Hag Directly Mediates the Adherence of *Moraxella catarrhalis* to Human Middle Ear Cells

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***Moraxella catarrhalis* is a human pathogen that causes otitis media in young children and lung infections in patients with chronic obstructive pulmonary disease. In this study, the role of the surface protein Hag in the adherence of multiple *M. catarrhalis* strains was examined. The *hag* genes of four clinical isolates were disrupted with a spectinomycin resistance cassette, and the binding of isogenic mutants to primary cultures of human middle ear epithelial cells (HMEC), as well as A549 pneumocytes, was measured. These experiments revealed that the attachment of most mutants to both cell types was 10-fold less than that of their wild-type progenitors. To determine whether Hag directly mediates adherence to human cells, the *hag* genes from three *M. catarrhalis* isolates were cloned and expressed in a nonadherent *Escherichia coli* cloning strain. At least 17-fold more *E. coli* bacteria expressing Hag attached to HMEC cells than an adherence-negative control. Surprisingly, Hag expression did not increase the binding of recombinant *E. coli* to A549 monolayers. Our data demonstrate that the involvement of Hag in *M. catarrhalis* adherence to A549 and HMEC cells is conserved among isolates and that Hag directly mediates binding to HMEC cells.**

Moraxella catarrhalis is a gram-negative bacterium that causes respiratory tract infections in humans. In adults, this organism causes up to 35% of all infectious exacerbations in patients suffering from chronic obstructive pulmonary disease, contributing to the progression of this fourth leading cause of death in the United States (48, 49). *M. catarrhalis* also causes ~20% of all cases of otitis media (middle ear infection) in children of developed countries (6, 9, 12, 23–26, 36, 45). More than 80% of children have at least one ear infection by the age of 3 years, and these episodes can cause significant delays in development of language and learning skills (24–26). More than 90% of *M. catarrhalis* strains isolated from patients are β -lactam resistant (21, 27, 34), and there is currently no vaccine protective against this organism. Clearly, *M. catarrhalis* is a significant health concern and the development of a vaccine and novel therapeutic approaches is desirable.

Adherence is a necessary step of pathogenesis by most infectious agents (4, 20, 51). The proteins mediating this adherence (adhesins) are surface located, making them attractive vaccine candidates. Studies with FimH, a major adhesin of uropathogenic *Escherichia coli*, have shown that vaccination with purified FimH and passive transfer of FimH-specific antibodies are protective in animal models of infection (31, 32). Moreover, the *Bordetella pertussis* adhesins FHA and Pertactin are components of three acellular pertussis vaccines currently licensed for use in the United States (7). Thus, adhesins are proven effective vaccine antigens. Several *M. catarrhalis* adhesins have been identified, including UspA1 (1, 28), UspA2H (28), OMPCD (18), and McaP (54), all of which have been shown to directly mediate adherence to human epithelial cells

in vitro. Hag, a 200-kDa outer membrane protein, is critical for hemagglutination, autoagglutination, and binding of human immunoglobulin D by the *M. catarrhalis* isolate O35E (42). In addition, our laboratory has shown that Hag expression plays an important role in the binding of strain O35E to A549 human pneumocytes and to primary cultures of human middle ear epithelial (HMEC) cells (19). A purified and radiolabeled recombinant protein corresponding to residues 764 to 913 of MID, a Hag ortholog expressed by *M. catarrhalis* strain Bc5, was shown by Forsgren and coworkers to bind A549 monolayers. In addition, immunization with this MID⁷⁶⁴⁻⁹¹³ polypeptide yielded antibodies that decreased adherence of *M. catarrhalis* to A549 cells by ~65% (13). These data suggest that Hag mediates the binding of *M. catarrhalis* to A549 and HMEC cells.

Building on past research, we have generated isogenic *hag* mutants in four *M. catarrhalis* clinical isolates of various geographic and clinical origins to determine whether Hag expression is important for adherence to HMEC and A549 cells. We also report the successful cloning and expression of *hag* genes in *E. coli* in order to demonstrate that this protein directly mediates binding to middle ear cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, tissue culture cell lines, and culture conditions.

A549 and primary HMEC cells were cultured as previously described (18, 19, 55). HMEC cells were kindly provided by Thomas DeMaria (Ohio State University). These cultures were obtained at passage 5, a stage at which they were shown to be free of fibroblast contamination via immunochemical staining with an anti-vimentin monoclonal antibody (MAb) (55), and aliquots were immediately frozen. At 4 to 6 days prior to performing adherence assays, aliquots of frozen HMEC cells were thawed and cultured in fresh minimal essential medium α (MEM α) as described by Tong et al. (55). When HMEC cells reached confluency, they were immediately seeded into the wells of 24-well tissue culture plates to perform adherence assays. HMEC cells were also passaged once into fresh medium in order to obtain more cells for repeating the adherence assays. When grown

