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Moraxella catarrhalis binds plasminogen to evade host innate immunity

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Abbreviations used in this article: \(\varepsilon\)-ACA, \(\varepsilon\)-aminocaproic acid; COPD, chronic obstructive pulmonary disease; C3b, third component of the complement; C5, fifth component of the complement; DGVB, dextrose gelatin veronal buffer; ECM, extracellular matrix; Fn, fibronectin; Ln, laminin; MAC, membrane attack complex, NHS, normal human serum; pAb, polyclonal antibodies; uPA, urokinase plasminogen activator; UspA2, Ubiquitous surface protein A2; UspA2H, Ubiquitous surface protein A2 hybrid; Vn, vitronectin; YfeA, Yersinia periplasmic iron binding protein.
Abstract

Several bacterial species recruit the complement regulators C4b binding protein, Factor H and vitronectin resulting in resistance against the bactericidal activity of human serum. It has recently been demonstrated that bacteria also bind plasminogen, which is converted to plasmin that degrades C3b and C5. In this study, we found that a series of clinical isolates \((n=58)\) of the respiratory pathogen *M. catarrhalis*, which is commonly isolated from pre-school children and adults with chronic obstructive pulmonary disease (COPD), significantly binds human plasminogen. Ubiquitous surface protein (Usp) A2 and A2 hybrid (UspA2H) was identified as the plasminogen-binding factor in the outer membrane proteome of *Moraxella*. Furthermore, expression of a series of truncated recombinant UspA2 and UspA2H followed by a detailed analysis of protein-protein interactions suggested that the N-terminal head domains bound to the kringle domains of plasminogen. The binding affinity constant \((K_D)\) for UspA2\(^{30-539}\) and UspA2H\(^{50-720}\) to immobilized plasminogen was \(4.8 \times 10^{-8}\) M and \(3.13 \times 10^{-8}\) M, respectively, as measured by Biolayer interferometry. Plasminogen bound to intact *M. catarrhalis* or to recombinant UspA2/A2H was readily accessible for urokinase plasminogen activator that converted the zymogen into active plasmin as verified by the specific substrate S-2251, and a degradation assay comprising fibrinogen. Importantly, plasmin bound at the bacterial surface also degraded C3b and C5 that consequently may contribute to a reduced bacterial killing. Our findings suggest that binding of plasminogen to *M. catarrhalis* may lead to increased virulence and hence more efficient colonization of the host.
Introduction

*Moraxella catarrhalis* is a Gram-negative human respiratory pathogen that is associated with acute otitis media in children and exacerbations in patients with chronic obstructive pulmonary disease (COPD) (1-3). It is most commonly found in a polymicrobial community with other pathogens such as *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae*. An efficient vaccine against *M. catarrhalis* has not yet been developed. However, several outer membrane proteins of *M. catarrhalis* have been shown to be immunogenic, and are thus suggested as potential components in a future subunit vaccine (4).

The complement system is the first line of immune defense and consists of more than 50 different proteins and effector components that clear invading pathogens through the formation of the membrane attack complex or by assisting macrophages in opsonophagocytosis. Complement activation consists of a cascade of reactions that are triggered by formation of antigen/ antibody complex (classical pathway), recognition of bacterial surface carbohydrates (lectin-mediated), or spontaneous activation (alternative pathway). Activation of either pathway leads to the cleavage of C3 into C3a and C3b, where C3b is deposited on the surface of the pathogen. Deposition of C3b activates the C5 convertase to cleave C5 into C5a and C5b. C5a and C3a induces a proinflammatory host cell response, whereas C5b initiates the formation of the membrane attack complex (MAC) (5, 6). *M. catarrhalis* clinical isolates are highly serum resistant and this is accomplished by mainly hijacking complement regulators in order to inhibit formation of the MAC (4). *M. catarrhalis* recruits vitronectin from serum and ultimately inhibits the assembly of the C5b-C7 complex and polymerization of C9 (7-10). In addition, C4b binding protein (C4BP) is utilized by *M. catarrhalis* for inhibition of the classical pathway of complement activation (11). *M. catarrhalis* binds factor H, one of the regulators of the
alternative pathway (12), and directly interacts with complement (13, 14), strategies that help to increase bacterial survival.

*M. catarrhalis* ubiquitous surface proteins (Usp) belong to a family of trimeric autotransporters that are found in several Gram negative species. This group of proteins are composed of a membrane anchor, stalk, neck and head domain, and thus appear like “lollipops” on the bacterial outer membrane (4, 15, 16). Ubiquitous surface proteins are generally categorized into three distinct subgroups; UspA1 is present in all clinical isolates, whereas UspA2 and UspA2H are found in 75% and 25% of strains, respectively. UspA1 and UspA2 both function as adhesins and interact with extracellular matrix (ECM) proteins such as laminin and fibronectin (17, 18). In addition, UspA1 and UspA2 neutralize C3 and C3d, and attract C4BP and vitronectin to protect *M. catarrhalis* from the bactericidal activity of serum (8, 11).

Plasminogen is a 92-kDa single chain glycoprotein that circulates in blood as a zymogen. The conversion of plasminogen into plasmin (active protease) is catalyzed by tissue type urokinase plasminogen activator (tPA) or urokinase plasminogen activator (uPA) (19). The basic function of plasmin is to degrade fibrin (fibrinolysis), which is involved in homeostatic processes such as blood coagulation, cell migration, wound repair, and remodeling of the ECM. Excess activity of plasmin is regulated by alfa-2 antiplasmin and alfa-2 macroglobulin (20). Plasminogen was recently also reported as a regulator of the complement system efficiently degrading complement factors including C3b and C5 (21). In addition, plasminogen activates host pro-collagenases and matrix metalloproteinases that subsequently degrade various components of the ECM. The protease activity mediated by plasminogen is occasionally utilized by bacterial pathogens in order to promote virulence (20, 22).
In this study, we show that a series of *M. catarrhalis* clinical isolates had the capacity to attract plasminogen from human serum and use it for inactivation of complement components. Plasminogen-binding at the bacterial surface occurred via UspA2/A2H, and was easily activated by urokinase plasminogen activator. The interaction of UspA2/2H with plasminogen consisted of ionic interactions and the lysine-binding kringle domains of plasminogen. Active plasmin degraded complement components C3b and C5 that ultimately enhanced the survival of serum-susceptible *M. catarrhalis*. Taken together, our results thus shed light upon how *M. catarrhalis* acquires serum resistance through recruitment of plasminogen.

