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Review

MID and UspA1/A2 of the human respiratory pathogen *Moraxella catarrhalis*, and interactions with the human host as basis for vaccine development*

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***Moraxella catarrhalis* IgD-binding protein MID is a 200 kDa autotransporter protein that exists as a oligomer and is governed at the transcriptional level. The majority of *M. catarrhalis* clinical isolates expresses MID. Two functional domains have been attributed to MID; MID764-913 functions as an adhesin and promotes the bacteria to attach to epithelial cells, whereas the IgD-binding domain is located within MID962-1200. In parallel, MID is stimulatory for B lymphocytes through the IgD B cell receptor. *M. catarrhalis* ubiquitous surface proteins A1 and A2 (UspA1/A2) are multifunctional outer membrane proteins that can bind complement and extracellular matrix proteins such as vitronectin and fibronectin. An interaction between the complement fluid phase regulator of the classical pathway, C4b binding protein (C4BP), and UspA1/A2 has also been observed. Moreover, UspA1/A2 has a unique feature to interfere with the innate immune system of complement by binding C3. Taken together, a growing body of knowledge on *M. catarrhalis* outer membrane proteins MID and UspA1/A2 and their precise interactions with the human host make them promising vaccine candidates in a future multicomponent vaccin.**

Keywords: adhesion, bacteria, *Moraxella catarrhalis*, non-immune Ig binding, respiratory pathogen

INTRODUCTION

Moraxella catarrhalis is an uncapsulated Gram-negative diplococcus that can be detected in nasopharyngeal cultures in 66% of children during the first year of life and in approximately 4% of adults at any given time. Despite *M. catarrhalis* often is considered as a commensal, the bacterium plays an important role in respiratory tract infections in both children and adults (Catlin, 1990; Murphy, 1996; Karalus *et al.*, Verduin, 2002). More than 80% of children under the age of three years will be diagnosed with acute otitis media. After *Haemophilus influenzae* and pneumococci, *M. catarrhalis* is the third most common bacterial species causing acute otitis media. In adults and the elderly, *M. catarrhalis* is a common cause of lower respiratory tract infections particularly in those with predisposing conditions, e.g., chronic obstructive pulmonary disease (COPD). Moreover, *M. catarrhalis* is often implicated as a cause of sinusitis in both children and adults.

Recent years focus of research on *Moraxella* has been on the outer membrane proteins and their interactions with the human host (Bartos & Murphy, 1988; McMichael, 2000). Some of these outer membrane proteins appear to have adhesive functions including amongst others, *M. catarrhalis* IgD binding protein (MID, also designated Hag), protein CD, *M. catarrhalis* adherence protein (McaP) and the ubiquitous surface proteins (Usp) (Aebi *et al.* 1998; Pearson *et al.*, 2002; Forsgren *et al.*, 2003; Timpe *et al.*, 2003; Holm *et al.*, 2004). In this review, we compile

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Abbreviations: BCR, B cell receptor; COPD, chronic obstructive pulmonary disease; Hag, hemagglutinin; LOS; lipooligosaccharide; MEE, middle-ear effusion; MAC, major attack complex; MID, *Moraxella* IgD-binding protein; mIgD, membrane bound IgD; NHS, normal human serum; OMP, outer membrane protein; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; UspA1/A2, ubiquitous surface protein A1 and A2.

available data on *M. catarrhalis* and interactions with the immune system with focus on the IgD-binding and adhesive protein MID and the multifunctional UspA1/A2.

MORAXELLA CATARRHALIS IgD-BINDING PROTEIN (MID)

Immunoglobulin D (IgD) was isolated in 1965 from the serum of a myeloma patient (Rowe & Fahey, 1965) and can be found at scarce concentrations in serum (≈ 30 μg/ml) that is considerably lower as compared to IgG (12 mg/ml). One explanation for this is that IgD is highly susceptible to proteases and therefore has a short serum half-life of 2.8 days that is in contrast to 23 days for IgG (Griffiths & Gleich, 1972). IgD also plays a role as a part of the B cell receptor complex (van Boxel *et al.*, 1972). Mature non-stimulated B-lymphocytes co-express IgD and IgM, but even immature, tolerance-susceptible B-cells express IgM and varying levels of IgD. Crosslinking of IgD and/or IgM with either antigens or class-specific antibodies results in the activation of protein tyrosine kinases and phosphorylation of the same substrate proteins, although the kinetics and intensity of the phosphorylation were shown to be quite different for these two receptor molecules (Kim & Reth, 1995).

Almost three decades ago it was shown that *M. catarrhalis* is an effective human B-cell mitogen (Banck & Forsgren, 1978) and capable of inducing polyclonal antibody production (Forsgren & Grubb, 1980). Moreover, *M. catarrhalis* strongly binds to human soluble IgD in a non-immune fashion (Forsgren *et al.*, 1979). Therefore, it was postulated that *M. catarrhalis* most likely delivers the mitogenic signal of the B cell through surface bound IgD (Forsgren *et al.*, 1988). In order to localise the region on IgD responsible for the binding Fab and Fc fragments were produced and analysed for bacterial binding. The Fab fragments bound to *M. catarrhalis* to the same degree as intact IgD, whereas the Fc fragment displayed only a weak binding. The authors suggested that the C_H1 region of the IgD molecule is responsible for the binding to *M. catarrhalis*.

