed a *kdsA* gene encoding Kdo-8-phosphate synthase and found a *kdsA* mutant consisting only of lipid A on its LOS molecule [22] while Peng et al. identified a *kdtA* gene encoding Kdo transferase during the LOS biosynthesis [18]. Edwards et al. revealed a cluster of three LOS glycosyltransferase genes (*lgt*) for extension of OS chains to the inner core [23] and an *lgt4* in serotype A and C strains [24]. Subsequently Wilson et al. found the *lgt5* gene encoding an α -galactosyltransferase for addition of the terminal galactose of the LOS [25] while Schwingel et al. found the *lgt6* gene involved in the initial assembly of the LOS [26]. However, as for the lipid A biosynthesis of the *M. catarrhalis* LOS, only an *lpxA* gene encoding UDP-N-acetylglucosamine acyltransferase responsible for the first step of lipid A or LOS biosynthesis in *M. catarrhalis* was identified and characterized [19]. Little is known regarding the late steps of the lipid A biosynthesis, particularly in the addition of the decanoyl and dodecanoyl acyloxyacyl residues.

Our knowledge of the enzymology and molecular genetics of the lipid A biosynthesis is based mainly on the studies of the LPS expressed by the enteric bacterium, especially *Escherichia coli*. The last steps of *E. coli* lipid A biosynthesis involve the addition of lauroyl and myristoyl residues to the distal glucosamine unit, generating acyloxyacyl moieties. The *E. coli* lauroyl and the myristoyl transferases are encoded by *lpxL* and *lpxM*, respectively, known as *htrB* and *msbB* prior to elucidation of their functions [20]. In this study, we identified two late acyltransferase genes encoding decanoyl transferase and dodecanoyl transferase from *M. catarrhalis* serotype A strain O35E and constructed the corresponding isogenic mutants. Analysis of physiochemical and biological features of both mutants was performed to study the functions of these genes and the structures of their resultant LOSs *in vitro* and *in vivo*.

Results

Identification of putative late acyltransferase genes of *M. catarrhalis* O35E

Two putative late acyltransferase genes in *M. catarrhalis* strain O35E were identified by BLAST searching from the *M. catarrhalis* partial genome sequence (AX067448 and AX067465). According to the sequence analysis results and structural data of each lipid A, these two genes were named as *lpxX* and *lpxL*. Analysis of promoter and ORF showed that the *lpxX* or *lpxL* DNA fragment contained a single ORF of 924 or 978 bp with a predicted gene product of 307 or 325 amino acids (Fig. 1). Upstream sequence analysis of the *lpxX* gene revealed the presence of a gene encoding aspartyl-tRNA synthetase (*ats*), while sequence analysis for the downstream region of the *lpxX* revealed the presence of a glycosyltransferase (*lgt 6*) gene (Fig. 1A) [26]. The upstream gene of *lpxL* was *atr* (encoding ABC transporter related protein), and the downstream gene was *asd* (encoding aspartate 1-decarboxylase) (Fig. 1B). The deduced amino acid sequences of *lpxX* and *lpxL* showed 19–32% identity and 39–50% similarity compared with the identified late acyltransferase homologues of other Gram-negative bacteria (Table 2). However, the identity and similarity between *lpxX* and *lpxL* were only 22% and 37%, respectively. Protein sequence analysis of *M. catarrhalis* LpxX and LpxL revealed that both contained membrane-spanning regions anchoring the proteins to the inner membrane but not in the cytoplasm of the bacterium (Data not shown), which is consistent to those of defined *E. coli* late acyltransferases [27]. The transmembrane helix locations and topology structures of LpxX and LpxL were also similar with that of *E. coli* late acyltransferases.

Construction and characterization of *lpxX* and *lpxL* knockout mutants

The *lpxX* mutant was constructed by allelic exchange of a 53-bp deletion within the induced EcoRI sites of the *lpxX* coding region with a substitution of a zeocin-resistant (*Zeo*^r) cassette, and the *lpxL* knockout mutant was constructed by allelic exchange of a 454 bp deletion between two PstI sites of the *lpxL* coding region with a substitution of a kanamycin-resistant (*Kan*^r) cassette (Fig. 1). Nucleotide sequence analysis of PCR products confirmed that the cassettes had been inserted into the chromosomal DNA at the predicted positions. The mutant bacteria were named as O35ElpxX and O35ElpxL.

Southern blot was performed to test if a single copy of the *Zeo*^r or *Kan*^r gene was inserted into the genome, the O35ElpxX or O35ElpxL genomic DNA was digested with EcoRV (Fig. 2A/C) and probed with DIG-labeled *Zeo*^r gene or DIG-labeled *Kan*^r gene, respectively (Fig. 2B/D). Only one band was detected in the chromosomal DNA of the O35ElpxX or O35ElpxL (Fig. 2B, lane 2; Fig. 2D, lane 4), but none in that of the parental O35E (Fig. 2B/D, lane 1), showing a single insertion in the genome of each mutant.

To determine if the insertion had a polar effect on the upstream or downstream gene, total RNA isolated from the parental O35E, O35ElpxX or O35ElpxL was subjected to RT-PCR analysis using primer sets designed for genes *ats* [*ats1/ats2*], *lpxX* [*b1SP/b1AP*] and *lgt 6* [*lg1/lg2*]; or genes *atr* [*atr1/atr2*], *lpxL* [*b2SP/b2AP*] and *asd* [*asd1/asd2*], respectively. When compared to the parental O35E, the insertion of the *Zeo*^r gene in the O35ElpxX only disrupted the *lpxX* gene transcription (Fig. 3A, lane 4b), and the insertion of the *Kan*^r gene in the O35ElpxL only disrupted the *lpxL* gene transcription (Fig. 3B, lane 7e).

Determination of LOSs in *lpxX* and *lpxL* mutants

An attempt was made to isolate LOS from proteinase K-treated cell lysates of O35E, O35ElpxX and O35ElpxL. Silver staining analysis after SDS-PAGE with three extracts revealed a different migration manner for the mutant LOS as compared to that of the parental LOS. In particular, for the O35ElpxX mutant LOS, the band was located below the

O35E band (Fig. 4, lane 2), and had a reduced intensity and a conversion from a black to a brown coloration while the LOS migration of the O35ElpxL (Fig. 4, lane 4) was slightly below that of the parental LOS. After complementation of the parental lpxX or lpxL gene by pWlpxX or pWlpxL (Table 1), silver staining analysis with the revertant O35ElpxX or O35ElpxL strain showed that an LOS band migrated in a manner identical to that of the parental LOS. The LOS band of the revertant O35ElpxX strain also had a conversion from a brown to a black coloration (Fig. 4, lanes 3, 5).

Composition and MALDI-TOF MS analysis of lipid A in *lpxX* and *lpxL* mutants

The fatty acid compositions of the lipid A liberated from strain O35E, O35ElpxX and O35ElpxL are shown in Fig. 5. The published lipid A structure of the *M. catarrhalis* serotype A strain ATCC 25238 is acylated with four molecules of 3OH-C12:0, two of C10:0 and one of C12:0 [10]. When compared to this structure, the lipid A of the O35ElpxX lacks two decanoic acyl (C10:0) substituents, and the O35ElpxL lacks one lauroyl acid (C12:0) substituents.

