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Amy Z. Buscher
Washington University School of Medicine in St. Louis
Katie Burmeister
Washington University School of Medicine in St. Louis
Stephen J. Barenkamp
Washington University School of Medicine in St. Louis
Joseph W. St. Geme III
Washington University School of Medicine in St. Louis

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Evolutionary and Functional Relationships among the Nontypeable *Haemophilus influenzae* HMW Family of Adhesins†‡

Amy Z. Buscher,[1] Katie Burmeister,[1] Stephen J. Barenkamp,[2] and Joseph W. St. Geme III[1]*

Edward Mallinckrodt Department of Pediatrics and Department of Molecular Microbiology, Washington University School of Medicine,[1] and Department of Pediatrics, St. Louis University School of Medicine, and The Pediatric Research Institute, Cardinal Glennon Children's Hospital,[2] St. Louis, Missouri

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Nontypeable *Haemophilus influenzae* (NTHi) is a common cause of localized respiratory tract disease and initiates infection by colonizing the nasopharynx. Approximately 75 to 80% of NTHi clinical isolates produce proteins that belong to the HMW family of adhesins, which are believed to facilitate colonization. The prototype HMW adhesins are designated HMW1 and HMW2 and were identified in NTHi strain 12. HMW1 and HMW2 are 71% identical and 80% similar overall, yet display differing cellular binding specificities. In the present study we set out to define more clearly the relationships between HMW1 and HMW2 and other members of the HMW family of adhesins. PCR analysis of 49 epidemiologically distinct isolates revealed that all strains possessing *hmw* genes as determined by Southern analysis contain two *hmw* loci in conserved, unlinked physical locations on the chromosome. Functional analysis of the HMW adhesins produced by three unrelated strains demonstrated that each isolate possesses one protein with HMW1-like adherence properties and another with HMW2-like adherence properties. These findings suggest that the *hmw1* and *hmw2* loci may have arisen via a gene duplication event in an ancestral strain. In addition, they support the hypothesis that the distinct binding specificities of HMW1 and HMW2 emerged early and have persisted over time, suggesting an ongoing selective advantage.

Nontypeable *Haemophilus influenzae* (NTHi) strains are commensal organisms in the nasopharynx and are also a frequent cause of localized respiratory tract disease, including otitis media, conjunctivitis, sinusitis, pneumonia, and exacerbations of chronic bronchitis (13, 29, 38). The pathogenesis of NTHi disease begins with colonization of the nasopharynx, followed by contiguous spread within the respiratory tract. Successful colonization requires that the organism overcome the mucociliary escalator, a task accomplished in part by adherence to respiratory epithelium (29, 38). NTHi adherence is mediated by both pilus and nonpilus adhesins. In experiments with cultured human epithelial cells, the major nonpilus adhesins were found to be HMW1/HMW2 and Hia (20, 22, 33, 35). Based on examination of several collections of epidemiologically distinct NTHi strains, approximately 75 to 80% of isolates produce HMW1/HMW2-like proteins, while most of the remaining isolates produce Hia (7, 20, 36). Of note, isolates produce either HMW1/HMW2-like proteins or Hia, but not both (7, 20, 36).

The HMW adhesins were first identified as major targets of the human serum antibody response during acute otitis media (4). The prototype proteins are designated HMW1 and HMW2 and are produced by NTHi strain 12, the strain from which they were originally cloned and sequenced (5). HMW1 and HMW2 are encoded by separate chromosomal loci, with each locus consisting of three genes, designated *hmwA*, *hmwB*, and *hmwC*. The *hmwA* genes encode the surface-exposed adhesins (HMW1 and HMW2), and the *hmwB* and *hmwC* genes encode accessory proteins required for processing and secretion of the adhesins (5, 6, 15, 16, 32, 35). Based on the predicted amino acid sequences, HMW1 and HMW2 exhibit 71% identity and 80% similarity, while HMW1B/HMW2B and HMW1C/HMW2C are 99 and 97% identical, respectively (5, 6). Despite the high degree of amino acid sequence homology, HMW1 and HMW2 differ in their binding specificities in assays assessing adherence to a variety of cultured epithelial cell types. In assays with isogenic strain 12 derivatives lacking one or both of the HMW proteins and with *Escherichia coli* transformants expressing HMW1 or HMW2, HMW1 mediates high levels of adherence to most human epithelial cell lines examined while HMW2 mediates appreciable levels of adherence to only a subset of epithelial cell lines (11, 33, 35).

Investigation to date has focused primarily on HMW1 and HMW2 from strain 12, and thus relatively little is known about the HMW adhesins produced by other NTHi isolates. In a recent study, we examined 59 epidemiologically distinct NTHi isolates by Southern analysis and found that 47 (80%) had sequence that hybridized with a 5′ fragment of *hmw1A*. Of these 47, 45 were capable of adherence to Chang epithelial cells and expressed at least one protein that reacted with an HMW-specific antiserum (36). In a similar study, van Schilfgaarde et al. examined 58 NTHi isolates obtained from patients with otitis media or chronic obstructive pulmonary disease and from healthy volunteers for the ability to adhere to two epithelial cell types (40). Thirty-two of the isolates were capable of adherence to two different epithelial cell lines, and 23 of the 32 were HMW protein or *hmw* gene positive. Addi-

* Corresponding author. Mailing address: Department of Pediatrics, Washington University Medical School, 660 S. Euclid Ave., Campus Box 8208, St. Louis, MO 63110. Phone: 314-286-2887. Fax: 314-286-2895. E-mail: stgeme@borcim.wustl.edu.

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‡ Dedicated to the memory of Katie Burmeister.

tional experimentation revealed five different adherence patterns based on the inhibiting effect of dextran sulfate (40). However, neither of these studies addressed whether diverse NTHi isolates typically contain two distinct HMW adhesins and whether these proteins have binding properties similar to those described for the prototype HMW1 and HMW2 adhesins of strain 12.

In the present study we set out to define more clearly the evolutionary and functional relationships between the NTHi strain 12 HMW1 and HMW2 proteins and other members of the HMW family of adhesins. Using a PCR approach, we found that all strains possessing sequence homologous to *hmw1A* contain two *hmw* loci. These loci are present at conserved, but unlinked, locations on the chromosome. Further analysis revealed that heterogeneous strains express one protein with HMW1-like adherence properties and another with HMW2-like adherence properties. These findings support the hypothesis that the distinct cellular binding specificities of HMW1 and HMW2 emerged early in evolution and have persisted over time, suggesting ongoing selective pressure.

