Serial isolates of persistent Haemophilus influenzae in patients with chronic obstructive pulmonary disease express diminishing quantities of the HMW1 and HMW2 adhesins

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In patients with chronic obstructive pulmonary disease (COPD), the lower respiratory tract is commonly colonized by bacterial pathogens, including nontypeable *Haemophilus influenzae*. The *H. influenzae* HMW1 and HMW2 adhesins are homologous proteins that promote bacterial adherence to respiratory epithelium and are the predominant targets of the host immune response. These adhesins undergo graded phase variation, controlled by the numbers of 7-bp repeats upstream of the HMW1 and HMW2 structural genes (*hmw1A* and *hmw2A*, respectively). In this study, we examined the levels of HMW1 and HMW2 expressed by *H. influenzae* isolates collected serially from patients with COPD. We found that expression of HMW1 and HMW2 in a given strain decreased over time in a majority of patients, reflecting progressive increases in the numbers of 7-bp repeats and associated with high serum titers of HMW1/HMW2-specific antibodies. We speculate that the presence of high titers of antibodies against the HMW1 and HMW2 adhesins and other immune factors in the lower respiratory tracts of patients with COPD may result in gradual selection for bacteria with reduced levels of HMW1 and HMW2.

Nontypeable *Haemophilus influenzae* is a common commensal organism in the human upper respiratory tract and an important cause of human respiratory tract disease (20). The pathogenesis of *H. influenzae* respiratory tract disease begins with bacterial colonization of the nasopharynx followed by contiguous spread to the middle ear, the sinuses, or the lower airways, resulting in localized disease at these sites (14).

Colonization of the respiratory epithelium by bacterial pathogens requires adhesive molecules. The majority of nontypeable *H. influenzae* clinical isolates express adhesins that belong to a family of high-molecular-weight proteins called the HMW1/HMW2 family (3). The prototype HMW1 and HMW2 proteins from *H. influenzae* strain 12 are 71% identical and 80% similar and are the predominant targets of the serum antibody response during *H. influenzae* disease (2, 3). HMW1 and HMW2 are encoded by separate chromosomal loci, with each locus consisting of three genes, designated *hmwA*, *hmwB*, and *hmwC* (4, 5). The *hmwA* genes encode the surface-exposed adhesins, and the *hmwB* and *hmwC* genes encode accessory proteins required for proper processing and secretion of the adhesins (4, 8, 17, 19). Based on examination of a large collection of epidemiologically distinct isolates, all strains with *hmw* genes appear to contain two *hmw* loci in conserved, unlinked physical locations on the chromosome, including one adjacent to open reading frame (ORF) HI1598 and one adjacent to ORF HI1679 (5). Functional analysis of the HMW adhesins produced by a subset of these strains has demonstrated that each isolate possesses one protein with HMW1-like adherence properties and one with HMW2-like adherence properties (5). Interestingly, in some strains the HMW1 adhesin is encoded by the *hmw* locus adjacent to ORF HI1598, and in others, the HMW1 adhesin is encoded by the *hmw* locus adjacent to ORF HI1679 (5). As a corollary, in some strains the HMW2 adhesin is encoded by the *hmw* locus adjacent to ORF HI1679, and in others the HMW2 adhesin is encoded by the *hmw* locus adjacent to ORF HI11598 (5).

Although the HMW1/HMW2 adhesins are critical for mediating attachment to human epithelial cells, these proteins may also contribute to bacterial clearance by the host via their immunogenicity. In earlier work, we discovered that HMW1 and HMW2 undergo phase variation in a graded fashion (7). The levels of expression of HMW1 and HMW2 are influenced by the numbers of tandem 7-bp repeats located upstream of *hmw1A* and *hmw2A*, the structural genes that encode these adhesive proteins. High numbers of repeats result in low levels of protein expression, and low numbers of repeats result in high levels of protein expression. To extend these observations, we examined paired *H. influenzae* isolates from the nasopharynx and middle ear regions of two children with acute middle ear infections (7). In both patients, low numbers of *hmw1A* and *hmw2A* repeats and high levels of HMW1 and HMW2 were observed in the nasopharyngeal isolates, while high numbers of *hmw1A* and *hmw2A* repeats and low levels of HMW1 and HMW2 were observed in the middle ear isolates, demonstrating that phase variation of HMW1 and HMW2 occurs during...
H. influenzae disease. During in vitro cultivation, the rate of variation in 7-bp repeats is approximately $10^{-3}$, with some variation from strain to strain and locus to locus (D. M. Cholon and J. W. St. Geme III, unpublished data).

In patients with chronic obstructive pulmonary disease (COPD), H. influenzae is frequently present in the lower airways, both at the time of acute clinical exacerbations and during clinically stable periods (10, 12). In these patients, H. influenzae infection of the lower respiratory tract often persists for extended periods of time. Although patients with COPD have abnormalities in mucociliary clearance, a full understanding of the factors influencing H. influenzae persistence in the lower respiratory tract is lacking.