MALDI-TOF MS analysis showed differences in the mass of lipid A from both mutants as compared to that of their parental strain (Fig. 6). The MS of the lipid A from strain O35E LOS (Fig. 6A) revealed the presence of three minor species of ions at m/z 1907.94, 1930.33, 1953.05 and a major ion at m/z 1784.75. The 1907.94 ion represented a lipid A that had the composition of published lipid A structure; i.e. P₂-PEA-GlcNAc₂-3OHC12:0₄-C10:0₂-C12:0₁ and its mono- and di-sodiated forms at m/z 1930.33 and 1953.05, respectively. The major observed ion at m/z 1784.75 is due to this structure that lacks a phosphoethanolamine (PEA) group (i.e. less 123 amu). The loss of the PEA group likely occurs due to the lability of its pyrophosphate bond, which can hydrolyze to the lipid A phosphate under mild acid hydrolysis conditions.

Compared to the lipid A of the parental LOS, the spectrum of lipid A from the O35ElpxX mutant (Fig. 6B) is consistent with a structure that lacks decanoic acid (C10:0). This result is consistent with data from fatty acid methyl esters (FAMES) analysis (Fig. 5B). The lipid A from O35ElpxX revealed the presence of three major ions at m/z 1476.06, 1498.08, and 1520.17. These ions represented the structure P₂-GlcNAc₂-3OHC12:0₄-C12:0₁ lacked PEA group and its mono- and di-sodiated forms. The cluster of ions at m/z 1599.26, 1623.29 and 1646.28, respectively, represented the structure having the compositions: P₂-PEA-GlcNAc₂-3OHC12:0₄-C12:0₁ (1599.26) and its mono- and di-sodiated forms. The other ions at m/z 1395.97, 1417.86 and 1440.96 were due to a monophosphorylated structure P-GlcNAc₂-3OHC12:0₄-C12:0₁ (1395.97) and its mono- and di-sodiated forms. The ion at m/z 1293.62 was due to a monophosphorylated tetraacylated structure P-GlcNAc₂-3OHC12:0₄ and the ions at m/z 1315.67 and 1337.84 were mono- and di-sodiated forms, respectively.

Consistent with observation from FAMES analysis for the lipid A from O35ElpxL which lacks lauric acid (C12:0), the MALDI-TOF spectrum (Fig. 6C) shows an ion at m/z 1725.62 which corresponds to a composition of P₂-PEA-GlcNAc₂-3OHC12:0₄-C10:0₂. Its mono- and di-sodiated forms are also present at m/z 1748.63 and 1770.65. The ion at m/z 1602.42 is due to a lipid A structure that lacks a PEA group (a loss of 123 mass units from m/z 1725.62) and corresponds to a composition of P₂-GlcNAc₂-3OHC12:0₄-C10:0₂. The ions at m/z 1625.44 and 1645.53 are mono- and di-sodiated species. The ion at m/z 1448.05 is due to a structure that has a composition of P₂-GlcNAc₂-3OHC12:0₄-C10:0₁, and the m/z 1470.08 and 1492.20 ions are its mono- and di-sodiated forms, respectively (Fig. 6C).

Composition and structural analysis of OSs in LOSs of *lpxX* and *lpxL* mutants

The OS portion of each LOS was obtained after mild acid hydrolysis and analyzed for its glycosyl compositions and by MALDI-TOF MS (Fig. 7). Glycosyl composition analyses of the OS from either O35ElpxX or O35ElpxL LOS all show a glycosyl residue ratio of Gal₂Glc₅GlcNAc₁Kdo, which is consistent with the glycosyl components of the published serotype A structure for the parental strain [10]. These results are consistent with the conclusion that the OS from O35ElpxX and O35ElpxL mutants have the same structure as that of the parental strain O35E.

Morphology and growth rate of *lpxX* and *lpxL* mutants

The O35ElpxX formed small and transparent colonies on the chocolate agar plates when compared with the parental strain. When it grew in BHI broth, its growth rate was slower than the parental strain in logarithmic phase (Fig. 8A). The colonies of reverted O35ElpxX with pWlpxX (Table 1) were similar to those of the wild type strain (data not shown). For the O35ElpxL mutant, the colonies on the chocolate agar plates were similar to those of the parental strain, and the growth rate in BHI broth in logarithmic phase was also similar to that of the parental strain (Fig. 8A).

Susceptibility of *lpxX* and *lpxL* mutants

A broad range of hydrophobic agents and a hydrophilic glycopeptide were used to determine the susceptibility of both mutants. Both mutants, especially O35ElpxX, exhibited more susceptibility to most hydrophobic antibiotics and reagents than that of the parental strain, except that O35ElpxL was more resistant to deoxycholate than the parental strain. Both O35ElpxX and O35ElpxL showed a similar resistance to the hydrophilic glycopeptide, vancomycin, as the parental strain (Table 3).

Biological activities of *lpxX* and *lpxL* mutants

The O35ElpxX and O35ElpxL mutants were tested for LOS-associated biological activity. In an LAL assay, whole cell suspensions ($A_{620} = 0.1$) gave 2.24×10^3 endotoxin units (EU)/ml for O35E, 7.26×10^3 EU/ml for O35ElpxX and 6.05×10^3 EU/ml for O35ElpxL, respectively.

In a bactericidal assay, 87.7% and 87.4% of cells from strain O35E survived at 12.5% and 25% of normal human serum, respectively (Fig. 8B). However, only 50.3% or 34.5% ($p < 0.05$) of cells from the O35ElpxX mutant survived at 12.5% or 25% of normal human serum while no difference was found between the O35ElpxL mutant and the parental strain, indicating a reduced resistance to the normal human serum from the O35ElpxX.

In a murine respiratory clearance model after an aerosol challenge with each viable bacterium, the O35ElpxL showed a similar bacterial clearance pattern as the parental strain. However, the number of O35ElpxX cells presented in mouse lungs was approximately 5-fold lower than that of the parental O35E cells right after the challenge (Fig. 8C), and the O35ElpxX mutant also showed an accelerated bacterial clearance at 3 h (86.5% vs. 61.3%, $p < 0.01$) or 6 h (96.8% vs. 88.9%, $p < 0.05$).

Discussion

In our previous study, an *lpxA* gene encoding UDP-N-acetylglucosamine acyltransferase responsible for the first step of lipid A biosynthesis in *M. catarrhalis* serotype A strain O35E was identified and an isogenic knockout mutant exhibited with a loss of LOS structure [19]. Here, two late acyltransferase genes responsible for lipid A biosynthesis were identified and their isogenic knockout mutants were constructed. Structural analysis revealed

that O35ElpxX mutant lacked two decanoic acid (C10:0) chains while O35ElpxL mutant did not acylate lipid A with a dodecanoic (lauric) acid (C12:0). In the literature, the nomenclature for the *lpxL/M* or *htrB/MsbB* is inconsistent among other bacteria. In *E. coli* LPS biosynthesis, the late acyltransferase *lpxL* was found to be responsible for the addition of a secondary laurate (C12:0) moiety to the 2' position of lipid A [27] while an *lpxM* is responsible for the addition of a secondary myristate (C14:0) chain at the 3' position of lipid A [36,37]. In *H. influenzae*, the *htrB* (*lpxL*) gene product was shown to be responsible for the addition of a secondary myristate (C14:0) chain at the 2' and 3' positions of lipid A [31] while in meningococci, the *lpxL1* (*msbB*) and *lpxL2* were responsible for the addition of secondary laurate (C12:0) chains at the 2 and 2' positions of lipid A [29,38]. In our case, the *lpxX* stands for acylating lipid A with decanoic acids (C10:0), which has not been identified in other species. The *lpxX* in *M. catarrhalis* O35E was responsible for the addition of two secondary decanoate (C10:0) chains at the 2' (reducing end) and 3 (non-reducing end) positions of lipid A while the *lpxL* was responsible for the addition of a secondary laurate (C12:0) chain at the 2 (non-reducing end) position of lipid A, suggesting that the roles of *lpxX* and *lpxL* in *M. catarrhalis* are not exactly the same as those of *lpxL/M* in *E. coli*, *htrB* (*lpxL*) in *H. influenzae* or *lpxL/MsbB* in meningococci at both acylation positions and secondary moieties, which match each unique LOS/LPS structure of different species [10].

