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The Respiratory Pathogen *Moraxella catarrhalis* Adheres to Epithelial Cells by Interacting with Fibronectin through Ubiquitous Surface Proteins A1 and A2

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***Moraxella catarrhalis* ubiquitous surface protein (Usp) A1 has been reported to bind fibronectin and is involved in adherence. In this study, using *M. catarrhalis* mutants derived from clinical isolates, we show that both UspA1 and UspA2 bind fibronectin. Recombinant truncated UspA1/A2 proteins, together with smaller fragments spanning the entire molecule, were tested for binding to fibronectin. Both UspA1 and UspA2 bound fibronectin, and the fibronectin-binding domains were located within UspA1^{299–452} and UspA2^{165–318}. These 2 truncated proteins inhibited binding of *M. catarrhalis* to Chang conjunctival epithelial cells to an extent similar to that by anti-human fibronectin antibodies. Our observations show that both UspA1 and UspA2 are involved in adherence to epithelial cells via cell-associated fibronectin. The biologically active sites within UspA1^{299–452} and UspA2^{165–318} have therefore been suggested to be potential candidates to be included in a future vaccine against *M. catarrhalis*.**

Moraxella catarrhalis is a leading bacterial cause of acute otitis media in children, after *Streptococcus pneumoniae* and *Haemophilus influenzae* [1–3]. It is also a common cause of sinusitis and lower respiratory tract infections in adults with chronic obstructive pulmonary disease (COPD).

In recent years, the focus of research on *Moraxella* species has been on the outer membrane proteins (OMPs) and their interactions with the human host [4, 5]. Some of these OMPs—including, among others, *M. catarrhalis* IgD binding protein (also designated as “Hag”), protein CD, *M. catarrhalis* adherence protein, and the

ubiquitous surface proteins (Usps)—appear to have adhesive functions [6–10].

The UspA family consists of UspA1 (molecular weight, 88), UspA2 (molecular weight, 62), and the hybrid protein UspA2H (molecular weight, 92) [11, 12]. The amino acid sequences of UspA1 and UspA2 are 43% identical and have 140 aa, of which 93% are identical [11]. These proteins are relatively conserved and, hence, are important vaccine candidates. In a series of *M. catarrhalis* isolates from children with otitis media, *uspA1* and *uspA2* genes were almost universally detected (99% and 100%, respectively); 21% of isolates were identified as having the hybrid variant gene *uspA2H* [13]. Moreover, naturally acquired antibodies to UspA1 and UspA2 are bactericidal [14].

Several functions have been attributed to the UspA family of proteins. Expression of UspA1 is essential for the attachment of *M. catarrhalis* to Chang conjunctival epithelial cells, Hep-2 laryngeal epithelial cells, and A549 lung epithelial cells [9, 12, 15, 16]. Purified UspA1 has also been shown to bind fibronectin in dot-blot experiments, whereas purified UspA2 has not [15]. Both

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Table 1. Clinical strains of *Moraxella catarrhalis* used in the present study.

Strain	Clinical source	Reference
BBH18	Sputum	[18]
D1	Sputum	[18]
Ri49	Sputum	[18]
C10	Sputum	[19]
F16	Sputum	[19]
Bro2	Respiratory tract	[18]
Z14	Pharynx	[19]
S6-688	Nasopharynx	[20]
Bc5	Nasopharynx	[21]
RH4	Blood	[18]
RH6	Blood	[18]
R14	Unknown	[19]
R4	Unknown	[19]
SÖ-1914	Tympanic cavity aspirate	[20]

NOTE. The strains C10 and R4 lacked the *uspA1* gene, whereas F16, R14, and Z14 lacked the *uspA2* gene. The remaining strains contained both the *uspA1* and the *uspA2* genes (data not shown).

UspA1 and UspA2 may play important roles in *M. catarrhalis* resistance to the bactericidal activity of human serum [9, 17].

The aims of the present study were to confirm that binding to fibronectin is indeed determined by UspA1, elucidate the binding motif, and, finally, study interactions between *M. catarrhalis* and epithelial cells. We demonstrate that both UspA1 and UspA2 are determinants of *M. catarrhalis* binding to fibronectin in the clinical isolates *M. catarrhalis* BBH18 and RH4. Interestingly, recombinant UspA1 and UspA2 derived from *M. catarrhalis* Bc5 bound fibronectin to the same extent. The binding domains were found within aa 299–452 of UspA1 and aa 165–318 of UspA2. These 2 domains share a sequence identity of 31 aa. Importantly, truncated protein fragments containing these residues in UspA1 and UspA2

were able to inhibit *M. catarrhalis* binding to Chang conjunctival epithelial cells, suggesting that the interactions were via cell-associated fibronectin.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The sources of the clinical *M. catarrhalis* strains are listed in table 1. *M. catarrhalis* BBH18 and RH4 mutants were constructed as described elsewhere [17, 18]. These 2 strains have a relatively higher expression of UspA2 than of UspA1 [17]. The *M. catarrhalis* strains were routinely cultured in brain heart infusion (BHI) broth or on BHI agar plates, at 37°C. The UspA1-deficient, UspA2-deficient, and double mutants were cultured in BHI supplemented with antibiotics, as described elsewhere [17].

DNA methods. To detect the presence of *uspA1*, *uspA2*, and *A2H* genes in those strains in which their presence was unknown, the primers and polymerase chain reaction (PCR) conditions described by Meier et al. [13] were used. Partial sequencing with the UspA1^{299–452} and UspA2^{165–318} 5' and 3' primers of the respective *uspA1* and *uspA2* genes of RH4 and BBH18 was also performed. Confirmation of the presence of the amino acid residues DQKADIDNNINNIYELAQQQDQHSSDIK-TLK was also performed by PCR with a primer (5'-CAAAGCT-GACATCCAAGCACTTG-3') designed from the 5' end of this sequence and 3' primers of *uspA1* and *uspA2*, as described by Meier et al. [13].

Construction and expression of recombinant proteins. Recombinant UspA1^{50–770} and UspA2^{30–539}, which are devoid of their hydrophobic C-termini, have recently been described [17]. Genomic DNA was extracted from *M. catarrhalis* Bc5 by use of a DNeasy tissue kit (Qiagen). In addition, recombinant proteins corresponding to multiple regions spanning UspA1^{50–770} and UspA2^{30–539} were also constructed by the same method. The prim-

Table 2. Primers used in the present study.