Moraxella IgD-binding protein (MID) was discovered by us in 2001 (Forsgren *et al.*, 2001). MID was extracted from an outer membrane protein (OMP) preparation using Empigen® and isolated on ion exchange chromatography and gel filtration. The purified protein product appeared as a 200 kDa protein, but also as a multimer complex when subjected to SDS/PAGE. MID was shown to bind myeloma serum IgDs, but not any of the other Ig subclasses. Totally MID (in different experiments) was shown to bind 7 out of 7 IgD myeloma sera or preparations and none out of 22 non-IgDs (IgG, IgA, IgM).

In order to find the *mid* gene, purified MID from the *M. catarrhalis* strain Bc5 was digested with the proteases trypsin and chymotrypsin. Resulting fragments were subjected to Edman degradation and based upon the amino-acid sequences, degenerate primers were constructed and used in a protocol with inverse PCR and standard molecular methods. The deduced amino-acid sequence consisted of 2,123 residues. The N-terminal amino-acid sequence showed the typical characteristics of a signal peptide with a potential cleavage site between amino acids 66 and 67. MID consists of several amino-acid repeats, an organization that can be found in an array of other various bacterial OMPs, e.g., the UspA family of *M. catarrhalis* (Aebi *et al.*, 1998). The KTRASS repeat is one of the largest sequences that is conserved in several *Moraxella* strains (Möllenkvist *et al.*, 2003). This repeat can also readily be detected using specific anti-KTRASS polyclonal antibodies (Forsgren *et al.*, 2003).

To detect MID on the protein level we developed a flow cytometry assay based on the IgD-binding. The majority (84%) of the clinical *M. catarrhalis* strains had MID-dependent IgD-binding activity (Möllenkvist *et al.*, 2003). The IgD-binding showed strain-specific patterns. Firstly, most strains consisted of a mixed population: some bacteria produced MID as indicated by a high intensity peak, whereas others did not, as shown by a low intensity peak. The relative proportions of MID-producing in relation to non-producing bacteria varied from strain to strain. Thus, 15 (16%) of the 91 strains belonged to the low MID-producing category (< 1% high intensity peak) and 34 (37%) of the 91 strains could be classed as high MID-producing strains (> 99 high intensity peak). The rest of the strains produced MID at various intermediate levels. No specific pattern in MID production was found that correlated with the clinical or geographical isolation site of the strains.

The number of MID molecules present per bacterium correlated with the degree of hemagglutination of the individual strains (unpublished observations). Fitzgerald had already described a similar phenomenon using TEM and a hemagglutination assay of *M. catarrhalis* (Fitzgerald *et al.*, 1999). However, an on-off regulatory mechanism corresponding to phase variation was found directing expression of protein MID (Möllenkvist *et al*, 2003). This could be coupled to a homopolymeric poly(G) box located at the 5′ end of the mid gene within the open reading frame (ORF). Sequence analysis of the poly(G) tract from 23 *M. catarrhalis* strains in combination with MID expression data led us to the conclusion that only a number of guanine residues that are a multiple of three allows for MID. A

poly(G) box within an ORF usually indicates translational regulation, whereas similar regulatory element upstream the ORF is a sign of transcriptional regulation (Lafontaine *et al.*, 2001). Different experiments performed suggested that the *mid* gene can either be regulated at the DNA level by *trans*-acting factors binding to the poly(G) tract or at the RNA level by regulation of the stability (half-life) of the MID mRNA.

MID is related to the autotransporter group of proteins (Henderson *et al*, 2004; Cotter *et al.*, 2005), the largest family of the Gram-negative OMPs including more than 700 proteins. These proteins do not require energy or accessory factors for secretion through the bacterial outer membrane, hence the name autotransporter. Autotransporters are both functionally and structurally highly heterogenous (Desvaux *et al.*, 2004; Henderson *et al.*, 2004). However, an important common structural feature is the translocator domain that comprises an even number of β-strands folded into a pore through which the passenger domain can be transported out to the bacterial surface. Bacterial OMPs that have been structurally determined contain between 8 and 22 β-strands and form monomeric or multimeric channels through the membrane (Schulz, 2000; 2002; 2003). However, multimeric autotransporters do exist and currently there are two different models explaining their structure (Veiga *et al.*, 2002; Cotter *et al.*, 2005). One model is based upon the study of the *Neisseria gonorrhoeae* IgA protease and consists of a minimum of six channels to assemble into a ring-like structure with a central cavity that conduits the export of the passenger domain. The other model proposed for multimeric autotransporters has only been applied to trimers so far, all of which belong to the oligomeric coiled-coil adhesin (oca) family (Hoiczyk *et al.*, 2000; Surana *et al.*, 2004) also termed trimeric autotransporters (Cotter *et al.*, 2005). Typical members of this family are the *Yersinia enterocolitica* YadA and the *Haemophilus influenzae* Hia (Cotter *et al.*, 2005). proteins, both with a very short translocator domain of approx. 70 aa that forms 4 β-strands (Hoiczyk *et al.*, 2000; Wollman *et al.*, 2006)). MID has sequence similarities with the subfamily of trimeric autotransporters, sharing sequence similarities with the OMP YadA and the Hia adhesin (Cotter *et al.*, 2005). This subgroup has also been referred to as the oligomeric coiled-coil adhesin (oca) family. In fact, the MID molecule contains a coiled coil structure within the adhesive fragment MID764-913 (Forsgren *et al.*, 2003). When native MID purified from wild type *Moraxella catarrhalis* was analysed by ultracentrifugation using sucrose gradients, MID migrated as a multimeric complex with a molecular mass above 800 kDa, suggesting that MID is a multimer (Nordström *et al.,* 2002). This was also supported by similar experiments using the IgD-binding fragment MID962-1200.