With respect to the physicochemical properties, the *E. coli lpxL* mutation does not affect the mobility of LPS on SDS-PAGE gels, but the silver-stained LPS has a dramatically reduced intensity and a conversion from a black to a brown coloration [39]. The LOS isolated from *H. influenzae* strain 2019 *htrB* mutants migrates faster than the wild type LOS and its color changed from black to brown on the silver-stained gels [31]. Our data suggested that the migration and the staining of the LOS from O35ElpxX were similar to the patterns of *H. influenzae* [31] while those of the LOS from O35ElpxL were different from those of *H. influenzae* or *E. coli* [39].

The O35ElpxX that lacks two decanoic acid substituents on its lipid A was very susceptible to most hydrophobic reagents while the O35ElpxL that lacks the single dodecanoic acid substituent on its lipid A was slightly susceptible, except for deoxycholate, as compared to the parental strain. These results imply that the susceptibility of the mutants to hydrophobic reagents depends on the fatty acylation pattern of their lipid A, and that the O35ElpxX, which contains pentaacylated lipid A, allowed more diffusion of hydrophobic solutes than the O35ElpxL, which contains hexacylated lipid A. The fact that both mutants and their parental strain were resistant to a hydrophilic glycopeptide which was normally excluded by an intact enterobacterial outer membrane [40] might indicate that, even though the lipid A of the *M. catarrhalis lpxL* mutants are altered, their ability to have a normal OS allows them to form an outer membrane that can still resist the hydrophilic glycopeptide. It was not clear why the O35ElpxL was resistant to deoxycholate, as were the *E. coli lpxL* mutants [27]. The mechanism of hydrophobic reagents susceptibility in the *M. catarrhalis* mutants needs to be further studied.

LOS toxicity was assumed to be associated mostly with the lipid A moiety. We analyzed the toxicity of *M. catarrhalis* mutants by an *in-vitro* LAL assay. Both the C10:0 acyl chain deficient O35ElpxX and the C12:0 acyl chain deficient O35ElpxL mutants did not show reductions of the toxicity by LAL assay; however, an LOS null-mutant [19] showed decreased toxicity (0.14 EU/ml) as compared with the parental strain (3.7×10^3 EU/ml). Future studies need to evaluate the toxicity of both mutants *in vivo* to confirm the results from the LAL assay.

In addition, the O35ElpxX was sensitive to the bactericidal activity of a normal human serum when compared to the parental strain, but less sensitive than that of the LOS-null

mutant [19]. These results suggested that the permeability change on the outer membrane barrier of the *M. catarrhalis* mutants might increase their sensitivity to the complement killing of the serum and this permeability varied with the impairment extent of its lipid A or LOS.

In a mouse challenge model, the O35ElpxX mutant strain showed a significantly enhanced clearance than O35ElpxL mutant strain or the parental strain from mouse lungs after an aerosol challenge with viable bacteria. The difference between these two mutants in the bacterial clearance might reflect the difference in the integrity of their outer membrane, binding activity and the sensitivity of the murine complement-mediated killing.

In conclusion, the *lpxX* and *lpxL* genes responsible for two late acyltransferases, decanoyl and dodecanoyl transferases, were identified in *M. catarrhalis*. The acyloxyacyl linked secondary acyl chains of lipid A moiety of the LOS are important in some biological activities of *M. catarrhalis*. Elucidation of lipid A/LOS biosynthesis, structure and functions *in vitro* and *in vivo* may provide insights into the mechanisms of *M. catarrhalis* pathogenesis and the immune response to infection.

Experimental procedures

Bioinformatics

Two putative late acyltransferase genes were predicted from the partial *M. catarrhalis* genome (AX067448 and AX067465, NCBI patent number WO0078968). To determine the gene sequences, the putative promoter sequences were predicted by a neural network based program [41] and the ORFs of these two genes were determined with Glimmer method [42]. Topology predictions of the deduced proteins were performed using Tmpred, TopPred and PredictProtein methods [43–45]. Similarities of these two proteins with late acyltransferase homologues in several other Gram-negative bacteria were searched by BLAST.

Strains, plasmids, primers and growth conditions

Bacterial strains, plasmids and primers are listed 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, zeocin 5 µg/ml or spectinomycin 15 µg/ml. Growth rates of wild type strain and mutants were measured as follows: an overnight culture was inoculated in 10 ml of BHI media (adjusted OD₆₀₀ = 0.05) and shaken at 37°C at 250 rpm. The bacterial cultures were monitored spectrophotometrically at OD₆₀₀ for 8 h. The data represented averages of three independent assays. *E. coli* was grown on Luria-Bertani agar plates or broth with appropriate antibiotic supplementation. The antibiotic concentrations used for *E. coli* were as follows: kanamycin, 30 µg/ml; zeocin, 25 µg/ml and 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 plasmids, 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).

Cloning of *lpxX* gene and construction of the knockout mutant O35ElpxX

DNA sequence containing the *lpxX* gene was amplified from the chromosomal DNA of *M. catarrhalis* strain O35E using primers b1X and b1B (Table 1, Fig. 1A). The PCR product was cloned into pCR2.1 using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) to obtain pCRlpxX. The insert was released by XhoI-BamHI digestion and then subcloned into an XhoI-BamHI site of pBluescript || SK (+) to form pSlpxX. To clone the *Zeo^r* gene into the *lpxX* gene, the *lpxX* PCR product was amplified from the pSlpxX using primers b1E1 and b1E2 and the *Zeo^r* gene was amplified from the pEM7/*Zeo* using primers *zeo* EcoRI-1 and *zeo* EcoRI-2, then these two PCR products were digested with EcoRI respectively, and ligated to form pSlpxX-*zeo*. After verification by sequence analysis, the disrupted *lpxX* gene containing the inserted *Zeo^r* gene in pSlpxX-*zeo* was amplified by PCR and purified for electroporation to O35E competent cells as described previously [19]. After 24 h incubation, the resulting *Zeo^r* colonies were selected for PCR identification using primers b1X and b1B and the inactivated *lpxX* mutant was verified by sequencing.

Cloning of *lpxL* gene and construction of the knockout mutant O35ElpxL

DNA sequence containing the *lpxL* gene was amplified from chromosomal DNA of strain O35E using primers b2E and b2B (Table 1, Fig. 1B), and cloned into pCR2.1 using a TOPO TA cloning kit to obtain pCRlpxL. The insertion was released by EcoRI-BamHI digestion, and then subcloned into an EcoRI-BamHI site of pBluescript || SK (+) to form pSlpxL. The *Kan^r* cassette (1240 bp) obtained from pUC4K after PstI digestion was subsequently cloned into the *lpxL* gene using a PstI site to form pSlpxL-*kan*. After verification by sequence analysis, the disrupted *lpxL* gene with the inserted *Kan^r* gene in pSlpxL-*kan* was amplified by PCR using primers b2E and b2B. The PCR product was purified and used for electroporation to O35E competent cells [19]. The resulting *Kan^r* colonies were selected for PCR analysis using primers b2E and b2B and the inactivated *lpxL* mutant was verified by sequencing.

