Late acyltransferase genes \(\textit{lpxX}\) and \(\textit{lpxL}\) jointly contribute to the biological activities of \textit{Moraxella catarrhalis}

Song Gao,\(^1\)† Dabin Ren,\(^1\)† Daxin Peng,\(^1\)† Wenhong Zhang,\(^1\)§ Artur Muszyński,\(^2\) Russell W. Carlson\(^2\) and Xin-Xing Gu\(^1\)¶

\(^1\)Vaccine Research Section, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Rockville, MD 20850, USA

\(^2\)Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, USA

Lipo-oligosaccharide (LOS) is a major surface component and virulence factor of the human respiratory pathogen \textit{Moraxella catarrhalis}. Two late acyltransferase genes, \textit{lpxX} and \textit{lpxL}, have been identified involved in the incorporation of acyloxyacyl-linked secondary acyl chains into lipid A during \textit{M. catarrhalis} LOS biosynthesis. In this study, a double mutant with a deletion of both the \textit{lpxX} and \textit{lpxL} genes in \textit{M. catarrhalis} strain O35E was constructed and named O35ElpxXL. Structural analysis of lipid A showed that the O35ElpxXL mutant lacked two decanoyl acids (10:0) and one dodecanoyl (lauric) acid (12:0). In comparison with the O35E parental strain and the single mutants O35ElpxX and O35ElpxL, the double mutant O35ElpxXL displayed prominently decreased endotoxin content, reduced resistance to normal human serum and accelerated bacterial clearance at 0, 3 and 6 h after an aerosol challenge in a mouse model of bacterial pulmonary clearance. These results indicate that these two genes encoding late acyltransferases responsible for lipid A biosynthesis jointly contribute to the biological activities and pathogenicity of \textit{M. catarrhalis}. The double mutant O35ElpxXL with dramatically reduced toxicity is proposed as a potential vaccine candidate against \textit{M. catarrhalis} infections for further investigation.

\textbf{INTRODUCTION}

\textit{Moraxella catarrhalis} is a common causative agent of otitis media in infants and young children (Karalus & Campagnari, 2000). In developed countries >80 % of children under the age of 3 are diagnosed at least once with otitis media, and \textit{M. catarrhalis} is responsible for 15–25 % of otitis media infections (Karalus & Campagnari, 2000). For adults, \textit{M. catarrhalis} is the second most common cause, accounting for ~7 million cases, of acute exacerbation of chronic obstructive pulmonary disease, which is the fourth leading cause of death in the USA (Murphy et al., 2005). However, despite being such a significant human respiratory pathogen, the molecular pathogenesis of \textit{M. catarrhalis} is not largely understood so far.

Lipo-oligosaccharide (LOS) is a major outer-membrane component of \textit{M. catarrhalis}, with three main LOS serotypes, A, B and C (Vanechoutte et al., 1990). LOS has been shown to be an important virulence factor for some respiratory pathogens such as \textit{Neisseria meningitidis} and \textit{Haemophilus influenzae} (Gorter et al., 2003, Song et al., 2000). Among these, \textit{M. catarrhalis} LOS is implicated to play a pivotal role in the pathogenesis of respiratory tract infections (Peng et al., 2005a, b). Distinct from the LOS or LPS molecules of most other Gram-negative bacteria, \textit{M. catarrhalis} LOS consists of only an oligosaccharide (OS) core and a lipid A moiety (Edebrink et al., 1994). Seven shorter fatty acid residues (decanoyl, 10:0, or dodecanoyl, 12:0) comprise the lipid A portion of \textit{M. catarrhalis} LOS (Holme et al., 1999; Masoud et al., 1994).

\textit{M. catarrhalis} LOS biosynthesis mechanisms have primarily been delineated by uncovering some of the key genes involved in the process to date. Among these, the \textit{lpxX} and \textit{lpxL} genes have been identified as encoding two late acyltransferases, decanoyl and dodecanoyl transferase,
which catalyse the addition of two decanoate (10:0) chains and one laurate (12:0) chain, respectively, into the lipid A group (Gao et al., 2008). By constructing the lpxX and lpxL single-knockout mutants O35ElpxX and O35ElpxL, we have shown previously that these two genes, especially lpxX, play an important role individually in the biological activities of M. catarrhalis (Gao et al., 2008).