Protein	5' primer	3' primer
UspA1 ^{50–770}	gcgtctgaggatccagtaggcaaggcaacc	cctgaagcttagtgataacctaattg
UspA1 ^{50–491}	gcgtctgaggatccagtaggcaaggcaacc	ttgagcaagcttagcttggttttagcg
UspA1 ^{50–197}	gcgtctgaggatccagtaggcaaggcaacc	acctgtggcaagcttctctctgcc
UspA1 ^{50–321}	gcgtctgaggatccagtaggcaaggcaacc	ggtgtcactaagcttacctgaccaacatgaac
UspA1 ^{299–452}	ggattgaggtgcatcgatcctgtaattggtact	gtctttgtaagatcaagctttgatcaat
UspA1 ^{433–580}	catagctctgataggatcacttaaaaaac	catgctgagaagcttacctagattgg
UspA1 ^{557–704}	gccaaagcacaagcggatccaaataaagac	ggtcttattgtagtaagcttagcttggtttg
UspA1 ^{680–770}	ggtgagcaaaaggatcccatcaatcaagag	cctgaagcttagtgataacctaattg
UspA2 ^{30–539}	cgaatgaggatcctaaaaatgatataactttagagg	cattaagcttggtgtctaatgcagttac
UspA2 ^{30–177}	cgaatgaggatcctaaaaatgatataactttagagg	ctcatgacaaaatcaagcttatcttcgatagactc
UspA2 ^{101–240}	gatattgaggatccggaagatgattgaaac	gatcaataagcttaccgcttagattgaatgtcttc
UspA2 ^{101–318}	gatattgaggatccggaagatgattgaaac	gtcaatcgcttcaagcttctttgagcactctg
UspA2 ^{165–318}	gagattgagaaggatccagatgctattgct	gtcaatcgcttcaagcttctttgagcactctg
UspA2 ^{302–458}	gctcaaaaccaagcggatcccaagatctg	ggtgagcgtttcaagctttgatcagcatcgcc
UspA2 ^{446–539}	gcaagtgctgaggatcctgatgattgct	cattaagcttggtgtctaatgcagttac

NOTE. Usp, ubiquitous surface protein.

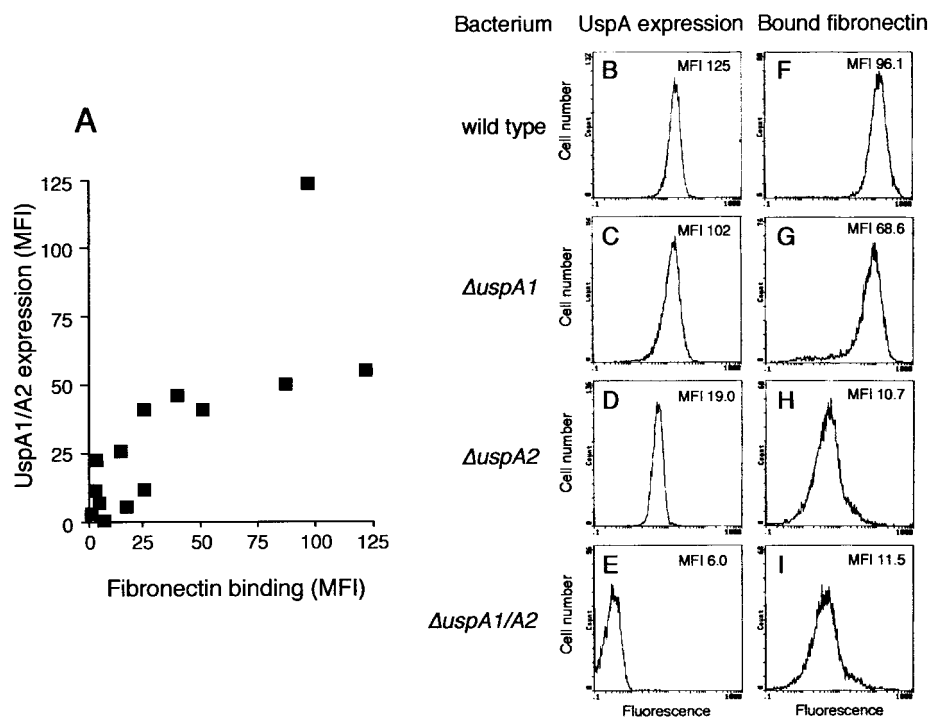


Figure 1. Binding of clinical isolates of *Moraxella catarrhalis* to fibronectin. The interaction depends on ubiquitous surface protein (Usp) A1 and UspA2. *A*, Testing of 14 *M. catarrhalis* strains for binding to fibronectin. Strong binding to fibronectin correlated with expression of UspA1/A2, as detected by anti-UspA1/A2 polyclonal antibody (PAb). *B–I*, Flow-cytometric profiles of *M. catarrhalis* BBH18 wild-type (*wt*) and UspA1/A2-deficient mutants, showing UspA1/A2-dependent binding to soluble fibronectin. The profiles of the *wt* clinical isolate (*B* and *F*) and its corresponding mutants lacking UspA1 (*C* and *G*) or UspA2 (*D* and *H*) and of double mutants (*E* and *I*) lacking both UspA1 and UspA2 are shown. Bacteria were incubated with rabbit anti-UspA1/A2 PAb or fibronectin, followed by an anti-human fibronectin PAb. Fluorescein isothiocyanate-conjugated anti-rabbit PAb was subsequently added, followed by flow-cytometric analysis. For each profile, 1 typical experiment, with the mean fluorescence intensity (MFI) of 3 experiments performed, is shown.

ers used are listed in table 2. All constructs were sequenced in accordance with standard methods. Expression and purification of the recombinant proteins were performed as described elsewhere [22]. Proteins were purified by use of columns containing a nickel resin (Novagen), in accordance with the manufacturer's instructions for native conditions. The recombinant proteins were analyzed by SDS-PAGE, as described elsewhere [21].