Southern blot

The *Zeo^r* gene or *Kan^r* gene was amplified from pEM7/*Zeo* or pUC4K as a probe with primers *zeo* EcoRI-1/*zeo* EcoRI-2 or *kan* RP/*kan* FP (Table 1) by using a PCR digoxigenin (DIG) probe synthesis kit (Roche, Indianapolis, IN). Southern blot analyses of the chromosomal DNA from strains O35E, O35ElpxX and O35ElpxL were performed using a DIG DNA labeling and detection kit (Roche) according to the instruction manual. The hybridization temperature of the Southern blot was at 42°C and the blots were washed under high stringency condition (65°C in 0.5 × SSC with 0.1% SDS).

RT-PCR

Total RNA was isolated from log-phase bacteria of each strain using an RNeasy Mini kit and treated with an on-column RNase-Free DNase set (Qiagen). The first-strand synthesis of cDNA was primed with random primers using a high capacity cDNA archive kit (Applied Biosystems). Primer sets for PCR amplification of target genes *ats*, *lpxX* and *lgt 6* in cDNA samples were *ats*1/*ats*2, *b1SP*/*b1AP*, and *lg1*/*lg2*, respectively (Table 1, Fig. 1A). Primer sets for PCR amplification of target genes *atr*, *lpxL*, and *asd* in cDNA samples were *atr*1/*atr*2, *b2SP*/*b2AP*, and *asd*1/*asd*2, respectively (Table 1, Fig. 1B). In parallel, PCRs were performed with chromosomal DNA as positive controls and cDNA samples without activation of the reverse-transcription reaction as negative controls. The PCR products were resolved on 0.8% agarose gels and visualized by ethidium bromide staining.

Reversion of O35ElpxX and O35ElpxL mutants

Primers b1B1/b1S or b2B1/b2S (Table 1; Fig. 1) were used to amplify native *lpxX* or *lpxL* gene from chromosomal DNA of the wild type strain O35E. The resulting PCR products were purified and subcloned into pWW115 plasmid [47] using BamHI and SacI, and the constructs were transformed into O35E competent cells by electroporation [47]. The bacteria were plated onto BHI agar containing 15 µg/ml spectinomycin. Plasmids were extracted from the spectinomycin-resistant colonies, identified by digestion with BamHI and SacI as well as by sequence analysis, and named as pWlpxX and pWlpxL. The pWlpxX and pWlpxL were used to transform O35ElpxX and O35ElpxL competent cells by electroporation, and the resulting cell suspensions were plated onto BHI agars containing spectinomycin. Potential revertant colonies were identified and chosen for further analyses.

LOS determination

A crude LOS extraction was performed from strain O35E, O35ElpxX or O35ElpxL using a method of proteinase-K treated whole cell lysates [48]. The resulting extracts from each bacterial suspension (1.9 µg of protein concentration) were resolved by 15% SDS-PAGE and visualized by silver staining [49].

Structural analysis of lipid A

The LOS from 30–35g of wet cells of each strain was prepared by phenol-water extraction [50]. Lipid A was released from each LOS using the SDS mild acid hydrolysis method [51]. Briefly, LOS samples were hydrolyzed in 10 mM NaOAc in 1% SDS, pH 4.5 buffer at 100°C for 1.5 h, with constant stirring. Hydrolysates were then freeze-dried, washed with 95% acidified ethanol, and centrifuged at 3500 rpm. Obtained sediments were washed twice with ethanol, following by centrifugation, then re-suspended in water and lyophilized. The remaining traces of oligosaccharides and SDS were removed by threefold extraction with chloroform:water (1:1). Organic phases, after concentration, were used for composition and MS analyses.

Lipid A structure was analyzed by MALDI-TOF MS. Spectra of the lipid A preparations were acquired using Applied Biosystems 4700 Proteomics System Spectrometer in reflector mode. Samples were dissolved in a chloroform:methanol mixture (3:1) and 1 µl was then mixed with 1 µl of the 0.5 M 2,4,6-trihydroxyacetophenone monohydrate (THAP) matrix in methanol and spotted onto the stainless steel MALDI plate. Spectra were recorded in negative ion mode.

The fatty acid compositions of the lipid A preparations were done by the preparation of FAMES and GC-MS analysis. The FAMES were obtained by methanolysis of lipid A using methanolic 1M HCl at 80°C for 18 h. The FAMES were recovered into organic phase by a threefold extraction with 50% NaCl:chloroform (1:1 v/v). A new volume of chloroform was used for each of the three extractions. Finally the organic phase was re-extracted with deionized water to remove traces of salts, and the volume of the organic phase was reduced prior GC-MS analysis. Analysis was performed on a Hewlett-Packard HP5890 gas chromatograph equipped with a mass selective detector 5970 MSD. A DB-1 fused silica capillary column (30 m×0.25 mm I.D.; J & W Scientific, Folsom, CA) was used for this analysis with helium as the carrier gas. The temperature program was: 70°C for 2 min, then ramping to 220°C at 8°C/min with a 10 min hold, then ramping to 280°C at 10°C/min.

Structural analysis of oligosaccharides (OSs)

For OS analysis, core OSs were released from LOS by mild hydrolysis with 1% (v/v) HOAc at 100°C for 1.5 h and further purified using Bio-Gel P-2 gel-filtration chromatography with water as the eluant. The eluting fractions were monitored with Shimadzu Refractive Index

Detector (RID-10A). Purified OSs were analyzed by MALDI-TOF MS using AB Proteomics Analyzer 4700 (Applied Biosystems). The OS samples were dissolved in nanopure water and mixed with 0.5 M 2,5-dihydroxy benzoic acid matrix in methanol in 1:1 (v/v) ratio. Samples were then applied onto a stainless steel MALDI plate and spectra were acquired in a positive reflector mode.

***Limulus* amebocyte lysate (LAL) assay**

The chromogenic LAL assay for endotoxin activity was performed using the QCL-1000 kit (Bio-Whittaker Inc., Walkersville, MD). Overnight cultures of the parental strains and two derived mutants from chocolate agar plates were suspended in BHI broth to OD₆₂₀ of 0.1 and serial dilutions of these stocks were tested based on the instruction of the manufacture.

Susceptibility determination

The sensitivity of strains to a panel of hydrophobic agents or a hydrophilic glycopeptide was performed using standard disk-diffusion assays [52]. 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) containing 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 to normal human serum

A complement-sufficient normal human serum was prepared and pooled from 8 healthy adult donors. A bactericidal assay was performed in a 96-well plate [18]. Normal human serum was diluted to 0.5, 2.5, 12.5, and 25% in pH 7.4 Dulbecco's phosphate-buffered saline containing 0.05% gelatin (DPBSG). Bacteria [10 µl containing 10⁶ CFU (colony-forming units)] 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.

Pulmonary clearance patterns in animal model

Female BALB/c mice (6–8 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 OD₅₄₀ of 0.4 for wild type strain O35E (1.3×10⁹ CFU/ml) or the OD₅₄₀ of 0.43 for the mutant O35ElpxX (1.0×10⁹ CFU/ml) or the A₅₄₀ of 0.4 for the O35ElpxL (1.8×10⁹ CFU/ml) in a 10 ml DPBSG [53]. The number of bacteria present in the lungs was measured at various time points post-challenge. The minimum detectable number of viable bacteria was 100 CFU per lung. Clearance of *M. catarrhalis* was expressed as the percentage of bacterial CFU at each time point compared with the number deposited at time zero.

