INSTITUT PASTEUR

Microbes and Infection 10 (2008) 3–11

www.elsevier.com/locate/micinf

Original article

# Outer membrane protein UspA1 and lipooligosaccharide are involved in invasion of human epithelial cells by *Moraxella catarrhalis*

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Received 3 July 2007; accepted 24 September 2007
Available online 2 October 2007

**Abstract**

Invasion of non-professional phagocytes is a strategy employed by several mucosal pathogens, but has not been investigated in detail for *Moraxella catarrhalis*, a major cause of human respiratory tract infections. We investigated the role of outer membrane protein (OMP) UspA1 and lipooligosaccharide (LOS) in *M. catarrhalis* invasion into epithelial cells. An isogenic mutant of strain O35E, which lacked expression of the UspA1 adhesin, demonstrated not only severely impaired adherence (86%) to but also reduced invasion (77%) into Chang conjunctival cells in comparison with the wild-type strain. The isogenic, LOS-deficient mutant strain O35E.*lpxA* was attenuated in adherence (93%) and its capacity to invade was severely reduced (95%), but not abolished. Inhibition assays using sucrose and cytochalasin D, respectively, demonstrated that clathrin and actin polymerization contribute to internalization of *M. catarrhalis* by Chang cells. Furthermore, inhibition of UspA1-mediated binding to cell-associated fibronectin and α5β1 integrin decreased invasion of *M. catarrhalis* strain O35E (72% and 41%, respectively). These data indicate that OMP UspA1 and LOS profoundly affect the capacity of *M. catarrhalis* to invade epithelial cells.


*Keywords:* *Moraxella catarrhalis*; Invasion; Outer membrane protein; Lipooligosaccharide; UspA1

## 1. Introduction

*Moraxella catarrhalis* is a human mucosal pathogen responsible for acute otitis media in children and lower respiratory tract infections in adults with chronic obstructive pulmonary disease [1]. Mucosal infection is preceded by colonization [2], which occurs in a two-step process consisting of adherence mediated by bacterial surface components and host cell receptors and, in some instances, invasion of host cells. Entry into epithelial cells may protect bacteria against host clearance mechanisms and plays an important role in overcoming the epithelial barrier for subsequent spreading [3]. *M. catarrhalis* was recently found to be capable of invading human epithelial cells in vitro [4]. Molecules involved in this process, however, have not been identified.

In gram-negative organisms, bacterial surface components interacting with host cells include outer membrane proteins (OMP) and lipooligosaccharide (LOS). In *M. catarrhalis*, the best characterized OMP belong to the family of the ubiquitous surface proteins A (UspA), which are trimeric autotransporters [5,6]. UspA1 mediates adherence to host cells, whereas expression of UspA2 is essential for the serum-resistant phenotype [5]. OMP as well as putative secretory and excretory products also induce the release of various proinflammatory cytokines and chemokines, including interleukin 1β (IL-1β), IL-6, IL-8 and prostaglandin $E_2$, which contribute to the pathogenesis and may promote internalization of bacteria [7]. In addition, LOS, a major trigger of the inflammatory response [8], plays a role in both adherence and invasion of host cells

*Abbreviations:* OMP, outer membrane protein; LOS, lipooligosaccharide; UspA1, ubiquitous surface protein A1; *lpxA*, UDP-*N*-acetylglucosamine acyltransferase; Fn, fibronectin.

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doi:10.1016/j.micinf.2007.09.014

by related bacteria such as *Haemophilus influenzae* [9], *Neisseria meningitidis* [10] and *Neisseria gonorrhoeae* [11]. Inactivation of the *lpxA* gene of *M. catarrhalis* blocks the initial step of the lipid A biosynthesis pathway, resulting in a LOS-deficient mutant [12]. LOS-deficient *M. catarrhalis* is impaired in its capacity to adhere to epithelial cells.

Several host cell surface receptors are involved in pattern recognition and uptake of bacteria. One way of introducing bacteria into host cells is mediated by α5β1 integrin, which binds via endogenous fibronectin to bacterial fibronectin-binding proteins [13]. This binding induces a cytoskeletal rearrangement and ingestion of pathogens such as S*treptococcus pyogenes* [14] and *Yersinia* spp. [15]. The *M. catarrhalis* OMP UspA1 and, in some strains, UspA2 have been shown to mediate adherence by binding to cell-associated fibronectin [16,17]. The *M. catarrhalis* isolate used in this study, strain O35E, expresses an UspA1 protein, which is able to bind endogenous fibronectin, while UspA2 is not [17]. UspA2 from this strain binds vitronectin and confers serum resistance [18].