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description ^a	Source or reference
Strains		
<i>M. catarrhalis</i>		
O35E	WT isolate from middle ear effusion (Dallas, TX)	2
O35E.ZCS	Zeo ^r Spec ^r Cm ^r ; isogenic <i>uspA1 uspA2 hag</i> mutant of strain O35E	42
O35E.TN2	Kan ^r ; <i>hag</i> transposon mutant of strain O35E	19
O12E	WT isolate from middle ear effusion (Dallas, TX)	28
O12E.Hag	Spec ^r ; <i>hag</i> isogenic mutant of strain O12E	This study
TTA37	WT isolate from transtracheal aspirate (Johnson City, TN)	28
TTA37.Hag	Spec ^r ; <i>hag</i> isogenic mutant of strain TTA37	This study
V1171	WT isolate isolated from the nasopharynx of healthy child (Chapel Hill, NC)	28
V1171.Hag	Spec ^r ; <i>hag</i> isogenic mutant of strain V1171	This study
McGHS1	Wild-type isolate from patient with respiratory infection (Toledo, OH)	This study
McGHS1.Hag	Spec ^r ; <i>hag</i> isogenic mutant of strain McGHS1	This study
P44	WT isolate from transtracheal aspirate (Johnson City, TN)	28
<i>E. coli</i> EPI300	Cloning strain	Epicentre
Plasmids		
pCC1	Cloning vector	Epicentre
pCC1.3	Adherence-negative plasmid control	19
pMHmcmA	Adherence-positive control	29
pELO35.Hag	Complete O35E- <i>hag</i> gene cloned into pCC1	This study
pBBO12.Hag	Complete O12E- <i>hag</i> gene cloned into pCC1	This study
pSV1171.Hag	Complete V1171- <i>hag</i> gene cloned into pCC1	This study

^a Zeo^r, zeomycin resistance; Kan^r, kanamycin resistance; Spec^r, spectinomycin resistance; Cm^r, chloramphenicol resistance.

under these conditions, HMEC cells were shown to be free of fibroblast contamination (55).

Table 1 lists the bacterial strains and plasmids used in the present study. *M. catarrhalis* was cultured by using Todd-Hewitt medium (TH; Difco) as previously described (18, 19, 54). For isogenic *hag* mutants, the medium was supplemented with spectinomycin at a concentration of 15 µg/ml with the exception of O35E.TN2, which was grown in the presence of 20 µg of kanamycin/ml. Recombinant *E. coli* clones were cultured using Luria-Bertani medium (LB; Fisher Scientific) supplemented with 15 µg of chloramphenicol/ml.

M. catarrhalis cells used for genomic DNA preparation, whole-cell lysates, and adherence assays were grown on solidified medium (1.5% agar). For the preparation of whole-cell lysates, plasmid DNA, and adherence assays, recombinant *E. coli* were cultured overnight in 5 ml of broth. These cells were then seeded into 20 ml of fresh medium supplemented with Epicentre's copy control induction solution at a final concentration of 10× and subsequently grown for 2 h (adherence assays and whole-cell lysates) or 5 h (plasmid DNA) with vigorous shaking (300 rpm). All agar plates were incubated at 37°C and 7.5% CO₂, whereas broth cultures were grown at 37°C with agitation only (no CO₂ supplementation). *Hag* was found to be expressed under these conditions.

Recombinant DNA methodology. Standard molecular biology techniques were implemented as described elsewhere (46). *M. catarrhalis* genomic DNA was obtained with the Invitrogen Easy-DNA kit according to the manufacturer's guidelines. Plasmid DNA was purified from *E. coli* recombinant clones with the QIAprep Spin Miniprep kit (QIAGEN) with minor variations from the manufacturer's instructions (two additional washes with PE buffer and an elution volume of 75 µl).

PCR. Unless stated otherwise, all PCRs were performed by using the Platinum Pfx DNA polymerase as recommended by the manufacturer (Invitrogen). Amplicons containing entire *hag* genes were generated with the oligonucleotides Hag minus 2 (5'-GTC AGC ATG TAT CAT TTT TTA AGG-3') and Hag R4 (5'-TGA GCG GTA AAT GGT TTA AGT G-3'). These PCR products ranging in size from 7.0 to 8.5 kb were used in cloning experiments, for the construction of isogenic mutants, and as templates for sequencing. To identify isogenic *hag* mutants, *Taq* DNA polymerase (Invitrogen) was substituted for Pfx (see below). Genomic DNA was used as a template in all PCRs.

Construction of isogenic *hag* mutations. A PCR product of ~8-kb, corresponding to the entire O35E *hag* gene interrupted by the insertion of a spectinomycin resistance (Spec^r) cassette near the middle of the open reading frame (ORF), was amplified from the genomic DNA of strain O35E.ZCS by using primers Hag minus 2 and Hag R4. This amplicon was purified with Epicentre's PCR precipitation solution according to the manufacturer's recommendations,

