Late acyltransferase genes *lpxX* and *lpxL* jointly contribute to the biological activities of *Moraxella catarrhalis*

Song Gao,^{1†} Dabin Ren,^{1‡} Daxin Peng,^{1†} Wenhong Zhang,^{1§}
Artur Muszyński,² Russell W. Carlson² and Xin-Xing Gu^{1¶}

Correspondence

Xin-Xing Gu

guxx@mail.nih.gov

Dabin Ren

dabin.ren@rochestergeneral.org

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

²Complex 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 *Moraxella catarrhalis*. Two late acyltransferase genes, *lpxX* and *lpxL*, have been identified involved in the incorporation of acyloxyacyl-linked secondary acyl chains into lipid A during *M. catarrhalis* LOS biosynthesis. In this study, a double mutant with a deletion of both the *lpxX* and *lpxL* genes in *M. catarrhalis* strain O35E was constructed and named O35ElpxXL. Structural analysis of lipid A showed that the O35ElpxXL mutant lacked two decanoic acids (10:0) and one dodecanoic (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 *M. catarrhalis*. The double mutant O35ElpxXL with dramatically reduced toxicity is proposed as a potential vaccine candidate against *M. catarrhalis* infections for further investigation.

Received 25 December 2012

Accepted 3 March 2013

INTRODUCTION

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 *M. catarrhalis* is responsible for 15–25% of otitis media infections (Karalus & Campagnari, 2000). For adults, *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 *M. catarrhalis* is not largely understood so far.

Lipo-oligosaccharide (LOS) is a major outer-membrane component of *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 *Neisseria meningitidis* and *Haemophilus influenzae* (Gorter *et al.*, 2003; Song *et al.*, 2000). Among these, *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, *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 *M. catarrhalis* LOS (Holme *et al.*, 1999; Masoud *et al.*, 1994).

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 *lpxX* and *lpxL* genes have been identified as encoding two late acyltransferases, decanoyl and dodecanoyl transferase,

[†]Present address: School of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu 225009, PR China.

[‡]Present address: Rochester General Hospital Research Institute, 1425 Portland Ave, Rochester, NY 14621, USA.

[§]Present address: National Institute of Biological Sciences, Beijing, 102206, PR China.

[¶]Present address: National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892, USA.

Abbreviations: EU, endotoxin units; LAL, *Limulus* amoebocyte lysate; LOS, lipo-oligosaccharide; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NHS, normal human serum; OD, optical density; OS, oligosaccharide; RT-PCR, reverse transcription-PCR.

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 features 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^r) cassette and a kanamycin-resistant (Kan^r) cassette, respectively, as described previously (Gao *et al.*, 2008). The disrupted *lpxX* gene containing the inserted Zeo^r 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^r and Kan^r double-positive colonies were selected for PCR identification using primers 5'-CTCCTCGAGAGCTCATCAGTGCA-GTCG-3' and 5'-CTCGGATCCCTTTGACATGGCTTGAAG-3' for Zeo^r insertion and 5'-CTCGAATTCGAGTTGCCATCATCAGCA-3' and 5'-CTCGGATCCAATTGGTGTTCATCGGCTT-3' for Kan^r insertion. The disrupted *lpxX* and *lpxL* genes were verified by sequencing, and the *lpxX* and *lpxL* double mutant was named O35ElpxXL. The inserted Zeo^r and Kan^r 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.

Structural analyses of lipid A and OSs. The LOS from 30–35 g of wet cells of the O35ElpxXL mutant was prepared by phenol/water extraction (Gu *et al.*, 1998). Lipid A was released from the LOS using the SDS/mild acid hydrolysis method (Caroff *et al.*, 1988). As the LOS from the O35ElpxXL mutant did not sediment during ultracentrifugation, we treated the LOS material that was extracted into the water phase with mild acid hydrolysis in 1% acetic acid for 1 h. The fatty acid content and molecular mass of the lipid A were analysed by GC-MS analysis of the fatty acid methyl esters and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS in negative ionization mode, respectively. For structural analysis of the O35ElpxXL OS, the O35ElpxXL mutant LOS was further purified using polymyxin B affinity chromatography (Forsberg & Carlson,

1998) and was subjected to MALDI-MS analysis in positive reflectron mode, as described previously (Gao *et al.*, 2008).

SDS-PAGE and silver staining. Crude LOS from the O35ElpxXL mutant was obtained from proteinase K-treated whole-cell lysates (Tzeng *et al.*, 2002). The resulting extracts from the bacterial suspension with 1.9 µg total protein content was resolved by SDS-PAGE (15% acrylamide), visualized by silver staining (Tsai & Frasch, 1982) and compared with the LOSs from strain O35E and the single mutants O35ElpxX and O35ElpxL (Gao *et al.*, 2008).

***Limulus* amoebocyte lysate (LAL) assay.** O35ElpxXL colonies cultured overnight on a chocolate agar plate were suspended in BHI broth to an optical density (OD) at 620 nm of 0.1. The chromogenic LAL assay for endotoxin activity was performed based on the instructions of the manufacturer (QCL-1000 kit; Bio-Whittaker).