THE ACTIVE IgD-BINDING SITE IS LOCATED IN THE CENTRAL PART OF THE MID MOLECULE

To investigate which part of MID is involved in the IgD binding, several recombinant fragments covering the entire molecule were produced (Nordström *et al.,* 2002). The smallest fragment with essentially preserved IgD binding was located between the amino-acid residues 962–1200 and hence was designated MID962-1200 (Fig. 1). Thus, the IgD-binding capacity of MID is located within the passenger domain. However, the IgD binding domain is distinct from the domain with adhesive capacity, which is described in detail below (Forsgren *et al.*, 2003). In an attempt to pinpoint the IgD-binding domain further, MID962-1200 was systematically narrowed in the N- and C-terminal ends. However, the IgD-binding ability gradually decreased with the size of the molecule. As compared with Ig-binding regions derived from other Ig-binding proteins, MID962-1200 is relatively large (238 amino acids). For example, the Ig-binding domains of the streptococcal protein A and protein L from *P. magnus* are 58 and 72–76 amino-acid residues, respectively (Uhlen *et al.*, 1984; Kastern *et al.*, 1992; Sakurada *et al.*, 1994), whereas

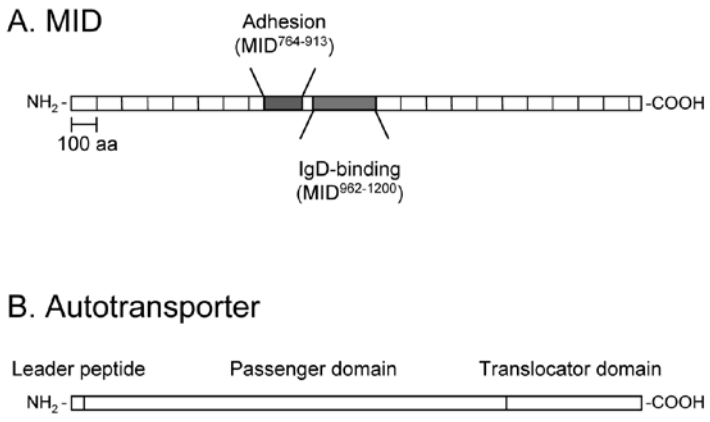

Figure 1. *Moraxella* IgD-binding protein is an autotransporter with functional domains in the central part of the molecule. A. The MID molecule with the adhesive and IgD-binding domains indicated between amino acids MID764-913, and MID962-1200 (Nordström *et al.*, 2002; Möllenkvist *et al.*, 2003; Forsgren *et al.*, 2003). B. Schematic drawing of an autotransporter protein.

the protein G:s binding site has been pinpointed to 11 amino acids (Frick *et al*., 1992). To further analyse the binding we utilised transposons randomly introducing five extra amino acids throughout the MID962-1200 sequence and tested whether the resulting clones retained their IgD-binding capacity using colony blotting. The IgD-binding capacity was shown to require the amino-acid sequences 982–1059 and 1100–1145. The amino acid residues 1060–1099 were not involved in the IgD binding. Because both the amino-acid sequences 982–1059 and 1100–1145 contained α-helix structures, these were suggested to be of importance to IgD binding.

The length of the IgD-binding region suggests that the interaction between MID962-1200 and IgD is most likely dependent on a definite conformational structure of MID962-1200. In addition to the secondary structure, we also determined the quaternary structure of MID962-1200 by native gels and ultracentrifugations. The results evidenced that MID962-1200 is formed as a oligomer at native conditions. Most interestingly, by comparing the monomeric and oligomeric form of MID962-1200, we could determine that the oligomer bound IgD 23-fold more efficiently. This implies that a stable oligomer formation is required to achieve preserved IgD binding. The flanking regions are most likely highly important for the formation of a stable oligomer, explaining why the binding decreases when the IgD-binding region is shortened. Moreover, intact α-helix structures located within the amino-acid sequences 982–1059 and 1100–1145 might also be required for the formation of the oligomer.

MORAXELLA CATARRHALIS AND MID ACTIVATE HUMAN B CELLS

M. catarrhalis has for long been known to be an effective human B-cell mitogen, i.e., the bacterium causes B cells to undergo cell division (Banck & Forsgren, 1978) and has been found to induce polyclonal antibody production (Forsgren *et al*., 1980). *M. catarrhalis* can directly stimulate B cells without any recognition of T cells and can thus be established to be a T-cell independent Ag (TI Ag) (Banck & Forsgren, 1978; Calvert & Calogeras, 1986). Huston and co-workers (Huston *et al*., 1996) reported that *M. catarrhalis* is able to stimulate purified B cells to produce IgM in the absence of exogenous cytokines. However, the IgM-production remarkably increases with the addition of interleukin-5 (IL-5) and/or IL-2.

To analyse whether MID is the only IgD-binding protein of *M. catarrhalis* we also used a MID-deficient *M. catarrhalis* mutant strain (Nordström *et al*, 2006). Using flow cytometry, we could determine that MID was the sole IgD-binding molecule of *M. catarrhalis*. We were also able to demonstrate that the mutated *M. catarrhalis* strains devoid of MID showed a 75% decreased activation of human B cells as compared with the wild type counterpart.