In this work, we constructed an lpxX and lpxL double-knockout mutant from strain O35E to further investigate their joint roles in the pathogenicity and virulence of M. catarrhalis. The structural and physicochemical properties of the double mutant LOS were analysed, and the biological and pathogenic activities of this mutant were investigated using both in vitro and in vivo studies.

**METHODS**

**Construction and characterization of the lpxX and lpxL double mutant for strain O35E.** The O35ElpxX and O35ElpxL single mutants were constructed by disrupting the lpxX and lpxL genes in the M. catarrhalis O35E genome by inserting a zeocin-resistant (Zeo') cassette and a kanamycin-resistant (Kan') cassette, respectively, as described previously (Gao et al., 2008). The disrupted lpxX gene containing the inserted Zeo' gene was amplified by PCR and purified for electroporation of O35ElpxL-competent cells as described previously (Peng et al., 2005b). After 24 h of incubation, the resulting Zeo' and Kan' double-positive colonies were selected for PCR identification using primers 5'-CTCCCTGAGAGCTCATCAGTGCAGTCG-3' and 5'-CTCCATCTCCAGGCCCCCCGCTTGAAAG-3' for Zeo' insertion and 5'-CTCGAATTCAGGTTGCCATCATCACGA-3' and 5'-CTCGGGATCCATACTTGTGTCATGCCTT-3' for Kan' insertion. The disrupted lpxX and lpxL genes were verified by sequencing, and the lpxX and lpxL double mutant was named O35ElpxXL. The inserted Zeo' and Kan' genes of the O35ElpxXL double mutant were detected with a Southern blot assay. A reverse transcription (RT) PCR assay was employed to determine whether the insertions affected the expression of the upstream and downstream genes as described previously (Gao et al., 2008).

**Reversion of the O35ElpxXL mutant.** The native lpxX gene was amplified from wild-type O35E and subcloned into plasmid pWW115. Recombinant plasmids were extracted from the spectinomycin-resistant colonies, identified by enzyme digestion as well as by sequence analysis, and named pWlpxX (Gao et al., 2008). The plasmid pWlpxX was transformed into O35ElpxXL-competent cells by electroporation, and the resulting cell suspensions were plated onto brain–heart infusion (BHI) agar containing spectinomycin. Potential revertant colonies were identified and chosen for further analysis. The disrupted lpxX and lpxL genes were verified by sequencing, and the lpxX and lpxL double mutant was named O35ElpxXL. The inserted Zeo' and Kan' genes of the O35ElpxXL double mutant were detected with a Southern blot assay. A reverse transcription (RT) PCR assay was employed to determine whether the insertions affected the expression of the upstream and downstream genes as described previously (Gao et al., 2008).

**Statistical analysis.** The numbers of viable bacteria recovered from mouse lungs were expressed as the geometric mean c.f.u. of eight independent observations ± SD. The significance of the clearance rate was analysed using a χ² test (two-tailed). One-way analysis of variance was employed for multiple point comparisons.