Bactericidal assay with normal human serum (NHS). A complement-sufficient NHS was prepared and pooled from eight healthy adult donors. The bactericidal assay was performed in 96-well plates (Peng *et al.*, 2005a). NHS was diluted to 0.5, 2.5, 5.0, 12.5 and 25.0% in Dulbecco's PBS (pH 7.4) containing 0.05% gelatin (DPBSG), respectively. A 10 µl volume of bacteria containing 10⁶ c.f.u. was inoculated into each reaction well containing 190 µl of the diluted NHS, DPBSG alone or 25.0% heat-inactivated NHS as a complement control and was incubated at 37 °C for 30 min. Serial 1:10 dilutions of each well were plated onto chocolate agar plates. The resulting colonies were counted after 24 h of incubation.

Mouse model of pulmonary clearance of bacteria. Female BALB/c mice (6–8 weeks of age) were obtained from Taconic Farms. All experiments involving mice were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were reviewed and approved by institutional review boards at the National Institutes of Health (permit no. 1158). Bacterial aerosol challenges were carried out for mice using wild-type strain O35E (OD₅₄₀ of 0.4; 1.3 × 10⁹ c.f.u. ml⁻¹) and the mutant O35ElpxXL (OD₅₄₀ of 1.0; 2 × 10⁹ c.f.u. ml⁻¹) in 10 ml DPBSG (Hu *et al.*, 2000). The number of bacteria present in the lungs was counted at 0, 3 and 6 h post-challenge. The minimum detectable number of viable bacteria was 100 c.f.u. per lung. Clearance of *M. catarrhalis* was expressed as the percentage of bacterial c.f.u. at each time point against that at 0 h.

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 χ^2 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^r and a Kan^r 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^r and Kan^r 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.

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

Ion observed*	Calculated FM*	Proposed composition†
1014.32	1015.21	P-GlcN-GlcN-[12:0(3OH)] ₃
1094.39	1095.19	P ₂ -GlcN-GlcN-[12:0(3OH)] ₃
1213.09	1213.51	P-GlcN-GlcN-[12:0(3OH)] ₄
1293.13	1293.49	P ₂ -GlcN-GlcN-[12:0(3OH)] ₄
1416.60	1416.54	P ₂ -PEA-GlcN-GlcN-[12:0(3OH)] ₄

*Analysis was carried out in negative ionization mode. FM, formula mass.

†P, phosphate; GlcN, glucosamine; PEA, phosphoethanolamine.

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) substituents (Fig. 1a). The result of composition analysis was supported by MALDI-TOF MS analysis (Fig. 1b and Table 1). The spectrum of lipid A from the O35ElpxXL mutant was consistent with a structure that lacks decanoic acid (10:0) and lauric 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)]₃, 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 (Gao *et al.*, 2008). 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 molecule during MALDI-TOF MS.

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 Gal₂Glc₅GlcNAc₁Kdo (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 proteinase 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 2. Proposed composition for major OS ions observed in MALDI-TOF analysis of *M. catarrhalis* O35ElpxXL mutant (Fig. 1c)

Ion observed*	Calculated FM*	Proposed composition†
1536.5	1535.2	Gal ₂ -Glc ₅ -GlcNAc-Kdo(-CO ₂)-Na ⁺ (anhydro)
1580.5	1581.3	Gal ₂ -Glc ₅ -GlcNAc-Kdo-Na ⁺ (anhydro)
1598.5	1599.2	Gal ₂ -Glc ₅ -GlcNAc-Kdo-Na ⁺
1620.5	1622.1	Gal ₂ -Glc ₅ -GlcNAc-Kdo-Na ⁺ ₂

*Analysis was carried out in a positive ionization mode. FM, formula mass.

†Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; Kdo, 3-deoxy-D-manno-2-octulosonic acid.

Fig. 2. Silver-stained SDS-PAGE pattern of LOS. Extracts from proteinase K-treated whole-cell lysates of wild-type strain O35E (lane 1), O35ElpxX (lane 2), O35ElpxL (lane 3), O35ElpxXL (lane 4) and O35ElpxXL revertant with an *lpxX* expression plasmid, pWlpxX (lane 5) (1.9 µg protein content) were subjected to SDS-PAGE, followed by silver staining. The positions of molecular mass markers (Mark12; Life Technologies) are indicated on the left.

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

Fig. 3. Bactericidal resistance and mouse pulmonary clearance of parental strain O35E and the O35ElpxXL mutant. (a) The bactericidal activity of NHS against strain O35E (open bars) and O35ElpxXL (filled bars) are shown. HI indicates 25.0 % heat-inactivated (56 °C, 30 min) NHS used as a control for each strain tested. The data represent the means $\pm$ SD of three independent experiments. (b) Time course of bacterial recovery from mouse lungs after aerosol challenges with the parental O35E (□) and mutant O35ElpxXL (■). Each time point represents the geometric mean $\pm$ SD of bacterial c.f.u. from eight mice. * $P < 0.05$; ** $P < 0.01$.