Native MID purified from *M. catarrhalis* specifically binds to human B cells (Forsgren *et al*., 2001; Gjörloff-Wingren *et al*., 2002) and purified native MID, or MID (range of 0.01–0.1 μg/ml) conjugated to cyanogen bromide (CNBr)-activated Sepharose (MID-Sepharose), induces a proliferative response in human PBL (Gjörloff-Wingren *et al*., 2002). For comparison, formalin-fixed *M. catarrhalis*-preparations induced similar proliferative responses in peripheral blood lymphocytes (PBL) cultures. The next question was whether MID could activate purified native, peripheral IgD^+ human B cells. PBLs were isolated and B cells were purified by either positive or negative selection. The isolated B cells were cultured for 3 days with MID or MID-Sepharose. The purified $CD19^+$ B cells responded to MID and MID-Sepharose, but not to the T cell mitogen phytohemagglutinin (PHA). Interestingly, MID-Sepharose together with IL-4 specifically induced a high level of IL-6 secretion from the B cells. Moreover, IgM-secretion was detected in B cell cultures stimulated with MID or MID-Sepharose and IL-2 for 10 days. Secretion of IgG and IgA, but not IgE, was efficiently induced in cultures from purified B cells stimulated with the combination of MID or MID-Sepharose and IL-4, IL-10 and soluble CD40L. These initial results suggested that T_H2-derived cytokines are required for optimal plasma cell generation in the presence of MID.

Our results derived from *in vitro* experiments (Forsgren *et al*., 2001; Gjörloff-Wingren *et al*., 2002) not only imply that B cells are the major cell type in peripheral blood that responds to MID, but also that MID is dependent on the IgD-binding to be able to function as a B cell stimulatory antigen. However, we found that isolated human peripheral B cells were more responsive to the purified MID protein in the presence of T cells or T cell derived molecules such as CD40L.

The IgD-binding domain of MID is located within amino acids MID962-1200 (as described above) and binds specifically to human B cells (Nordström *et al*., 2002). Since full length MID, when conjugated to Sepharose, strongly activated B cells (Gjörloff-Wingren *et al*., 2002), we also tested whether the smallest IgD-binding fragment, MID962-1200, was able to stimulate B cells. Human PBL containing 5–15% B cells were isolated and incubated with MID962-1200. To achieve an efficient cross-linking of the IgD BCRs, MID962-1200 was conjugated to Sepharose beads. The results showed that MID962-1200-Sepharose readily induces lymphocyte proliferation, with the strongest response observed at 96 h. The results also indicated that the optimal concen-

tration of MID962-1200-Sepharose to activate PBL was 0.5 μg/ml.

To analyse whether MID962-1200-Sepharose has the capacity to induce B cells to proliferate without physical T cells, pure B cells were isolated. MID962-1200-Sepharose was then incubated with B cells in the presence of a cell-free supernatant obtained from stimulated T cells containing various T-cell derived cytokines. A vigorous stimulation of the B cells was obtained with 0.5 or 1.0 μg/ml MID962-1200-Sepharose in the presence of the T-cell supernatant. In contrast, only a minor proliferation was observed with MID962-1200-Sepharose alone, whereas the T-cell supernatant did not induce any proliferation. Addition of IL-4 or IL-2 together with MID962-1200-Sepharose resulted in a strong proliferation, whereas no activation could be detected when the B cells were incubated with MID962-1200-Sepharose and IL-l0. The requirement for cytokines is in analogy with experiments with B cell activation and full length native MID (Gjörloff-Wingren *et al*., 2002).

MID FUNCTIONS AS AN ADHESIN

An interesting observation is that MID-producing *M. catarrhalis* strains agglutinate human erythrocytes and bind to type II alveolar epithelial cells (Forsgren *et al*., 2003). In contrast, *M. catarrhalis* isolates with low MID levels and two MID-deficient mutants, but with readily detectable UspA1, do not agglutinate erythrocytes and have a 50% lower adhesive capacity. In parallel to our findings and experiments with MID-deficient mutants, an isogenic *hag/mid* mutant was unable to agglutinate human erythrocytes and lost its ability to autoagglutinate (Pearson *et al*., 2002). The *hag* mutation also eliminated the ability of this mutant to bind human IgD. However, Pearson *et al*. (2002) found that the Hag-deficient mutant was still attached at wild-type levels to several human epithelial cell lines including Chang conjunctival epithelial cell line. In contrast, a mutant deficient in UspA1 had little or no ability to attach to the same cells. The discrepancy between our results and the results by Pearson *et al.* (2002) on the importance of MID/Hag for attachment might be explained by the different cell lines used. In fact, Holm and colleagues showed that the MID/Hag protein was required for *M. catarrhalis* adherence to A549 human lung and middle ear cells (Holm *et al*., 2003). These results were recently strengthened by cloning and expression of the *mid/hag* gene in *Escherichia coli* (Bullard *et al*., 2005). The recombinant *E. coli* producing MID/Hag attached to human middle ear cells 17-fold more efficiently than the control bacteria without MID/Hag. Thus, MID/Hag is an important adhesin for *M. catarrhalis*.

