Outer Membrane Protein OlpA Contributes to Moraxella catarrhalis Serum Resistance via Interaction With Factor H and the Alternative Pathway

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Factor H is an important complement regulator of the alternative pathway commonly recruited by pathogens to achieve increased rates of survival in the human host. The respiratory pathogen Moraxella catarrhalis, which resides in the mucosa, is highly resistant to the bactericidal activity of serum and causes otitis media in children and respiratory tract infections in individuals with underlying diseases. In this study, we show that M. catarrhalis binds factor H via the outer membrane protein OlpA. M. catarrhalis serum resistance was dramatically decreased in the absence of either OlpA or factor H, demonstrating that this inhibition of the alternative pathway significantly contributes to the virulence of M. catarrhalis.

Keywords. factor H; Moraxella catarrhalis; serum resistance.

The complement system is a crucial component of the immune response that results in direct lysis of pathogens or opsonization for increased phagocytosis. Therefore, complement resistance is an important virulence trait of many pathogens that consequently increases the survival rate among bacteria within the human host [1]. The complement system is activated via 3 different routes, the classical pathway, the lectin pathway, and the alternative pathway, all of which leads to the terminal pathway resulting in formation of the bactericidal membrane attack complex. All pathways are tightly controlled by human fluid-phase or membrane-bound regulators, and one of the most well-described complement-resistance mechanisms consists of hijacking of such regulators. Factor H (FH) is a 150-kDa fluid-phase protein and an important regulator of the alternative pathway, facilitating the decay of C3b [2]. FH consists of 20 short consensus repeat domains of approximately 60 amino acids.

Moraxella catarrhalis is a human commensal and an emerging respiratory pathogen causing otitis media in children and lower respiratory tract infections in patients with chronic obstructive pulmonary disease. The vast majority of clinical M. catarrhalis isolates recovered from both adults and children are complement resistant [3]. We have previously shown that M. catarrhalis ubiquitous surface proteins (Usp) A1 and A2 that reside in the outer membrane interact with the C4b binding protein (C4BP), thereby inhibiting the classical pathway [4]. In addition, we and others have described an interaction of vitronectin and UspA2/UspA2H that results in increased survival of M. catarrhalis due to inhibition of the terminal pathway [5, 6]. A noncovalent interaction of C3 and UspA1/A2 has also been described to have a protective effect against the bactericidal activity of serum [7]. In the present study we reveal an additional complement resistance mechanism of M. catarrhalis, the Opa-like protein A (OlpA)–dependent interaction with FH.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions
M. catarrhalis strains were obtained and cultured as described elsewhere [8]. Appropriate antibiotics were supplemented to the M. catarrhalis mutants.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blots
Whole bacterial lysates of M. catarrhalis strains were analyzed by 12% SDS-PAGE and separated proteins were transferred to an Immobilon-P membrane (Millipore). Membranes were blocked in phosphate-buffered saline with 0.05% Tween 20 containing 2.5% milk powder (blocking buffer) and incubated with 3 µg/mL human complement FH (Quidel) in blocking buffer for 2 hours at room temperature. Membranes were thereafter incubated with a monoclonal mouse anti-human complement FH antibody (AbD Serotec) diluted 1:1000, followed by horse-radish peroxidase–conjugated rabbit anti-mouse polyclonal antibodies (Dako). Development of membranes was done...
using Pierce ECL Western blotting detection reagents (Thermo Fisher Scientific).

2D-gel Electrophoresis and Mass Spectrometry
Bacterial outer membrane vesicles (OMVs) were prepared as described elsewhere [9]. To digest nucleic acids, OMVs (1 mg) were incubated with 50 U Benzonase (Sigma-Aldrich) in 100 μL at 25°C for 30 minutes. Thereafter, samples were solubilized for 1 hour in 400 μL of rehydration buffer with alternate vortexing and were subsequently spun down. Separation in the first and second dimension was performed by isoelectric focusing (IEF) and gel electrophoresis, respectively [10]. Protein spots were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Construction and Complementation of OlpA- and OMP J–Deficient M. catarrhalis
To construct isogenic mutants of M. catarrhalis unable to express OlpA or the outer membrane protein J (OMPJ), the corresponding genes were disrupted with an erythromycin resistance cassette. The olpA/ompJ upstream flanking region, the erythromycin resistance cassette, and the olpA/ompJ downstream flanking region were fused by overlapping extension polymerase chain reaction (PCR). In the construction of the RH4ΔolpAΔompJ double mutant, the OMPJ-encoding gene was abolished with a kanamycin resistance cassette.