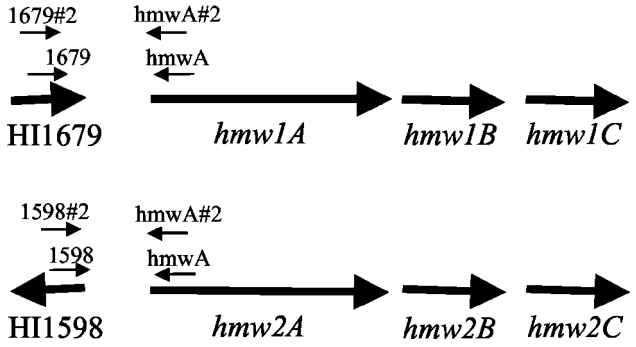

FIG. 1. Schematic representation of PCR primer location used for analysis of numbers and chromosomal locations of *hmw* loci from 49 epidemiologically and genetically distinct NTHi clinical isolates. Diagram is based on locations and organization of the *hmw1* and *hmw2* loci in NTHi strain 12. Small arrows represent primers used in PCR analysis. Arrowheads designate direction of ORF transcription. HI1679 and HI1598 are designations based on the published sequence of *H. influenzae* strain Rd.

MATERIALS AND METHODS

Culture and storage conditions. *H. influenzae* strains were grown on chocolate agar supplemented with 1% IsoVitaleX or in brain heart infusion broth supplemented with hemin and NAD and were stored at −80°C in brain heart infusion broth with 20% glycerol. *E. coli* strains were grown on Luria-Bertani (LB) agar or in LB broth and were stored at −80°C in LB broth plus 50% glycerol. For *E. coli*, antibiotic concentrations used to select for plasmids included ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, and chloramphenicol at 30 μg/ml. For *H. influenzae*, kanamycin was used at 25 μg/ml to select for transformants.

Bacterial strains. Nontypeable *H. influenzae* strains 12, 5, and 15 are clinical isolates recovered from patients with acute otitis media. Strain 12 is the strain from which the *hmw* loci were first cloned and sequenced. Derivatives of strain 12 and strain 5 that lack expression of HMW1, HMW2, or both HMW1 and HMW2 have been described previously (35). Derivatives of strain 15 that lack expression of the HMW1-like, the HMW2-like, or both the HMW1-like and HMW2-like adhesins were constructed by transformation with pHMW1-16 (35) linearized with XbaI and then selecting for kanamycin-resistant colonies. The presence of single or double kanamycin inserts was confirmed by PCR, by Southern analysis using a labeled kanamycin cassette as a probe, and by Western analysis of whole-cell sonicates using anti-HMW antiserum. As a source of additional NTHi strains, we used a collection of 47 epidemiologically and genetically diverse clinical isolates that have been characterized by multilocus enzyme electrophoresis (25) and are known to contain genomic sequence that hybridizes with an intragenic fragment of *hmw1A* (36). Within this collection, 64% of strains were cultured from middle ear effusions, 32% of strains were recovered from blood, and 4% were recovered from cerebrospinal fluid (25). NTHi strain 11 is the strain from which *hia* was first cloned and sequenced and lacks *hmw* genes (7). *H. influenzae* strain Rd is a nonencapsulated former serotype d laboratory strain that has been sequenced in its entirety and lacks the *hmw* genes (31). DH5α is a laboratory strain of *E. coli* that is nonadherent to Chang, HaCaT, HEp-2, and NCI-H292 epithelial cells (Life Technologies).

Recombinant DNA methods. Chromosomal DNA extractions, DNA ligations, restriction endonuclease digestions, and gel electrophoresis were performed according to standard techniques (30). Plasmids were introduced into *E. coli* by electroporation (12). *H. influenzae* was made competent for transformation by the MIV method of Herriott et al. (17).

PCR analysis of *hmw* loci. Nucleotide sequences of the regions upstream of the *hmw1* and *hmw2* gene clusters were determined by sequencing lambda phage clones from a lambda library of NTHi strain 12 (5). In NTHi strain 12, open reading frame (ORF) HI1679 is located upstream of the *hmw1* locus, and ORF HI1598 is located upstream of the *hmw2* locus (ORFs HI1679 and HI1598 were originally identified in *H. influenzae* strain Rd). In order to determine the presence and physical locations of the *hmw* loci in other strains, PCR assays were performed using chromosomal DNA as the template and a 5′ primer which anneals to the 3′ end of ORF HI1679 (corresponding to nucleotides 361 to 385 of the strain Rd HI1679 coding sequence) or the 3′ end of ORF HI1598 (corresponding to nucleotides 451 to 475 of the strain Rd HI1598 coding sequence) and a 3′ primer which anneals to conserved sequence in the 5′ region of both *hmw1A* and *hmw2A* (corresponding to nucleotides 490 to 514 of the strain 12 *hmw1* and *hmw2* loci) (Fig. 1). PCR conditions included initial denaturation at 92°C for 2 min, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min and 30 s, with a final extension at 72°C for 7 min. Products were resolved by electrophoresis on 1% agarose gels. NTHi strain 12 served as a positive control, and strains 11 and Rd served as negative controls. Initially, PCR analysis of strain 3184A yielded a product with the HI1679-hmwA primer set but not with the HI1598-hmwA primer set, and PCR analysis of strain 1276 was negative with both primer sets. To determine whether sequence variation at the primer binding sites was the reason for the lack of product, additional primers were used, namely primer HI1679#2 (corresponding to nucleotides 195 to 219 of the strain Rd HI1679 coding sequence), primer HI1598#2 (corresponding to nucleotides 652 to 673 of the strain Rd HI1598 coding sequence), and primer hmwA#2 (corresponding to nucleotides 384 to 409 of the strain 12 *hmw1* and *hmw2* loci) (Fig. 1).

DNA and protein sequence analysis. DNA sequencing was performed using the BigDye terminator cycle sequencing kit following the manufacturer's instructions (Applied Biosystems, Foster City, Calif.).

Comparisons of the amino acid sequences of the HMW adhesins from NTHi strains 12, 5, and 15 were performed using the BLAST 2 sequence (bl2seq) interface on the National Center for Biotechnology Information (NCBI) website. The following parameters were used for amino acid comparisons: Alignments, BlastP; Matrix, Blosum62; Open gap, 11; extension gap penalties, 1; gapx_dropoff, 50; filter, off.

For generation of phylogenetic trees, sequences were first aligned by the using T-Coffee method (26). Phylogenetic trees were constructed with MEGA version 2.1 (21), using neighbor joining with Poisson-corrected distances. Trees were unrooted or midpoint rooted, and bootstrapping was performed using 1,000 replicates.