To examine the adhesive part of MID, the OMP was dissected into 9 fragments covering the entire molecule (Forsgren *et al*., 2003). The truncated MID proteins were produced in *E. coli*, purified and used for raising polyclonal antibodies in rabbits. Interestingly, by using recombinant fragments of MID extending over the whole 2139 amino-acid residues long molecule, we show that the hemagglutinating and adhesive part of MID is localized within the 150 amino-acid residues MID764-913. Polyclonal antibodies directed against full length MID1-2139, MID764-913, or the consensus sequence MID775-804 (that starts with KTRASS) significantly inhibited attachment of the high MID-producing isolates to alveolar epithelial cells. The similarity and identity of MID764-913 between 6 different MID sequences (Sasaki *et al*., 1998; Forsgren *et al*., 2001; Möllenkvist *et al*., 2003) were 96% and 97%, respectively. Thus, experiments with antibodies confirmed MID764-913 to be responsible for adhesion. Interestingly, in our study, antibodies directed to UspAl also inhibited adhesion to cells to the same degree as antibodies to MID. Since UspAl is produced by essentially all *M. catarrhalis* isolates, this observation suggested that a synergism existed between UspAl and MID, i.e., that both proteins were required for optimal adhesion. Thus, our experiments indicate that despite that the hemagglutinating determinant is located on MID, both outer membrane proteins MID and UspAl are likely to play a role in adhesion to epithelial cells.

To further analyze the importance of MID as an adhesin, *mid*-deficient *M. catarrhalis* were manufactured and examined in a mouse pulmonary clearance model (Forsgren *et al*., 2004). *M. catarrhalis* devoid of MID was cleared more efficiently as compared to the wild type counterparts suggesting that MID is important for bacterial adhesion and consequently survival also *in vivo*. Interestingly, animals immunized with MID764-913 cleared *M. catarrhalis* much more efficiently as compared to mice immunized with bovine serum albumin (BSA). Thus, experiments performed both *in vivo* (Forsgren *et al*., 2004) and *in vitro* (Forsgren *et al*., 2003) suggest MID764-913 as a promising candidate in a future *M. catarrhalis* vaccine.

BACTERIA AND THE COMPLEMENT SYSTEM

Pathogens have developed specific mechanisms to overcome the immune system. The pathogenesis of many microbes relies on the capacity of pathogens to avoid, resist or neutralise the host defence. Because the complement system is in the first line of defence against invading agents, complement evasion is very important to conquer for a pathogen.

The interference of the complement may be mediated through different strategies and at a variety of steps in the cascade. Pathogens can avoid being recognised by the complement, shedding the complement components from their surfaces or inhibit the activation of the complement (Wurzner, 1999; Hornef *et al.,* 2002). Moreover, some pathogens are able to destroy the complement component by degradation or consume it far from the surface of the microbe. Finally, a number of pathogens are able to use the complement components for their own benefit, whereas others have employed the ability to mimic the complement regulators.

The complement system is traditionally classified as a component of the serum, however, an increasing number of studies have shown that complement is present in various sites in the body. Knowledge about the occurrence of complement components in the respiratory tract of healthy individuals is scarce, though there are several reports about the conditions during infections. The complement components C3, C4 and factor B, however, have been detected in human saliva and a haemolytic assay indicated that these complement molecules in saliva were functionally active (Andoh *et al.*, 1997). It has been shown that during the inflammation process, exudation of plasma into the airway lumen occurs (Persson *et al.*, 1991; Greiff *et al.*, 1993). This plasma exudation brings, among others, complement proteins to the mucosal surface. Because the mucosal layer is the first the microbes encounter, the plasma exudation has been suggested as the first line of respiratory defence mechanisms. Furthermore, patients with COPD have increased concentrations of anaphylatoxins (C3a, C5a) in their sputum (Marc *et al.*, 2004), indicating an active complement response in the upper airways. However, several studies have shown that middle-ear effusions (MEEs) from patients with chronic secretory otitis media contain complement components. For example, Meri and colleagues (Meri *et al.,* 1984) have reported that MEEs from children had strongly elevated concentrations of C3 products, suggesting complement activation in the MEEs.

COMPLEMENT RESISTANCE OF *M. CATARRHALIS*

Complement resistance is considered a virulence factor of *M. catarrhalis*. An epidemiological study found that 62% of the *M. catarrhalis* isolates in sputum samples from patients with lower respiratory tract infections were serum resistant and 27% were determined as intermediate serum resistant (Hol *et al.,* 1995). A significant lower proportion of serum resistant and intermediate (33 and 8.5%, respectively) isolates were found in sputum samples obtained from healthy individuals. In another report it was shown that *M. catarrhalis* strains isolated from elderly patients with respiratory tract infections were predominantly more resistant to serum killing than those isolated from healthy individuals (Murphy *et al.*, 1997).

Experiments by Verduin and co-authors (1994) indicated that the serum resistant *M. catarrhalis* strains inhibited complement killing at the level of the major attack complex (MAC) formation. UspA2 is known to be involved in the serum resistance of *M. catarrhalis* (Aebi *et al.*, 1998). This was shown by comparing a UspA2 deficient *M. catarrhalis* mutant with its wild type counterpart in a human serum-killing assay. The *uspA2* mutant was nearly instantly killed in the serum, whereas the wild type producing UspA2 on its surface survived. Later, it was shown that UspA2 binds to the fluid-phase inhibitor vitronectin (McMichael *et al.*, 1998) and *M. catarrhalis* serum resistance was suggested to be due to this interaction. More recently, the biological significance for the moraxella-dependent vitronectin binding was shown (Attia *et al.,* 2006). However, most pathogens are killed by phagocytosis rather than *via* MAC lysis, and Onofrio *et al.* (1981) found a 400-fold increase in numbers of granulocytes in mice lung infected by *M. catarrhalis*. These findings in parts question the importance of *M. catarrhalis* vitronectin binding in that vitronectin inhibits the terminal pathway.