In this study, we investigated the role of OMP and LOS in *M. catarrhalis* invasion into epithelial cells using UspA1- and LOS-deficient mutants. We demonstrate that variations in the expression of UspA1 and LOS, respectively, affect the capacity of *M. catarrhali*s to invade host cells. Furthermore, we show that fibronectin-bound α5β1 integrin receptors are involved in UspA1-promoted invasion.

## 2. Materials and methods

### 2.1. Reagents

Cytochalasin D and a mouse monoclonal anti-human fibronectin antibody were purchased from Sigma (St. Louis, MO). A monoclonal rat anti-human α5 antibody (BIIG2) was obtained from Hybridoma Bank (University of Iowa, USA). Tetramethylrhodamine isothiocyanate-phalloidine was obtained from Molecular Probes (Eugene, OR).

### 2.2. Bacterial strains and culture conditions

*M. catarrhalis* O35E and its isogenic *uspA1* (O35E.*uspA1*) mutant have been described [5]. All strains were cultured at 37 °C and 200 rpm in brain heart infusion (BHI) broth (Difco, Detroit, MI) or on BHI agar plates in an atmosphere containing 5% $CO_2$. Media were supplemented with kanamycin (20 μg/ml) for culturing of isogenic mutant O35E.*uspA1*. *Escherichia coli* DH5α was grown on Luria-Bertani (LB) agar plates or in LB broth.

### 2.3. DNA methods

Plasmids were isolated using the Wizard Plus SV Minipreps DNA purification system (Promega Corp., Madison, WI). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Electrocompetent *M. catarrhalis* was prepared and DNA was electroporated as described [19]. DNA sequencing was performed by using an ABI PRISM 310 genetic analyzer (PE Biosystems, Rotkreuz, Switzerland) with the Big Dye Terminator cycle sequencing ready reaction kit (PE Biosystems). Sequences were analyzed with the DNASTAR software (DNASTAR, Madison, WI).

### 2.4. Construction of the lpxA mutant O35E.lpxA

Our first approach to construct a *lpxA* mutant was to replace the *lpxA* gene by allelic replacement using the 774-bp *lpxA* ORF with an inserted antibiotic resistance cassette as described [12]. We failed to obtain recombinants, which suggested that the homologous flanking regions were too short for effective recombination. We next constructed a fragment with flanking regions of at least 1 kb on either side of *lpxA*. This time, we failed to obtain clones after transformation into *E. coli* DH5α. Assuming that the fragment was toxic for *E. coli*, our next strategy was to divide *lpxA* into two parts and clone each part separately. The 5′-portion of *lpxA* (*lpxA*1) from strain O35E was amplified with forward primer *lpxA*1F (5′-GCTGATTGACCGAGTGACC-3′) and reverse primer *lpxA*1R (5′-CGATACGACAAAACTGATGC-3′). The PCR product was ligated into pGEM-T-Easy (Promega). The 3′-portion of *lpxA* (*lpxA*2) was amplified with forward primer *lpxA*2F (5′-AAAGATGTTGCGGCGTATG-3′) and reverse primer *lpxA*2R (5′-GCCCAGCTTAGAACAGACG-3′) and ligated as described. A kanamycin resistance cassette (pUC4k) was ligated into the NcoI restriction site upstream of *lpxA*2. The insertion was released using SphI-NdeI digestion and subcloned into an NdeI site of pGEM-T-Easy-*lpxA*1. Correct orientation was verified by digestion analysis and PCR. The construct Δ*lpxA*::*kan* was amplified by using the primers *lpxA*1F and *lpxA*2R, purified and used for electrotransformation of strain O35E. After electroporation the cell suspension was mixed with 1 ml of BHI, shaken at 200 rpm at 37 °C for 6 h, and plated on BHI containing kanamycin. After 24 h of incubation, kanamycin-resistant colonies were selected for PCR analysis of chromosomal DNA using the primers *lpxA*1F and *lpxA*2R. Insertional inactivation of *lpxA* was confirmed by sequence analysis and Southern blotting.

### 2.5. Preparation of M. catarrhalis outer membrane proteins

OMP were prepared by the EDTA buffer method as described [20]. Bacteria were harvested from a stationary phase culture, resuspended in EDTA buffer (0.05 M $Na_2HPO4$, 0.15 M NaCl, 0.01 M EDTA, pH 7.4), homogenized and incubated at 56 °C and 300 rpm for 1 h. Cells and cell debris were eliminated by centrifugation at 10,000 × *g* for 15 min at 4 °C. Finally, the proteins were collected by ultracentrifugation at 100,000 × *g* for 3 h at 4 °C and resuspended in PBS.

### 2.6. Western blot analysis

Samples were resolved by SDS-PAGE using a 7.5% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore Corp., Bedford,