Statistical analysis

The number of viable bacteria were expressed as the geometric mean CFU of eight (mice) independent observation ± standard deviation (SD). The significance of the clearance rate was analyzed by a Chi-square test (two tailed). One-way ANOVA was employed for multiple point comparison.

Nucleotide sequence accession number

The nucleotide sequences of the *lpxX* and *lpxL* genes in *M. catarrhalis* strain O35E were deposited at GenBank under accession number EU155137 and EU155138, respectively.

Acknowledgments

We thank Eric J. Hansen for providing strain O35E/plasmid pWW115, Shengqing Yu for advice in pulmonary clearance assay, Robert Morell for help in DNA sequencing, Yandan Yang for help in Southern blotting, and Lina Zhu and Yili Chen for help in manuscript preparation. This research was supported by the Intramural Research Program of the NIDCD/NIH and a Department of Energy grant (DE-FG09-93-ER20097) to the CCRC.

Abbreviations

LOS	lipooligosaccharide
LPS	lipopolysaccharide
OS	oligosaccharide
Kdo	3-deoxy-D -manno-octulosonic acid
BHI	brain-heart infusion
Zeo^r	zeocin-resistant
Kan^r	kanamycin-resistant
FAMEs	fatty acid methyl esters
LAL	<i>Limulus</i> ameocyte lysate
EU	endotoxin units
CFU	colony-forming units

References

1. Catlin BW. *Branhamella catarrhalis*: an organism gaining respect as a pathogen. Clin Microbiol Rev. 1990; 3:293–320. [PubMed: 2121328]
2. Karalus R, Campagnari A. *Moraxella catarrhalis*: a review of an important human mucosal pathogen. Microbes Infect. 2000; 2:547–559. [PubMed: 10865200]
3. Murphy TF. *Branhamella catarrhalis*: epidemiology, surface antigenic structure, and immune response. Microbiol Rev. 1996; 60:267–279. [PubMed: 8801433]
4. Faden H, Hong J, Murphy T. Immune response to outer membrane antigens of *Moraxella catarrhalis* in children with otitis media. Infect Immun. 1992; 60:3824–3829. [PubMed: 1500191]
5. Ruuskanen O, Heikkinen T. Otitis media: etiology and diagnosis. Pediatr Infect Dis J. 1994; 13:S23–26. discussion S50–24. [PubMed: 8159511]
6. Murphy TF, Brauer AL, Grant BJ, Sethi S. *Moraxella catarrhalis* in chronic obstructive pulmonary disease: burden of disease and immune response. Am J Respir Crit Care Med. 2005; 172:195–199. [PubMed: 15805178]
7. Verduin CM, Hol C, Fleer A, van Dijk H, van Belkum A. *Moraxella catarrhalis*: from emerging to established pathogen. Clin Microbiol Rev. 2002; 15:125–144. [PubMed: 11781271]
8. Holm MM, Vanlerberg SL, Sledjeski DD, Lafontaine ER. The Hag protein of *Moraxella catarrhalis* strain O35E is associated with adherence to human lung and middle ear cells. Infect Immun. 2003; 71:4977–4984. [PubMed: 12933840]
9. Edebrink P, Jansson PE, Rahman MM, Widmalm G, Holme T, Rahman M, Weintraub A. Structural studies of the O-polysaccharide from the lipopolysaccharide of *Moraxella (Branhamella) catarrhalis* serotype A (strain ATCC 25238). Carbohydr Res. 1994; 257:269–284. [PubMed: 7516823]

10. Holme T, Rahman M, Jansson PE, Widmalm G. The lipopolysaccharide of *Moraxella catarrhalis* structural relationships and antigenic properties. *Eur J Biochem.* 1999; 265:524–529. [PubMed: 10504382]
11. Masoud H, Perry MB, Richards JC. Characterization of the lipopolysaccharide of *Moraxella catarrhalis*. Structural analysis of the lipid A from *M. catarrhalis* serotype A lipopolysaccharide. *Eur J Biochem.* 1994; 220:209–216. [PubMed: 8119289]
12. Vaneechoutte M, Verschraegen G, Claeys G, Van Den Abeele AM. Serological typing of *Branhamella catarrhalis* strains on the basis of lipopolysaccharide antigens. *J Clin Microbiol.* 1990; 28:182–187. [PubMed: 2107197]
13. Gorter AD, Oostrik J, van der Ley P, Hiemstra PS, Dankert J, van Alphen L. Involvement of lipooligosaccharides of *Haemophilus influenzae* and *Neisseria meningitidis* in defensin-enhanced bacterial adherence to epithelial cells. *Microb Pathog.* 2003; 34:121–130. [PubMed: 12631473]
14. Song W, Ma L, Chen R, Stein DC. Role of lipooligosaccharide in Opa-independent invasion of *Neisseria gonorrhoeae* into human epithelial cells. *J Exp Med.* 2000; 191:949–960. [PubMed: 10727457]
15. Swords WE, Chance DL, Cohn LA, Shao J, Apicella MA, Smith AL. Acylation of the lipooligosaccharide of *Haemophilus influenzae* and colonization: an htrB mutation diminishes the colonization of human airway epithelial cells. *Infect Immun.* 2002; 70:4661–4668. [PubMed: 12117980]
16. Attia AS, Lafontaine ER, Latimer JL, Aebi C, Syrogiannopoulos GA, Hansen EJ. The UspA2 protein of *Moraxella catarrhalis* is directly involved in the expression of serum resistance. *Infect Immun.* 2005; 73:2400–2410. [PubMed: 15784586]
17. Hu WG, Chen J, McMichael JC, Gu XX. Functional characteristics of a protective monoclonal antibody against serotype A and C lipooligosaccharides from *Moraxella catarrhalis*. *Infect Immun.* 2001; 69:1358–1363. [PubMed: 11179299]
18. Peng D, Choudhury BP, Petralia RS, Carlson RW, Gu XX. Roles of 3-deoxy-D-manno-2-octulosonic acid transferase from *Moraxella catarrhalis* in lipooligosaccharide biosynthesis and virulence. *Infect Immun.* 2005; 73:4222–4230. [PubMed: 15972513]
19. Peng D, Hong W, Choudhury BP, Carlson RW, Gu XX. *Moraxella catarrhalis* bacterium without endotoxin, a potential vaccine candidate. *Infect Immun.* 2005; 73:7569–7577. [PubMed: 16239560]
20. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem.* 2002; 71:635–700. [PubMed: 12045108]
21. Zaleski A, Scheffler NK, Densen P, Lee FK, Campagnari AA, Gibson BW, Apicella MA. Lipooligosaccharide P(k) (Galalpha1-4Galbeta1-4Glc) epitope of *Moraxella catarrhalis* is a factor in resistance to bactericidal activity mediated by normal human serum. *Infect Immun.* 2000; 68:5261–5268. [PubMed: 10948153]
22. Luke NR, Allen S, Gibson BW, Campagnari AA. Identification of a 3-deoxy-D-manno-octulosonic acid biosynthetic operon in *Moraxella catarrhalis* and analysis of a KdsA-deficient isogenic mutant. *Infect Immun.* 2003; 71:6426–6434. [PubMed: 14573664]
23. Edwards KJ, Allen S, Gibson BW, Campagnari AA. Characterization of a cluster of three glycosyltransferase enzymes essential for *Moraxella catarrhalis* lipooligosaccharide assembly. *J Bacteriol.* 2005; 187:2939–2947. [PubMed: 15838019]
24. Edwards KJ, Schwingel JM, Datta AK, Campagnari AA. Multiplex PCR assay that identifies the major lipooligosaccharide serotype expressed by *Moraxella catarrhalis* clinical isolates. *J Clin Microbiol.* 2005; 43:6139–6143. [PubMed: 16333114]
25. Wilson JC, Collins PM, Klipic Z, Grice ID, Peak IR. Identification of a novel glycosyltransferase involved in LOS biosynthesis of *Moraxella catarrhalis*. *Carbohydr Res.* 2006; 341:2600–2606. [PubMed: 16934238]
26. Schwingel JM, Michael FS, Cox AD, Masoud H, Richards JC, Campagnari AA. A Unique Glycosyltransferase Involved in the Initial Assembly of *Moraxella catarrhalis* Lipooligosaccharides. *Glycobiology.* 2008; 8:447–55. [PubMed: 18337458]