resuspended in sterile deionized water, and used to electroporate *M. catarrhalis* competent cells, which were prepared as previously reported (19). Upon electroporation, *M. catarrhalis* cells were spread onto agar plates supplemented with spectinomycin, and resistant colonies were analyzed by PCR with *Taq* DNA polymerase. The introduction of the Spec^r cartridge into the *hag* gene was demonstrated by the following three distinct PCRs. First, we used the primers HagF 3760 (5'-ACA TTG ACC AGT ACT GGC ACA G-3') and HagR 4153 (5'-GGC GTT GTC ATT GAT GCC TTG-3'), which anneal to regions within the *hag* gene that are adjacent to the intended insertion site of the Spec^r cassette. In wild-type (WT) strains, these primers amplified DNA fragments of 0.4 kb. In contrast, PCR products of 1.6 kb were generated in isogenic mutants tested with HagF 3760 and HagR 4153 (data not shown). This 1.2-kb difference in the size of amplicons is consistent with the size of the Spec^r cassette (56). To confirm that the Spec^r cassette is inserted within the *hag* gene, we used primer sets in which one primer anneals within the *hag* gene, whereas the other binds to one of the terminal regions of Spec^r. The first primer pair, HagR 4153 and Spec 5' (5'-AGT CCA CTC TCA ACT CCT GAT CCA-3') generated an amplicon of 0.8-kb in isogenic mutants. As expected, no PCR products were amplified in WT strains with these primers (data not shown). These reactions demonstrated the insertion of the Spec^r cassette in the *hag* genes. These results were confirmed with a second set of primers, HagF 3231 (5'-CCA TCA CAG GAC TAA GCA ACA C-3') and Spec 3' (5'-TAT TGC GGG AAA TGC AGT GGC TGA-3'), which yielded a DNA fragment of 0.7-kb in mutants. No products were observed in WT strains (data not shown).

Adherence assays. Adherence assays were performed as reported (18, 19, 54) with the exception that *M. catarrhalis* strains were incubated with monolayers of epithelial cells for 5 min prior to washing off unbound bacteria. For adherence assays with recombinant *E. coli* clones, bacteria were incubated with human cell lines for 1 h. Bacterial growth was found to be negligible during these incubation periods (data not shown).

Protein preparation and analysis. Western blots were performed as previously described (18, 19, 42, 54). Briefly, whole-cell lysates were prepared by suspending bacteria in ~5 ml of phosphate-buffered saline (PBS) plus 0.15% gelatin (PBSG) to an optical density of 300 Klett units. These suspensions were then centrifuged, and the pelleted bacteria were resuspended in 1 ml of PBS plus 0.5 ml of 3× sodium dodecyl sulfate (SDS) loading buffer. Lysates were electrophoresed on a 7.5% SDS-polyacrylamide gel, and proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) via electrotransfer in a solution containing no alcohol. After transfer, membranes were incubated for 1 h in PBS supplemented with 0.05% (vol/vol) Tween 20 and 3% (wt/vol) skim milk. Detection of proteins was accomplished with mouse MAb 5D2 (*Hag* specific)

FIG. 1. Western blot analysis of *M. catarrhalis* strains. Proteins present in whole-cell preparations were resolved by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and analyzed by Western blotting with the Hag-specific MAb 5D2 (arrow), and the reactivity of polyclonal antibodies specific for the 62-kDa McaP protein (open arrow head) was used for loading comparison. The numbers on the left indicate molecular masses in kilodaltons.

(42), 1D3 (OMPCD specific) (37), 24B5 (USPA1 specific) (11), and 17C7 (UspA1-, UspA2- and UspA2H-reactive) (2), followed by incubation with a secondary goat anti-mouse antibody that was conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc.). McaP expression was detected using polyclonal antibodies specific for the synthetic peptide PEP1 (see below). Signals were then detected by using the SuperSignal West Pico chemiluminescence kit from Pierce according to the manufacturer's recommendations.

To obtain antibodies directed against the *M. catarrhalis* protein McaP (54), the synthetic peptide PEP1 (KDNNKTVETAVLSNRDYHRS) was synthesized and conjugated to keyhole limpet hemocyanin (Sigma Genosys). The sequence of PEP1 corresponds to residues 577 to 598 of the *M. catarrhalis* O35E-McaP. The keyhole limpet hemocyanin-PEP1 conjugate was used to immunize mice as previously reported (30). Murine antibodies were shown to specifically bind to McaP by Western blotting (data not shown).

Nucleotide sequence analysis. Purified PCR products and plasmid DNA were used as templates in sequencing reactions. Sequencing services were provided by the University of Michigan sequencing core (<http://seqcore.brcf.med.umich.edu/>). Chromatograms were analyzed and assembled by using the ChromaTool software (BioTools, Inc.). Sequence analysis was conducted with the various tools provided by the ExPasy Proteomics Server (<http://us.expasy.org/>), as well as with the BioEdit analysis tool (16). The nucleotide sequences of the *hag* genes from strains P44 (AY862882), McGHS1 (AY862881), V1171 (AY862884), and TTA37 (AY862883) were deposited in GenBank under the indicated accession numbers.

Statistical analysis. All statistical analyses were performed with the Mann-Whitney test in the GraphPad Prism 4.0 software. *P* values of <0.05 were considered statistically significant.