To reconstitute OlpA expression, M. catarrhalis RH4 OlpA was amplified by PCR and ligated into pWW115 (a kind gift from Prof Eric Hansen, Southwestern Medical Center, Dallas, TX). The resulting plasmid, pWWolpA, was transformed into the RH4ΔolpA mutant and the OlpA-negative M. catarrhalis Bc5, yielding strains RH4ΔolpA(pWWolpA) and Bc5(pWWolpA). The backbone plasmid pWW115 was used to generate RH4ΔolpA(pWW115) and Bc5(pWW115) as controls.

Flow Cytometry Analysis
Bacteria (0.5 × 10^6 colony-forming units) were incubated at 37°C for 1 hour with 10 μg/mL FH in incubation buffer (phosphate-buffered saline and 1% bovine serum albumin). After 2 washing steps, bacteria were incubated with fluorescein isothiocyanate (FITC)–conjugated anti-FH antibody (Abcam), followed by washings and flow cytometry analysis (FACSCanto; BD Bioscience).

Serum Bactericidal Assay
Normal human serum (NHS) from 5 healthy adult volunteers was pooled. Heat-inactivated serum (HIS) was manufactured by treatment of NHS at 56°C for 30 minutes. FH-depleted human serum was purchased from Complement Technologies. Human FH (Quidel; 100 μg/mL) was used to reconstitute the FH-depleted serum. To inactivate the classical pathway, 10 mM Mg–ethylene glycol tetraacetic acid (EGTA) was included, whereas 10 mM ethylenediaminetetraacetic acid (EDTA) was used for complete complement inactivation. The serum bactericidal assay was performed as described elsewhere [4].

Fluorescence Microscopy
Bacteria were incubated with FH (10 μg/mL) for 1 hour at 37°C followed by incubation with a FITC-conjugated anti-FH antibody. After washing, bacteria were transferred on poly-L-lysine (Sigma-Aldrich)–coated microscopy glass slides and fixed with 4% paraformaldehyde. Binding was visualized using an Olympus fluorescence microscope.

Statistical Analysis
Two-way analysis of variance (GraphPad Prism) was used to analyze findings of the bactericidal serum resistance assays, and the Mann–Whitney test was used to analyze flow cytometry results.

RESULTS
We have previously shown that M. catarrhalis interacts with both the classical and alternative pathways through binding of C4BP and C3, respectively. To further elucidate M. catarrhalis serum resistance, the interaction with the abundant and important alternative pathway inhibitor FH was studied. A randomly chosen collection of M. catarrhalis strains was screened by far Western blotting. As can be seen in Figure 1A, 9 of 10 M. catarrhalis strains bound FH, although to a different level. The high-binding strain RH4 and the nonbinding strain Bc5 were chosen for further analysis.

To identify the FH-binding proteins of M. catarrhalis RH4, OMVs were separated by 2-dimensional gel electrophoresis followed by far Western blotting using human complement FH and a monoclonal anti-FH antibody. Two putative FH-binding proteins with sizes of approximately 24 kDa and 20 kDa were identified (Figure 1B). Subsequent MALDI-TOF mass spectrometry identified the larger protein as OlpA and the smaller protein as OMP J.

The FH-binding capacity and the particular role of OlpA and OMP J expression were assessed using different mutants. The binding of FH was abolished in OlpA-deficient mutants, as shown by flow cytometry analysis (Figure 1C). In contrast, deletion of OMP J did not decrease the FH binding; that is, the RH4ΔompJ mutant showed a similar FH-binding capacity as compared to the wild-type counterpart. In addition, the double mutant did not have any reduced binding of FH relative to the single OlpA mutant. These results were consistent with the far Western blot analysis of OMV derived from the wild-type and mutant strains (Figure 1D). OlpA expression was reconstituted in the RH4 mutant devoid of OlpA, resulting in RH4ΔolpA (pWWolpA). The specificity of the OlpA-dependent FH-binding was confirmed using far Western blotting (data not shown) and fluorescence microscopy (Figure 1E). Importantly, introduction
of OlpA in *M. catarrhalis* Bc5, which did not bind FH (Figure 1A), resulted in a significant FH binding (Figure 1F). Taken together, our results show that OMP J does not contribute to the interaction with FH, whereas OlpA is a new FH-binding protein of *M. catarrhalis*.