**Material and Methods**

*Bacterial strains and culture conditions*

*M. catarrhalis* RH4 and Bc5 and other *Moraxella* clinical isolates (8) were cultured on chocolate agar plates or in brain heart infusion (BHI) followed by incubation at 37°C in a humid atmosphere at 5% CO₂. Details of *M. catarrhalis* clinical isolates selected for this study have been described by Su *et al.* (8). *M. catarrhalis* uspA1, uspA2, mid single and multiple mutants were from our lab (11). *M. catarrhalis* mutants were grown in 1.5 µg ml⁻¹ chloramphenicol, 7 µg ml⁻¹ zeocin, or 20 µg ml⁻¹ kanamycin, respectively (10). Double and multiple mutants were grown in combinations of antibiotics. *E. coli* DH5α and *E. coli* BL21 (DE3) were cultured in Luria Bertani (LB) broth or on LB agar plates at 37°C. *E. coli* containing pET26b expression vectors with various truncated *uspA2/A2H* genes (8, 9) were grown in LB medium supplemented with 50 µg ml⁻¹ kanamycin.
Plasminogen direct binding assay

Plasminogen binding at the surface of bacteria was analyzed by a direct ligand binding assay. For this purpose, plasminogen was labeled with $^{125}$I by using Chloramine-T as described elsewhere (23). App. $10^7$ M. catarrhalis were blocked with PBS-2.5% BSA and added to microtiter plates. $[^{125}]$-plasminogen at increasing concentrations was added to bacteria and incubated for 1 h at 37°C. In inhibition experiments, cold ligands were added to samples prior to addition of $[^{125}]$-plasminogen. In the next step, bacteria were washed 3 times with PBS to remove unbound $[^{125}]$-plasminogen. Plates were harvested in a 96-well plate harvester (Tomtec, Hamden, CT), and counted in a liquid scintillation counter (Trilux, Microbeta 1450; Perkin Elmer, Waltham, MA).

Protein purification and ELISA

UspA2 and A2H full length proteins and fragments were expressed and purified as described (8, 17). An iron binding periplasmic protein (YfeA) from Yersinia pestis was used a negative control. Similar to UspA2 and UspA2H fragments this protein was also expressed in the pET26b(+) expression vector and purified by using Ni-NTA affinity chromatography. Purified UspA2, UspA2H (both at 50 nM) were coated on PolySorp microtiter plates (Nunc-Immuno, Roskilde, Denmark) in the presence of Tris-HCl, pH 9.0 for 15 h at 4°C. Plates were washed 3 times with PBS and blocked with PBS-2.5 % BSA for 1 h at RT. Plasminogen or kringle domains (24) at increasing concentrations was added to plates and incubated for 1 h at room temperature. In blocking assays, NaCl, εACA, laminin, fibronectin, and vitronectin were supplemented to the wells prior to addition of plasminogen. Unbound plasminogen was removed by washing with PBS containing 0.05 % Tween-20 (PBS-T). Thereafter, sheep anti-human
plasminogen pAb (AbD Serotec, Oxford, UK) (dilution 1:1000) in PBS containing 2.5% BSA (PBS-BSA) was added. After 1 h of incubation at RT, plates were washed 4 times with PBS-T and incubated with secondary horseradish peroxidase (HRP)-conjugated donkey anti-sheep pAb (dilution 1:1000; AbD Serotec) in PBS-BSA. Finally plates were washed 4 times with PBS-T and developed. Plates were read in a 96-well microtiter plate reader (Sunrise Tecan, Männedorf, Germany) at 450 nm.

Measurement of protein-protein interactions by Biolayer interferometry

The interaction between UspA2, UspA2H and plasminogen was analyzed using a Biolayer interferometry technique (Octet Red96; ForteBio, CA). Plasminogen was immobilized in an amine reactive (AR2G) sensor. Sensors were activated by submerging them into a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.05 M N-hydroxysulfosuccinimide (NHS) solutions followed by loading the sensors with plasminogen. The sensor thickness was achieved between 2-4 nm. Unoccupied groups on the sensor layer were quenched with 2 M ethanolamine. An empty sensor neutralized by ethanolamine and a plasminogen-bound sensor in PBS were considered as controls. UspA2 and UspA2H were dialyzed in PBS and 0.1-2.0 µM were used for analysis of the binding kinetics. Similar to UspA2 and UspA2H, we also included YfeA as a negative control. Data were collected by the Octet Data acquisition software and analysis of the data was performed by using the Data analysis 8.1 module.

Measurement of plasmin activity
Plasminogen bound to the bacterial surface or to recombinant UspA2/A2H were activated by using urokinase plasminogen activator (uPA). The activity of plasmin was measured by using the S-2251 chromogenic substrate (Chromogenix, Bedford, MA). Bacteria (10^7) or 100 nM UspA2/2H coated on microtiter plates were allowed to bind 5 µg plasminogen in the presence of 2.5% BSA in PBS. The unbound fraction was washed and samples were incubated in a plasmin activity assay buffer containing 32 mM Tris-HCl, pH 7.5 and 175 mM NaCl. Finally, 5U uPA was added to this solution and incubated at 37 °C. The absorbance of solutions was measured in a spectrophotometer at 405 nm for 30 min up to 6 h. Controls without uPA and plasminogen were prepared in parallel. We also measured the plasmin activity by using a fibrinogen degradation assay and Western blotting. Bacteria (10^7) or UspA2/2H bound to plasminogen were added to 2 µg fibrinogen and 5U uPA. Controls were also prepared without plasminogen and uPA. The degradation of fibrinogen (fibrin) was monitored by stopping the reaction at different time points (10-30 min) by adding SDS-loading dye. Samples were boiled at 95 °C for 10 min and supernatants were separated by 12% SDS-PAGE. Subsequently, the gels were blotted onto a PVDF membrane. Blots were blocked with 5% milk for 1 h at RT and incubated with primary sheep anti-human fibrinogen pAb (Sigma) diluted 1:1000 in 5% milk. After 1 h of incubation at RT, membranes were washed with PBS-T. Subsequently, the membranes were incubated with HRP-conjugated donkey anti-sheep/goat pAb (AbD Serotec) diluted 1:1000 in 5% milk. Finally membranes were washed in PBS-T and developed by using ECL western blotting kit (Pierce, IL).