Construction of plasmids containing *hmwA* genes from NTHi isolates. The *hmwA* genes from NTHi strains 12, 5, and 15 were amplified by using primer HI1679-BglII (primer HI1679 with a BglII site engineered on the 5′ end) or primer HI1598-BglII (primer HI1598 with a BglII site engineered on the 5′ end) as a 5′ primer and primer hmwB-BglII or primer hmwB-SalI (corresponding to nucleotides 5154 to 5175 of the strain 12 *hmw1* locus and nucleotides 4973 to 4995 of the strain 12 *hmw2* locus) as a 3′ primer. The PCR products were digested with the appropriate restriction enzyme(s) and ligated into BamHI- or BamHI/SalI- digested pACYC184 (9). The presence of the insert was confirmed by PCR and restriction analysis. Plasmids containing *hmwA* genes downstream of either HI1598 or HI1679 were transformed into DH5α containing pHMW1BC, which encodes the *hmw1B* and *hmw1C* accessory genes, as described previously (32).

Adherence assays. Adherence assays were performed as described previously (35). Briefly, Chang (human conjunctiva; ATCC CCL20.2 [Wong-Kilbourne derivative, clone 1-5c-4]), HaCaT (derived from human keratinocytes) (8), HEp-2 (human laryngeal epidermoid carcinoma; ATCC CCL 23), and NCI-H292 (human lung mucoepidermoid carcinoma; ATCC CRL1848) cells were seeded into wells of 24-well tissue culture plates and grown to confluency. Bacteria were inoculated into broth and allowed to grow to a density of approx-

imately 2×10^9 CFU per ml. Approximately 2×10^7 CFU was inoculated onto viable epithelial cell monolayers, and plates were gently centrifuged at 165 x *g* for 5 min to facilitate contact between the bacteria and the epithelial cells. After incubation for 30 min at 37°C in 5% CO_2, monolayers were rinsed four times with phosphate-buffered saline to remove nonadherent organisms. Trypsin-EDTA (0.05% trypsin, 0.5% EDTA) was added to the wells to release epithelial cells and adherent bacteria. Dilutions of adherent organisms were plated on solid medium to determine the number of adherent bacteria per monolayer. Accordingly, 100% adherence corresponds to $\sim 2 \times 10^7$ CFU per monolayer. Percent adherence was calculated by dividing the number of adherent CFU per well by the number of inoculated CFU. Each strain was examined in triplicate in a given assay, and assays were performed a minimum of three times.

Inhibition of adherence with MAA. Inhibition of HMW1 and HMW1-like mediated adherence with *Maackia amurensis* agglutinin (MAA) was carried out essentially as described previously (37). Briefly, epithelial cells were seeded into wells of a 24-well tissue culture plate. Confluent monolayers were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C for 2 h and then rinsed three times with Tris-buffered saline. Fixed monolayers were incubated with or without MAA (Sigma) at a concentration of 5 μg/ml in PBS–1% bovine serum albumin–0.25% Triton X-100 at 4°C overnight. The MAA solution was removed and replaced with serum-free minimal essential medium, and adherence assays were carried out as described above.

Western blots. Whole-cell sonicates of DH5α transformants expressing HMW proteins and of NTHi derivatives were prepared by resuspending bacterial pellets in 10 mM HEPES, pH 7.4, and sonicating to clarity. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5 or 10% polyacrylamide gels. Western blotting was performed with guinea pig polyclonal antiserum GP75, which was raised against purified HMW1 and recognizes both HMW1 and HMW2 from NTHi strain 12.

Whole-cell (dot) immunoblots. In order to examine HMW1 and HMW2 associated with the bacterial surface in a qualitative manner, whole-cell immunoblotting was performed using guinea pig polyclonal antiserum GP75, as previously described (16).

Nucleotide sequence accession numbers. Nucleotide sequences of the *hmw1A* and *hmw2A* genes from NTHi strains 5 and 15 have been submitted to GenBank and assigned the accession numbers AY497551, AY497552, AY497554, AY497553, respectively.

RESULTS

Examination of the number and physical location of *hmw* loci in genetically distinct NTHi strains. In previous work, Southern hybridization studies performed on a collection of genetically diverse NTHi isolates described by Musser et al. revealed that approximately 80% of the isolates contain sequence that hybridizes with an intragenic fragment of *hmw1A* (25, 36). In the current study, we were interested in determining whether all of these strains contain two *hmw* loci and whether the physical locations of the *hmw* loci are conserved. In addition, we elected to examine NTHi strains 5 and 15. NTHi strain 12 lambda library clones containing the *hmw1* and *hmw2* loci were used as templates to determine sequences upstream of the gene clusters (5). Comparison of these upstream sequences to the genome of *H. influenzae* strain Rd (the laboratory strain that has been sequenced in its entirety) revealed that the *hmw1* locus in strain 12 is situated downstream of an ORF corresponding to HI1679 of Rd and that the *hmw2* locus of strain 12 is located downstream of an ORF corresponding to HI1598 of Rd. Based on this information, we designed primers specific for HI1598 and HI1679 and for a region conserved in *hmw1A* and *hmw2A* (Fig. 1) and performed PCR. Initially, 47 of 49 strains (96%) examined were positive for *hmw* loci downstream of HI1679 and HI1598 using primer sets HI1679-hmwA and HI1598-hmwA. Strains Rd and 11 are known to lack sequence homologous to the *hmw1A* locus of strain 12 and served as negative controls. PCR analysis of strain 3184A yielded a product with the HI1679-hmwA primer set but not with the HI1598-hmwA primer set, and strain 1276 yielded no product with either primer set. To determine whether sequence heterogeneity at the primer binding sites might be the reason for the inability to amplify the intergenic regions in these two strains, additional primers were designed based on sequence upstream of the original primers (Fig. 1), and reactions were repeated. With the new primer sets (HI1679#2-hmwA#2 and HI1598#2-hmwA#2), PCR products were generated for strains 3184A and 1276, indicating the presence of two *hmw* loci in these strains and suggesting that sequence variation and a lack of primer binding is a plausible explanation for the preliminary negative results. These data indicate that genetically diverse NTHi strains with sequence homologous to *hmw1A* by Southern hybridization uniformly contain two *hmw* loci (Table 1). Furthermore, the two *hmw* loci are present at conserved, but physically distinct, locations on the chromosome.