RESULTS

Construction of isogenic *hag* mutants. Previous work demonstrated that Hag is involved in the binding of *M. catarrhalis* strain O35E to primary cultures of HMEC cells and to A549 human lung cells (19). To determine whether Hag's involvement in adherence is conserved among *M. catarrhalis* strains, isogenic *hag* mutations were constructed in four additional isolates. Mutagenesis was accomplished via homologous recombination of a PCR product, which consisted of the entire O35E-*hag* gene disrupted by a Spec^r cassette, into the chromosome of target strains. This ~8-kb DNA fragment was amplified from strain O35E.ZCS and electroporated into *M. catarrhalis* isolates McGHS1, O12E, V1171, and TTA37. Spec^r colonies were selected, and the isogenic *hag* mutations were verified as described in Materials and Methods.

To confirm the lack of Hag expression in isogenic mutants, whole-cell preparations were analyzed by Western blotting with the Hag-specific MAb 5D2 (Fig. 1). As expected, the WT

isolates O12E, V1171, TTA37, and McGHS1 all expressed Hag, whereas none of the isogenic mutants did. We noted that the Hag proteins of strains TTA37, V1171, O12E, and McGHS1 migrated more slowly than O35E-Hag, suggesting larger molecules. We also observed that TTA37, V1171, and particularly McGHS1 appeared to express less Hag than strains O35E and O12E. Upon sequence analysis (see below), it was noted that the Hag peptide to which MAb 5D2 binds (e.g., DNADGNQVNIADIKKDPNSGSSSNR [42]) has a valine (underlined)-to-isoleucine substitution in McGHS1-Hag (data not shown). Thus, reduced antibody affinity or binding may account for the apparent lower level of Hag expression in McGHS1. The binding of polyclonal antibodies to the adhesin McaP was used to demonstrate that equivalent amounts of cell lysates were analyzed (Fig. 1). The panel of mutants was also shown to express wild-type levels of the adhesins UspA1, UspA2H (in the case of strain TTA37), and OMPCD by Western blotting with appropriate MAbs (data not shown).

Hag is associated with the adherence of *M. catarrhalis* isolates to HMEC and A549 human cells. The adherence of the aforementioned isogenic mutants to HMEC cells was compared to that of their corresponding parent strain by the use of a quantitative viable cell count attachment assay. The four isogenic mutants constructed in the present study bound substantially less than their respective WT strains during a 5-min incubation with HMEC monolayers (Fig. 2A). O12E.Hag adhered 27-fold less than its parent strain O12E, and this was the greatest decrease observed among mutants (Fig. 2A). Disruption of the *hag* ORF in the isogenic strains TTA37.Hag and McGHS1.Hag resulted in ~21-fold decrease in adherence to HMEC cells (Fig. 2A). V1171.Hag also showed substantially decreased binding (sevenfold), albeit to a lesser extent than what was observed for the other isogenic mutants (Fig. 2A). However, the adherence of the parent strain V1171 was also slightly less than that of other WT isolates (see Fig. 2A), possibly due to lower expression of Hag (see Fig. 1). The *hag* mutant O35E.TN2, which we previously reported to bind poorly to HMEC cells (19), was used as a negative control; it exhibited a 22-fold reduction in adherence compared to its parent strain O35E (Fig. 2A). All *hag* mutants bound poorly, with values ranging from 1.1 to 3.1% of input bacteria. These results indicate that Hag's involvement in adherence to HMEC cells is conserved among the *M. catarrhalis* strains that were tested.

It has been previously demonstrated that Hag expression plays a role in the binding of strain O35E to A549 human type II alveolar pneumocytes (19) and that *M. catarrhalis* isogenic mutants lacking MID, a Hag ortholog, also exhibit decreased adherence to A549 cells (13). When we tested the binding of *hag* mutants to A549 monolayers, a substantial reduction in adherence was measured for three of the four isogenic strains (Fig. 2B). Interestingly, the WT strain V1171 bound poorly to A549 cells (<1.5%), and the adherence of the V1171.Hag mutant was not significantly lower (data not shown). TTA37.Hag, O12E.Hag, and the negative control O35E.TN2 bound poorly to A549 cells, with percent adherence ranging from 0.8 to 3.6%. McGHS1.Hag adhered at levels slightly higher (6.2%) than the other isogenic strains, but still exhibited a 6.1-fold decrease in binding compared to McGHS1 (Fig. 2B). These data demonstrate that the role of Hag in adherence to

FIG. 2. Adherence of *M. catarrhalis* strains to HMEE (A) and A549 (B) cells. The results are expressed as the mean percentage ($\pm$ the standard error) of inoculated bacteria binding to monolayers after 5-min incubation. Duplicate assays were performed on at least three separate occasions. Hag expression (+) or lack thereof (–) is indicated below each strain name. Asterisks indicate that the difference between the adherence of the mutant strain and that of its parental WT is statistically significant.

human pneumocytes is conserved in three of the *M. catarrhalis* strains that were tested.

Hag directly mediates adherence to middle ear cells. To determine whether Hag directly mediates binding to A549 and HMEE cells, the *hag* genes from isolates O35E, O12E, and V1171 were cloned and expressed in the recombinant background of the nonadherent *E. coli* cloning strain EPI300. Recombinant plasmids were sequenced to verify that no mutations were introduced during PCR and that each ORF contained the appropriate number of guanine residues in their poly(G) tracts (see below). To ensure that all recombinant clones expressed their respective proteins, Western blots were also performed with the Hag-specific MAb 5D2 (Fig. 3).