_C3b and C5 degradation by plasmin_
Plasmin modulates the activity of the complement system by degrading complement factors C3b and C5 (21, 25). We analyzed degradation of C3b and C5 by plasminogen bound at the surface of *M. catarrhalis* or bound to UspA2/A2H by Western blotting. Plasminogen was added together with 5U uPA, 2 µg C3b or C5 to microtiter plates coated with bacteria (10⁷)/ or UspA2/2H followed by incubation at 37 °C. Reactions were terminated by addition of SDS-loading dye at different time intervals (1 to 20 h). Reaction mixtures were boiled for 10 min at 95 °C. Supernatants were separated on 10% or 12% SDS-PAGE and blotted to a PVDF membrane. One set of gels were stained with Coomassie R-250 stain in order to monitor the loading control. Blots were developed by using sheep anti-human C3b (CompTech, Tyler, TX), or sheep anti-human C5 mAbs (CompTech) primary antibodies, both diluted at 1:1000. Finally, the membranes were incubated with diluted (1:1000) HRP-conjugated donkey anti-sheep/goat pAb and blots were developed by using ECL western blotting kit (Pierce, IL).

**Serum resistance assay**

We also tested whether normal human serum (NHS) that had been pre-treated with UspA2/2H would have a decreased bactericidal activity. UspA2/2H was coated on Dynabeads (Novex, Life Technologies, Norway) and washed thoroughly with PBS. Protein-coated Dynabeads (10 µl) were incubated with 10 µg plasminogen for 1 h at 37 °C, and unbound plasminogen was thoroughly washed by PBS. Beads were resuspended in dextrose-GVB (DGVB⁺⁺) buffer, pH 7.3 containing 140 mM glucose, 0.1 % (w/v) gelatin, 1 mM MgCl₂, and 0.15 mM CaCl₂. Subsequently, 10 % normal human serum (NHS) together with 20 U uPA was added to this solution. The reaction mixture was incubated at 37 °C for 2 h. This NHS preparation was used
for the bactericidal activity assay. The negative control protein YfeA (*Yersinia* iron binding protein) was also coated on Dynabeads and processed in a similar way as the UspA2/A2H-coated beads. In parallel, control beads without any coating (beads only) were treated with 2.5% BSA and washed in PBS were also included.

**Results**

*UspA2 and A2H are the major plasminogen-binding proteins in Moraxella*

We observed that plasminogen binds to the surface of *M. catarrhalis*, and therefore wanted to identify the plasminogen binding protein(s) of this particular pathogen. An outer membrane protein (OMP) fraction of *M. catarrhalis* wild type (WT) and Δ*uspA1*, Δ*uspA2* mutants were analyzed by native PAGE and far-Western blotting with plasminogen as bait. The result clearly indicated UspA2 as a high molecular weight protein that bound plasminogen (Fig. 1A). The loading controls are shown in Figure S1A. *M. catarrhalis* Bc5 and isogenic mutants were analyzed for plasminogen binding by using a pull down assay. Bacteria were incubated with purified plasminogen or normal human serum. The unbound proteins were washed and total proteins were separated in SDS-PAGE (Fig. S1B-C) and blotted. The presence of plasminogen was analyzed by using an anti-plasminogen pAb. Interestingly, deletion of UspA2 from the bacterial surface resulted in a reduced binding of purified or serum-derived plasminogen (Fig. 1B). *M. catarrhalis* WT and isogenic mutants were also tested for [125I]-plasminogen by a direct binding assay. Deletion of *uspA2* from *M. catarrhalis* Bc5 caused a 35.6% decrease in [125I]-plasminogen-binding as can be seen in Fig. 1C. In contrast to the Δ*uspA2* mutants, no decreased plasminogen binding was observed with the Δ*uspA1* and Δ*mid* mutants. *Moraxella* IgD binding
protein (MID) (26) mutants were also included as control. The Δmid mutants of *M. catarrhalis* RH4 and Bc5 did not show any reduced plasminogen-binding.

One fourth of *M. catarrhalis* clinical isolates express UspA2H at their surface. Therefore, we selected *M. catarrhalis* RH4, which carries UspA2H, and its isogenic mutants to detect plasminogen-binding proteins by far Western blotting. In parallel to UspA2, the hybrid UspA2H was identified as a major plasminogen-binding protein in *M. catarrhalis* RH4 (Fig. 1D to F). Taken together, our direct binding assays and Western blots strongly suggested that *M. catarrhalis* recruits plasminogen at its surface by using the trimeric autotransporters UspA2 or UspA2H.

**Moraxella catarrhalis clinical isolates bind plasminogen at their surface**

To prove that plasminogen binding is a common characteristic for all *M. catarrhalis* isolates, we analysed a series of *Moraxella* by using the direct binding assay. *M. catarrhalis* (*n*=58) were incubated with [125I]-plasminogen and bound radioactivity was measured. All *M. catarrhalis* tested bound plasminogen, but the binding capacity varied between isolates. The majority (=50 %) were high-binding isolates, that is, ≥10% of added plasminogen bound to bacteria. Of note, 6 clinical isolates displayed an extraordinary high plasminogen-binding capacity (Fig. 2A). Since both UspA2 and UspA2H were involved in interactions with plasminogen, *M. catarrhalis* Bc5 and RH4 were selected for further detailed analysis as representatives for UspA2 and UspA2H, respectively. A dose-dependent binding of [125I]-plasminogen was seen with both *M. catarrhalis* RH4 and Bc5 (Fig. 2B and C). The binding of [125I]-plasminogen to bacteria was not completely saturable. The reason for this could be due to other low affinity plasminogen receptors at the surface of *M. catarrhalis*. Multiple bacteria-host protein interactions are often observed (9, 27).
The N-terminal head domains of UspA2 and UspA2H bind plasminogen