Antibodies. Rabbit anti-UspA1/A2 polyclonal antibodies (PAbs) were recently described in detail [17]. The other antibodies used were rabbit anti-human fibronectin PAb, swine fluorescein isothiocyanate (FITC)-conjugated anti-rabbit PAb, swine horseradish peroxidase (HRP)-conjugated anti-rabbit PAb, and, finally, a mouse anti-human CD54 (intercellular adhesion molecule 1 [ICAM1]) monoclonal antibody (MAb). Antibodies were purchased from Dakopatts.

Flow-cytometric analysis. The expression of UspA1/A2 and the capacity of *M. catarrhalis* to bind fibronectin were analyzed by flow cytometry. Wild-type (*wt*) strains and UspA1/A2-deficient mutants were grown overnight and washed twice in PBS containing 3% fish gelatin (PBS-gelatin). The bacteria (10^8) were then incubated with either anti-UspA1/A2 antiserum or

5 μ g of fibronectin (Sigma). They were then washed and incubated for 30 min at room temperature with either FITC-conjugated anti-rabbit PAb (diluted according to the manufacturer's instructions) or a 1:100 dilution of rabbit anti-human fibronectin PAb (if fibronectin was first added) for 30 min at room temperature, before incubation with the FITC-conjugated anti-rabbit PAb. After 3 additional washes, the bacteria were analyzed by flow cytometry (EPICS, XL-MCL; Coulter). All incubations were kept in a final volume of 100 μ L of PBS-gelatin, and the washings were performed with the same buffer. Anti-human fibronectin PAb and FITC-conjugated anti-rabbit PAb were added separately, as negative controls for each strain analyzed. Inhibition studies were performed by preincubating 0.25 μ mol of UspA fragments with 2 μ g of fibronectin before incubation for 1 h with *M. catarrhalis* bacteria (10^8). The residual-free amount of fibronectin that bound to *M. catarrhalis* was determined by flow cytometry, as outlined above.

Binding of *M. catarrhalis* to immobilized fibronectin. Glass slides were coated with 30- μ L aliquots of fibronectin (1 mg/mL) and air-dried at room temperature. After being washed once with PBS, the slides were incubated in petri dishes with

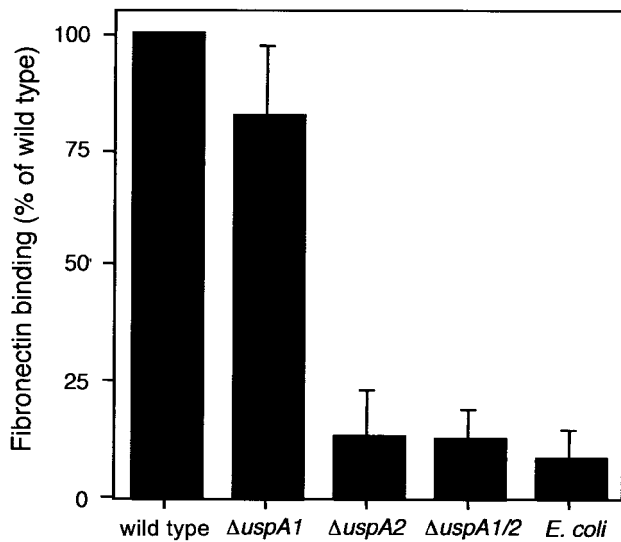


Figure 2. No binding of *Moraxella catarrhalis* RH4 ubiquitous surface protein (Usp) A2-deficient mutants to ^{125}I -labeled fibronectin. *Escherichia coli* BL21 was included as a negative control that did not bind fibronectin. Bacteria were incubated with ^{125}I -labeled fibronectin, followed by several washes, and were analyzed by use of a gamma counter. Binding of RH4 wild type expressing both UspA1 and UspA2 to fibronectin was set at 100%. Mean values of 3 separate experiments are shown, and error bars indicate SDs. Similar results were obtained with *M. catarrhalis* BBH18.

prechilled bacteria at late exponential phase ($\text{OD}_{600} = 0.9$). After incubation for 2 h at room temperature, glass slides were washed once with PBS, followed by Gram staining.

Protein labeling and radio immunoassay. Fibronectin was ^{125}I labeled (0.05 mol of iodine/mol of protein) (Amersham) by the Chloramine T method [23]. *M. catarrhalis* strains BBH18 and RH4, together with their corresponding mutants, were grown overnight on solid medium and washed in PBS with 2% bovine serum albumin (BSA). Bacteria (10^8) were incubated for 1 h at 37°C with ^{125}I -labeled fibronectin (1600 kcpm/sample) in PBS with 2% BSA. After 3 washings with PBS with 2% BSA, the ^{125}I -labeled fibronectin that bound to bacteria was measured by use of a gamma counter (Wallac).

ELISA. Microtiter plates (Nunc-Immuno Module) were coated with 40 nmol/L purified recombinant UspA1⁵⁰⁻⁷⁷⁰ and UspA2³⁰⁻⁵³⁹ in 75 mmol/L Na_2CO_3 (pH 9.6) overnight at 4°C . Plates were washed 4 times with washing buffer (50 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 0.1% Tween 20 [pH 7.5]) and were blocked for 2 h at room temperature with washing buffer containing 3% fish gelatin. After 4 additional washings, the wells were incubated for 1 h at room temperature with fibronectin at different dilutions in 1.5% fish gelatin (in washing buffer). Thereafter, the plates were washed and incubated with rabbit anti-human fibronectin PAb for 1 h. After additional washings, HRP-conjugated anti-rabbit PAb was added, and the plates were incubated for 1 h at room temperature. Both the anti-human

fibronectin PAb and the HRP-conjugated anti-rabbit PAb were diluted 1:1000 in washing buffer containing 1.5% fish gelatin. The wells were washed 4 times, and the plates were developed and measured at an optical density of 450 nm. ELISAs were performed with truncated proteins spanning aa 50–770 of UspA1 and aa 30–539 of UspA2, by use of fixed doses of fibronectin (80 $\mu\text{g}/\text{mL}$ and 120 $\mu\text{g}/\text{mL}$, respectively).

