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The outer membrane protein OlpA contributes to *Moraxella catarrhalis* serum resistance via interaction with factor H and the alternative pathway

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ABSTRACT

Factor H is an important complement regulator of the alternative pathway commonly recruited by pathogens for increased survival in the human host. The respiratory pathogen *Moraxella catarrhalis* that resides in the mucosa is highly serum resistant 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*.

Keyword List: Factor H, *Moraxella catarrhalis*, serum resistance

INTRODUCTION

The complement system is a crucial component of the immune response that results in direct lysis of pathogens or opsonisation for increased phagocytosis. Therefore, complement resistance is an important virulence trait of many pathogens that consequently increases the bacterial survival within the human host [1]. The complement system is activated via three different routes, *i.e.*, the classical, the lectin or the alternative pathway, all three leading to the terminal pathway resulting in the bactericidal membrane attack complex (MAC). 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]. Factor H consists of 20 short consensus repeat (SCR) 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 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 protein (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 presented 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 non-covalent 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 [8]. Appropriate antibiotics were supplemented to the *M. catarrhalis* mutants.

SDS-Page and Western blots

Whole bacterial lysates of *M. catarrhalis* strains were analysed on 12 % SDS-PAGE and separated proteins were transferred to an Immobilon-P membrane (Millipore). Membranes were blocked in PBS with 0.05 % Tween 20 (PBST) containing 2.5 % milk powder (blocking buffer) and incubated with 3 µg/ml human complement FH (Quidel) in blocking buffer for 2 h at room temperature (RT). Membranes were thereafter incubated with a monoclonal mouse anti-human complement factor H antibody (AbD Serotec) diluted 1/1000 followed by horseradish peroxidase (HRP) -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 (OMV) were prepared as described [9]. To digest nucleic acids, OMV (1 mg) were incubated with 50 U Benzonase (Sigma-Aldrich) in 100 µl at 25^{o}C for 30 min. Thereafter, samples were solubilized for 1 h in 400 µl of rehydration buffer with alternate vortexing, and 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/ionisation-time of flight mass spectrometry (MALDI-TOF-MS).

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 finally the *olpA*/*ompJ* downstream flanking region were fused by overlapping extension PCR. In the construction of the RH4Δ*olpA*Δ*ompJ* 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, pWW*olpA*, was transformed into the RH4Δ*olpA* mutant and the OlpA-negative *M. catarrhalis* Bc5, yielding strains RH4Δo*lpA*(pWW*olpA*) and Bc5(pWW*olpA*). The backbone plasmid pWW115 was used to generate RH4Δ*olpA*(pWW115) and Bc5(pWW115) as controls.

Flow cytometry analysis

Bacteria ($0.5x10^6$ CFU) were incubated at 37°C for 1 h with 10 µg/ml FH in incubation buffer (PBS, 1% bovine serum albumin). After two washing steps, bacteria were incubated with Flourescein 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 five healthy adult volunteers was pooled. Heat inactivated serum (HIS) was manufactured by treatment at 56°C for 30 min. 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-EGTA was included, whereas 10 mM EDTA was used for complete complement inactivation. The serum bactericidal assay was as described [4].

Fluorescence microscopy

Bacteria were incubated with FH (10 µg/ml) for 1 h 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

A two-way ANOVA test (GraphPad Prism®) was used for the bactericidal serum resistance assays and Mann-Whitney test for the flow cytometry results.

RESULTS

We have previously shown that *M. catarrhalis* interacts with both the classical and alternative pathway 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, nine out of ten *M. catarrhalis* strains bound FH, although to a different level. The high-binding strain RH4 and the non-binding strain Bc5 were chosen for further analysis.

To identify the FH-binding proteins of *M. catarrhalis* RH4, outer membrane vesicles (OMV) were separated by 2D-gel electrophoresis followed by far Western blot using human complement FH and a monoclonal anti-FH antibody. Two putative FH-binding proteins of approximately 24 kDa and 20 kDa were identified (Figure 1B). Subsequent MALDI-TOF mass spectrometry analysis 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; lower left histogram). In contrast, deletion of OMP J did not decrease the FH binding, *i.e.*, the RH4Δ*ompJ* 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 Factor H 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 RH4Δ*olpA*(pWW*olpA*). 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, *i.e.*, the *M. catarrhalis* RH4 wild type and the complemented OlpA mutant (RH4Δ*olpA*(pWW*olpA*) survived equally well (Figure 2; white bars), whereas the OlpA mutant and the transcomplemented control (RH4Δ*olpA*(pWW115)) displayed a significantly decreased survival. Interestingly, selective blocking of the classical pathway using the Ca^{2+}-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 a decreased survival in FH-depleted serum, and when the serum was reconstituted with FH the survival was significantly increased (Figure 2B). In contrast, the

survival of the OlpA-deficient RH4 mutant was unaffected by addition of FH. Taken together, *M. catarrhalis* FH-binding OlpA significantly contributes to serum resistance.