**RESULTS**

**Composition and structural analysis of lipid A and OS from O35ElpxXL LOS.** A Zeo' and a Kan' cassette were inserted into the lpxX and lpxL genes in the M. catarrhalis strain O35E genome, respectively, to construct an lpxX and lpxL knockout-double mutant, named O35ElpxXL. Nucleotide sequence analysis and a Southern blot assay confirmed that single copies of both the Zeo' and Kan' cassettes were inserted in the predicted positions of the O35ElpxXL genome. The insertions had no polar effect on the upstream and downstream genes in RT-PCR analyses (data not shown).
**Fig. 1.** Composition and structural analysis of the O35ElpxXL LOS. (a) GC-MS profile of the fatty acid methyl esters obtained from lipid A of *M. catarrhalis* mutant O35ElpxXL. Lipid A of O35ElpxXL did not contain decanoic acid (10:0) or dodecanoic (lauric) acid (12:0). The asterisk indicates impurities. tr, Trace. (b) MALDI-TOF analysis of lipid A from O35ElpxXL and its proposed structure. The analysis was carried out in negative mode, and all ions are represented as deprotonated [M-H]⁻ ions. Lipid A of the O35ElpxXL mutant was tetra-acylated and lacked two 10:0 residues and one 12:0 residue with a structure at m/z 1416.60. (c) MALDI-TOF MS spectrum for the OS from the O35ElpxXL mutant. The analysis was carried out in positive reflectron mode.
Lipid A from the O35ElpxXL mutant was subjected to fatty acid composition analysis (Fig. 1a). In comparison with the published lipid A structure of the *M. catarrhalis* serotype A strain 25238 (Holme et al., 1999) and the parental strain O35E (Gao et al., 2008), lipid A of O35ElpxXL lacked decanoic acid (10:0) and lauroyl acid (12:0) (Fig. 1b) in comparison with lipid A of the parental O35E LOS (Gao et al., 2008), and is in agreement with the data from fatty acid methyl ester analysis (Fig. 1a). Lipid A from O35ElpxXL revealed the presence of three major ions at mass-to-charge ratios (m/z) of 1416.60, 1293.13 and 1094.39 (Fig. 1b and Table 1). These ions represented the structures P2-PEA-GlcN2-[12:0(3OH)]4, P2-GlcN2-[12:0(3OH)]4 and P2-GlcN2-[12:0(3OH)]4, respectively (Table 1). Interestingly, we observed the presence of ions representing tri-acylated lipid A species from the LOS of the O35ElpxXL double mutant, which were not found in lipid A of the wild-type O35E or the single mutants O35ElpxX and O35ElpxL. It is possible that partial degradation had occurred during mild release of lipid A in the presence of 1% acetic acid or partial cleavage of the OS portion of LOS by MALDI-TOF MS analysis (Fig. 1b and Table 1). The OS portion of O35ElpxXL LOS, which was analysed using MALDI-TOF MS in positive ionization mode (Fig. 1c), demonstrated the presence of ions at m/z 1536.48, 1580.48, 1598.48 and 1620.46 (Table 2). This is consistent with the glycosyl components found in the published serotype A structure for the O35E strain having composition Gal2-Glc2-GlcNAc2Kdo (Holme et al., 1999). These results indicated that the OS portion of LOS from O35ElpxXL mutant had the same structure as that of the parental strain O35E (Gao et al., 2008).

### Characterization of O35ElpxXL LOS

LOS was isolated from protease K-treated cell lysate of the O35ElpxXL double mutant and compared with those from the parental O35E and the single mutants O35ElpxX and O35ElpxL. Silver staining analysis following SDS-PAGE of four extracts showed a trace LOS band for the O35ElpxXL mutant (Fig. 2, lane 4) compared with the LOSs of the parental O35E strain and single mutants O35ElpxX and O35ElpxL (Fig. 2, lanes 1–3). Because the lpxX gene significantly modulated the O35E LOS migration pattern in the SDS-PAGE (Gao et al., 2008), we reverted the O35ElpxXL mutant by introducing an lpxX expression plasmid, pWLpxX. Accordingly, the O35ElpxXL mutant complemented with pWLpxX displayed a LOS band migrating in a manner identical to O35ElpxL LOS (Fig. 2, lanes 5 and 3, respectively).

### Biological activities of the O35ElpxXL mutant

An LAL assay was applied to test the LOS-associated biological activity of the *M. catarrhalis* strains. A whole-cell suspension of O35E (OD620 of 0.1) exhibited 2.2 × 10^3 endotoxin units (EU) ml⁻¹, whereas O35ElpxXL showed 4.8 EU ml⁻¹ under the same conditions. The toxicity of the O35ElpxXL mutant showed a 500-fold reduction compared with that of the parental O35E. In contrast, the LOS toxicity of the two single mutants O35ElpxX (7.3 × 10^3 EU ml⁻¹) and O35ElpxL (6.1 × 10^3 EU ml⁻¹) did not exhibit any decrease when the single gene of either lpxX or lpxL was disrupted in the parental O35E.