In addition, both the outer membrane proteins CopB and Omp E have been suggested to be involved in *M. catarrhalis* serum resistance (Helminen *et al.*, 1993; Murphy *et al.*, 2000). *M. catarrhalis* mutants devoid of either CopB or Omp E were shown to be sensitive to killing by normal human serum in two different assays. Another study has shown that *M. catarrhalis* LOS might be involved in the serum resistance of *M. catarrhalis* (Zaleski *et al.*, 2000). Loss of two hexose residues in the LOS resulted in decreased survival in NHS. Taken together, the serum resistance of *M. catarrhalis* is most likely a multifactorial phenomenon from the perspective of both the host and the pathogen.

THE UBIQUITOUS SURFACE PROTEIN (Usp) FAMILY

The Usp family of proteins is one of the most extensively characterised OMPs of *M. catarrhalis*. The proteins were originally believed to be one protein partly because they co-aggregate and thus often migrate as a high molecular complex in SDS/PAGE. In addition, the proteins are recognised by the same monoclonal antibody named 17C7. It is now known that the Usp family consists of at least three proteins,

i.e., UspA1, UspA2 and a hybrid protein designated UspA2H (Aebi *et al.*, 1997; Lafontaine *et al.*, 2000). In a series of 108 *M. catarrhalis* nasopharyngeal isolates from young children with otitis media the *uspA1* gene was detected in 107 (99%) of the isolates, whereas all 108 (100%) contained either the *uspA2* gene (79%) or the hybrid variant gene *uspA2H* (21%) (Meier *et al.*, 2002). The UspA1, UspA2 and UspA2H proteins have molecular masses of 88, 62 and 92 kDa, respectively (Aebi *et al.*, 1997; Lafontaine *et al.*, 2000). The formation of the high molecular mass complexes detected in SDS/PAGE might be due to coiled coils formed within the proteins. The amino-acid sequences of UspA1 and UspA2 are only 43% identical, but the proteins bear a common epitope of 140 amino-acid residues with 93% identity (Aebi *et al.*, 1997). The monoclonal antibody 17C7 recognises this common epitope and passive immunisation with the monoclonal 17C7 has been observed to enhance pulmonary clearance of *M. catarrhalis* in a mouse model system (Helminen *et al.*, 1994). The same study further demonstrated that antibodies against the UspA family were detected in patients with *M. catarrhalis* lower respiratory tract infections, indicating that *M. catarrhalis* expresses the Usp *in vivo*.

UspA1 and UspA2H have been shown to be involved in the adhesion of *M. catarrhalis* to Chang conjunctival epithelial cells and HEp-2 laryngeal epithelial cells (Aebi *et al.*, 1998; Lafontaine *et al.*, 2000). This involvement was demonstrated by using *M. catarrhalis* strains devoid of UspA1 and/or UspA2H. UspA1 has been reported to bind to carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1) as produced in the lung epithelial cell line A549 (Hill & Virji, 2003). Purified UspA1 has also been found to bind to the extracellular matrix protein fibronectin (McMichael *et al.*, 1998), and recently, the fibronectin binding of UspA1 was confirmed in our laboratory (Fig. 2) (Tan *et al.*, 2005). We showed that both UspA1 and UspA2 are determinants for *M. catarrhalis* binding to fibronectin, where the binding domains in the proteins were identified and found to share a 31 amino-acid residues sequence. Notably, recombinant fragments containing the binding domain partly inhibited *M. catarrhalis* adhesion to Chang epithelial cells. Thus, both UspA1 and UspA2 were involved in the adhesion of *M. catarrhalis*. UspA2 has also been shown to be crucial for *M. catarrhalis* serum resistance (Aebi *et al.*, 1998). *M. catarrhalis* devoid of UspA2 was instantly killed in human serum. One suggestion to account for this observation has been the ability of UspA2 to bind vitronection (McMichael *et al.*, 1998), which functions as an inhibitor of the terminal pathway of the complement system. Both UspA1 and UspA2 are well conserved (Klingman & Murphy, 1994) and are considered promising vaccine candidates. Studies of healthy individuals revealed that these had acquired antibodies directed against both UspA1 and UspA2 (Chen *et al.*, 1999), supporting the use in a future vaccine.

UspA1 AND UspA2 INTERFERE WITH THE CLASSICAL PATHWAY BY BINDING C4BP

Protection against *M. catarrhalis* infections seems to be primarily due to the production of antibodies. Children that have low levels of antibodies against *M. catarrhalis* tend to have more frequent and persistent infections (Takada *et al.*, 1998). One important property of these antibodies is their ability to elicit the classical pathway leading to complement-mediated killing or opsonisation of the pathogen.