TABLE 1. PCR analysis of *hmw* loci from diverse NTHi isolates

PCR primer set	No. of strains (%)
HI1679-hmwA	49 (100%)
HI1598-hmwA	49 (100%)
~0.8-kb product with HI1679-hmwA	35 (71%)
~1.7-kb product with HI1679-hmwA	14 (29%)
~1.0-kb product with HI1598-hmwA	49 (100%)

Interestingly, the PCR product corresponding to the region between HI1679 and the *hmw* locus was ~1.7 kb in 29% (14 of 49) of the strains and ~0.8 kb in the remaining 71% (35 of 49) of the strains (Table 1). Nucleotide sequencing was carried out on the ~1.7-kb PCR product from eight strains and on the ~0.8-kb PCR product from five strains, and BlastX analysis was performed using the NCBI interface. All 1.7-kb sequences had evidence of transposase gene remnants. As an example, the HI1679-hmwA intergenic region in strain 3219C is predicted to encode sequence that shares 57% identity and 70% similarity with a 95-amino-acid region (E value, 5e-22) of IS200 transposase A of *Helicobacter pylori* (data not shown). In contrast, the ~0.8-kb products shared no homology with transposases. PCR products generated with the HI1598-hmwA primer set were all ~1.0 kb. Products from five strains were sequenced, and none had sequence homologous to transposase or insertion sequence element genes.

Examination of the binding specificities of HMW adhesins from diverse NTHi clinical isolates. In an effort to understand the functional relationships between HMW1 and HMW2 from strain 12 and other members of the HMW family of adhesins, we examined the binding properties of the HMW adhesins from two additional clinical isolates, using HMW1 and HMW2 from strain 12 as controls. The *hmwA* genes located downstream of HI1679 and HI1598 were PCR amplified from NTHi strains 12, 5, and 15 and cloned into pACYC184. These plasmid constructs were coexpressed with pHMW1BC in DH5α. Expression of the adhesin was verified by Western blotting (Fig. 2A), and localization of the adhesin on the bacterial surface was verified by whole-cell dot immunoblot analysis using a polyclonal antiserum that recognizes HMW1 and HMW2 from strain 12 (Fig. 2B). Expression levels of the HMW adhesins from strains 12, 5, and 15 were similar. The

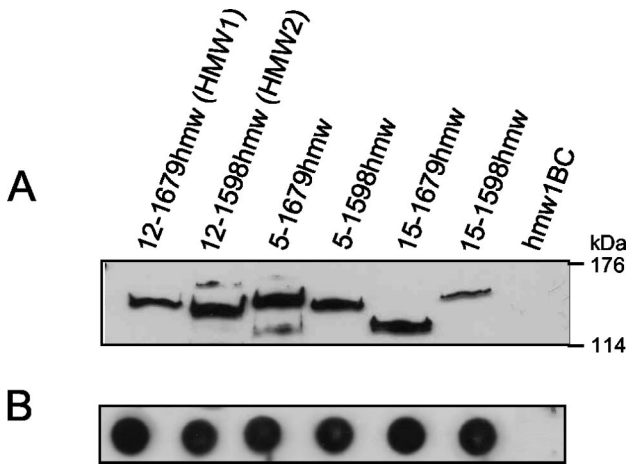

FIG. 2. Expression and surface localization of HMW adhesins expressed in DH5α. (A) Western blot of whole-cell sonicates of DH5α expressing 12-1679*hmw*, 12-1598*hmw*, 5-1679*hmw*, 5-1598*hmw*, 15-1679*hmw*, or 15-1598*hmw* coexpressed with *hmw*1BC were separated via SDS–PAGE, transferred to nitrocellulose, and probed with GP75 (polyclonal antiserum raised against HMW1 from strain 12). The blot shows the fully processed, mature adhesins. (B) Surface localization of the adhesins is shown via whole-cell dot immunoblotting of the strains depicted in panel A, probing with GP75.

binding specificity of each HMW adhesin was then examined in adherence assays with a panel of epithelial cells. Consistent with previous results, strain 12 HMW1 (12-1679*hmw*) mediated high-level adherence to Chang, HEp-2, HaCaT, and NCI-H292 epithelial cells, while strain 12 HMW2 (12-1598*hmw*) mediated high-level adherence to HaCaT and NCI-H292 cells but negligible adherence to Chang and HEp-2 cells (Fig. 3) (11, 33, 35). The adhesins cloned from strains 5 and 15 exhibited a similar pattern, with one adhesin mediating high-level adherence to all four cell lines (5-1598*hmw* and 15-1598*hmw*) and the second adhesin (5-1679*hmw* and 15-1679*hmw*) mediating high-level adherence to HaCaT and NCI-H292 cells and negligible adherence to Chang and HEp-2 cells (Fig. 3).

In previous work, our group found that HMW1 interacts with glycoprotein receptors containing α2,3-linked sialic acid on Chang and HaCaT epithelial cells (37). As a consequence, preincubation of epithelial cells with the lectin MAA results in a marked decrease in HMW1-mediated adherence, reflecting the fact that MAA binds to α2,3-linked sialic acid (11, 37). In contrast, MAA has minimal effect on HMW2-mediated adherence (11). In order to obtain additional evidence that the HMW adhesins from strains 5 and 15 exhibit either HMW1-like or HMW2-like binding specificity, we used MAA to specifically inhibit HMW1-mediated adherence. As shown in Fig. 4A, preincubation of Chang cells with MAA resulted in an ~80% reduction in strain 12 HMW1-mediated adherence. Similarly, adherence of *E. coli* derivatives expressing 5-1598*hmw* and 15-1598*hmw* to Chang cells was reduced by ~80% when compared with untreated controls (Fig. 4A). In experiments with HaCaT cells, MAA again inhibited adherence by strain 12 HMW1 (12-1679*hmw*), 5-1598*hmw*, and 15-1598*hmw* by ~80% (Fig. 4B). DH5α expressing strain 12