Quantitative attachment assays demonstrated that the binding of *E. coli* clones expressing O12E-Hag (pBBO12.Hag), O35E-Hag (pELO35.Hag), and V1171-Hag (pSV1171.Hag) was 17, 31, and 50 times greater, respectively, than that of the control strain (Fig. 4A). In contrast, Hag expression did not increase binding of *E. coli* to A549 pneumocytes. As an adherence-positive control, we used EPI300 cells carrying the plasmid pMHmcmA, which encodes a novel adhesin recently discovered by our laboratory (Fig. 4B) (29). The data clearly demonstrate that Hag directly mediates adherence to HMEE cells. In addition, our results suggest that Hag expression alone is not sufficient to enable recombinant *E. coli* to bind to A549 lung cells.

The lack of Hag expression does not adversely affect the autoagglutination of all *M. catarrhalis* isolates. Previous studies have shown that *hag* mutants of strain O35E no longer autoagglutinate, suggesting that Hag is involved in this phenotypic trait of *M. catarrhalis* isolates (19, 42). We therefore

tested whether disruption of *hag* in strains other than O35E would have the same effect. To this end, autoagglutination assays were performed by suspending the different *M. catarrhalis* strains and their respective *hag* mutants in PBS. The tur-

FIG. 3. Western blot analysis of recombinant *E. coli* clones. Proteins present in whole-cell preparations were resolved by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and analyzed by Western blotting with the Hag-specific MAb 5D2. The numbers on the left indicate molecular mass in kilodaltons.

FIG. 4. Adherence of *E. coli* recombinant bacteria to HMEE (A) and A549 (B) cells. The results are expressed as the mean percentage ($\pm$ the standard error) of inoculated bacteria binding to monolayers after 1 h of incubation. Duplicate assays were performed on at least three separate occasions. Hag expression (+) or lack thereof (–) is indicated below each strain name. Asterisks indicate that the difference between the adherence of the experimental clone and that of the negative control (pCC1.3) is statistically significant. Strain pMHmcmA is an adherence positive control for A549 cells.

bidity of these suspensions was then measured at various time points, with O35E and O35E.TN2 as positive and negative controls, respectively. Surprisingly, these assays revealed that all experimental isogenic *hag* mutants autoagglutinate at or near WT levels (Fig. 5). For the majority of strains, autoagglutination began to level out at 30 min after suspension. The V1171.Hag mutant stayed in suspension longer than most other isogenic strains but eventually autoagglutinated to the same extent as V1171 (Fig. 5).

FIG. 5. Clumping of *M. catarrhalis* WT (filled symbols) and Hag mutant (open symbols) strains. The results are expressed as the optical density of bacterial suspension taken at 15-min intervals. Error bars represent the standard error of the mean of duplicate assays.

Nucleotide sequence analysis of *hag* from strains TTA37, McGHS1, P44, and V1171. PCR products of 7 to 8 kb and containing entire *hag* genes were generated with oligonucleotides Hag minus 2 and Hag R4. Sequence analysis predicted that the *hag* ORFs specified proteins of 1,964 to 2,335 amino acid (aa) residues (Table 2). V1171-Hag was found to be the largest, whereas O35E-Hag was the smallest of the predicted molecules (Table 2). The sequences of the O35E (GenBank accession no. AY077637) and O12E (GenBank accession no. AY077638) *hag* ORFs, reported by Pearson et al. (42), as well as that of the *M. catarrhalis* strain Bc5 *mid* gene product, described by Forsgren et al. (14), were included in our comparative analysis. The *hag* ORFs of strains TTA37, McGHS1, V1171, and P44 contain homopolymeric tracts of guanine residues that are 9, 5, 6, and 6 nucleotides (nt) in length, respectively (Table 2). Variation in the length of this poly(G) tract, which in each case is located 80 nt downstream of the predicted ATG translational start codon, has been shown by several investigators to cause phase-variable expression of Hag/MID (35, 42, 47, 52). In these studies, it was shown that only poly(G) tracts in multiples of three allow for Hag expression. McGHS1-*hag* appears to have circumvented this requirement by replacing what would be the sixth guanine with an adenosine, thereby keeping the gene in the correct reading frame (data not shown).

The predicted amino acid sequences of the six Hag proteins, as well as that of Bc5-MID, were compared and found to share an average identity of ~73%. O35E-Hag and Bc5-MID displayed only 56% identity, whereas V1171-Hag and McGHS1-Hag were 89.1% identical (Table 3). We noted that most of the similarity between molecules was clustered in their C termini,