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* using several independent methods including far Western blot, flow cytometry and fluorescence microscopy. Initial results with 2D-SDS-PAGE revealed two FH-binding proteins, OlpA and OMP J that both have been described earlier [11]. In further downstream experiments we confirmed a specific FH-binding to OlpA only.

OlpA is a conserved 24 kDa outer membrane protein that has a high similarity with the nesserial Opa adhesins [11]. The neisserial Opa proteins are known to bind both Carcinoembryonic Antigen-related Cell Adhesion Molecule (CEACAM) and heparin sulphate proteoglycan (HSPG) receptors. However, no similar interactions or other additional functions have been assigned to OlpA. Recently, Vries and colleagues presented a detailed molecular profile of the complement resistance factors of *M. catarrhalis* [12]. They studied the transcriptional adaptation and performed a genome-wide targeted sequencing using transposon mutagenesis (Tn-seq) in the presence of human serum, and demonstrated an upregulation of 84 genes and a downregulation of 134 genes. The Usp-proteins have for long been regarded as the major complement resistance factor which was also confirmed in that study. However, the *olpA* gene together with several other genes were also identified as required for *M. catarrhalis* complement resistance. One of the main findings in that study was the importance of the disulphide bond formation (DSB) system in the resistance against the

classical pathway. Taken together, *M. catarrhalis* have 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 used in our study are still very serum resistant (data not shown).

Bacterial complement resistance is a well described and intriguing phenomenon for most respiratory pathogens, and consequently is 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 three 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 opsonisation 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 SCR number 20 [15]. Most interestingly, when FH is bound to bacterial proteins an enhanced FH-dependent interaction with C3b occurs, and thus FH mediates an 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 paper 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 OlpA also contributes to the serum resistance further increasing *Moraxella* pathogenesis.

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LEGENDS

Figure 1. *M. catarrhalis* binds FH and OlpA is the main FH-binding protein. A) Most *M. catarrhalis* strains bind FH as demonstrated by a far Western blotting. B) Identification of two FH-binding outer membrane proteins of approximately 24 kDa and 20 kDa were identified using in a 2D-SDS-PAGE. In A), B) and D), whole cell bacterial lysates or outer membrane vesicles of *M. catarrhalis* were separated by SDS-PAGE or 2D-SDS-PAGE and either stained by Coomassie blue or transferred to membranes followed by incubation with human complement FH and an anti-FH monoclonal antibody. C and F) *M. catarrhalis* strains were incubated with FH followed by FITC-conjugated anti-FH antibody and flow cytometry analysis. The negative controls (background) represent bacteria incubated with FITC-conjugated antibody only. E) Bacteria were incubated with FH (10 μg/ml) followed by a FITC-conjugated anti-FH antibody, and thereafter bacteria were fixed on glass slides. Representative experiments are shown. In F) the mean value out of three is presented and error bars represent standard deviations. ** $P<0.01$.

Figure 2. A) OlpA expression is important for the serum resistance of *M. catarrhalis*. B) Recruitment of FH by *M. catarrhalis* wild type contributes to the survival in human serum. Bacteria were incubated with 10 % of the indicated sera for 30 min at 37°C. Aliquots were plated out at time 0 (t=0) and after 30 min (t=30). The number of bacteria (CFU) at t=0 was defined as 100 % and the percentage relative to the initial CFU was calculated. Heat-inactivated 10 % NHS (HIS) and EDTA-treated serum was used as negative controls. The data represent the mean of three independent experiments, error bars indicate standard deviations. *** $P<0.001$.