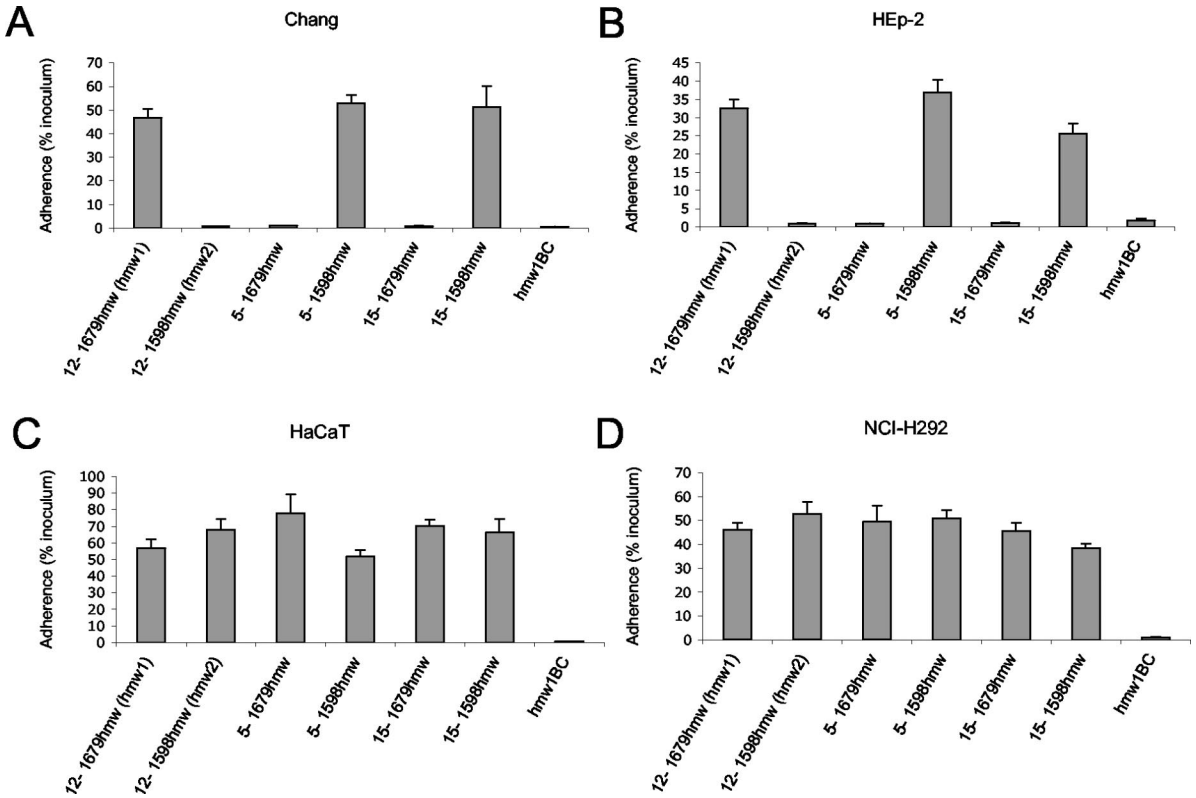

FIG. 3. Adherence properties of HMW adhesins from NTHi strains 12, 5, and 15 expressed in DH5α. Graphs represent adherence, as a percentage of the inoculum, to Chang cells (A), HEp-2 cells (B), HaCaT cells (C), and NCI-H292 cells (D). Values represent the averages ± standard errors of three experiments, each performed in triplicate.

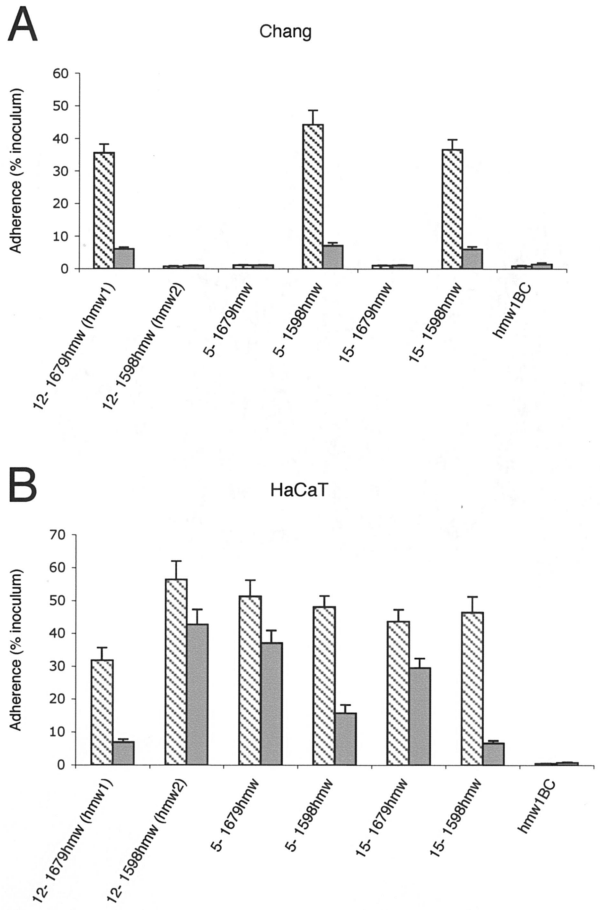

FIG. 4. Inhibition of adherence with MAA. Monolayers of Chang cells (A) and HaCaT cells (B) were untreated (hatched bars) or preincubated with 5 μg of MAA/ml (shaded bars). Adherence of DH5α expressing HMW adhesins from NTHi strains 12, 5, and 15 was assessed in a 30-min adherence assay. Adherence is expressed as a percentage of the inoculum. Values represent the averages ± standard errors from three experiments, each performed in triplicate.

HMW2 (12-1598*hmw*), 5-1679*hmw*, or 15-1679*hmw* adhered at high levels to HaCaT cells in the absence of MAA and exhibited only a small reduction in adherence in the presence of MAA (~25% reduction compared to the untreated control) (Fig. 4B). This partial reduction in adherence may reflect nonspecific steric effects of MAA on accessibility of the HMW2 receptor. These studies indicate that adherence by one of the HMW adhesins expressed by a given strain is significantly inhibited in the presence of MAA, while adherence by the other adhesin is only minimally affected, effectively distinguishing the two adhesins from each other.

Taken together, the patterns of adherence to Chang, HEp-2, HaCaT, and NCI-H292 cells and the MAA inhibition phenotypes indicate that strains 5 and 15 express one HMW adhesin with HMW1-like binding properties and a second HMW adhesin with HMW2-like binding properties. Interestingly, in both strains 5 and 15, the *hmw1*-like locus lies downstream of HI1598 and the *hmw2*-like locus resides downstream of HI1679, opposite to the arrangement in strain 12.

Examination of the binding properties of the HMW1-like and HMW2-like adhesins expressed in *H. influenzae*. To address whether the HMW1-like and HMW2-like adhesins behave the same in *H. influenzae* as they do in *E. coli*, we compared NTHi strains 12, 5, and 15 and isogenic mutants lacking the HMW1-like protein, the HMW2-like protein, or both. As shown in Fig. 5, Western analysis using a polyclonal antiserum reactive with both HMW1 and HMW2 confirmed the phenotypes of all of these strains. Examination of the wild-type strains in assays with Chang, HEp-2, HaCaT, and NCI-H292 cells revealed strain-to-strain variation in the levels of adherence (ranging from ~30 to ~75% of the inoculum) (Fig. 6). Despite this variation, comparison of *hmw2* and *hmw1 hmw2* mutants allowed assessment of the contribution of the HMW1-like adhesins to adherence, and comparison of *hmw1* and *hmw1 hmw2* mutants allowed assessment of the contribution of the HMW2-like adhesins to adherence. Consistent with our results with *E. coli* transformants, the HMW1-like adhesins from all three strains mediated high-level adherence to Chang, HEp-2, HaCaT, and NCI-H292 cells, and the HMW2-like adhesins from all three strains mediated high-level adherence to HaCaT and NCI-H292 cells (Fig. 6). Interestingly, while *E. coli* transformants expressing the HMW2-like adhesins were unable to adhere to Chang and HEp-2 cells above background levels (Fig. 3A and B), in *H. influenzae* the HMW2-like proteins mediated appreciable levels of adherence to these two cell types (ranging from ~10 to ~50% of the inoculum) (Figs. 6A and 6B). Derivatives lacking both the HMW1-like protein and the HMW2-like protein adhered at negligible levels to all four cell lines, emphasizing the important role of these proteins in adherence.