Cell-line adherence-inhibition assay. Chang conjunctival epithelial cells (ATCC CCL 20.2) were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, and 12 $\mu\text{g}/\text{mL}$ gentamicin. The day before adherence-inhibition experiments, cells were harvested, washed twice in gentamicin-free culture media, and added to 96-well tissue culture plates (Nunc) (10^4 cells/200 μL of gentamicin-free culture media). Thereafter, cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 . On the day of experiments, inhibition of *M. catarrhalis* adhesion was performed by preincubating increasing concentrations of either recombinant truncated UspA1/A2 proteins containing the fibronectin-binding domains UspA1²⁹⁹⁻⁴⁵² and UspA2¹⁶⁵⁻³¹⁸ or rabbit anti-human fibronectin PAb for 1 h. Recombinant non-fibronectin-binding proteins (UspA1⁴³³⁻⁵⁸⁰ and UspA2³⁰⁻¹⁷⁷) were used as controls. An anti-ICAM1 MAb (Chang conjunctival epithelial cells are known to express ICAM1 [24]) was used as a control antibody. Subsequently, *M. catarrhalis* RH4 (10^6) in PBS-gelatin was inoculated onto the confluent monolayers. The culture plates were then centrifuged at 3000 g for 5 min and incubated at 37°C in 5% CO_2 . After 30 min, infected monolayers were rinsed several times with PBS-gelatin, to remove nonadherent bacteria, and then were treated with trypsin-EDTA (0.05% trypsin and 0.5 mmol/L EDTA), to release the Chang conjunctival epithelial cells from the plastic support. Thereafter, the resulting cell-bacterium suspension was seeded in dilution onto BHI agar plates and incubated overnight at 37°C in 5% CO_2 .

Determination of expression of fibronectin in Chang conjunctival epithelial cells. Chang conjunctival epithelial cells were harvested by scraping; they were then resuspended in PBS-gelatin. Cells ($10^6/\text{mL}$) were labeled with rabbit anti-human fibronectin PAb, followed by washing and incubation with an FITC-conjugated anti-rabbit PAb. After 3 additional washes, the cells were analyzed by flow cytometry, as outlined above.

RESULTS

No binding of *M. catarrhalis* devoid of UspA1 and UspA2 to soluble or immobilized fibronectin. We selected a random series of *M. catarrhalis* clinical strains ($n = 14$) (table 1) and tested them, by flow-cytometric analysis, for binding to fibronectin in relation to their total expression of UspA1/A2. High total expression of UspA1/A2, as determined by high mean fluorescence intensity (MFI), correlated with high binding to

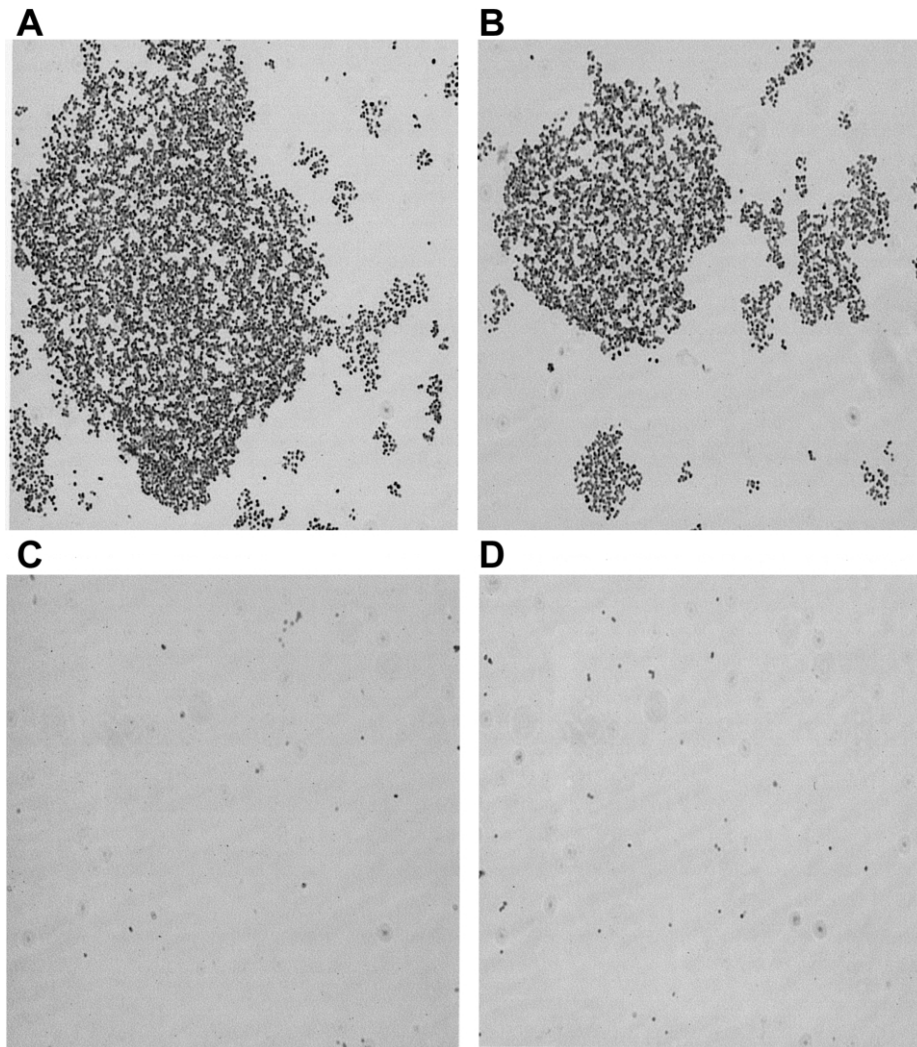


Figure 3. No binding of *Moraxella catarrhalis* mutants devoid of ubiquitous surface protein (Usp) A1 and UspA2 to immobilized fibronectin. *A*, Adherence of *M. catarrhalis* wild type, at a high density, on fibronectin-coated glass slides. *B*, Adherence of *M. catarrhalis* Δ *uspA1* mutant, which was also at a high density. *C* and *D*, Poor adherence of *M. catarrhalis* Δ *uspA2* and Δ *uspA1/A2* double mutants. Glass slides were coated with fibronectin and incubated with *M. catarrhalis* RH4 and its corresponding UspA1/A2 mutants. After several washes, bacteria were Gram stained.