UspA2 and UspA2H are trimeric autotransporters composed of head, neck, stalk and hydrophobic membrane anchoring domains (14). We mapped the plasminogen-binding region of UspA2 and UspA2H by using truncated proteins that were recombinantly expressed in E. coli. As shown in Fig. 3A, M. catarrhalis Bc5 full length UspA230-539 bound plasminogen in a dose-dependent manner as tested in ELISA. To pin-point the plasminogen-binding region of UspA2, a series of truncated recombinant proteins were used (8, 9, 14). Our results revealed that all UspA2 fragments, except for UspA2165-318 and UspA2302-458, significantly bound plasminogen in comparison with the negative control protein YfeA. However, UspA230-539, UspA230-177 and UspA2101-318 had the highest binding when compared to the other truncated fragments (Fig. 3B). Similarly to UspA230-539, UspA2H50-720 also bound plasminogen in a concentration-dependent manner (Fig. 3C). The recombinant full length UspA2H50-720 and the truncated UspA2H50-296 had the highest interaction with plasminogen in comparison to the other UspA2H fragments (Fig. 3D). These results indicated that the plasminogen-binding region was located at the N-terminal head domains of UspA2 (amino acids 100-177), and UspA2H (amino acids 50-296). The binding affinity constant (K_D) for UspA230-539 and UspA2H50-720 to immobilized plasminogen was 4.8x10^-8 M, and 3.13x10^-8 M, respectively, as measured by Biolayer interferometry (OctRed) (Fig. 3E and F).

We previously reported that M. catarrhalis UspA2 interacts with several ECM proteins including, vitronectin, fibronectin and laminin (9, 17, 18). The UspA2 N-terminal head domain binds to laminin and vitronectin, whereas the neck region of UspA2 binds to fibronectin. To analyse whether plasminogen binds to the same region as the ECM proteins, we did a
competitive inhibition experiment by ELISA. Full length UspA2\textsuperscript{30-539} was coated in microtiter plates, and specific binding of plasminogen was measured in the presence of fibronectin, vitronectin, laminin or UspA2\textsuperscript{30-539}, which all were added simultaneously. Laminin (2.5 nM) inhibited the binding of plasminogen to UspA2 with 59.3% (Fig. 4A), whereas addition of vitronectin (2.5 nM) inhibited plasminogen binding by 34.5%. In contrast, fibronectin (2.5 nM) only inhibited the binding of plasminogen to UspA2 by 19.9%, and there was no further significant reduction up to 100 nM fibronectin. In parallel with the data obtained with UspA2, similar results were found with UspA2H (Fig. 4B). YfeA did not show any decrease of plasminogen-binding to UspA2 or UspA2H. Our results thus clearly indicated that plasminogen shares the vitronectin- and laminin-binding regions on UspA2 and UspA2H as schematically outlined in Fig. 3B and D (upper parts). In contrast, fibronectin has distinct binding regions, that is, the neck and stalk of UspA2, which partially overlap and hence did not significantly compete with plasminogen.

**The interaction of Moraxella catarrhalis with plasminogen depends on lysine-binding residues and ionic strength**

Plasminogen consists of five kringle domains (K1-K5) that have affinity for lysine or lysine analogues. We have recently shown that lysine-binding residues of plasminogen are important for the interaction with protein E of *H. influenzae* (27). In addition, we noticed that ionic forces are involved in the interaction since binding of plasminogen to protein E was dependent on NaCl. To analyse whether UspA2 and A2H bind to plasminogen in a similar way as protein E, *M. catarrhalis* was incubated with $[^{125}\text{I}]$-plasminogen in the presence of the lysine analogue εAmino caproic acid (εACA). As shown in Fig. 5A, addition of εACA at 0.05 μM resulted in a
12.9% decrease in plasminogen-binding to *M. catarrhalis* RH4, whereas εACA at the same concentration significantly decreased binding (59.9%) to *M. catarrhalis* Be5. At 1 mM εACA, a 54.9% and 79.5% reduction in plasminogen-binding was observed to *M. catarrhalis* RH4 and Be5, respectively (Fig. 5A). Finally, addition of NaCl also resulted in an inhibited [125I]-plasminogen binding to both *M. catarrhalis* strains (Fig. 5B).

To further study the protein-protein interactions, we included recombinant UspA2H\textsuperscript{50-720} and UspA2H\textsuperscript{50-296} (both at 50 nM). Plasminogen (20 nM) was added in the presence of εACA at increasing concentrations. εACA (0.1 mM) reduced the binding of plasminogen to UspA2 and UspA2H (Fig. 5C). In parallel with whole bacteria, addition of NaCl also inhibited plasminogen-binding to both recombinant UspA2 and UspA2H (Fig. 5D).

Finally we used a set of recombinant plasminogen constructs encompassing different combinations of Kringle domains (Fig. 5E). The plasminogen K1-K5 domains interacted with UspA2 and UspA2H in a similar way as the commercial full length plasminogen (Fig. 5F). When K1 and K2 were deleted an almost completely reduced binding to UspA2 and UspA2H was observed. In conclusion, our results indicate that ionic interactions are important for plasminogen-binding to *M. catarrhalis* UspA2 and A2H, and also that the lysine-binding residues of plasminogen K1-K2 are crucial for this interaction.

*Plasminogen bound at the surface of bacteria is activated to plasmin*

Conversion of plasminogen into plasmin is regulated by tissue type plasminogen activator (tPA) or urokinase plasminogen activator (uPA). To analyse whether plasminogen can be converted into plasmin at the surface of *M. catarrhalis*, plasmin activity was measured by using the chromogenic substrate S-2251 that is cleaved by plasmin. Interestingly, most of the surface-
bound plasminogen was converted into plasmin within 3 h in the presence of uPA (Fig. 6A). We also tested activation of plasminogen bound to recombinant UspA2 and A2H (Fig. 6B). Here, plasmin activity was detected in the presence of UspA2 and A2H, whereas the negative control (recombinant YfeA) that did not bind plasminogen was without any proteolytic (plasmin) activity.