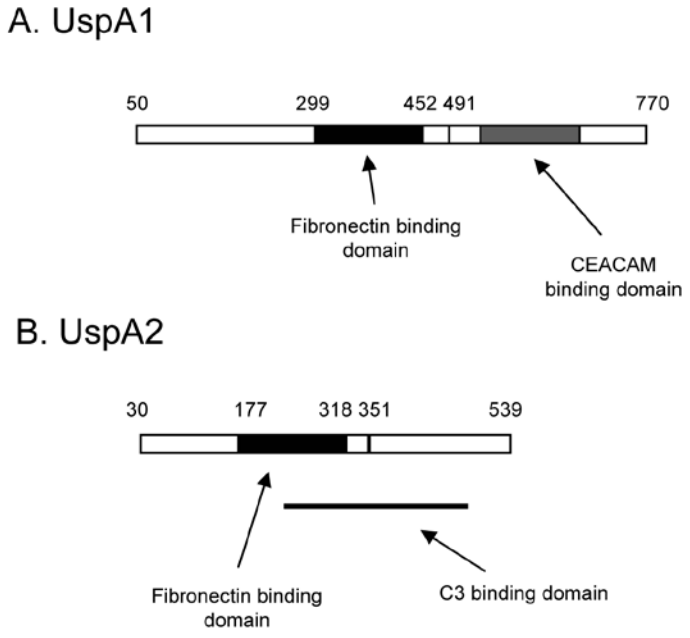

Figure 2. Schematic diagram with UspA1 and A2 with multiple binding sites for different ligands.
A. UspA1 binds fibronectin and CEACAM (Aebi *et al.*, 1998; Hill *et al.*, 2003; Tan *et al.*, 2005). B. UspA2 with binding sites for fibronectin and C3 (Nordström *et al.*, 2005; Tan *et al.*, 2005). C4BP binds to large parts of the molecules and is consequently not indicated in the figure (Nordström *et al.*, 2004).

However, even adults with production of antibodies and the possibility to activate the classical pathway can get infected by *M. catarrhalis* (Sethi & Murphy, 2001; Verduin *et al.*, 2002). Of interest is that the majority of the moraxella isolates from patients with lower respiratory tract infections have been shown to be complement resistant (Hol *et al.*, 1995). Because high concentrations of antibodies against *M. catarrhalis* can be found in adults (Black & Wilson, 1988; Mathers *et al.*, 1999) and because *M. catarrhalis* is serum resistant, we wanted to investigate whether *M. catarrhalis* has developed mechanisms to avoid a complement attack *via* the classical pathway.

Some pathogens have been shown to bind to the fluid-phase complement regulator C4BP and hence inhibit the classical pathway (Kraiczy & Wurzner, 2006). Therefore, *M. catarrhalis* was analysed for binding of C4BP using flow cytometry. Interestingly, *M. catarrhalis* strain BBH18 had a very high binding capacity to C4BP. This was not only a feature for BBH18 but all *M. catarrhalis* strains analysed were able to bind C4BP (not shown).

The outer membrane protein UspA2 of *M. catarrhalis* has been shown to be involved in serum resistance (Aebi *et al.*, 1998). Therefore, we wanted to investigate whether UspA2 was responsible for the C4BP binding. To analyse this possibility we constructed Usp-deficient mutants of two *M. catarrhalis* strains. The *M. catarrhalis* wild type was resistant to the bactericidal activity of normal human serum (NHS), whereas the UspA2-deficient mutants were instantly killed when exposed to NHS. The UspA1-deficient mutant was determined to be partially resistant to the bactericidal activity of NHS. Thus, our results were consistent with those obtained by Aebi *et al.* (1998), i.e., UspA2 surface production is crucial for survival in NHS. An intriguing finding was that UspA2 was the major binder of C4BP revealed by the analyses of the Usp-deficient mutants in flow cytometry and radioimmunoassay (RIA). To confirm these results the UspA1 and A2 were recombinantly produced in *E. coli* and purified using affinity chromatography. Both UspA1 and A2 are considered as autotransporter proteins with their signal peptide cleaved off in the periplasm and their hydrophobic C-terminal forming an anchor in the outer membrane (Aebi *et al.*, 1997; Henderson *et al.*, 1998). Because UspA1 and A2 were difficult to produce, we did not include the signal peptides and the hydrophobic C-termini. The recombinant proteins UspA1 and UspA2 were thus designated UspA1 150-770 and UspA2 230-539, respectively. UspA1 150-770 and UspA2 230-539 were analysed for C4BP binding in ELISA. This analysis indicated that both exhibit C4BP binding in a dose-dependent manner, even though the UspA1-deficient mutant only showed a minor decrease in C4BP binding in the flow cytometry and RIA experiments. This finding is most likely related to the difference in production of UspA1 and UspA2. Using an anti-UspA polyclonal antibody, the Western blot and the flow cytometry results both showed that our *M. catarrhalis* strains (BBH18 and RH4) produced UspA2 to a much higher degree than UspA1. This finding is in agreement with the results obtained by Pearson *et al.* (2002), they found that UspA2 was produced as a very dense layer, whereas the production of UspA1 was more sparsely.

The hypothesis on why *M. catarrhalis* binds to C4BP is that the bacterium utilises the capacity of C4BP to inhibit the classical pathway. However, to be able to do that, C4BP is required to retain its functions when bound to the surface of *M. catarrhalis*. One function of C4BP is its ability to act as a co-factor in the factor I mediated cleavage of C4b, resulting in the surface-bound C4d and the released C4c. Thus, the C4d/C4c ratio on the surface of *M. catarrhalis* after pre-incubation of C4BP was analysed by flow cytometry. In addition, the cleavage of C4b was also determined by the detection of C4d after separation in SDS/PAGE. Both assays indicated that C4BP retained its co-factor capacity to degrade C4b when it was bound to *M. catarrhalis*.

UspA1 AND UspA2 BIND C3 AND THUS INTERFERE WITH THE ALTERNATIVE PATHWAY

To investigate whether *M. catarrhalis* also interacts with the alternative pathway we performed a serum bactericidal assay with *M. catarrhalis* in the presence of EDTA or Mg^{2+}-EGTA. Chelation of Ca^{2+} with Mg^{2+}-EGTA effectively blocks the classical pathway while leaving the alternative pathway intact, whereas chelation of both Ca^{2+} and Mg^{2+} with EDTA blocks all pathways of the complement system. We found that both the classical and the alternative pathways were shown to be activated by *M. catarrhalis*. Hence, the serum resistant *M. catarrhalis* strains are resistant to both pathways.