Since FH inhibits the alternative pathway, we hypothesized that the bacterial binding of FH would contribute to *M. catarrhalis* serum resistance. Hence, a series of bactericidal assays using the RH4 wild type and the OlpA mutant strains were performed. OlpA expression promoted survival in human serum; that is, the *M. catarrhalis* RH4 wild-type strain and the complemented OlpA mutant (RH4ΔolpA[pWWolpA]) survived equally well (Figure 2A), whereas the OlpA mutant and the transcomplemented control (RH4ΔolpA[pWW115]) displayed a significantly decreased survival. Interestingly, selective blocking of the classical pathway by using the Ca²⁺-chelating agent EGTA, leaving the alternative pathway intact, resulted in a comparable killing of the OlpA-deficient mutants. This last experiment with EGTA thus indicated that OlpA expression plays a role, specifically in serum resistance related to the alternative pathway.

To further elucidate the role of FH in the OlpA-dependent survival of *M. catarrhalis* when exposed to human serum, a FH-depleted serum preparation was used in the bactericidal assay. *M. catarrhalis* exhibited decreased survival in FH-depleted serum, and when the serum was reconstituted with FH the survival rate was significantly increased (Figure 2B). In contrast, survival of the OlpA-deficient RH4 mutant was
carcinoembryonic antigen–related cell adhesion molecule and heparan sulfate proteoglycan receptors. However, no similar interactions or other additional functions have been assigned to OlpA. Recently, Vries et al presented a detailed molecular profile of the complement resistance factors of *M. catarrhalis* [12]. They studied transcriptional adaptation and performed genome-wide targeted sequencing using transposon mutagenesis in the presence of human serum, which demonstrated upregulation of 84 genes and downregulation of 134 genes. The Usp proteins have long been regarded as the major complement resistance factor, which was also confirmed in that study. However, the *olpA* gene and several other genes were also identified to be required for *M. catarrhalis* complement resistance. One of the main findings in that study was the importance of the disulfide bond formation system in the resistance against the classical pathway. Taken together, *M. catarrhalis* has several mechanisms that are involved in serum resistance, and it is difficult to judge their individual importance in vivo. The advantage for *Moraxella* of having several mechanisms is that the serum resistance is maintained although separate strains express different factors at various levels. For example the natural OlpA-deficient *M. catarrhalis* Bc5 strain used in our study is still very resistant to serum (data not shown).

Bacterial complement resistance is a well-described and intriguing phenomenon for most respiratory pathogens and an important virulence mechanism. Components of the complement system are detectable in the respiratory tract and the complement resistance of several pathogens indicates that complement components are active in the mucosa. The ability to bind the complement regulator FH with acquired protection of the alternative pathway is a characteristic feature of several bacteria, including human respiratory pathogens [1]. The 3 most common microbes causing acute otitis media are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *M. catarrhalis*. *S. pneumoniae* and *H. influenzae* have previously been shown to interact with FH, resulting in enhanced survival in serum [1,13]. The FH-binding also inhibited opsonization of *S. pneumoniae* and facilitated adherence to epithelial cells [13, 14]. It was previously demonstrated that all known FH-binding microbes interact with the same binding region on short consensus repeat number 20 [15]. Most interestingly, when FH is bound to bacterial proteins, an enhanced FH-dependent interaction with C3b occurs, and thus FH mediates efficient inhibition of complement activation at the bacterial surface.

In conclusion, *M. catarrhalis* possesses several mechanisms for mediating complement resistance. Interactions with C4BP and vitronectin have previously been reported, and in the present article we describe an additional mechanism resulting in inhibition of the alternative pathway. Serum resistance of *M. catarrhalis* has previously been described to be mainly dependent on the Usp family of proteins. However, we now propose that

**DISCUSSION**

Here we describe a novel mechanism of escaping complement-mediated killing by the alternative pathway that significantly contributes to *M. catarrhalis* serum resistance. We have demonstrated the specific binding of the important inhibitory complement regulator FH to *M. catarrhalis* by using several independent methods, including far Western blotting, flow cytometry, and fluorescence microscopy. Initial results with 2-dimensional SDS-PAGE revealed 2 FH-binding proteins, OlpA and OMP J, which have been described elsewhere [11]. In further downstream experiments, we confirmed specific FH binding to OlpA only.

OlpA is a conserved 24-kDa outer membrane protein that has a high similarity with the neisserial Opa adhesins [11]. The neisserial Opa proteins are known to bind both unaffected by the addition of FH. Taken together, *M. catarrhalis* FH–binding OlpA significantly contributes to serum resistance.
OlpA also contributes to the serum resistance further increasing *Moraxella* pathogenesis.

**Notes**

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