Microbiol. Immunol., **49**(10), 931–935, 2005

Role of Lipooligosaccharide in the Attachment of *Moraxella catarrhalis* to Human Pharyngeal Epithelial Cells

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Received April 4, 2005; in revised form, June 24, 2005. Accepted July 4, 2005

***Abstract*: The goal of this study was to determine the role of lipooligosaccharide in the attachment of *Moraxella catarrhalis* to human pharyngeal epithelial cells. Strain 2951 and its P^k mutant strain 2951 *galE* were used in this study. This study suggests that the P^k epitope of LOS is not an adhesin for *M. catarrhalis*, but plays a crucial role by its surface charge in the initial stage of attachment.**

***Key words*: Attachment, *Moraxella catarrhalis*, Lipooligosaccharide**

Moraxella catarrhalis is an important pathogen associated with respiratory and middle ear infections and has been shown to express a lipooligosaccharide (LOS) (7, 8, 15). An immune response against *M. catarrhalis* has been documented in patients with bronchopulmonary infections and otitis media (6, 11). Using sera from patients it has been shown that an antibody to LOS of *M. catarrhalis* mediates complement-dependent bacteriolysis against homologous strains (15). Most *M. catarrhalis* strains have been shown to be highly resistant to complement-mediated killing in normal serum (13, 17). Recently it has been reported that the P^k (Galα1-4Galβ1-4Glc) epitope of the LOS may be an important factor in the resistance of *M. catarrhalis* to the complement-mediated bactericidal effect of normal human serum (18). All of this evidence suggests that LOS is an important virulence factor and plays an important role in the pathogenesis of *M. catarrhalis* infections. Only three major antigenic types of *M. catarrhalis* LOS can be distinguished, and more than 90% of the 302 strains express one of three LOS serotypes (16). Therefore interest has grown concerning the use of LOS as a potential vaccine against *M. catarrhalis* infections. Promising results have been obtained in a mouse model using detoxified LOS conjugated to proteins (9).

Bacterial attachment to host cell constitutes the initial step in the pathogenesis of infections. A body of evidence has accumulated to show that the attachment of *M. catarrhalis* to human pharyngeal epithelial cells (HPECs) is the basis of colonization and subsequent infections. As a major constituent of surface structures it has been shown that the lipopolysaccharides (LPS) /LOS of several bacteria act as adhesins, which mediate the attachment of a bacterium to host cells (10). Therefore our interest has focused on the role of LOS in the attachment of *M. catarrhalis* to HPECs.

Strain 2951 a *M. catarrhalis* strain of serotype A and its *galE* mutant were used in this study. Strain 2951 *galE* mutant was made by introducing a deletion/insertion mutation of UDP-glucose-4-epimerase (*galE*) gene into *M. catarrhalis* strain 2951; resulting in the loss of P^k epitope expression on its LOS (18). The culture conditions have been described previously (18). An attachment assay was done to compare the attachment ability of wild and mutant strains to pharyngeal epithelial cells obtained from 10 normal healthy adult humans. Their

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Abbreviations: DNA, deoxyribonucleic acid; galE, UDP-glucose-4-epimerase; Gg_4Cer, asialoganglioside GM1; Gg_3Cer, asialoganglioside GM2; HPEC, human pharyngeal epithelial cell; LOS, lipooligosaccharide; LPS, lipopolysaccharide; mAb, monoclonal antibody; TEM, transmission electron microscopy; TLC, thin layer chromatography.

mean age was 22.5 years and the group comprised 3 females and 7 males. The attachment assay was done following previously described procedures using HPEC collected from the pharynx (1). Bacteria suspended in PBS at a concentration of 1×10^8 cfu/ml and pharyngeal epithelial cells at a concentration of 2.5×10^4 cell/ml were mixed in a 1:1 ratio. They were centrifuged at $750\times g$ for 10 min at room temperature and kept at 37 C for 30 min. Unattached bacteria were separated by a total five washing with PBS using centrifugation at $80\times g$ for 10 min at room temperature. Finally cells were collected on a glass slide by a cytospin (Thermo Shandon International, U.K.). Smears were Gram-stained and viewed under the oil-immersion lens of a light microscope to count the number of attached bacteria on 50 successive cells. For each assay the average results from duplicate experiments were taken. The attachment (mean±SE) of strain 2951 *galE* (25.2±2.9 bacteria/cell) was found to be significantly ($P<0.001$, Student's *t* test) less than that of strain 2951 (38.3±4.9 bacteria/cell).