Because the alternative pathway is part of the innate immunity, it exists also in children who have not been exposed to pathogens. However, otitis media that is caused by *M. catarrhalis* is still very common in pre-school children (Klein, 1994). Therefore, the pathogen has possibly developed a mechanism to avoid complement attack *via* the alternative pathway. Many pathogens have employed several mechanisms to evade host defences. For instance, *S. pyogenes* and *N. gonorrhoeae* have the ability to interact with both C4BP and factor H and hence interfere with both the classical and the alternative pathways (Horstmann *et al.*, 1985; Ram *et al.*, 2001; Kraiczy & Wurzner, 2006).

During complement activation, C3b is covalently deposited on the surface of a microbe resulting in amplification of the alternative pathway activation, phagocytosis or MAC formation. However, deposition of C3b cannot occur if the complement activation is inhibited in that the thiolester site is hidden in the C3 and C3(H_2O) molecules. Of considerable interest was that we could demonstrate that the *M. catarrhalis* strain RH4 bound C3 even if the complement was inactivated with EDTA, indicating that the binding could not be of a covalent fashion. To evaluate further the C3 binding we used methylamine treated C3 (C3met), which is an intact C3 molecule without the capacity to covalently attach to microbes. C3met was shown to bind *M. catarrhalis* in a dose-dependent manner as revealed by flow cytometry, and the binding was most likely due to ionic interactions. To ascertain that the C4BP binding was not only specific to our model strains we analysed a random series of clinical *M. catarrhalis* isolates. All *M. catarrhalis* strains (n = 13) bound C3met. Importantly, similar analyses with related moraxella subspecies (n = 13) as well as common human pathogens (n = 13) revealed that *M. catarrhalis* was the only pathogen with the capacity to bind C3 in EDTA-treated serum as well as C3met.

Different *M. catarrhalis* mutants were tested in flow cytometry to study whether the outer membrane proteins MID, UspA1 or UspA2 were responsible for the interaction with C3. The flow cytometry profiles showed that production of UspA2 was required for the binding of both C3 in EDTA-treated serum and C3met. The *M. catarrhalis* mutant devoid of UspA1 showed a slightly decreased C3 binding, whereas the MID-deficient mutant bound C3 to a similar extent as the corresponding wild type strain. The interaction between C3 and UspA1/A2 was further analysed using recombinant UspA1150-770 and UspA2230-539. UspA2230-539 bound strongly to C3met, whereas UspA1150-770 displayed a weaker binding.

Our experiments imply that C3 binds to the surface of *M. catarrhalis*, irrespective of complement activation. The question was whether this interaction could affect the alternative pathway. The UspA1/A2-deficient mutant was killed in Mg^{2+}-EGTA-treated serum because of the alternative pathway. However, we found that the addition of recombinant UspA1150-770 and UspA2230-539 in the normal human serum (NHS) inhibited the bactericidal effect. Moreover, flow cytometry analyses showed that the C3b deposition on the surface of the UspA1/A2-deficient mutant decreased in the presence of recombinant UspA1150-770 and UspA2230-539. These results suggest that the UspA1/A2 have the ability to absorb C3 in serum and prevent complement activation through the alternative pathway. This hypothesis was further confirmed in an alternative pathway haemolytic assay using rabbit erythrocytes. The assay showed that, in comparison with untreated NHS, NHS preincubated with UspA1150-770 and UspA2230-539 resulted in a decreased haemolysis. In an attempt to study whether the absorption of C3 could also inhibit the complement attack through the classical pathway we observed a slightly increased haemolysis. This was most likely due to absorption of C4BP (Nordström, 2004), which results in an accelerated complement cascade.

Although the moraxella-dependent C3 binding in parts is unique, a few examples of direct C3 binding to microbes exist. A C3-binding protein of *Streptococcus pneumoniae* identified as the pneumococcal choline-binding protein has been isolated and determined to bind C3 in a non-covalent manner (Cheng *et al.*, 2000). However, any beneficial consequences for the pathogen upon binding C3 have not been demonstrated thus far. Recently, a 19-kDa protein (designated Efb) secreted by *Staphylococcus aureus* was identified and shown to bind C3 and inhibit both the classical and alternative pathways (Lee *et al.*, 2004).

MORAXELLA CATARRHALIS MID AND UspA1/A2 AS VACCINE CANDIDATES?

M. catarrhalis is an emerging pathogen and the need for a vaccine is an issue the forthcoming decade. Several vaccine candidates have been discussed, but the adhesins MID and UspA1/A2 are currently the most interesting. Both MID and UspA1/2 have been shown to play an important role in *Moraxella* adhesion to lung and conjunctival epithelial cells. Precise mapping of the two different outer membrane proteins has revealed at least two interesting domains, i.e., the adhesive part of MID (Forsgren *et al.*, 2003) and the domain of UspA1/2 that binds both fibronectin and the two complement regulators C3 and C4BP (Nordström *et al.*, 2004; 2005; Tan *et al.*, 2005). Moreover, antibodies directed against these domains are effective in mouse pulmonary clearance models (Chen *et al.*, 1996; Forsgren *et al.*, 2004).

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