27. Clementz T, Bednarski JJ, Raetz CR. Function of the *htrB* high temperature requirement gene of *Escherichia coli* in the acylation of lipid A: HtrB catalyzed incorporation of laurate. *J Biol Chem.* 1996; 271:12095–12102. [PubMed: 8662613]
28. Carty SM, Sreekumar KR, Raetz CR. Effect of cold shock on lipid A biosynthesis in *Escherichia coli*. Induction At 12 degrees C of an acyltransferase specific for palmitoleoyl-acyl carrier protein. *J Biol Chem.* 1999; 274:9677–9685. [PubMed: 10092655]
29. Post DM, Ketterer MR, Phillips NJ, Gibson BW, Apicella MA. The *msbB* mutant of *Neisseria meningitidis* strain NMB has a defect in lipooligosaccharide assembly and transport to the outer membrane. *Infect Immun.* 2003; 71:647–655. [PubMed: 12540541]
30. Post DM, Phillips NJ, Shao JQ, Entz DD, Gibson BW, Apicella MA. Intracellular survival of *Neisseria gonorrhoeae* in male urethral epithelial cells: importance of a hexaacyl lipid A. *Infect Immun.* 2002; 70:909–920. [PubMed: 11796626]
31. Lee NG, Sunshine MG, Engstrom JJ, Gibson BW, Apicella MA. Mutation of the *htrB* locus of *Haemophilus influenzae* nontypable strain 2019 is associated with modifications of lipid A and phosphorylation of the lipooligosaccharide. *J Biol Chem.* 1995; 270:27151–27159. [PubMed: 7592970]
32. Sunshine MG, Gibson BW, Engstrom JJ, Nichols WA, Jones BD, Apicella MA. Mutation of the *htrB* gene in a virulent *Salmonella typhimurium* strain by intergeneric transduction: strain construction and phenotypic characterization. *J Bacteriol.* 1997; 179:5521–5533. [PubMed: 9287009]
33. McLendon MK, Schilling B, Hunt JR, Apicella MA, Gibson BW. Identification of LpxL, a late acyltransferase of *Francisella tularensis*. *Infect Immun.* 2007; 75:5518–5531. [PubMed: 17724076]
34. Rebeil R, Ernst RK, Jarrett CO, Adams KN, Miller SI, Hinnebusch BJ. Characterization of late acyltransferase genes of *Yersinia pestis* and their role in temperature-dependent lipid A variation. *J Bacteriol.* 2006; 188:1381–1388. [PubMed: 16452420]
35. Karow M, Georgopoulos C. Isolation and characterization of the *Escherichia coli msbB* gene, a multicopy suppressor of null mutations in the high-temperature requirement gene *htrB*. *J Bacteriol.* 1992; 174:702–710. [PubMed: 1732206]
36. Clementz T, Zhou Z, Raetz CR. Function of the *Escherichia coli msbB* gene, a multicopy suppressor of *htrB* knockouts, in the acylation of lipid A. Acylation by MsbB follows laurate incorporation by HtrB. *J Biol Chem.* 1997; 272:10353–10360. [PubMed: 9099672]
37. Raetz CR, Reynolds CM, Trent MS, Bishop RE. Lipid A modification systems in gram-negative bacteria. *Annu Rev Biochem.* 2007; 76:295–329. [PubMed: 17362200]
38. van der Ley P, Steeghs L, Hamstra HJ, ten Hove J, Zomer B, van Alphen L. Modification of lipid A biosynthesis in *Neisseria meningitidis* lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. *Infect Immun.* 2001; 69:5981–5990. [PubMed: 11553534]
39. Schnaitman CA, Klena JD. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol Rev.* 1993; 57:655–682. [PubMed: 7504166]
40. Vaara M. Antibiotic-supersusceptible mutants of *Escherichia coli* and *Salmonella typhimurium*. *Antimicrob Agents Chemother.* 1993; 37:2255–2260. [PubMed: 8285603]
41. Reese MG. Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. *Comput Chem.* 2001; 26:51–56. [PubMed: 11765852]
42. Delcher AL, Bratke KA, Powers EC, Salzberg SL. Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics.* 2007; 23:673–679. [PubMed: 17237039]
43. Hofmann K, Stoffel W. TMbase - A database of membrane spanning proteins segments. *Biol Chem Hoppe-Seyler.* 1993; 374:166.
44. Claros MG, von Heijne G. TopPred II: An Improved Software For Membrane Protein Structure Predictions. *CABIOS.* 1994; 10:685–686. [PubMed: 7704669]
45. Rost B, Yachdav G, Liu J. The PredictProtein Server. *Nucleic Acids Res.* 2003; 32(Web Server issue):W321–W326. [PubMed: 15215403]
46. Helminen ME, Maciver I, Latimer JL, Cope LD, McCracken GH Jr, Hansen EJ. A major outer membrane protein of *Moraxella catarrhalis* is a target for antibodies that enhance pulmonary

- clearance of the pathogen in an animal model. *Infect Immun.* 1993; 61:2003–2010. [PubMed: 7683000]
47. Wang W, Hansen EJ. Plasmid pWW115, a cloning vector for use with *Moraxella catarrhalis*. *Plasmid.* 2006; 56:133–137. [PubMed: 16757025]
48. Tzeng YL, Datta A, Kolli VK, Carlson RW, Stephens DS. Endotoxin of *Neisseria meningitidis* composed only of intact lipid A: inactivation of the meningococcal 3-deoxy-D-manno-octulosonic acid transferase. *J Bacteriol.* 2002; 184:2379–2388. [PubMed: 11948150]
49. Tsai CM, Frasch CE. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem.* 1982; 119:115–119. [PubMed: 6176137]
50. Gu XX, Chen J, Barenkamp SJ, Robbins JB, Tsai CM, Lim DJ, Battey J. Synthesis and characterization of lipooligosaccharide-based conjugates as vaccine candidates for *Moraxella (Branhamella) catarrhalis*. *Infect Immun.* 1998; 66:1891–1897. [PubMed: 9573066]
51. Caroff M, Tacken A, Szabo L. Detergent-accelerated hydrolysis of bacterial endotoxins and determination of the anomeric configuration of the glycosyl phosphate present in the “isolated lipid A” fragment of the *Bordetella pertussis* endotoxin. *Carbohydr Res.* 1988; 175:273–282. [PubMed: 2900066]
52. Ochsner UA, Vasil AI, Johnson Z, Vasil ML. *Pseudomonas aeruginosa* fur overlaps with a gene encoding a novel outer membrane lipoprotein, OmlA. *J Bacteriol.* 1999; 181:1099–1109. [PubMed: 9973334]
53. Hu WG, Chen J, Battey JF, Gu XX. Enhancement of clearance of bacteria from murine lungs by immunization with detoxified lipooligosaccharide from *Moraxella catarrhalis* conjugated to proteins. *Infect Immun.* 2000; 68:4980–4985. [PubMed: 10948114]

Fig.1.