To determine whether LOS is an adhesin of *M. catarrhalis* we performed an attachment inhibition assay using isolated LOS and a monoclonal antibody against the P^k antigen. Briefly, *M. catarrhalis* or HPECs were treated with monoclonal antibody against the P^k antigen or LOS for 30 min at 37 C in a rotator as described before (3) and an attachment assay was done as described above. Similarly handled untreated bacteria or cells were used as a control. Each experiment was done at least three times and each time, the average results of duplicate experiments was taken. LOS was isolated from the wild type strain of *M. catarrhalis* by lipopolysaccharide microextraction using proteinase K digestion (5).

Nucleic acid and protein contamination were also determined by spectral analysis by a UV measurement at 260 nm and the Bradford method (Bio-Rad Laboratories, Munchen, Germany) respectively. In quantitative terms, the isolated LOS was contaminated with 3% proteins and 8.4% nucleic acids.

For all attachment inhibition assays, HPECs were collected from the pharynx of a healthy adult female. Compared to the similarly handled untreated control, there was no significant decrease of attachment of strain 2951 when HPECs were treated with LOS at concentrations of 0.1, 1 and 10 μg/ml (Table 1). Since strain 2951 is a self-agglutinating strain, these results were confirmed using strain B-88-152, a non-agglutinating strain of *M. catarrhalis* isolated from the sputum of a patient with a respiratory tract infection (1). The isolated LOS from this strain showed 1.4% and 10.5% protein and DNA contamination, respectively. Compared to the similarly handled untreated control, there was no significant decrease of attachment of strain B-88-152 when HPECs were treated with LOS at concentrations of 0.1, 1 and 10 μg/ml (Table 1). These results indicate that LOS does not block the binding sites of *M. catarrhalis*, and as a result did not inhibit attachment. The limitation of these experiments is that the extracted LOS was contaminated with low levels of proteins and nucleic acids. To determine the effects of DNA contamination in the attachment, an attachment inhibition assay was done a second time with isolated DNA from strain B-88-152 by the standard method. HPECs were treated with 10 μg/ml of DNA. The attachment of bacteria to DNA-treated HPEC (66.5±17.2 bacteria/cell) was not significantly different from that for similarly handled untreated control cells (59.0±8.5 bacteria/cell).

Monoclonal antibody, mAb 4G5, an immunoglobulin G2a against the P^k epitope was a gift from Campagnari A.A. (18). Treatment of strain 2951 with a culture supernatant of mAb at a dilution of 1:5,000, 1:1,000 and 1:500 the attachment of bacteria was 27.7±4.3, 34.9±2.0 and 30.4±5.7 bacteria/cell, respectively. The attachment of a similarly handled untreated control was 31.8±2.1 bacteria/cell (Table 1). Therefore mAb binding with the P^k epitope had no effect on attachment. These findings indicate that the P^k epitope of LOS is not an adhesin of *M. catarrhalis*.

We sought to investigate whether any difference exists between the wild (strain 2951) and mutant strains (strain 2951 *galE*) in their binding ability with different glycolipids, using thin layer chromatography (TLC) (3). The following glycolipids, prepared from bovine brain, were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.): asialoganglioside GM1 (Gg_4Cer), GM1, asialoganglioside GM2 (Gg_3Cer), gangliosides GM2,

Table 1. Attachment inhibition assay of *M. catarrhalis* strain 2951 and B-88-152 after cells were treated with lipooligosaccharide (LOS)

Strain name	Control	0.1 μg/ml	1 μg/ml	10 μg/ml of LOS
Strain 2951	34.1±4.2	32.6±4.1	40.4±5.9	37.9±4.7
Strain B-88-152	30.0±14.7	43.2±18.9	44.9±23.3	47.4±22.3

Attachment is expressed as number of bacteria attached per human pharyngeal epithelial cell. For each concentration at least three experiments were performed in duplicate.