We also measured plasmin activity by including human fibrinogen, which is a natural substrate and has a high sensitivity for digestion by plasmin. *M. catarrhalis* Bc5 and RH4 were incubated with plasminogen followed by addition of fibrinogen together with uPA. The reaction was stopped at various time points, and the total protein content was analyzed by SDS-PAGE and Western blotting. One set of the gels were stained with Coomassie R-250 to visualize uniform loading of proteins (Fig. S2). The fibrinogen α-chain has an approximate molecular weight of 63.5 kDa, the β-chain 56 kDa, and the γ-chain 47 kDa. Plasmin predominantly digests the α and β chains in comparison to γ-chain. Therefore, in blots, degradation of α and β chains are shown. Importantly, *M. catarrhalis* RH4 bound high concentrations of plasminogen that degraded fibrinogen very efficiently (Fig. 6C), and intact fibrinogen disappeared after 20 min incubation. In contrast, *M. catarrhalis* Bc5, which bound less plasminogen (Fig. 2), caused a slower degradation of fibrinogen. Controls without bacteria were run into parallel and proved that the experimental set up was functional (Fig. 6D). Fibrinogen degradation was also analyzed when plasminogen was bound to purified recombinant UspA2 or A2H (Fig. 6E). A more efficient degradation of fibrinogen was observed in reactions with plasminogen and UspA2H in comparison to UspA2. Appropriate controls in the presence or absence of plasminogen are shown in Figure 6F. Taken together, our results directly revealed that plasminogen bound to the
bacterial surface via UspA2/2H is fully accessible to uPA, and hence can be activated to functional plasmin.

**C3b and C5 are degraded by plasmin bound at the bacterial surface**

C3 is an important component of the alternative complement pathway, and is cleaved into C3b and further on to C3a after activation. Plasminogen interacts with C3b and inactivates it by cleavage (21, 25). To analyze the degradation capacity of C3b when plasmin was bound to the surface of *M. catarrhalis*, plasminogen bound at the bacterial surface was activated with uPA followed by addition of pure C3b. Degradation of C3b was measured at different time points (2 to 20 h) by separation on SDS-PAGE followed by Western blotting. One set of gels were stained with Coomassie blue, in order to verify equivalent protein concentrations loaded (Fig. S3A). C3b constitutes of α’ and β chains (101 and 75 kDa, respectively) connected with disulfide bonds. Degradation of C3b chains by plasmin results in small molecular weight products of the α’ chain ranging from 30-68 kDa (21). In our hands, C3b degradation products appeared within 2 h, and the intensity of degraded C3b fragments increased with further incubation (Fig. 7A). RH4 has a higher plasminogen-binding capacity and hence showed a more efficient C3b degradation in comparison to Bc5 (Fig. 7A). Plasminogen bound to recombinant proteins UspA2\textsuperscript{30-539} and UspA2H\textsuperscript{50-720} in a microtiter plate also digested C3b after activated into plasmin (Fig. S4A).

We also tested plasmin-mediated C5 degradation related to plasminogen bound at the *M. catarrhalis* surface. Plasminogen bound to both *M. catarrhalis* RH4 and Bc5 degraded C5 within 2 h of incubation and small molecular products ranging from 65-25 kDa appeared. As expected, *M. catarrhalis* RH4 expressing UspA2H degraded the C5 α chain more intensely when compared to *M. catarrhalis* Bc5 (Fig. 7C). One set of gels was stained with Coomassie blue in
order to verify the same concentration of protein loaded (Fig. S3B). In addition, plasmin bound to recombinant UspA2 or A2H also degraded C5 (Fig. S4B). Taken together, plasminogen bound at the surface of *M. catarrhalis* is activated into plasmin and degrades components of the innate immune system.

**UspA2/A2H-dependent plasminogen-binding protects *M. catarrhalis* from the bactericidal activity of human serum**

Our experiments suggested that C3b and C5 were efficiently degraded by plasmin derived from plasminogen at the *M. catarrhalis* surface. We therefore tested whether bound plasminogen protected *Moraxella* from the bactericidal activity of serum. Recombinant UspA2, UspA2H or YfeA were coated on Dynabeads followed by addition of plasminogen. Thereafter, we supplemented beads to normal human serum (NHS) together with uPA and incubated for 2 h at 37°C. *M. catarrhalis* RH4 and Bc5 are serum-resistant, whereas UspA2- and A2H-deficient mutants are highly susceptible to the bactericidal effect of NHS (9). NHS pre-incubated with beads coated with the various proteins was tested for bactericidal activity against *M. catarrhalis* Bc5 Δ*uspA2* and RH4 Δ*uspA2H*, and two other serum-sensitive *M. catarrhalis* strains (KR509 and KR539). Importantly, beads with immobilized UspA2H/ plasminogen neutralized the bactericidal effect of NHS and rescued 20 to 100% of the bacteria in comparison to NHS or serum treated with the YfeA-coated control beads (Fig. 8). In parallel, UspA2/ plasminogen-coated beads protected the serum-sensitive *M. catarrhalis* 35-100%. Samples consisting of UspA2/ plasminogen- or UspA2H/ plasminogen-coated beads without uPA showed only a minor protection of the serum-sensitive *M. catarrhalis* (Fig. 8). Beads treated with NHS only also
showed similar results as the YfeA-coated beads. This result supported our hypothesis that \textit{M. catarrhalis} utilizes human plasminogen to survive lethal attacks from the complement system.

**Discussion**

The majority of \textit{M. catarrhalis} strains are considered to be non-invasive, and only in rare cases \textit{Moraxella} causes invasive disease. Importantly, \textit{M. catarrhalis} does not secrete any proteases or toxins that potentially would damage the epithelial cell layer and the extracellular matrix (ECM) for deep tissue penetration (4). Despite this fact \textit{M. catarrhalis} successfully adheres to and resides at the surface of the host epithelium, resulting in induction of a pro-inflammatory response. The bacterial species has a unique capacity to inactivate the acute phase reactant and protease inhibitor $\alpha_1$-antichymotrypsin, and hereby potentiate the effect of the protease chymotrypsin (28). This cunning strategy may induce excessive inflammation resulting in more exposed ECM that is beneficial for bacterial colonization. Another approach for microbes to degrade the ECM is to attract the zymogen plasminogen. It has been reported that when plasminogen is bound to pathogens it contributes to adherence to host tissues (29), degradation of ECM proteins resulting in increased invasion (30), and finally degradation of complement (25). Interestingly, our present results suggest that also \textit{M. catarrhalis} utilizes host plasminogen to escape from the innate host defense. It has previuosly been shown that plasminogen bound at the surface of the respiratory pathogen \textit{H. influenzae} degrades C3b (27). In parallel, \textit{Borrelia burgdorferi} recruits plasminogen and inactivates C3b as well as C5 to escape the innate immune system (25). Similar to those findings, we observed that \textit{M. catarrhalis} with plasmin at the bacterial surface also degrades C3b and C5 that most likely contributes to increased resistance against serum-mediated killing.
*M. catarrhalis* clinical isolates exhibit a high degree of the serum resistance (7-9). This serum resistance is dependent on the acquisition of host serum complement regulators such as vitronectin, factor H, C4BP, and complement components C3, C3d (9, 11-14). Clinical isolates of *M. catarrhalis* have variable serum resistance that is not possible to correlate with recruitment of a single complement inhibitor. Serum resistance is hence dependent on the recruitment of multiple complement regulators at the same time. For instance, one strain may acquire vitronectin substantially and less of the other factors, while another strain may acquire C4BP much more in comparison to vitronectin. In addition to the interaction of the well known complement inhibitors of *M. catarrhalis*, our study suggests that plasminogen can also be used as a tool to inactivate the host complement system.