Genetic organization of *lpxX* or *lpxL* locus in *M. catarrhalis* O35E genome. (A) The location of deletion in *lpxX* replaced by a zeocin resistance (Zeo^r) gene is between two EcoRI cleavage sites introduced by PCR reaction. A gene upstream from *lpxX* is an aspartyl-tRNA synthetase (*ats*) while a downstream gene encodes a glycosyltransferase (*lgt 6*). (B) The location of deletion in *lpxL* replaced by a kanamycin resistance (Kan^r) gene is between two PstI cleavage sites. A gene upstream from *lpxL* is an ABC transporter related (*atr*) while a downstream gene encodes an aspartate1-decarboxylase (*asd*). Large arrows represent the direction of transcription, and the sites of primers used are indicated with small arrows.

Fig.2.

Detection of Zeo^r gene inserted into O35ElpxX or Kan^r gene into O35ElpxL chromosomal DNA by Southern blotting. Lane 1, 2 or 4, 5 μg of chromosomal DNA from O35E, O35ElpxX or O35ElpxL plus EcoRV; lane 3, 0.1 μg of pEM7/ Zeo (a plasmid with a Zeo^r cassette as positive control) plus EcoRI+XhoI; lane 5, 0.1 μg of pUC4K (a plasmid with a Kan^r cassette as positive control) plus EcoRI. Each digested sample was resolved on a 0.7% agarose gel and visualized by ethidium bromide staining (A, C). Southern blotting was performed using DIG-labeled Zeo^r (B) or Kan^r gene probe (D). Lambda DNA/EcoRI-plus-HindIII molecular size standards (Fermentas) are shown in base pairs on the left (lane M).

Fig.3.

Detection of *lpxX* (A) and *lpxL* (B) gene expression by RT-PCR. The RT-PCRs were performed using the following nucleic acid templates and primers: total RNA from O35E (lanes 1 and 3), O35ElpxX (lanes 4 and 6) and O35ElpxL (lanes 7 and 9), and chromosomal DNA from O35E (lane 2), O35ElpxX (lane 5) and O35ElpxL (lane 8). Reaction sets contained the following primers: for lanes labeled “a”, *ats1/ats2*; lanes labeled “b”, *b1SP/b1AP*; lanes labeled “c”, *lg1/lg2*; lanes labeled “d”, *atr1/atr2*; lanes labeled “e”, *b2SP/b2AP*; and lanes labeled “f”, *asd1/asd2*. The controls (lanes 3, 6, 9) used total RNA as the nucleic acid template without activation of the RT. GenRuler DNA ladder mix (Fermentas) was used for the molecular size standards in base pairs (M).

Fig.4.

LOS patterns from SDS-PAGE followed by silver staining. *M. catarrhalis* wild type strain O35E (lane 1), O35ElpxX, O35ElpxX revertant (lanes 2, 3), O35ElpxL and O35ElpxL revertant (lanes 4, 5). Extracts from proteinase K-treated whole-cell lysates from each bacterial suspension (1.9 μ g of protein content) were used and molecular mass markers (Mark12; Invitrogen) indicated on the left.

Fig.5.

GC-MS profiles of the fatty methyl esters (FAME) obtained from lipid A of *M. catarrhalis* wild type strain O35E, O35ElpxX and O35ElpxL. When compared with the lipid A of the parental strain O35E (A), the lipid A of O35E lpxX mutant did not contain C10:0 (decanoic acid) (B) while the O35ElpxL showed no C12:0 [dodecanoic (lauric) acid] (C). * indicates impurities.

Fig.6.

MALDI-TOF analysis of the lipid A from *M. catarrhalis* wild type strain O35E, O35ElpxX and O35ElpxL, and their proposed structures. These analyses were done in the negative mode, and all ions were represented as deprotonated $[M-H]^-$ ions. The upper portion of the figure shows the structure of the major species of O35E lipid A at 1907.94 amu (A). In contrast, the lipid A of the O35ElpxX was pentaacylated and lacked two C10:0 residues with a structure at 1599.25 amu (B), while the O35ElpxL lipid A was hexaacylated and missing one C12:0 residue with a structure at 1725.62 amu (C).

Observed ion	Calculated MW	Proposed composition
1536.4	1535.2	Gal ₂ -Glc ₅ -GlcNAc-Kdo(-CO ₂)-Na ⁺ (anhydro)
1580.4	1581.3	Gal ₂ -Glc ₅ -GlcNAc-Kdo-Na ⁺ (anhydro)
1598.4	1599.2	Gal ₂ -Glc ₅ -GlcNAc-Kdo-Na ⁺
1620.4	1622.1	Gal ₂ -Glc ₅ -GlcNAc-Kdo-Na ⁺ ₂

Fig.7.

The MALDI-TOF MS spectra for the OSs released from the O35ElpxX (A) and O35ElpxL (B) mutants. The inset shows the compositions and the calculated ions for the observed ions in each of these spectra.

Fig.8.

Growth curves, bactericidal resistance and mouse clearance of *M. catarrhalis* wild type strain O35E, O35ElpxX and O35ElpxL. (A) Strain O35E (□), O35ElpxX (■) or O35ElpxL (▣) was grown in BHI broth at 37°C, respectively, and their optical density checked at different times. (B) Bactericidal activities of normal human serum against strain O35E (white bar), O35ElpxX (black bar) and O35ElpxL (gray bar) were shown. “HI” represents the group of 25% heat-inactivated normal human serum as controls for each strain tested. The data represent the averages of three independent assays. (C) Time courses of bacterial recovery in mouse lungs after an aerosol challenge with strain O35E (□), O35ElpxX (■) and

O35ElpxL (■). Each time point represents a geometric mean of eight mice. *: $p < 0.05$; **: $p < 0.01$