Examination of the relationship between HMW protein sequence and binding specificity. As it appears that *hmw*-containing strains express one adhesin with HMW1-like binding specificity and a second adhesin with HMW2-like binding specificity, we reasoned that protein sequence might be predictive of binding specificity. The mature HMW adhesins of strains 12, 5, and 15 (corresponding to fully processed adhesins lacking the signal sequence and cleaved at the predicted propeptide

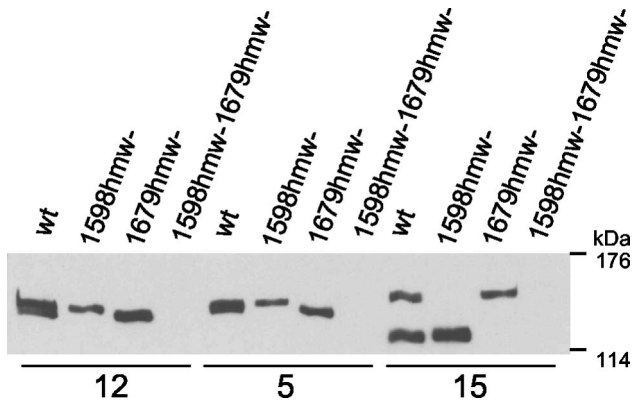

FIG. 5. Western blot of NTHi isogenic derivatives. Whole-cell sonicates of the parent strain (wt) and 1598*hmw*, 1679*hmw*, and 1598*hmw* 1679*hmw* derivatives of NTHi strains 12, 5, and 15 were separated via SDS–7.5% PAGE, transferred to nitrocellulose, and probed with GP75 (polyclonal antiserum raised against HMW1 from strain 12). The blot shows the fully processed, mature adhesins.

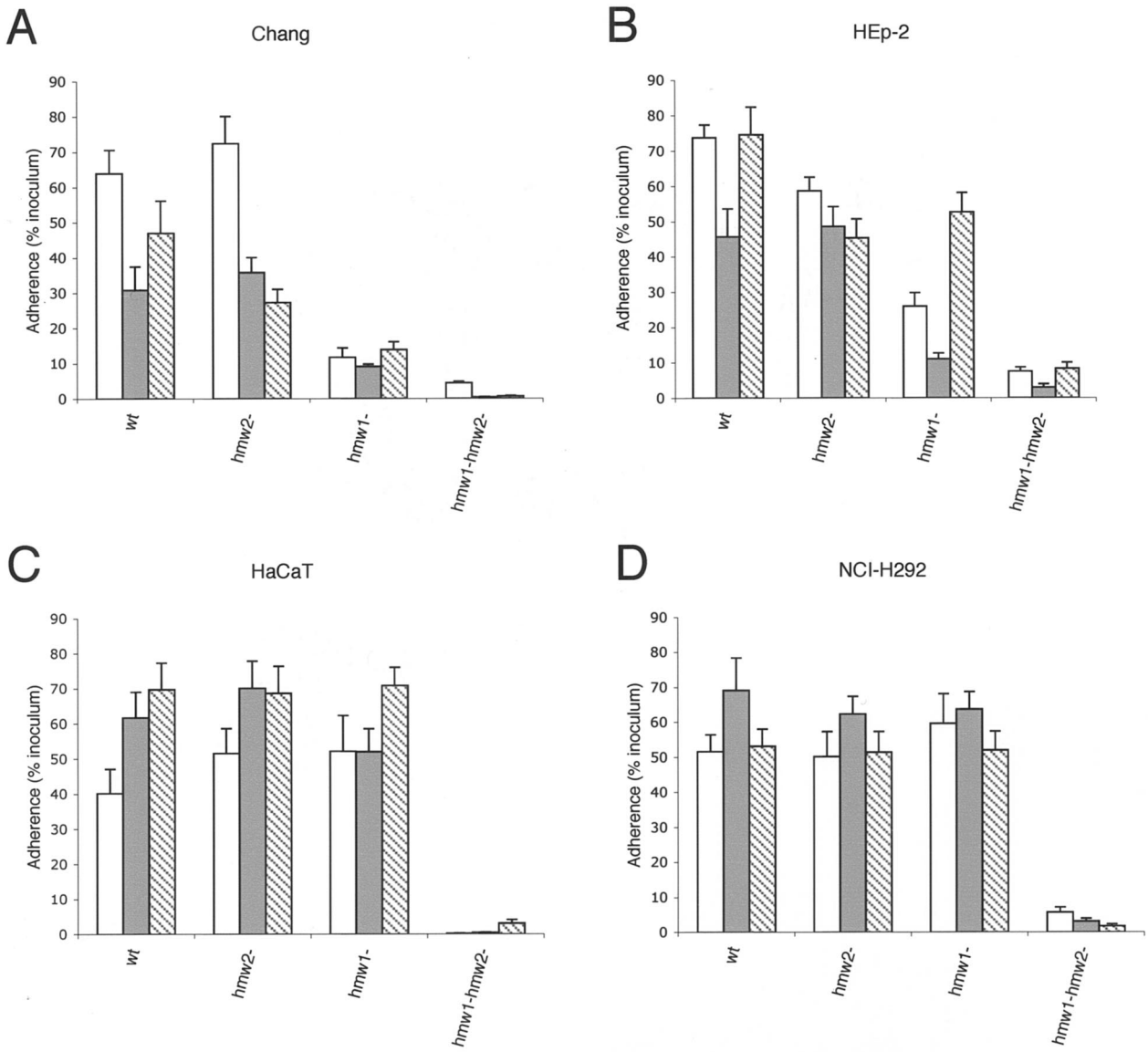

FIG. 6. Adherence properties of isogenic *hmw* derivatives in NTHi strains 12, 5, and 15. Graphs represent adherence of isogenic derivatives of strain 12 (open bars), strain 5 (shaded bars) and strain 15 (hatched bars), as a percent of the inoculum, to Chang cells (A), HEp-2 cells (B), HaCaT cells (C), and NCI-H292 cells (D). Values represent the averages ± standard errors of three experiments each performed in triplicate. *hmw2*, *hmw1*, and *hmw1 hmw2* designations are based on phenotypes of the adhesins as established for Fig. 3 and 4.

cleavage site) (5) were found to vary considerably in length both within individual strains and between different strains. The strain 15 HMW mature adhesins range in size from 992 amino acids (15-1679*hmw*) to 1,180 amino acids (15-1598*hmw*), a >15% size difference, while the strain 5 adhesins are 1,158 amino acids (5-1679*hmw*) and 1,161 amino acids (5-1598*hmw*) in length, a 0.26% difference.