fibronectin (Pearson correlation coefficient, 0.77; $P < .05$) (figure 1A). However, determination of whether UspA1 or UspA2 contributed more to binding was not possible with the anti-UspA1/A2 PAb that we used. That UspA2H contributed to binding was unlikely, since the *uspA2H* gene was not found in the strains used in the present study (data not shown). Two *M. catarrhalis* isolates (BBH18 and RH4) and their specific mutants—which lack UspA1, UspA2, or both proteins—were also analyzed by flow cytometry. UspA1 was expressed at a lower density than was UspA2 (figure 1C and 1D). *M. catarrhalis* BBH18 strongly bound fibronectin, with an MFI of 96.1 (figure 1F). In contrast, BBH18 Δ *uspA1* showed decreased binding to fibronectin, with an MFI of 68.6 (figure 1G). Binding of BBH18 Δ *uspA2* and the double mutant BBH18 Δ *uspA1/A2* to fibronectin revealed MFIs of only 10.7 and 11.5, respectively

(figure 1H and 1I). Similar results were obtained with UspA1/A2 mutants of the clinical strain *M. catarrhalis* RH4. Taken together, these results suggest that UspA1 and UspA2 bound fibronectin and that the ability of the bacteria to bind fibronectin strongly depended on expression of UspA1/A2.

To further analyze the interaction between fibronectin and *M. catarrhalis*, 125 I-labeled fibronectin was incubated with 2 clinical *M. catarrhalis* isolates (BBH18 and RH4) and their respective mutants. The *wt* *M. catarrhalis* RH4 strongly bound 125 I-labeled fibronectin, whereas the corresponding Δ *uspA1* mutant showed 80% binding of the *wt*. In contrast, the Δ *uspA2* (UspA2 was also predominantly expressed in *M. catarrhalis* RH4) and the double mutant bound 125 I-labeled fibronectin at 14% and 12%, respectively, which were just above the background levels (5.0%–10%) (figure 2). Similar results were ob-

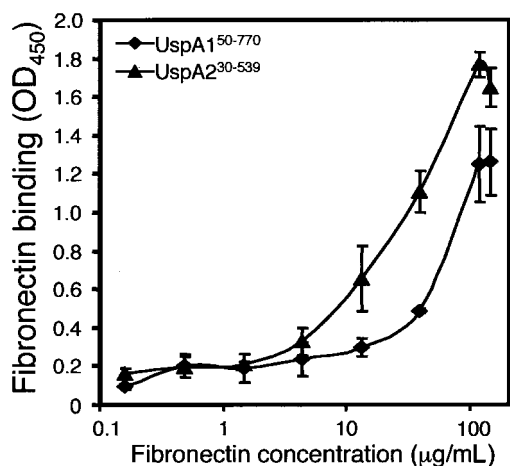


Figure 4. Dose-dependent binding of recombinant ubiquitous surface protein (Usp) A1 and UspA2 to fibronectin. Specific binding to fibronectin is shown for UspA1⁵⁰⁻⁷⁷⁰ and UspA2³⁰⁻⁵³⁹. Both UspA proteins (40 nmol/L) were coated on microtiter plates and incubated with increasing concentrations of fibronectin, followed by detection with rabbit anti-human fibronectin polyclonal antibody (PAb) and horseradish peroxidase-conjugated anti-rabbit PAb. Mean values of 3 separate experiments are shown, and error bars indicate SDs.

tained with *M. catarrhalis* BBH18 and the corresponding UspA1/A2 mutants. Thus, our results suggest that both UspA1 and UspA2 are required for the maximal binding of soluble fibronectin by *M. catarrhalis*.

To investigate the attachment of bacteria to immobilized fibronectin, *M. catarrhalis* RH4 and its corresponding Δ uspA1/A2 mutants were applied to fibronectin-coated glass slides, incubated, and washed. *M. catarrhalis* wt and the Δ uspA1 mutant were found to strongly adhere to the fibronectin-coated glass slides (figure 3A and 3B). In contrast, *M. catarrhalis* Δ uspA2 and Δ uspA1/A2 double mutants weakly adhered to the fibronectin-coated glass slides, with only a few bacteria left after washing (figure 3C and 3D, respectively). In addition, experiments with *M. catarrhalis* BBH18 and its derived mutants showed a similar pattern, indicating that UspA2 contributes strongly to binding of *M. catarrhalis* to immobilized fibronectin.

Inclusion of aa 299–452 of UspA1 and aa 165–318 of UspA2 in the fibronectin-binding domains. To further analyze the interactions of UspA1/A2 with fibronectin, UspA1⁵⁰⁻⁷⁷⁰ and UspA2³⁰⁻⁵³⁹ were recombinantly produced in *Escherichia coli*, coated on microtiter plates, and incubated with increasing concentrations of fibronectin. Bound fibronectin was detected by use of an anti-human fibronectin PAb, followed by incubation with an HRP-conjugated anti-rabbit PAb. Both recombinant UspA1⁵⁰⁻⁷⁷⁰ and UspA2³⁰⁻⁵³⁹ bound soluble fibronectin, and the interactions were dose dependent (figure 4).

To define the fibronectin-binding domain of UspA1, recombinant proteins spanning the entire molecule of UspA1⁵⁰⁻⁷⁷⁰ were manufactured. Fibronectin was incubated with immo-

bilized UspA1 fragments, and the interactions were quantified by ELISA. UspA1⁵⁰⁻⁴⁹¹ bound fibronectin almost as efficiently as did UspA1⁵⁰⁻⁷⁷⁰, suggesting that the binding domain was within this part of the protein. Among the other fragments, UspA1²⁹⁹⁻⁴⁵² efficiently bound fibronectin (figure 5A). In parallel, the interactions between fibronectin and several recombinant UspA2 fragments, including UspA2³⁰⁻⁵³⁹, were analyzed. UspA2¹⁰¹⁻³¹⁸ and UspA2¹⁶⁵⁻³¹⁸ strongly bound fibronectin (figure 5B). Our findings provide significant evidence that the binding domains include residues found within UspA1²⁹⁹⁻⁴⁵² and UspA2¹⁶⁵⁻³¹⁸. A sequence comparison between these 2 binding fragments revealed that the 31 aa, DQKADIDNNINNIYELAQQDQHQSSDIKTLK, were identical for UspA1 and UspA2 (figure 6). Moreover, this repeat sequence was also found in the *uspA1* and *uspA2* genes of *M. catarrhalis* BBH18 and RH4 (data not shown).