In a bactericidal assay, 87.7 and 87.4% of the O35E cells survived at 12.5 and 25.0% NHS, respectively (Fig. 3a). Compared with the parental O35E, only 53.1% (P<0.05) and 21.5% (P<0.05) of the O35ElpxXL mutant cells survived at 12.5 and 25.0% NHS, respectively (Fig. 3a),

### Table 1. Proposed composition for the major lipid A ions observed in MALDI-TOF analysis of the *M. catarrhalis* O35ElpxXL mutant (Fig. 1b)

<table>
<thead>
<tr>
<th>Ion observed*</th>
<th>Calculated FM*</th>
<th>Proposed composition†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1014.32</td>
<td>1015.21</td>
<td>P-GlcN-GlcN-[12:0(3OH)]4</td>
</tr>
<tr>
<td>1094.39</td>
<td>1095.19</td>
<td>P2-GlcN-GlcN-[12:0(3OH)]4</td>
</tr>
<tr>
<td>1213.09</td>
<td>1213.51</td>
<td>P-GlcN-GlcN-[12:0(3OH)]4</td>
</tr>
<tr>
<td>1293.13</td>
<td>1293.49</td>
<td>P2-GlcN-GlcN-[12:0(3OH)]4</td>
</tr>
<tr>
<td>1416.60</td>
<td>1416.54</td>
<td>P2-PEA-GlcN-GlcN-[12:0(3OH)]4</td>
</tr>
</tbody>
</table>

*Analysis was carried out in negative ionization mode. FM, formula mass. †P, phosphate; GlcN, glucosamine; PEA, phosphoethanolamine.

### Table 2. Proposed composition for major OS ions observed in MALDI-TOF analysis of *M. catarrhalis* O35ElpxXL mutant (Fig. 1c)

<table>
<thead>
<tr>
<th>Ion observed*</th>
<th>Calculated FM*</th>
<th>Proposed composition†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1536.5</td>
<td>1535.2</td>
<td>Gal2-Glc2-GlcNAc-Kdo(CO2)-Na(anhdro)</td>
</tr>
<tr>
<td>1580.5</td>
<td>1581.3</td>
<td>Gal2-Glc2-GlcNAc-Kdo-Na(anhdro)</td>
</tr>
<tr>
<td>1598.5</td>
<td>1599.2</td>
<td>Gal2-Glc2-GlcNAc-Kdo-Na(anhdro)</td>
</tr>
<tr>
<td>1620.5</td>
<td>1622.1</td>
<td>Gal2-Glc2-GlcNAc-Kdo-Na(anhdro)</td>
</tr>
</tbody>
</table>

*Analysis was carried out in a positive ionization mode. FM, formula mass. †Gal, galactose; Glc, glucose; GlcNAc, N-acetylgalosamine; Kdo, 3-deoxy-D-manno-2-octulosonic acid.
showing reduced resistance to NHS. Moreover, the double mutant O35ElpxXL exhibited lower resistance to 12.5 and 25.0 % NHS than the two single mutants O35ElpxX ($P<0.05$) and O35ElpxL ($P<0.05$) under the same conditions (Gao et al., 2008).

In a mouse lung clearance test, the number of O35ElpxXL cells recovered from mouse lungs was approximately tenfold lower than that of the parental O35E cells immediately (0 h) after challenge ($P<0.01$, Fig. 3b). The O35ElpxXL mutant also showed accelerated bacterial clearance at 3 h (92.5 versus 61.3 %, $P<0.01$) and 6 h (96.6 versus 88.9 %, $P<0.05$) after challenge in comparison with the parental O35E strain (Fig. 3b). Similarly, the double mutant O35ElpxXL displayed a higher clearance rate than the single mutants O35ElpxX and O35ElpxL in mouse lungs at 0 h ($P<0.01$), 3 h ($P<0.01$) and 6 h ($P<0.05$) after challenge (Gao et al., 2008).