UspA1 and UspA2/2H are major surface proteins of *M. catarrhalis* involved in interactions with fibronectin, vitronectin, laminin, C4BP, C3, C3d, and α1-antichymotrypsin (9, 11, 14, 17, 18, 28). In our hands, plasminogen-binding occurred particularly with UspA2 and UspA2H. The head domains of UspA2 and A2H bind plasminogen that overlaps with the vitronectin-, laminin-, and fibronectin-binding regions. Our inhibition experiments also suggested that plasminogen-binding to UspA2 and UspA2H is inhibited by laminin and vitronectin, whereas fibronectin has the least inhibitory effect (Fig. 4). Fibronectin binds below the head region of UspA2 (18), and therefore only partially blocks plasminogen-binding. Since the head regions of UspA2 and UspA2H bind multiple host proteins, binding to plasminogen might be conditional or dependent on the availability of putative ligands. When multiple ligands are present, as in the case when bacteria are incubated with NHS, UspA2- and UspA2H-mediated plasminogen binding to *M. catarrhalis* is still clearly observed (Fig. 1).
M. catarrhalis UspAs are also receptors for C3 and C3d. C3 binds to the stalk region of the UspA2 molecule, *i.e.*, within amino acids spanning 200-539. On the other hand, C3d is recognized within the stalk and neck region of UspA2 (amino acids 101-318). The target regions of C3 and C3d on UspA2 are thus separated from the plasminogen-binding site. Binding of C3 and C3d by UspA1 and A2 also results in increased serum resistance (13, 14). Since UspA2 attracts C3 or C3d, it may simultaneously result in degradation of C3b in case the bacterial protein acquires plasminogen in the close vicinity.

M. catarrhalis clinical isolates showed a variable plasminogen-binding capacity. This variability was not correlated to UspA2 or UspA2H. UspA2/A2H are diverse in their amino acid sequence and also in their size of the head region (8) that may further influence the plasminogen interaction. We have previously shown that UspA2 expression is variable in clinical isolates (14). Hence the expression levels of M. catarrhalis UspA2/A2H may also be a determining factor for plasminogen binding.

Respiratory pathogens including Streptococcus pneumoniae, Mycobacterium tuberculosis, Streptococcus pyogenes, Staphylococcus aureus, Neisseria meningitidis, H. influenzae, and Mycoplasma pneumoniae are known for hijacking plasminogen from the host (31). Bacterial plasminogen surface receptors are diverse in their structure and function. Some bacteria have multiple plasminogen-binding proteins at their surface, for example, *S. pneumoniae* that recruits plasminogen via choline-binding protein E (CBPE), PavB, PfbB, and PfbA. Moreover, *S. pyogenes* binds plasminogen via M and M-like proteins, and type I fimbriae. *S. aureus* attracts plasminogen to its surface by using immunoglobulin-binding protein (Sbi) and extracellular fibrinogen-binding protein (31). Similarly, *H. influenzae* protein E and aspartase also bind plasminogen (27, 32). While diversity is the most common feature among bacterial
plasminogen receptors, homologues of type I fimbriae, M-like proteins of *Streptococcus* spp., glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and enolase of multiple pathogens have the capacity to attract plasminogen (31). *M. catarrhalis* has previously not been tested for interactions with plasminogen. Our study shows that the trimeric autransporters UspA2 and UspA2H are major plasminogen-binding proteins. However, it cannot be excluded that other proteins with lower affinity for plasminogen might also be present at the surface of *M. catarrhalis*.

Plasminogen consists of five different homologous kringle domains (K1-K5). These kringle domains, except for K3 (31), have the capacity to bind free lysine or lysine-like compounds. The highest lysine binding affinity has been reported to K1 followed by the K4, K5, and K2 domains. Since lysine-like ligands, *e.g.*, εACA, and tranexamic acid, bind to kringle domains and mask them, these compounds have been used to verify bacterial interactions. We found that the binding of plasminogen to UspA2 and UspA2H can easily be inhibited by using εACA (Fig. 5). Moreover, the truncated fragments of plasminogen suggested that Kringles 1-2 are involved in binding to UspA2 and A2H (Fig. 5E and F).

In conclusion, we have shown that the multifunctional UspA2 and UspA2H recruit plasminogen to the surface of *M. catarrhalis*. The surface-bound plasminogen can be converted into highly active plasmin by host factors, and we postulate that this may contribute to bacterial virulence.
References


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**Legends**

**FIG 1** *M. catarrhalis* UspA2 and UspA2H are plasminogen-binding proteins. A. Whole cell proteins of *M. catarrhalis* Bc5 and mutants were separated in a native-PAGE and plasminogen-binding was analyzed by far-Western blotting. High molecular weight proteins (UspA2 and UspA2H) revealed binding to plasminogen that was absent in *M. catarrhalis* Bc5ΔuspA2 and ΔuspA1/A2 mutants. Control of loading of proteins is shown in Fig. S1A. B. Binding of pure plasminogen and plasminogen from serum (dilution 1:50) to *M. catarrhalis* Bc5 and isogenic mutants. The binding was detected by Western blotting using anti-plasminogen pAb. Similarly to the *M. catarrhalis* uspA2 mutant, the double (ΔuspA1/A2) and triple (ΔuspA1/A2/mid) mutants
had also a decreased plasminogen-binding. C. Comparative binding of $^{[125]}$I-plasminogen to *M. catarrhalis* Bc5 and corresponding isogenic mutants. D. Whole cell proteins of *M. catarrhalis* and mutants were separated in native PAGE. The high molecular weight UspA2H was detected as a plasminogen-binding protein by far-Western blotting. E. *M. catarrhalis* RH4 and corresponding Usp mutants were incubated with serum or purified plasminogen. F. Comparison of $^{[125]}$I-plasminogen binding to *M. catarrhalis* RH4 and isogenic mutants. Experiments in C and F were repeated three times in triplicates. Blots shown in A, B, D and E were repeated twice and one representative blot is shown. Statistical analysis for C and F were performed by one-way ANOVA, wild type was compared with each mutant. **, $p \leq 0.01$; ***, $p \leq 0.001$.