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. 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 *M. 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 *M. catarrhalis*. Due to the quick removal of *M. 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 *M. catarrhalis*. Targeting both of these genes together indicates a new path towards the prophylaxis and therapy of *M. 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 (NIH0010103632) and a Department of Energy grant (DE-FG09-93-ER20097) to the Complex Carbohydrate Research Center (CCRC), University of Georgia, USA.

REFERENCES

- Caroff, M., Tacken, A. & Szabó, L. (1988). 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* 175, 273–282.
- Edebrink, P., Jansson, P. E., Rahman, M. M., Widmalm, G., Holme, T., Rahman, M. & Weintraub, A. (1994). Structural studies of the O-polysaccharide from the lipopolysaccharide of *Moraxella* (*Branhamella*) *catarrhalis* serotype A (strain ATCC 25238). *Carbohydr Res* 257, 269–284.
- Forsberg, L. S. & Carlson, R. W. (1998). The structures of the lipopolysaccharides from *Rhizobium etli* strains CE358 and CE359. The complete structure of the core region of *R. etli* lipopolysaccharides. *J Biol Chem* 273, 2747–2757.
- Gao, S., Peng, D., Zhang, W., Muszyński, A., Carlson, R. W. & Gu, X.-X. (2008). Identification of two late acyltransferase genes responsible for lipid A biosynthesis in *Moraxella catarrhalis*. *FEBS J* 275, 5201–5214.
- Gorter, A. D., Oostrik, J., van der Ley, P., Hiemstra, P. S., Dankert, J. & van Alphen, L. (2003). Involvement of lipooligosaccharides of *Haemophilus influenzae* and *Neisseria meningitidis* in defensin-enhanced bacterial adherence to epithelial cells. *Microb Pathog* 34, 121–130.
- Gu, X.-X., Chen, J., Barenkamp, S. J., Robbins, J. B., Tsai, C. M., Lim, D. J. & Battey, J. (1998). Synthesis and characterization of lipooligosaccharide-based conjugates as vaccine candidates for *Moraxella* (*Branhamella*) *catarrhalis*. *Infect Immun* 66, 1891–1897.
- Holme, T., Rahman, M., Jansson, P. E. & Widmalm, G. (1999). The lipopolysaccharide of *Moraxella catarrhalis* structural relationships and antigenic properties. *Eur J Biochem* 265, 524–529.
- Hu, W. G., Chen, J., Battey, J. F. & Gu, X. X. (2000). Enhancement of clearance of bacteria from murine lungs by immunization with detoxified lipooligosaccharide from *Moraxella catarrhalis* conjugated to proteins. *Infect Immun* 68, 4980–4985.
- Karalus, R. & Campagnari, A. (2000). *Moraxella catarrhalis*: a review of an important human mucosal pathogen. *Microbes Infect* 2, 547–559.
- Lugade, A. A., Bogner, P. N. & Thanavala, Y. (2011). Murine model of chronic respiratory inflammation. *Adv Exp Med Biol* 780, 125–141.
- Masoud, H., Perry, M. B. & Richards, J. C. (1994). Characterization of the lipopolysaccharide of *Moraxella catarrhalis*. Structural analysis of the lipid A from *M. catarrhalis* serotype A lipopolysaccharide. *Eur J Biochem* 220, 209–216.
- Murphy, T. F., Brauer, A. L., Grant, B. J. & Sethi, S. (2005). *Moraxella catarrhalis* in chronic obstructive pulmonary disease: burden of disease and immune response. *Am J Respir Crit Care Med* 172, 195–199.
- Peng, D., Choudhury, B. P., Petralia, R. S., Carlson, R. W. & Gu, X.-X. (2005a). Roles of 3-deoxy-D-manno-2-octulosonic acid transferase from *Moraxella catarrhalis* in lipooligosaccharide biosynthesis and virulence. *Infect Immun* 73, 4222–4230.
- Peng, D., Hong, W., Choudhury, B. P., Carlson, R. W. & Gu, X.-X. (2005b). *Moraxella catarrhalis* bacterium without endotoxin, a potential vaccine candidate. *Infect Immun* 73, 7569–7577.
- Song, W., Ma, L., Chen, R. & Stein, D. C. (2000). Role of lipooligosaccharide in Opa-independent invasion of *Neisseria gonorrhoeae* into human epithelial cells. *J Exp Med* 191, 949–960.
- Tsai, C. M. & Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* 119, 115–119.
- Tzeng, Y. L., Datta, A., Kolli, V. K., Carlson, R. W. & Stephens, D. S. (2002). Endotoxin of *Neisseria meningitidis* composed only of intact lipid A: inactivation of the meningococcal 3-deoxy-D-manno-octulosonic acid transferase. *J Bacteriol* 184, 2379–2388.
- Vaneechoutte, M., Verschraegen, G., Claeys, G. & Van Den Abeele, A. M. (1990). Serological typing of *Branhamella catarrhalis* strains on the basis of lipopolysaccharide antigens. *J Clin Microbiol* 28, 182–187.