TABLE 2. Characteristics of *hag* genes and their encoded products

Strain	ORF (nt)	Length of poly(G) tract (nt)	Predicted Protein (aa)	Molecular mass (kDa)	Potential signal sequence cleavage sites ^c	No. of HIM-domains
O35E ^a	5,892	6	1,964	201,585	AVA ²⁰ -E, AYA ⁶⁶ -Q	5
Bc5 ^b	6,417	6	2,139	219,579	AVA ²⁰ -E, AYA ⁶⁶ -Q	4
P44	6,870	6	2,290	235,689	AVA ²⁰ -E, AYA ⁶⁶ -Q	6
TTA37	6,888	9	2,296	235,544	AVA ²⁰ -E, AYA ⁶⁷ -A	6
O12E ^c	6,942	6	2,314	237,981	AVA ²⁰ -E, AYA ⁶⁶ -Q	6
McGHS1	6,951	5	2,317	237,345	AVA ²⁰ -E, AYA ⁶⁶ -Q	5
V1171	7,005	6	2,335	239,253	AVA ²⁰ -E, AVS ⁷⁶ -Q	5

^a As reported previously (42).^b As reported previously (13).^c Amino acids indicated by the number correspond to the predicted signal sequence cleavage site.

and these observations are summarized in Fig. 6A. The last 454 residues were particularly well-conserved among these proteins, with an average identity of 97% (see red box in Fig. 6A). In contrast, a region of ~800 aa encompassing most of the N-terminal halves of these molecules displayed an average identity of only 35.8% (see green box in Fig. 6A). We also noted that O35E-Hag is missing a 326-aa domain that is highly conserved in all of the other proteins analyzed (97.5% identical; see gray box in Fig. 6A). Bc5-MID was found to be missing a moderately conserved region (64.8%) of 200 residues that is present in all other Hag proteins (aa 861 to 1,063 of O35E-Hag; see yellow box in Fig. 6A).

Selected structural features of the *hag* gene products. Analysis with the PSIPRED secondary structure prediction method (22) revealed that the last 52 aa of Hag and MID proteins are predicted to form four β -strands (data not shown), each connected by short loops of two to five residues (illustrated in Fig. 6B). This potential membrane-anchoring domain was immediately preceded by a helical region of ~40 residues (Fig. 6B; aa 1862 to 1902 of O35E-Hag). Two putative signal sequence cleavage sites were detected at the N terminus of each molecule by using the SignalP 3.0 server (38, 39), and those are as listed in Table 2. Interestingly, analysis through the Protein Families database (Pfam) service (3) identified a region of 25 aa that exhibits a high level of similarity with a domain designated HIM (Pfam accession no. PF05662), which is described as being associated with bacterial adhesins. This HIM motif corresponds to aa 312 to 336 of O35E-Hag (Fig. 6B) and is well conserved at the N terminus of all Hag and MID proteins (76% identity).

Upon closer examination, the last 14 aa residues of this HIM-like domain of Hag (e.g., AGxxxTDAVNVAQL) were

found to be 78 to 85% identical to a region located in the middle of the so-called “neck” of the *Yersinia enterocolitica* adhesin YadA, with the last nine residues (e.g., TDAVNV AQL) being perfectly conserved (17, 40, 44). Of note, this YadA neck region is preceded by degenerate repeated motifs that are generally 14 residues in length and composed of the consensus sequence xxxSVAIGxxSxAX (40). These repeats, termed β roll by Nummelin et al. (40), have been shown to be essential for the adhesive properties of YadA (17, 40, 44). When the region located directly upstream of the HIM-like domain of Hag was analyzed, 10 repeated motifs 14 residues in length and with the consensus GxxSIAIGxx[A/S]xAX were found (Fig. 6B; see aa 83 to 272).

In contrast to YadA, which contains only one β roll/neck (HIM) combination, all Hag and MID proteins contained a second HIM-like domain that is also immediately preceded by eight degenerate repeats (β roll-like) and located directly prior to the C-terminal helical region of ~40 residues (Fig. 6B; see aa 1680 to 1865 of O35E-Hag). Further analysis also revealed two to four additional HIM-like domains dispersed throughout the Hag/MID molecules. These additional HIM domains, however, were not associated with potential β rolls. These observations are summarized in Table 2 and Fig. 6B.

DISCUSSION

Previous research has demonstrated that the Hag protein of strain O35E forms elongated fibrillar structures covering the surface of *M. catarrhalis* (42), which are crucial for attachment to HMEE and A549 cells (19). In the present study, we sought to extend these findings by constructing isogenic *hag* mutations in four *M. catarrhalis* isolates of various geographical and clinical origins in order to test whether the involvement of Hag in adherence is conserved. We found that all mutants exhibited a substantial decrease in binding to HMEE cells (7- to 27-fold). Attachment assays with A549 pneumocytes yielded comparable results, with the exception of strain V1171.Hag. The parent strain V1171 did not attach well to A549 cells, and the basis for this low binding is under investigation. Our findings are consistent with those of Forsgren et al. with the *M. catarrhalis* protein MID, a Hag ortholog. When these investigators disrupted the *mid* gene of wild-type strains BBH18 and RH4, a two- to threefold reduction in attachment to A549 cells was measured (13). The role of Hag in adherence to HMEE and A549 cells is therefore conserved among *M. catarrhalis* strains.