Using the BLAST interface of the NCBI website, pairwise comparisons of each predicted amino acid sequence were made, allowing comparison of each sequence to all others. Designations of HMW1 or HMW2 were made based on the analysis of adherence properties presented in Fig. 3, 4, and 6. When amino acid sequences corresponding to the predicted mature, fully processed adhesin were compared, amino acid similarity and identity between HMW1-like adhesins (~72 and ~62%, respectively) and between HMW2-like adhesins (~69 and ~59%, respectively) were slightly greater than the similarity and identity observed between sequences of HMW1-like and HMW2-like adhesins (~63 and ~52%, respectively) (Table 2). In a previous study, Dawid et al. established that binding activity of strain 12 HMW1 and HMW2 resides in an ~360-amino-acid region, corresponding to the region of maximal dissimilarity between these proteins (11). The binding domains of the strain 5 and strain 15 HMW adhesins were defined as the amino acid sequences corresponding to the known HMW1 and HMW2 binding domains obtained by using ClustalW alignment. The HMW1-like binding domains were found to share ~74% similarity and ~63% identity, and the HMW2-like bind-

TABLE 2. Pairwise comparisons of predicted amino acid sequences of HMW adhesins from NTHi strains 12, 5, and 15

Comparison[a]	% Similarity ± SD	% Identity ± SD	% Gaps ± SD
HMW1 vs. HMW1 mature[b]	72 ± 4	62 ± 6	8 ± 1
HMW2 vs. HMW2 mature	69 ± 5	59 ± 5	11 ± 6
HMW1 vs. HMW2 mature	63 ± 7	52 ± 7	12 ± 5
HMW1 vs. HMW1 binding domain[c]	74 ± 4	63 ± 5	2 ± 1
HMW2 vs. HMW2 binding domain	69 ± 3	54 ± 3	2 ± 1
HMW1 vs. HMW2 binding domain	50 ± 2	35 ± 2	7 ± 1

[a] Comparisons were done using the BLAST interface on the NCBI website.
[b] Mature refers to the protein sequence after cleavage at the known and predicted cleavage sites (corresponding to the peptide bond between amino acids 441 and 442 of strain 12 HMW1 and HMW2).
[c] Binding domain refers to the protein sequence corresponding to the binding domains of strain 12 HMW1 and HMW2 as defined by Dawid et al. (11).

ing domains were found to share ~69% similarity and ~54% identity. In contrast, comparison of the HMW1-like and the HMW2-like binding domains revealed ~50% amino acid similarity and ~35% identity. These observations suggest conservation of sequence critical for a particular binding specificity.

To extend this analysis, we generated phylogenetic trees. Sequences were aligned by the using T-Coffee method (26), and phylogenetic trees of the mature HMW adhesins and the HMW binding domains were made by using neighbor joining with Poisson-corrected distances implemented in MEGA version 2.1 (21). Initially, unrooted trees were generated with 1,000 bootstrap replicates, revealing separation of the HMW1-like mature proteins from the HMW2-like mature proteins and the HMW1-like binding domains from the HMW2-like binding domains (data not shown). With this information in mind, for the sake of clarity, midpoint-rooted trees were generated. As shown in Fig. 7A, the mature HMW1 protein sequences clustered together with a bootstrap confidence level of 100, and the mature HMW2 protein sequences clustered together with bootstrap confidence levels of 100 and 99. Similarly, the sequences of the HMW1-like binding domains clustered together with bootstrap confidence levels of 100 and 86, and the sequences of the HMW2-like binding domains clustered together with a bootstrap confidence level of 98 (Fig. 7B).

DISCUSSION

In this study, we analyzed a collection of 49 epidemiologically and genetically distinct NTHi isolates containing sequences homologous to an intragenic fragment of *hmw1A* from strain 12. In all isolates, we found evidence of two *hmw* loci, with one locus downstream of ORF HI1598 and the other locus downstream of ORF HI1679. In addition, we examined the adherence properties of the HMW adhesins from three different strains and in all three identified one adhesin with HMW1-like binding specificity and a second adhesin with HMW2-like binding specificity.

The presence of two closely related *hmw* loci in individual isolates of NTHi suggests that a gene duplication event occurred. Furthermore, the finding that the *hmw* loci are present at conserved physical locations on the chromosome in all *hmw*-containing isolates examined to date suggests that movement of the duplicated locus may have occurred early in the evolution of NTHi. In this context, it is interesting to note the similarity between our observations with the HMW adhesins and studies of the *Helicobacter pylori* BabA and BabB adhesins, which share striking homology with each other and appear to have arisen by an analogous ancestral gene duplication event (1, 28).

It is notable that transposon remnants are present between HI1679 and the downstream *hmw* locus in nearly 30% of strains, indicating a possible mechanism for acquisition of the original *hmw* locus or movement of the duplicated *hmw* locus. Using the genomic sequence of *H. influenzae* strain Rd for orientation, the strain 12 *hmw1* locus is flanked upstream by HI1679 and downstream by HI1680. In contrast, the strain 12 *hmw2* locus is flanked upstream by HI1598 and downstream by unrecognizable sequence, at least over the first ~225 nucleotides. However, closer examination of the *hmw2* downstream sequence reveals a 31-nucleotide stretch that corresponds to the end of HI1680. This finding suggests that the HI1679-HI1680 *hmw* junction may represent the location of the progenitor *hmw* locus, with duplication including the end of HI1680.