GD1a, GD2, GT1b and GQ1b. In both strains, a positive reaction was obtained with Gg_4Cer and Gg_3Cer, but no reactivity was observed with gangliosides GM1, GM2, GD1a, GD2, GT1b or GQ1b.

Transmission electron microscopy (TEM) was done to observe any change that may have occurred on the surface structures of the mutant strain according to a previously described procedure (1). All specimens for TEM were examined with a JEM 100CX electron microscope (JEOL Ltd., Tokyo) operated at 80 kV. The

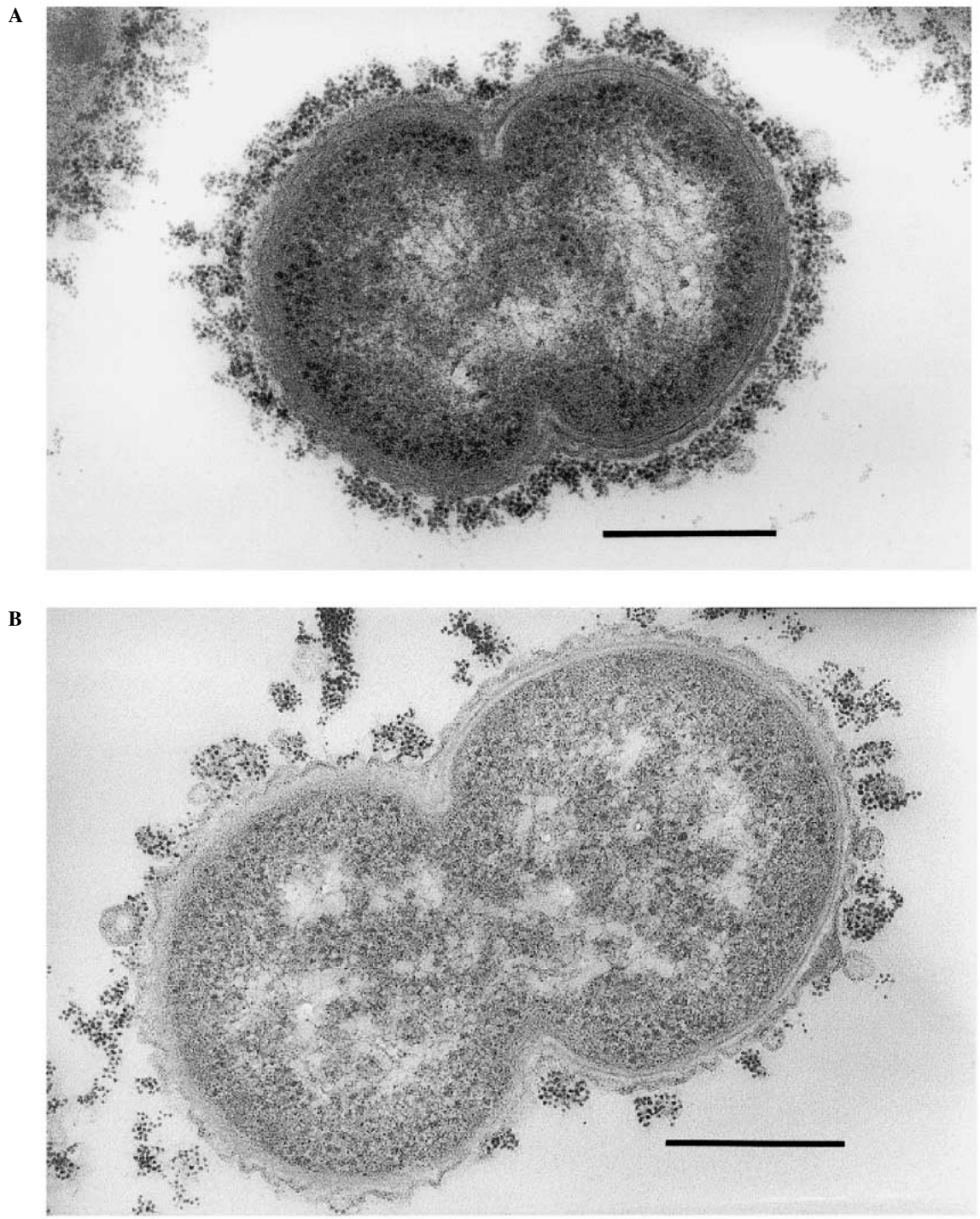

Fig. 1. Transmission electron microscopic photo of *M. catarrhalis* after treatment with cationized ferritin. Cationized ferritin particles are attached homogenously to the surface of strain 2951, indicating a net negative charge (A). On some areas the cationized ferritin particles are attached to the surface of the P^k mutant of *M. catarrhalis* (strain 2951 *galE*), while most areas are devoid of ferritin particles, indicating that some areas are negatively charged while most of the area is devoid of negative charge (B). Bar: 300 nm.

TEM observation demonstrated that the morphology and fimbriation of the P^k mutant was indistinguishable from that of the wild type.

To characterize the surface charge, TEM was done using cationized ferritin particles. All strains of *M. catarrhalis* were treated with cationized ferritin particles and uncharged ferritin particles according to a previously described method (2). At least one hundred bacterial cells were observed to determine the result. TEM observation showed that cationized ferritin particles were attached homogenously on the surface of all of the cells of the wild type strain (Fig. 1A), indicating a net negative surface charge. In all of the cells of the mutant strain, either the whole or most of the surface was devoid of cationized ferritin particles (Fig. 1B), indicating a loss of negative charge from the surface. Uncharged ferritin particles did not bind with the surface of either the wild or mutant strains.