**FIG 2** *M. catarrhalis* clinical isolates interact with plasminogen. A. Direct binding assay showing binding of $^{[125]}$I-labeled plasminogen to *Moraxella* clinical isolates ($n=58$). B. Dose-dependent binding of $^{[125]}$I-plasminogen to *M. catarrhalis* Bc5 that expresses UspA2. C. Binding of $^{[125]}$I-plasminogen to the UspA2H-expressing *M. catarrhalis* RH4 is also dose-dependent. Mean values of triplicates from three independent experiments are presented and error bars indicate standard deviations. According to the plasminogen binding, isolates that bound $\geq 10\%$ of the added radioactivity ($\geq 6000$ cpm) were considered as high binding strains, and between 4000-6000 cpm medium binding strains, and $\leq 4000$ cpm were considered as low plasminogen binding *Moraxella*.

**FIG 3** The head regions of UspA2/A2H interact with plasminogen. A. Dose-dependent binding of plasminogen to UspA2$_{30-539}$ as revealed by ELISA. B. Schematic representation of constructs used for recombinant expression of UspA2 in *E. coli*. The right panel shows ELISA results
demonstrating binding of the plasminogen to UspA2 fragments. C. Dose-dependent binding of plasminogen to UspA2H<sup>50-720</sup> as revealed by ELISA. D. Schematic representation of constructs used for recombinant expression of UspA2H in <i>E. coli</i> and binding of those proteins to plasminogen. E-F. Binding affinities between plasminogen and UspA2, UspA2H were measured by Biolayer interferometry (Octet Red96). In B-D results represent means of triplicates of three independent experiments and error bars indicate standard deviations. Data shown in panel A and C were analyzed by a two-way ANOVA, and panels B and C by an one-way ANOVA. Plasminogen binding was significantly (<i>p</i> ≤ 0.001) higher in comparison to controls (panel A and C). *, <i>p</i> ≤ 0.05; **, <i>p</i> ≤ 0.01; ***, <i>p</i> ≤ 0.001.

**FIG 4** Plasminogen binds to the same regions of UspA2 and UspA2H as vitronectin and laminin. 

A. ELISA showing inhibition of plasminogen binding to UspA2<sup>30-539</sup> by increasing concentrations of vitronectin, laminin, and UspA2<sup>30-539</sup>. In contrast, fibronectin inhibited the interaction partially. B. ELISA showing inhibition of plasminogen-binding to UspA2H<sup>50-720</sup> in the presence of increasing concentrations of vitronectin, fibronectin, laminin, and UspA2H<sup>50-720</sup>. Statistical analyses were performed by a two-way ANOVA. Data shown are the means of triplicates of three independent experiments and error bars indicate standard deviations. All ligand concentrations between 2.5-20 nM showed statistical significant decrease in plasminogen binding to UspA2<sup>30-539</sup> and UspA2H<sup>50-720</sup> when compared with samples without ligands (0 nM). Fibronectin (2.5 nM) showed significant inhibition (<i>p</i>≤0.001) in comparison to the control. However, further addition of fibronectin did not reveal any significant decrease when compared with 2.5 nM fibronectin.
FIG 5 Plasminogen binds to *M. catarrhalis* via kringle domains and involves ionic interactions. 

A. $^{125}$I-plasminogen binding to *M. catarrhalis* strains was inhibited by addition of increasing concentrations of εACA. B. Increasing concentrations of NaCl inhibit the $^{125}$I-plasminogen interaction with *M. catarrhalis*. Binding was set as 100% at 150 mM NaCl, which represents the physiological conditions. C. εACA inhibits the plasminogen-UspA2/2H interaction as shown by ELISA. D. The binding of plasminogen to UspA2/A2H is inhibited by NaCl as demonstrated by ELISA. Mean values of three replicates from three independent experiments are shown, and error bars indicate standard deviations. E. Schematic outline of truncated plasminogen fragments used in this study. F. Binding of different plasminogen kringle domains to UspA2 and UspA2H as demonstrated by ELISA. Means of three independent experiments in triplicate were performed, error bars denote standard deviation. Statistical analyses were performed by a two-way ANOVA. Plasminogen, and K1-K5 samples were compared with other kringle domains. Plasminogen binding was significantly ($p \leq 0.001$) inhibited at 0.05-10 mM εACA (panel A and C) and at 0.25- 2.15 M NaCl (panel B and D).

FIG 6 Plasminogen bound at the bacterial surface can be converted into plasmin. A. Chromogenic assay that shows a time-dependent conversion of plasminogen to plasmin at the surface of *M. catarrhalis* strains. The positive control consisted of 1 µg plasminogen and 5U uPA. Negative controls without uPA or without plasminogen (uPA only) were included. B. Time-dependent conversion of plasminogen to plasmin when plasminogen was bound to UspA2 and UspA2H. The positive control consisted of 50 ng plasminogen and 2U uPA. Experiments were repeated twice with triplicate wells, and results from one typical experiment are shown here. C. Plasmin activity is retained at the bacterial surface as analyzed by fibrinogen
degradation. Bacteria \((10^7)\) with plasminogen bound at their surface were incubated with 2 µg fibrinogen and uPA. Samples were collected at different time points and analysed by Western blots. The fibrinogen \(\alpha\)-chain has an approximate molecular weight of 63.5 kDa, the \(\beta\)-chain 56 kDa, and the \(\gamma\)-chain 47 kDa. Plasmin degrades \(\alpha\) and \(\beta\) chains predominantly. Therefore, in blots the degradation of \(\alpha\) and \(\beta\) chains are shown. D. control reactions performed in absence of bacteria. E. Plasmin activity is retained when it is bound to recombinant UspA2 or UspA2H. Recombinant proteins were coated in a microtiter plate, and plasminogen was added. In the next step, uPA and fibrinogen were supplemented and degradation was monitored by Western blotting. F. Control reactions performed in absence of UspA2 and UspA2H. The blots shown in C-F were repeated twice and one set of blots is shown here. Plg, plasminogen; Fgn, fibrinogen.