Competitive inhibition of binding of *M. catarrhalis* to fibronectin by UspA1⁵⁰⁻⁴⁹¹ and UspA1²⁹⁹⁻⁴⁵². To further validate

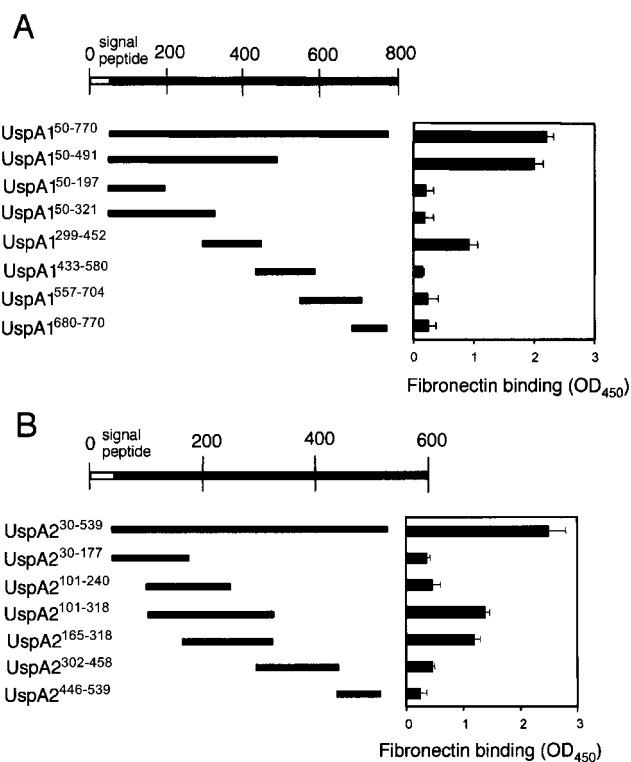


Figure 5. The active fibronectin-binding domains of ubiquitous surface protein (Usp) A1 and UspA2, which are located between aa 299 and 452 and aa 165 and 318, respectively. Truncated proteins derived from UspA1 (A) and UspA2 (B) are shown. All fragments were tested for binding to fibronectin by ELISA; 40 nmol/L of each fragment was coated on microtiter plates and incubated with 80 and 120 µg/mL fibronectin for UspA1 and UspA2, respectively. Bound fibronectin was detected with rabbit anti-human fibronectin polyclonal antibody (PAb), followed by horseradish peroxidase-conjugated anti-rabbit PAb. Results are representative of 3 sets of experiments, and error bars indicate SDs.

UspA1 ²⁹⁹⁻⁴⁵²	TGNGTIVSVGKKGKERQIVHVGAGEISDTST
UspA2 ¹⁶⁵⁻³¹⁸	-KDAIAKNNESIED-LYDFGH-EVAES-
UspA1 ²⁹⁹⁻⁴⁵²	DAVNGSQLHVLATVVAQNKADIKDLDEVG
UspA2 ¹⁶⁵⁻³¹⁸	- - - I G - E I H A H N - - E A Q N E T - - - - L K - - - G
UspA1 ²⁹⁹⁻⁴⁵²	LLGEEINSLEGEIFNNQDAIAKNQADIKTL
UspA2 ¹⁶⁵⁻³¹⁸	LI - - - T N S I E - - N T N N - - - I T K N K A D I Q A L
UspA1 ²⁹⁹⁻⁴⁵²	ESNVEEGLLDLSGRLLDQKADIDNNINNIY
UspA2 ¹⁶⁵⁻³¹⁸	ENNVEELFNLSGRLIDQKADIDNNINNIY
UspA1 ²⁹⁹⁻⁴⁵²	ELAQQDQHS SDIKTLKNNVEEGLLDLSGR
UspA2 ¹⁶⁵⁻³¹⁸	ELAQQDQHS SDIKTLKKNVEEGLLELSDH
UspA1 ²⁹⁹⁻⁴⁵²	LIDQ - - - - - - - - - - - - - - - - - - -
UspA2 ¹⁶⁵⁻³¹⁸	IIDQKTDIAQNQANIQLLATYNELQDQYQA
UspA1 ²⁹⁹⁻⁴⁵²	-
UspA2 ¹⁶⁵⁻³¹⁸	K

Figure 6. Sequence homology between ubiquitous surface protein (Usp) A1²⁹⁹⁻⁴⁵² and UspA2¹⁶⁵⁻³¹⁸. The 31 identical amino acid residues are within boxes.

our findings on the UspA1/A2 fibronectin-binding domains, recombinant truncated UspA1 proteins were tested for their capacity to block binding of *M. catarrhalis* to fibronectin. Fibronectin (2 μg) was preincubated with 0.25 μmol of recombinant UspA1 fragments and subsequently incubated with *M. catarrhalis*. Finally, *M. catarrhalis* UspA-dependent binding to fibronectin was measured by flow cytometry. Preincubation with UspA1⁵⁰⁻⁴⁹¹ and UspA1²⁹⁹⁻⁴⁵² resulted in decreased binding to fibronectin, with a 95% reduction for UspA1⁵⁰⁻⁴⁹¹ and a 63% reduction for UspA1²⁹⁹⁻⁴⁵² (figure 7). When fibronectin was preincubated with the UspA2¹⁰¹⁻³¹⁸, an inhibition of 50% was obtained. Thus, the fibronectin-binding domains of UspA1 and UspA2 blocked the interactions between fibronectin and *M. catarrhalis*.