**DISCUSSION**

Our prior study identified two late acyltransferase genes of *M. catarrhalis*, *lpxX* and *lpxL*, which are responsible for the biosynthesis of acyloxyacyl-linked secondary acyl chains in the lipid A moiety of the LOS (Gao et al., 2008). By constructing *lpxX* and *lpxL* single-knockout mutants, we found that each of these two genes is individually involved in the biological activities of *M. catarrhalis* (Gao et al., 2008). In this work, we constructed an *lpxX* and *lpxL* double-knockout mutant, O35ElpxXL, to observe the joint role of these two genes in the biological and pathogenic behaviour of *M. catarrhalis* by comparison with the parental strain O35E and the single mutants O35ElpxX and O35ElpxL.

O35ElpxXL colonies were large, flat and transparent, and their altered opacity was similar to that of an O35ElpxA mutant (Peng et al., 2005b). Both *lpxX* and *lpxL* appeared to act together to affect the biophysical features of the *M. catarrhalis* colonies, as the characteristics of the colonies of O35ElpxXL were different from those of the parental O35E and the single mutants O35ElpxX and O35ElpxL (Gao et al., 2008). The lipid A moiety is assumed to be primarily responsible for the LOS toxicity of bacteria. By LAL assay, the O35ElpxXL mutant showed a 500-fold reduction in endotoxin activity compared with the parental strain O35E (Gao et al., 2008). The lipid A moiety is assumed to be primarily responsible for the LOS toxicity of bacteria. By LAL assay, the O35ElpxXL mutant showed a 500-fold reduction in endotoxin activity compared with the parental strain O35E. Two 10:0 acyl chains plus one 12:0 acyl chain jointly contribute to a major part of LOS toxicity in *M. catarrhalis*, as the 10:0 acyl chain-deficient mutant O35ElpxX and the 12:0 acyl chain-deficient mutant O35ElpxL did not show decreased endotoxin activity. Due to its significantly low endotoxin activity, the O35ElpxXL double-mutant strain is thus suggested as a potential vaccine candidate against O35E infections. Further extensive investigations on the immunological protection properties of the O35ElpxXL double mutant are warranted.
The O35ElpxXL mutant displayed more sensitivity to complement-mediated killing by NHS than the parental O35E strain, indicating that the LOS integrity of the outer membrane plays a role in the resistance of Moraxella catarrhalis against host immune attack responses. Furthermore, the O35ElpxXL mutant showed significantly higher clearance than the parental O35E strain in mouse lungs after an aerosol challenge with viable bacteria. Thus, two decanoic acids (10:0) and one dodecanoic (lauric) acid (12:0) in the LOS lipid A moiety synergistically enhance the pathogenicity and virulence of Moraxella catarrhalis. Due to the quick removal of Moraxella catarrhalis from the respiratory tract after the challenge, our current acute model could not display features under chronic lung infection conditions. A recent mouse model of chronic respiratory inflammation (Lugade et al., 2011) could be applied to further address both the pathobiological behaviour and the vaccine properties of our O35ElpxXL double mutant in future studies.

Taken together, the lpxX and lpxL genes encoding two late acyltransferases, decanoyl and dodecanoyl transferase, for LOS lipid A moiety biosynthesis jointly contribute to the biological activities and pathogenicity of Moraxella catarrhalis. Targeting both of these genes together indicates a new path towards the prophylaxis and therapy of Moraxella catarrhalis-caused respiratory infections. The O35ElpxXL double mutant with dramatically reduced toxicity is proposed as a prospective vaccine candidate for further tests.

ACKNOWLEDGEMENTS

We thank Dr Eric J. Hansen (University of Texas, Dallas, TX, USA) for providing strain O35E and plasmid pWW115. We also thank Shengqing Yu for advice in the pulmonary clearance assay, Robert Morell for help with DNA sequencing, Yandan Yang for help with Southern blotting and Qingqing Gao for help with manuscript preparation. This research was supported by the Intramural Research Program of the National Institute on Deafness and Other Communication Disorders (NIDCD), National Institutes of Health, Bethesda, MD, USA (NIH010103632) and a Department of Energy grant (DE-FG09-93-ER20097) to the Complex Carbohydrate Research Center (CCRC), University of Georgia, USA.

REFERENCES