FIG 7 Plasminogen bound to the surface of \textit{M. catarrhalis} cleaves the complement protein C3b and C5. A. \textit{M. catarrhalis} Bc5 and RH4 loaded with plasminogen degrade C3b. Bacteria \((10^7)\) bound to plasminogen were incubated with 5 µg C3b and 5U uPA 37 ºC. At the indicated time points, the cleavage of C3b was analyzed by Western blotting. When plasminogen is activated into plasmin it cleaves the \(\alpha´\) chain of C3b in to \(\alpha´68, \alpha´40, \alpha´36, \alpha´30\) molecular weight products as illustrated with arrows. B. Degradation of C5 by plasmin bound to \textit{M. catarrhalis} surface. Similar settings as in experiment in panel A was used, except for that C3b was replaced with C5. C5 is composed of a 115 kDa \(\alpha\) chain and 75 kDa \(\beta\) chain. The \(\alpha\) chain is degraded by plasmin to produce 25-65 kDa \(\alpha´\) fragments as illustrated. The degradation was monitored at different time points by using Western blot. Positive controls consisted of 1 µg plasminogen and negative controls contained only uPA or only plasminogen. All experiments were repeated twice, and one representative blot is shown. The loading controls are shown in figure S3.
FIG 8 Plasminogen bound to UspA2 or UspA2H impairs the serum bactericidal activity. Recombinant UspA2, UspA2H, or YfeA were immobilized on Dynabeads and incubated with plasminogen. NHS (10 %) was added to beads, and supplemented with uPA (20 U) followed by incubation for 2 h at 37 °C. The serum was tested for bactericidal activity against serum sensitive *M. catarrhalis* Bc5ΔuspA2, RH4ΔuspA2H, KR509, and KR539. Beads were also coated with the control protein YfeA that does not bind plasminogen. Bacterial survival in the absence of serum was set as 100 %. In parallel, UspA2, UspA2H, or YfeA immobilized on Dynabeads and incubated with NHS in the absence of uPA were included as controls. Dynabeads were treated with BSA (2.5% BSA in PBS and washed with PBS) and then added to NHS. The results shown are means of three independent experiments in triplicate samples and bars denote standard error of the means. Statistical analyses were performed by a two-way ANOVA. Statistical significance between NHS and other serum samples are shown. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
Figure 4

A

Binding of plasminogen to UspA2\(^{30-539}\) (% of control)

YfeA
Vitronectin
Laminin
Fibronectin
UspA2\(^{30-539}\)

Ligands [nM]

B

Binding of plasminogen to UspA2\(^{50-720}\) (% of control)

YfeA
Vitronectin
Laminin
Fibronectin
UspA2H\(^{50-720}\)

Ligand [nM]
Figure 7

A

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Bc5

RH4

Positive ctrl

α
β
α'
β

Figure 8

Survival (CFU, % of control)

Bc5ΔuspA2

RH4ΔuspA2H

KR509

KR539

uPA;
UspA2;
UspA2H;
YfeA;
Beads;

** p < 0.001
Fig. S1. SDS-PAGE gels stained with Coomassie blue showing loading controls for the blots presented in Figure 1. A. Gel showing separation of total proteins of *M. catarrhalis* WT and mutants. A similar gel was used to perform the far-Western blot (Fig.1A and D). B. *M. catarrhalis* and mutants were treated with 1 µg pure plasminogen. The unbound fraction was washed and total bacterial proteins separated. This is the loading control for Figure 1B and E (upper panels). C. *Moraxella catarrhalis* and mutants were treated with normal human serum (dilution 1:50). Unbound proteins were washed and total proteins separated. This is a loading control for Figure 1B and E (lower panels).
Fig. S2. SDS-PAGE gels stained with Coomassie blue showing loading controls for the blots presented in Figure 6. A. Gel showing separation of total proteins of *M. catarrhalis* RH4 and Bc5. A similar gel was used to perform a far-Western blot (Fig. 6C). B. A standard assay showing degradation of fibrinogen by plasminogen in presence or absence of uPA. This is the loading control for Figure 6D. The fibrinogen α-chain has an approximate molecular weight of 63.5 kDa, the β-chain 56 kDa, and the γ-chain 47 kDa. The degradation of the α and β chains is shown in the blots (Fig. 6C-F).
Fig. S3. SDS-PAGE gels stained with Coomassie blue showing loading controls for the blots presented in Figure 7. A. Gel showing separation of total proteins of *M. catarrhalis* RH4 and Bc5 for digestion of C3b. A similar gel was used to perform the Western blot (Fig. 7A). B. Positive control showing degradation of C3b by plasmin (Fig. 7A). This is the loading control for Figure 6D. C. Degradation of C5 by Bc5 and RH4 surface-bound plasmin. It is the loading control for Figure 7B. D. Standard assay showing degradation of C5 by plasmin. This is the control gel for Figure 7B.
**Fig S4, Singh et al.**

**A.** Recombinant UspA2\textsuperscript{30-539} and UspA2H\textsuperscript{50-720} were coated on microtiter plates and plasminogen (5 µg) was added. Unbound plasminogen was washed away and C3b along with uPA was added. The degradation of C3b was monitored by Western blotting. When plasminogen is activated into plasmin it cleaves the α' chain of C3b into α'68, α'40, α'36, α'30 molecular weight products as illustrated with arrows.

**B.** Degradation of C5 by plasmin bound to UspA2\textsuperscript{30-539} or UspA2H\textsuperscript{50-720}. Similar settings were used as in the experiment outlined in panel A, except for that C3b was replaced with C5. C5 is composed of a 115 kDa α chain and a 75 kDa β chain. The α chain is degraded by plasmin to produce 25-65 kDa α' fragments as illustrated. The degradation was monitored at different time points by using an anti-C5 pAb in a Western blot. Positive controls consisted of plasminogen (1 µg) and negative controls contained only uPA or plasminogen only.