Inhibition of *M. catarrhalis* adherence to Chang conjunctival epithelial cells by UspA1²⁹⁹⁻⁴⁵² and UspA2¹⁶⁵⁻³¹⁸. Many bacteria attach to epithelial cells via cell-associated fibronectin [25–27]. Previous studies have shown that *M. catarrhalis* adhere to epithelial cells [12, 15]. We analyzed Chang conjunctival epithelial cells, which have frequently been used in adhesion experiments with respiratory pathogens. These cells expressed fibronectin, as revealed by flow-cytometric analysis (figure 8A). To analyze whether the UspA-dependent binding to fibronectin was important for bacterial adhesion, Chang conjunctival epithelial cells were preincubated with anti-human fibronectin PAb or with UspA1²⁹⁹⁻⁴⁵² and UspA2¹⁶⁵⁻³¹⁸. Thereafter, *M. catarrhalis* RH4 was added, and bacterial adhesion was analyzed. The relative adherences (measured by the number of colony-forming units) after preincubation with 0.4 μmol (per 200 μL of media) of UspA1²⁹⁹⁻⁴⁵², UspA2¹⁶⁵⁻³¹⁸, or anti-human fibronectin PAb (1:50 dilution) were 36%, 35%, and 32%, respec-

tively. Higher concentrations of recombinant peptides did not result in further inhibition. In contrast, the non-fibronectin-binding fragments UspA1⁴³³⁻⁵⁸⁰ and UspA2³⁰⁻¹⁷⁷ did not inhibit the interactions between *M. catarrhalis* and Chang conjunctival epithelial cells (figure 8B). Thus, on Chang conjunctival epithelial cells, fibronectin may function as a receptor for *M. catarrhalis*, and aa 299–452 of UspA1 and 165–318 of UspA2 contain the ligand responsible for the interactions.

DISCUSSION

In the present study, we have shown that a series of clinical *M. catarrhalis* strains bind soluble fibronectin and that expression of UspA1/A2 correlates with binding to fibronectin. A previous study showed that UspA1, but not UspA2, purified from *M. catarrhalis* bound fibronectin [15]. However, *M. catarrhalis* BBH18 and RH4 mutants devoid of UspA2 resulted in nearly abolished binding to fibronectin. When UspA1 (expressed at a lower density) was deleted, a decrease in binding of only 20%–

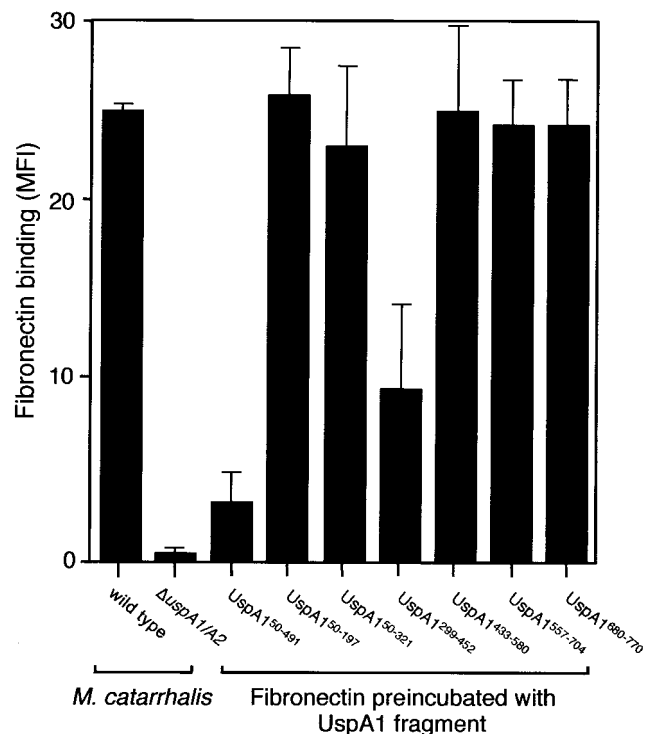


Figure 7. Competitive inhibition of *Moraxella catarrhalis* ubiquitous surface protein (Usp) A-dependent binding to fibronectin by UspA1⁵⁰⁻⁴⁹¹ and UspA1²⁹⁹⁻⁴⁵². *M. catarrhalis* ΔuspA1/A2 double mutants, which do not bind fibronectin, were included as negative controls. Recombinant UspA1 proteins were preincubated with 2 μg/100 μL of fibronectin before incubation with *M. catarrhalis*. The mean fluorescence intensities (MFIs) of *M. catarrhalis*, with bound fibronectin detected by use of fluorescein isothiocyanate-conjugated anti-human fibronectin polyclonal antibody in flow-cytometric analysis, are shown. UspA1⁵⁰⁻⁴⁹¹ and UspA1²⁹⁹⁻⁴⁵² caused 95% and 63% inhibition, respectively. Mean values of 3 separate experiments are shown, and error bars indicate SDs.

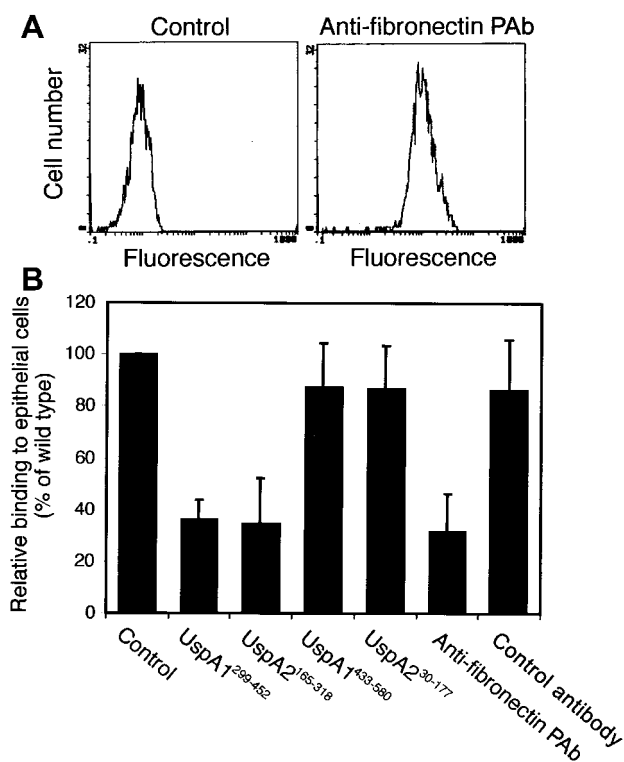


Figure 8. Inhibition of *M. catarrhalis* adherence to Chang conjunctival epithelial cells via cell-associated fibronectin by ubiquitous surface protein (Usp) A1²⁹⁹⁻⁴⁵² and UspA2¹⁶⁵⁻³¹⁸. *A*, Chang conjunctival epithelial cells expressed fibronectin on the surface, as revealed by use of an anti-human fibronectin polyclonal antibody (PAb) in flow-cytometric analysis. *B*, Preincubation with the fibronectin-binding proteins UspA1²⁹⁹⁻⁴⁵², UspA2¹⁶⁵⁻³¹⁸, and anti-human fibronectin PAb resulted in significantly reduced binding by *M. catarrhalis* RH4, compared with that induced by control recombinant proteins (UspA1⁴³³⁻⁵⁸⁰ and UspA2³⁰⁻¹⁷⁷) and a control antibody (anti-ICAM1 monoclonal antibody). *P* < .05, 2-tailed paired Student's *t* test. Mean values of 3 separate experiments are shown, and error bars indicate SDs.