Based on examination of strains 12, 5, and 15, it appears that chromosomal location of a given *hmw* locus is not predictive of the binding specificity of the associated adhesin. In particular, in strain 12 the *hmw1*-like locus is downstream of HI1679 and the *hmw2*-like locus is downstream of HI1598, while in NTHi strains 5 and 15 the opposite is true. Given that the HMW adhesins share a high degree of amino acid identity at their N-terminal and C-terminal ends, it is possible that the strain-to-strain variability in physical locations of the *hmw1*-like and

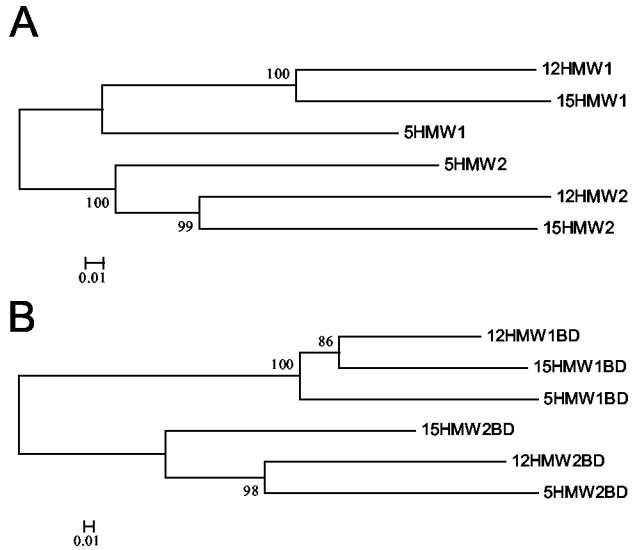

FIG. 7. Phylogenetic analysis of HMW mature and binding domain amino acid sequences from NTHi strains 12, 5, and 15. Midpoint rooted phylogenetic trees were constructed with MEGA version 2.1 (21) using the neighbor joining method with Poisson-corrected distances. Bootstrap confidence values are shown at the branches. Scale bars represent the number of amino acid substitutions per site. (A) Phylogenetic tree based on the mature HMW amino acid sequences as defined in Table 2. (B) Phylogenetic tree based on the binding domain of the HMW adhesins from strains 12, 5, and 15 as defined in Table 2.

the *hmw2*-like loci is due to swapping of coding sequence for the binding domains, potentially via an intrastrain recombination event, as has also been proposed to explain the opposite locations of the *H. pylori babA* and *babB* genes in strains J99 and 26695 (1, 2, 18).

Recognizing that the strain 12 HMW1 and HMW2 adhesins have distinct cellular binding specificities, we wondered whether all adhesins in the HMW family can be classified as HMW1-like or HMW2-like based on binding properties. Our analysis of *E. coli* transformants expressing the *hmw* loci from NTHi strains 5 and 15 indicates that both of these isolates possess one protein with HMW1-like adherence properties and another with HMW2-like adherence properties, suggesting that the HMW adhesins indeed fall into two distinct subfamilies. Consistent with these results, phylogenetic analysis of the binding domains of seven additional HMW adhesins reveals clustering into an HMW1-like group and an HMW2-like group (See Fig. S1 in the supplemental material). Nevertheless, given the relatively limited scope of our studies, we cannot exclude the possibility that hybrid HMW proteins exist, analogous to the *Moraxella catarrhalis* UspA2H protein, which is present in approximately 20% of *M. catarrhalis* isolates and possesses the properties of both the UspA1 adhesin and the UspA2 serum resistance factor (23).

In adherence assays examining *E. coli* transformants, HMW2 mediated negligible adherence to Chang and HEp-2 cells. In contrast, in assays examining *H. influenzae* derivatives, HMW2 was capable of promoting appreciable adherence to Chang and HEp-2 cells. In considering this discrepancy, it is possible that levels of HMW2 expression are higher in *H. influenzae* than in *E. coli*. However, comparison of strains by Western analysis failed to support this idea. In a recent report, our group demonstrated that HMW1 and HMW2 are glycosylated (15). It is possible that the HMW adhesins are differentially glycosylated when expressed in *E. coli* and that these differences influence the ability of the adhesin to interact with some, but not all, cell types. Alternatively, *H. influenzae* may possess a coadhesin or some other factor that augments HMW2-mediated adherence to certain cell types, either by interacting cooperatively with the HMW2 receptor or by triggering up-regulation of the HMW2 receptor. Along these lines, in *Bordetella pertussis* the RGD motif in filamentous hemagglutinin interacts with the leukocyte response integrin/integrin-associated protein receptors on monocytes and up-regulates expression of CR3, allowing enhanced binding between a separate domain on filamentous hemagglutinin and the CR3 receptor (19). Similarly, in *H. pylori*, chronic infection of the gastric mucosa stimulates upregulation of sialyl-Lewis x antigen, which is recognized by the SabA adhesin, resulting in intimate adherence (24).

There appears to be some strain-to-strain variation in the levels of adherence among NTHi derivatives expressing the HMW adhesins, variation that is less apparent when the same adhesins are expressed in *E. coli*. The variation is most marked in assays with HEp-2 cells examining NTHi derivatives expressing only HMW2, with levels ranging from ~10% (strain 5) to ~50% (strain 15). The differences in adherence were not due to variation in the quantity of protein, as examination of the promoter region of the *hmwA* genes in strains 12, 5, and 15 revealed a similar number of 7-bp repeats, which are known to influence the quantity of HMW protein produced (data not shown) (10). Differential glycosylation of the HMW adhesins by NTHi may provide a partial explanation for the variation in adherence patterns between strains and is currently under investigation. Alternatively, the observed differences in adherence of the NTHi derivatives may reflect variation in other surface molecules typically capable of promoting low-level adherence, such as lipopolysaccharide, OapA, and Hap (27, 34, 39). Beyond promoting adherence, these surface molecules may interfere sterically with HMW-mediated adherence.

Based on sequence data and phenotypic analysis, it appears that the HMW proteins are under two forms of selective pressure, with different consequences. On the one hand, the HMW adhesins stimulate an antibody response that selects for amino acid sequence divergence (4). At the same time, all strains appear to have one HMW adhesin with HMW1-like adhesive properties and a second HMW adhesin with HMW2-like adhesive properties, suggesting selective pressure to maintain both adhesive specificities. In this context, it is noteworthy that efforts to develop a vaccine effective against NTHi disease have been impeded by extensive genetic diversity among strains and among potential vaccine antigens (13, 14). However, Barenkamp has demonstrated that purified HMW1 and HMW2 are partially protective against challenge by strain 12 in the chinchilla otitis media model and may be useful as components of a multicomponent NTHi vaccine (3). One possibility would be to include the binding domains of both HMW1 and HMW2, aiming to stimulate antibody that blocks adherence.

In summary, all NTHi clinical isolates examined to date with *hmw* sequences by Southern analysis harbor two *hmw* loci. These two loci encode adhesins with distinct cellular binding specificities, similar to those described for NTHi strain 12. We speculate that the *hmw1* and *hmw2* loci were generated by a gene duplication event that occurred early in the evolution of NTHi. This duplication and subsequent divergence of the *hmw* sequences resulted in HMW1-like and HMW2-like binding properties, providing a broader adhesive potential for the organism. Expression of both HMW1-like and HMW2-like adhesins has persisted over time, suggesting that broader adhesive potential is associated with an ongoing selective advantage.

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