Figure 1, Bernhard *et al.*

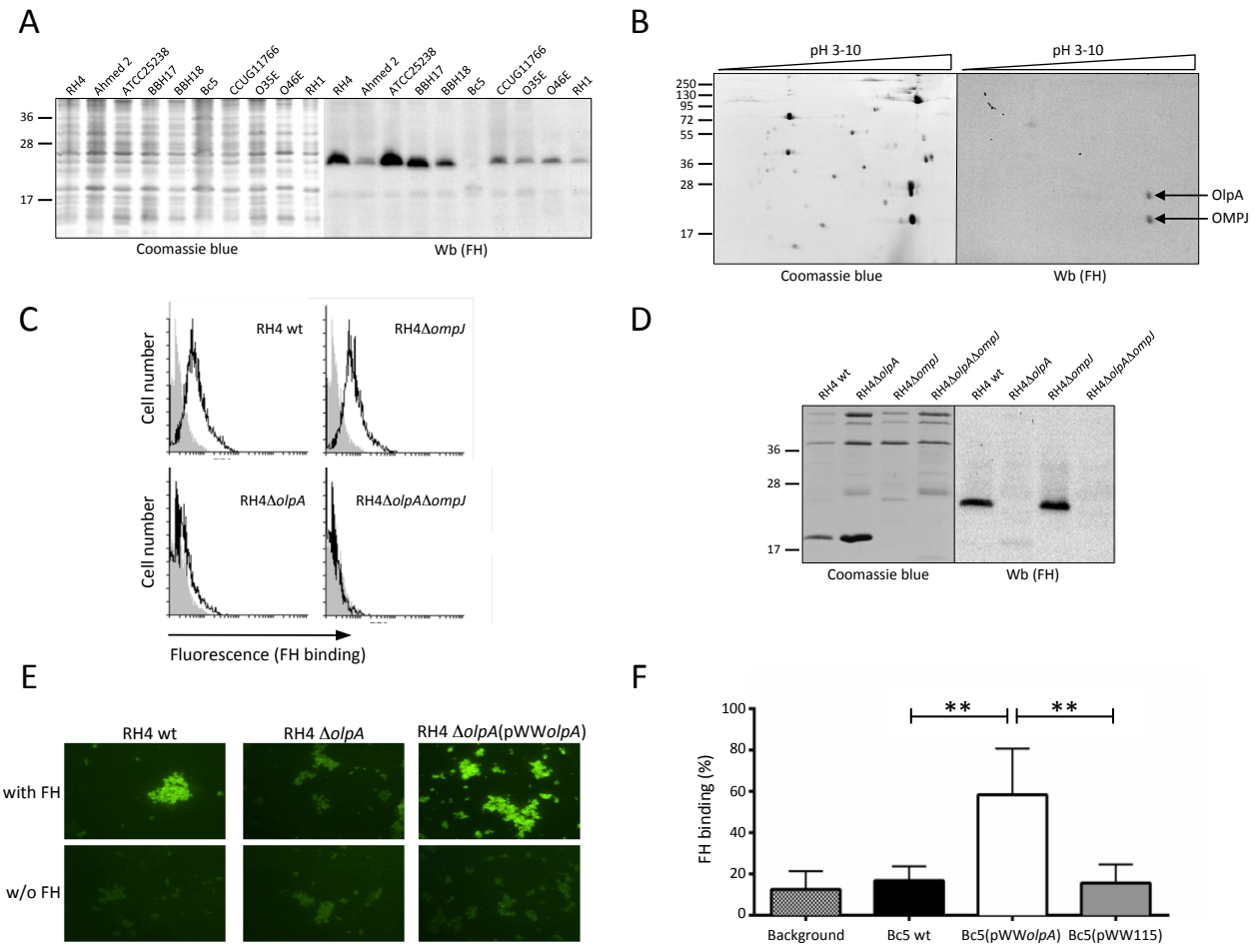
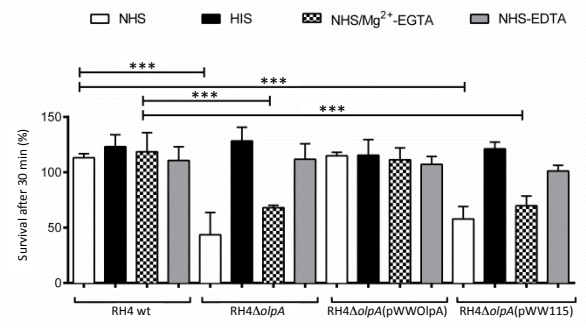


Figure 2*, Bernhard et al.*

A



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