30% was observed (figures 1G and 2). These results suggest that both UspA1 and UspA2 can be determinants of binding to fibronectin. In addition, equimolar amounts of immobilized recombinant UspA1 and UspA2 bound soluble fibronectin to a similar extent when tested by ELISA (figure 4).

The ability to bind fibronectin is of great importance for several bacterial species [28]. For example, *Staphylococcus aureus* and *Streptococcus pyogenes* possess fibronectin-binding proteins (FnBPs) with related sequence organization. These FnBPs are known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). They mediate bacterial adhesion and invasion of host cells. UspA1 and UspA2, however, do not have any sequence similarity to these FnBPs of gram-positive bacteria, as has been revealed by BLAST searches performed by us and by others [15]. They do share sequence similarity to a novel class of nonfimbrial adhesins, of which YadA of *Yersinia enterocolitica* is the prototype protein. An oligomeric structure of coiled coils, a conserved N-terminal

secretion signal, a neck domain, a stalk domain that varies in length, and a C-terminal anchor domain characterize these proteins [29]. They bind to eukaryotic cell-surface and extracellular matrix (ECM) proteins. By electron microscopic analysis, both UspA1 and UspA2 appear as distinct lollipop-shaped surface projections, similar to YadA [30]. Likewise, UspA1 and UspA2 also exist as heat-stable oligomers in SDS-PAGE and are believed to exist as oligomers on the cell surface [11, 29]. The function of each part of the UspA molecule has yet to be defined. Thus, localizing the fibronectin-binding domains is an important first step.

UspA1²⁹⁹⁻⁴⁵² and UspA2¹⁶⁵⁻³¹⁸ from the clinical *M. catarrhalis* strain Bc5 were the shortest fragments that still bound fibronectin. Longer fragments encompassing the amino acid sequence found within UspA1²⁹⁹⁻⁴⁵² and UspA2¹⁶⁵⁻³¹⁸ displayed more-efficient binding to fibronectin (figure 5A and 5B). This may mean that these 2 regions represent partial binding domains or that the binding site is highly dependent on a specific molecular structure. UspA1²⁹⁹⁻⁴⁵² and UspA2¹⁶⁵⁻³¹⁸ share a sequence of 31 identical amino acid residues, including the 23 aa NNINNIYELAQQDQHSSDIKTL (NNINNIY sequence). This sequence contains the epitope for the protective MAb 17C7, for which there is universal reactivity [11, 13, 31]. In a mouse model, passive immunization with MAb 17C7 provided protection and improved pulmonary clearance of *M. catarrhalis* [31]. It is, hence, most interesting that UspA1/A2 fibronectin-binding domains contain these residues and highlights the importance of this region in the pathogenesis of *M. catarrhalis* respiratory tract infection.

The fibronectin-binding *M. catarrhalis* BBH18 and RH4 strains used in our experiments also carry the 31 aa in their UspA1/A2 protein. Most *M. catarrhalis* strains have a part of this sequence (i.e., the NNINNIY sequence). However, strains such as O35E, which has the NNINNIY sequence in its *uspA2* gene, do not express a fibronectin-binding UspA2 protein [15]. A likely explanation would be that the variations in the flanking regions might affect the interaction with fibronectin. Also, the conserved NNINNIY sequence itself can have minor single amino acid base changes [32]. Binding to fibronectin would thus depend not just on expression of UspA1/A2 but also on the individual makeup of each UspA protein. Interestingly, an almost identical amino acid sequence can be found in the hybrid UspA2H protein, with adhesive properties (*M. catarrhalis* TTA37 and O46E) [12]. This gives support to our findings that the 31-aa sequence is important in adhesion.

Many bacteria adhere to epithelial cells via fibronectin-binding MSCRAMMs [25-27]. Blocking the bacteria-fibronectin protein interactions may help the host tissue to overcome the infection. For example, antibodies against an *S. aureus* FnBP caused rapid clearance of the bacteria in infected mice [33]. In our last set of experiments, we tested whether the adherence

of *M. catarrhalis* to Chang conjunctival epithelial cells could be inhibited by the fibronectin-binding fragments UspA1^{299–452} and UspA2^{165–318} (figure 8B). Preincubation with UspA1^{299–452}, UspA2^{165–318}, or an anti-human fibronectin PAb resulted in decreased binding to Chang conjunctival epithelial cells. These results confirm the importance of these binding domains in the interactions of UspA1/A2 with Chang conjunctival epithelial cells and further suggest that fibronectin is an important receptor for UspA.

FnBPs facilitate the adherence of bacteria to undifferentiated and injured airways [26, 27]. Expression of fibronectin by lung fibroblasts is also increased by cigarette smoke extract [34]. Therefore, the role that binding of *M. catarrhalis* UspA1/A2 to ECM fibronectin or epithelial cell-associated fibronectin plays is of great importance in patients with COPD and may explain the common occurrence of *M. catarrhalis* infection in this group of patients [2].

In conclusion, we have shown that UspA1/A2 of *M. catarrhalis* BBH18, RH4, and Bc5 are crucial FnBPs. Recombinant UspA1 and UspA2 derived from Bc5 bind fibronectin, with a binding domain sharing identical amino acid residues. Furthermore, an interaction of *M. catarrhalis* UspA1/A2 with epithelial cells is via cell-associated fibronectin. The definition of these fibronectin-binding domains is therefore an important step forward in the development of a vaccine against *M. catarrhalis*.

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