The identification of molecules involved in the attachment of bacteria to host cells is important for understanding the pathogenesis as well as to develop preventive and treatment strategies for infections. LPS/LOS is known to be involved in the attachment of various microorganisms (10). Although the P^k mutant attached significantly less than the wild type strain, however, isolated LOS and mAb against P^k did not inhibit the attachment of *M. catarrhalis* to HPECs. The only difference noted in the P^k mutant strain, which may contribute to attachment, is the decrease of negatively charged surface area. A model for the attachment of *M. catarrhalis* suggests that this bacterium, with its net negative charge, binds to the positively charged domain of HPECs (2). Since the area with negative charge on the surface of the mutant strain is decreased, the above model may explain why it attaches less strongly compared with the wild type. However the quantitative difference between the surface charge of wild and mutant strains needs to be explored in the future.

It has been suggested that the absence of LOS may affect the membrane integrity of the bacteria, thus altering the localization and structural formation of proteins either by impeding the proper insertion of membrane proteins into the membrane or by affecting the general charge on the bacterial surface (4). It is difficult to explain how the deletion of the P^k epitope would change the charge on the bacterial surface. Similar to *M. catarrhalis* P^k antigen, *Helicobacter pylori* also express Le^x and Le^y as constituents of the O antigen of its LPS. It has been shown that the *H. pylori* LPS plays a minor role in attachment (12) or Le expression is not necessary for *H. pylori* attachment to epithelial cells (14). This study suggests that, although the P^k epitope of the LOS of *M. catarrhalis* is not an adhesin but it contributes to the surface charge of this bacterium, which is critical for attachment to HPECs. It appears that surface structures must be coordinately expressed to enable *M. catarrhalis* to establish an infection in the respiratory tract.

A portion of this project was supported by Bilkent University Research Fund (MBG-01-04) and Bilkent University Research Development Grant Program 2002. We thank Michael A. Apicella (the University of Iowa) for providing us with strain 2951 and strain 2951 *galE* and Anthony A. Campagnari (State University of New York at Buffalo) for monoclonal antibody. We also thank Kazunori Oishi and Hiroshi Watanabe (Nagasaki University) for their advice.

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