TABLE 3. Percent identity shared by *M. catarrhalis* Hag and Bc5-MID proteins

Strain	% Identity						
	O35E	Bc5	P44	TTA37	O12E	McGHS1	V1171
O35E	100	56.6	63.9	64.5	68.8	65.6	65.9
Bc5		100	68.4	68.6	68.4	73.6	71.3
P44			100	80.7	79.3	75.8	75.3
TTA37				100	78.8	76.6	75.7
O12E					100	80.7	80.1
McGHS1						100	89.1
V1171							100

FIG. 6. Similarity and structural features of selected Hag domains. (A) Different regions of the O35E-Hag protein are depicted with the position of amino acid residues defining these regions listed above and boxed. The average percent identity of each of these regions among the Hag and MID proteins is also shown. The yellow box corresponds to a region present in all Hag proteins analyzed but missing in Bc5-MID. The gray box corresponds to a region missing only in O35E-Hag but present in all other Hag/MID proteins analyzed. The purple box represents the previously proposed Bc5-MID adherence epitope for A549 cells (13). (B) Selected structural features of O35E-Hag. The lines connecting panels A and B indicate the approximate location of these features. The numbers below panel B represent key amino acid residues associated with these structural features.

Furthermore, the very low binding capabilities of our isogenic mutants indicate that nonexpression of Hag has profound effects on attachment (Fig. 2).

To determine whether Hag directly mediates adherence, the *hag* genes of three *M. catarrhalis* strains were cloned and expressed in *E. coli*, allowing recombinant bacteria to attach to HMEC cells. These experiments conclusively demonstrate that Hag is sufficient to mediate attachment to middle ear cells. In contrast, we discovered that Hag expression alone is not sufficient to permit recombinant *E. coli* to bind A549 cells. Our results are interesting in light of a previous report demonstrating that purified recombinant Bc5-MID binds to A549 monolayers (13). These observations suggest that Hag/MID expression by itself may not be sufficient for *M. catarrhalis* adherence to A549 pneumocytes. Alternatively, Hag's adherence epitope for A549 cells may not be properly displayed on the surface of *E. coli*. Adherence to A549 cells may also require posttranslational modification of Hag that is possibly not achieved in the heterologous genetic background of *E. coli*. For instance, glycosylation of the diarrheagenic *E. coli* protein AIDA modulates its ability to function as an adhesin and requires the expression of the heptosyltransferase *aah* gene, which is located directly upstream of the *aidA* ORF (5, 50). We believe, however, that binding to A549 pneumocytes requires coexpression of another *M. catarrhalis* protein. UspA1 has previously been proposed to act synergistically with Hag in attachment to A549 cells (13). Another adhesin potentially acting in concert with Hag is OMPCD, which was recently shown by our labo-

ratory to mediate adherence to A549 monolayers. Interestingly, isogenic *ompCD* mutants have also been shown to bind poorly to these lung cells (18). The hypothesis that coexpression of OMPCD and Hag is necessary and sufficient for attachment to A549 cells is currently being investigated.

Another phenotypic trait associated with Hag/MID expression is autoagglutination (19, 35, 42, 47). Pearson et al. were the first to show that an isogenic *hag* mutant of strain O35E no longer autoaggregated when suspended in PBS (42). Mollenkvist et al. also isolated nonflocculating variants of two *M. catarrhalis* strains that expressed very low levels of MID (35). We therefore tested the ability of *hag* mutants to fall out of solution when suspended in PBS. Surprisingly, the only mutant that did not flocculate was that of strain O35E; all other strains autoagglutinated at or near WT levels (Fig. 5). It should be noted that Mollenkvist et al. obtained their nonautoagglutinating variants after six serial passages over a period of 4 weeks, each time recovering nonflocculating variants (35). During this type of progressive selection, other mutations probably accumulated in the *M. catarrhalis* genome, and those mutations might have collectively caused the strong nonflocculating phenotype. In the case of strain O35E, it is possible that Hag does not mediate autoagglutination but rather lack of its expression affects the proper surface display of another molecule responsible for autoagglutination. This possibility is supported by the fact that none of our Hag-expressing *E. coli* strains gained the ability to autoagglutinate, including EPI300(pELO35.Hag), which we would have expected to flocculate based on the

autoagglutination phenotypes of O35E and its *hag* mutant (Fig. 5) (42). Although these results suggest that Hag is not a flocculation factor, we cannot exclude the possibility that Hag-mediated autoagglutination requires posttranslational modification not achieved in *E. coli*. Our results suggest, however, that Hag is not the sole contributor to autoagglutination. This is consistent with data presented by Stutzmann Meier et al., in which six isogenic *hag* mutants were found to autoagglutinate at WT levels (52). Interestingly, *M. catarrhalis* was recently shown to express a type IV pilus (Tfp) (33), a structure associated with autoagglutination in other bacterial species (8, 41, 57). Whether Tfp is involved in autoaggregation by *M. catarrhalis* cells remains to be determined.