TABLE 1

Strains, plasmids and primers used in this study

Description		Source
Strain		
<i>M. catarrhalis</i> O35E	Wild-type strain	46
<i>M. catarrhalis</i> O35ElpxX	Decanoyl transferase-deficient strain	This study
<i>M. catarrhalis</i> O35ElpxL	Dodecanoyl transferase-deficient strain	This study
<i>E. Coli</i> TOP10	Cloning strain	Invitrogen
Plasmid		
pCR2.1	TOPO TA cloning vector	Invitrogen
pCRlpxX	<i>lpxX</i> cloned into pCR2.1	This study
pCRlpxL	<i>lpxL</i> cloned into pCR2.1	This study
pBluescript II SK(+)	Cloning vector	Fermentas
pSlpxX	XhoI-BamHI <i>lpxX</i> fragment cloned into SK(+)	This study
pSlpxL	EcoRI-BamHI <i>lpxL</i> fragment cloned into SK(+)	This study
pEM7/Zeo	Zeocin-resistant cassette	Invitrogen
pSlpxX-zeo	Zeocin-resistant gene inserted into pSlpxX	This study
pUC4K	Kanamycin-resistance cassette	Amersham
pSlpxL-kan	Kanamycin-resistance gene inserted into pSlpxL	This study
pWW115	Cloning vector for use with <i>M. catarrhalis</i>	47
pWlpxX	BamHI-SacI <i>lpxX</i> fragment cloned into pWW115	This study
pWlpxL	BamHI-SacI <i>lpxL</i> fragment cloned into pWW115	This study
Primer		
B1SP	5'-AGC TCA TCA GTG CAG TCG-3' (<i>lpxX</i> sense)	This study
B1AP	5'-CTT TGA CAT GGC TTG AAG-3' (<i>lpxX</i> antisense)	This study
B1X	5'-CTC <u>CTC GAG</u> AGC TCA TCA GTG CAG TCG-3' (<i>lpxX</i> sense; XhoI site underlined)	This study
b1B	5'-CTC <u>GGA TCC</u> CTT TGA CAT GGC TTG AAG-3' (<i>lpxX</i> antisense; BamHI site underlined)	This study
b1E1	5'-CTC <u>GAA TTC</u> GTA TCA TAC TGC CCG ACC-3' (Inserting <i>zeo</i> gene; EcoRI site underlined)	This study
b1E2	5'-CTC <u>GAA TTC</u> GTG GGT ACA AGG CTG GCA-3' (Inserting <i>zeo</i> gene; EcoRI site underlined)	This study
b1B1	5'-CTC <u>GGA TCC</u> GTG CTT GGT TTT TTA AGA TAT GTA CC-3' (<i>lpxX</i> sense; BamHI site underlined)	This study
b1S	5'-CTC <u>GAG CTC</u> TCA CTC ATA ACT ATC CTT TGA CAT GG-3' (<i>lpxX</i> antisense; SacI site underlined)	This study
ats1	5'-GCT CAA TCC GTG ATG TGA-3' (<i>ats</i> sense)	This study
ats2	5'-CGA CTG CAC TGA TGA GCT-3' (<i>ats</i> antisense)	This study
lg1	5'-CTT CAA GCC ATG TCA AAG-3' (<i>lgt 6</i> sense)	This study
lg2	5'-CGA ATA ATC ATC ACA CTG-3' (<i>lgt 6</i> antisense)	This study
<i>zeo</i> EcoRI-1	5'-CTC <u>GAA TTC</u> CAC GTG TTG ACA ATT AAT-3' (zeocin sense; EcoRI site underlined)	This study
<i>zeo</i> EcoRI-2	5'-CTC <u>GAA TTC</u> TCA GTC CTG CTC CTC GGC-3' (zeocin antisense; EcoRI site underlined)	This study
b2SP	5'-GAG TTG CCA TCA TCA GCA-3' (<i>lpxL</i> sense)	This study

	Description	Source
b2AP	5'-AAT TGG TGT CAT CGG CTT-3' (<i>lpxL</i> antisense)	This study
b2E	5'-CTC <u>GAA TTC</u> GAG TTG CCA TCA TCA GCA-3' (<i>lpxL</i> sense; EcoRI site underlined)	This study
b2B	5'-CTC <u>GGA TCC</u> AAT TGG TGT CAT CGG CTT-3' (<i>lpxL</i> antisense; BamHI site underlined)	This study
b2B1	5'-CTC <u>GGA TCC</u> TTG ACA GAT ACT CAT AAA CAA AGT AGC-3' (<i>lpxL</i> sense; BamHI site underlined)	This study
b2S	5'-CTC <u>GAG CTC</u> TTA ATG TTG ATA GTA ATT GGT GTC A-3' (<i>lpxL</i> antisense; SacI site underlined)	This study
atr1	5'-TGC TTG ATG AGC CTA CCA-3' (<i>atr</i> sense)	This study
atr2	5'-TGC TGA TGA TGG CAA CTC-3' (<i>atr</i> antisense)	This study
asd1	5'-AAG CCG ATG ACA CCA ATT-3' (<i>asd</i> sense)	This study
asd2	5'-GCA GGT TCA TAG TGC ATG-3' (<i>asd</i> antisense)	This study
<i>Kan</i> RP	5'-GGT GCG ACA ATC TAT CGA-3' (kanamycin sense)	19
<i>Kan</i> FP	5'-CTC ATC GAG CAT CAA ATG-3' (kanamycin antisense)	19

TABLE 2

Comparison of *M. catarrhalis* LpxX and LpxL with the late acyltransferase homologues identified in other Gram-negative bacteria

Bacterium	Protein (Accession no.)	Identity	Similarity	Reference
LpxX				
<i>Escherichia coli</i> str. K12	LpxL (NP_415572)	24% (68/281)	41% (117/281)	27
substr. W3110	LpxP (NP_416879)	21% (53/243)	44% (109/243)	28
<i>Neisseria meningitidis</i>	MsbB (AAL74160)	24% (70/281)	43% (121/281)	29
<i>Neisseria gonorrhoeae</i>	MsbB (AAL24441)	24% (68/278)	43% (122/278)	30
<i>Haemophilus influenzae</i>	HtrB (P45239)	23% (66/283)	41% (118/283)	31
Rd KW20				
<i>Salmonella typhimurium</i>	HtrB (NP_460126)	23% (64/273)	39% (108/273)	32
LT2				
<i>Francisella tularensis</i>	HtrB (YP_666416)	22% (57/253)	43% (110/253)	33
subsp. <i>Tularensis</i>				
<i>Yersinia pestis</i> KIM	MsbB (AAM85807)	19% (54/272)	39% (105/272)	34
LpxL				
<i>Escherichia coli</i> str. K12	LpxL (NP_415572)	31% (96/305)	49% (150/305)	27
substr. W3110	LpxP (NP_416879)	31% (96/306)	50% (156/306)	28
	LpxM (AP_002475)	27% (87/319)	46% (147/319)	35
<i>Neisseria meningitidis</i>	MsbB (AAL74160)	26% (67/252)	44% (111/252)	29
<i>Neisseria gonorrhoeae</i>	MsbB (AAL24441)	27% (74/270)	44% (121/270)	30
<i>Haemophilus influenzae</i>	HtrB (P45239)	32% (101/308)	50% (156/308)	31
Rd KW20	MsbB (NP_438368)	28% (90/316)	48% (154/316)	
<i>Salmonella typhimurium</i>	HtrB (NP_460126)	31% (96/304)	48% (146/304)	32
LT2	MsbB (AAL20805)	26% (85/319)	46% (147/319)	
<i>Francisella tularensis</i>	HtrB (YP_666416)	26% (81/309)	45% (142/309)	33
subsp. <i>Tularensis</i>				
<i>Yersinia pestis</i> KIM	MsbB (AAM85807)	27% (88/321)	46% (149/321)	34

TABLE 3

Susceptibility of *M. catarrhalis* wild-type O35E and its *lpxX* and *lpxL* mutants to a panel of hydrophobic reagents or hydrophilic glycopeptide

Compound	Zone of growth inhibition (mm) for strain ^a		
	O35E	O35ElpxX	O35ElpxL
Clindamycin (2μg)	11.5±0.5	14.5±0.5	11.5±0.8
Fusidic acid (10mg/ml)	22.2±0.3	28.8±0.3	24.9±0.7
Novobiocin (5μg)	13.8±0.3	16.7±0.6	14.7±0.3
Polymyxin B (300iu)	12.0±0.2	14.0±0.3	13.8±0.3
Rifapin (5μg)	21.5±0.5	32.3±0.6	25.7±0.6
Vancomycin (5μg)	<6.0 ^b	<6.0	<6.0
Deoxycholate (100mg/ml)	19.2±0.3	22.8±0.3	17.8±0.8
Triton X-100 (5% [wt/vol])	18.3±0.3	25.0±0.5	20.3±0.3
Tween 20 (5% [vol/vol])	15.8±0.3	21.2±0.8	18.2±0.8
Azithromycin (15μg)	22.3±0.6	34.2±0.8	29.4±0.5

^a Sensitivities were assessed by measuring the diameters of the zones of growth inhibition on two axes, and the mean values were calculated. The data represent the averages of three separate experiment ± SD.

^b No inhibition.