Structural analysis of the Hag sequence revealed that it resembles members of a family of autotransporter proteins designated Oca (for oligomeric coiled-coil adhesion) (17). This family is represented by *Yersinia enterocolitica* YadA and also includes *Haemophilus influenzae* Hia (53), *Neisseria meningitidis* NadA (10), and the recently described *Bartonella henselae* proteins BadA (43) and VompA, VompB, and VompC (58). These molecules share the following structural features: (i) a C-terminal membrane anchor consisting of four β -strands, (ii) a helical region of ~ 40 residues that links the membrane anchor to a region of variable size, and (iii) an N-terminal signal sequence. Previous studies have suggested that the signal sequence cleavage site for Hag/MID is located between aa 66 and 67 (AYA⁶⁶Q) (35, 42). Our analysis revealed that all Hag/MID proteins display a second potential cleavage site (AVA²⁰E; see Table 2). Upon aligning Hag/MID sequences, it was also noted that the first 20 amino acids are perfectly conserved (data not shown). Furthermore, V1171-Hag does not have the potential signal sequence cleavage site AYA⁶⁶Q due to an insertion of ten residues after aa 62 (data not shown). This insertion places the next potential signal sequence cleavage site at serine 76 (AVS⁷⁶Q; see Table 2). The hypothesis that the Hag signal sequence cleavage site is located between residues 20 and 21 is being tested.

The N-terminal region of several Oca adhesins has also been reported to contain a series of degenerate repeats, which in YadA have been shown to form a nine-coiled left-handed parallel structure termed β roll (40). We found that the N terminus of all Hag/MID proteins exhibit a potential β roll that contains 10 14-mer degenerate repeats with the consensus sequence GxxSIAIGxx[A/S]xAx. A related sequence was previously reported for O35E-Hag and O12E-Hag by Pearson et al. (IAIGxxxxxxxxIAIG) (42), and we believe that this sequence contains the end of one 14-mer repeat, as well as the beginning of a second one. This belief is based on the fact that the consensus sequences for the Hag (GxxSIAIGxx[A/S]xAx) and YadA (xxxSVAIGxxSxAx) repeats are very similar and that the glycine at position 8 is perfectly conserved (underlined). Following this potential β roll near the N terminus of Hag, sequence analysis identified a region of amino acids that is similar to a Pfam domain designated HIM. YadA also contains a HIM domain that is found in a so-called "neck" region located immediately after the aforementioned β roll. This β roll-neck combination is essential for the adhesive properties of YadA (40, 44) and has been observed in other bacterial adhesins (17, 40, 43, 58). Based on these observations, we propose that this

N-terminal region of Hag forms a similar β roll-neck structure which could be important for adherence.

It is also interesting that YadA has only one HIM-like domain, whereas the Hag/MID proteins have at least four interspersed throughout their entire lengths (Table 2 and Fig. 6B). The presence of multiple potential neck (HIM) regions in Oca adhesins has previously been observed (40, 43, 58). For instance, the *B. henselae* adhesin BadA displays as many as 24 potential neck (HIM) regions, which have been proposed to be important for the structural stability of this large molecule (340 kD) (43). Given its size, it is possible that the multiple HIM (neck) regions of Hag may serve a similar purpose. We also noted that one of these additional HIM domains, located near the C terminus of Hag/MID, was preceded by eight degenerate repeats resembling those associated with a β roll (see Fig. 6B), an observation also made by Pearson et al. with the IAIGxxxxxxxxIAIG motif (42). This stretch of 112 residues is 97.3% identical in all proteins analyzed, and its contribution to the adhesive properties of Hag is under investigation.

M. catarrhalis is one of the major causative agents of otitis media. Thus, the findings that Hag directly mediates adherence to human middle ear cells has important implications for vaccine development. The Hag/MID proteins exhibit several characteristics of potential vaccine antigen. Most isolates tested to date contain a *hag/mid* gene, and a majority express the proteins on their surface (35, 42, 52). However, our data and those of others (35, 42) clearly demonstrate that although certain domains are highly conserved (e.g., aa 1063 to 1964 of O35E-Hag, see Fig. 6A), other regions of Hag/MID vary substantially (e.g., aa 66 to 860 of O35E-Hag, see Fig. 6A). Furthermore, some regions appear to be completely deleted. For instance, a domain of 326 aa located near the C terminus of all other Hag/MID proteins analyzed in the present study is missing in O35E-Hag (see gray box in Fig. 6A). Interestingly, this domain that is missing from O35E has the highest degree of identity (97.5%) among the remaining proteins analyzed. A moderately conserved domain of ~ 200 aa was also found to be missing in Bc5-MID but is present in all other Hag isotypes examined (see yellow box in Fig. 6A). Thus, a detailed structure-function analysis of Hag is warranted to identify domains of the molecules with the best vaccinogenic potential. The need for a functional study was highlighted by a recent report showing that immunization with MID⁷⁶⁴⁻⁹⁶³ (proposed adherence epitope for A549 cells) increased the clearance of *M. catarrhalis* from the lungs of mice in a pulmonary challenge model (15); this region of MID shares the highest degree of identity with O35E-Hag at 54.1%.

Although Hag's participation in attachment to both A549 and HMEC cells is conserved, it is clear that the molecular basis for adherence differs for these cell lines, reemphasizing the need for a detailed structure-function analysis of the Hag protein in order to understand this important step in pathogenesis by *M. catarrhalis*. These studies will also facilitate the identification of Hag's ligands on the surface middle ear and lung epithelial cells, which may lead to novel therapeutic approaches.

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