In this study, we characterized the HMW1 and HMW2 protein levels and the corresponding hmw1A and hmw2A repeat numbers in H. influenzae isolates collected serially from patients with COPD. In addition, we examined the titers of antibodies against HMW1 and HMW2 in serum samples obtained at the same time as the sputum isolates. We found that expression of HMW1 and HMW1 decreased over time in most patients, associated with high serum titers of HMW1/HMW2-specific antibodies and reflecting progressive increases in the numbers of 7-bp repeats.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** H. influenzae strains and serum samples were recovered from patients with COPD at serial visits to an outpatient clinic in the Buffalo Veterans Affairs Medical Center, as described previously (12). Clinical information regarding the status of the patients' chronic respiratory symptoms (shortness of breath, cough, and sputum production) was obtained during each visit in order to assess whether the patients were experiencing stable disease or an exacerbation. A patient was defined as having an exacerbation when the symptoms of shortness of breath, cough, sputum volume, and sputum purulence were increased compared to baseline symptoms. A patient was defined as having stable COPD when the symptoms were no different from those at baseline levels. This study protocol was approved by the Human Subjects Subcommittee of the Veterans Affairs Western New York Healthcare System.

To determine whether serial isolates from a given patient were the same strain, outer membrane protein profiles were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and chromosomal digests were examined by agarose gel electrophoresis (9).

**H. influenzae strain 12 is a prototype nontypeable strain that was originally recovered from the middle ear fluid of a patient with acute otitis media and is the strain from which the hmw1 and hmw2 loci were first cloned. As controls in experiments assessing levels of HMW1 and HMW2, we used derivatives of H. influenzae strain Rd/HMW1 that contain various numbers of 7-bp repeats upstream of the hmw1A gene (Rd/HMW1-17R, Rd/HMW1-20R, and Rd/HMW1-27R). Strain Rd/HMW1 contains the intact hmw1 locus and has been described previously (8).**

**Bacteria were grown on chocolate agar plates and were stored at $-80^\circ$C in brain heart infusion broth with 20% glycerol (13).**

**Western blotting.** To determine the relative amounts of HMW1 and HMW2 produced by serial isolates of nontypeable H. influenzae, bacteria were grown on chocolate agar plates and were then resuspended in 10 mm HEPES buffer, pH 7.4, and sonicated. Protein concentrations in whole-cell sonicates were quantified using the Bio-Rad reagent and were normalized for equivalent loading on a 7.5% SDS-polyacrylamide gel electrophoresis gel. Western immunoblots were performed using polyclonal antiseraum GP75, which was raised against HMW1 from strain 12 and is broadly reactive with the HMW1/HMW2 family of adhesins (11). Given the high level of homology and the variability in size among members of the HMW1/HMW2 family (3, 5, 18), Western analysis does not allow differentiation of HMW1 from HMW2 in a given strain. Accordingly, band intensities for immunoreactive proteins were considered in aggregate and were quantified using Alphalager spot density tools. With this information, relative intensities for serial isolates of a given strain were assigned values of $+/-$, $+/-$, or $+/-$. For purposes of display, the level of protein in the isolate with the highest expression level in a given series was arbitrarily defined as $+/-$, and comparison levels were designated $+$/ for 75 to 100% of the maximum, + for 50 to 75% of the maximum, $+/-$, for 25 to 50% of the maximum, and $+$/ for <25% of the maximum.

**Repeat number analysis.** To determine the numbers of 7-bp repeats upstream of the hmw1A and hmw2A genes, individual bacterial colonies were resuspended in sterile water and used as DNA templates for PCRs that amplified fragments specific for the hmw1A and hmw2A upstream regions. These reactions exploited primers that anneal to the unique regions upstream of the hmw1 and hmw2 loci (primer 1679 anneals to the ORF upstream of hmw1A in strain 12, and primer 1598 anneals to the ORF upstream of hmw2A in strain 12) and a common downstream primer, H1A (H1A anneals to sequences in both hmw1A and hmw2A). The hmw1A- and hmw2A-specific products were used as templates for reactions with internal primers called Repeat5’ and Repeat3’, which anneal to sequences that immediately flank the hmw1A repeat region in both the hmw1 and the hmw2 loci (7). The products of these secondary reactions were separated on 3% MetaPhor agarose (Cambrex) gels, allowing resolution of 7-bp changes (7).

Given that the location of the gene encoding HMW1 varies from strain to strain as either adjacent to ORF HI1679 or adjacent to ORF HI1598, with the gene encoding HMW2 adjacent to the other ORF, we refer to the repeats in a given strain as the 1679 repeats and the 1598 repeats.

**ELISA methodolgy.** Titers of human serum antibody directed against the HMW1 and HMW2 proteins were determined by an enzyme-linked immunosorbent assay (ELISA) according to the method of Barenkamp, with minor modifications (1). In brief, 96-well flat-bottomed assay plates (Falcon; Becton Dickinson) were coated with 2.5 μg/ml of purified HMW1 protein recovered from *H. influenzae* strain 12 in 0.1 M carbonate buffer, pH 9.6, and were incubated for 2 h at 37°C and then overnight at 4°C. The next day, wells were washed three times with 150 μl of phosphate-buffered saline (PBS)-0.05% Tween 20, and then human serum was added. All serum samples were serially diluted in PBS-0.05% Tween 20-1% bovine serum albumin (Calbiochem) with a starting dilution of 1:100 (with the exception of the patient 31 samples, which were serially diluted with a starting dilution of 1:800) and were incubated for 2 h at 37°C and then overnight at 4°C. After three washes with 150 μl of PBS-0.05% Tween 20, the wells were incubated with a 1:100,000 dilution of monoclonal anti-human immunoglobulin G conjugated to horseradish peroxidase (Biosource) for 2 h at 37°C. Subsequently, the wells were washed three times with 150 μl of PBS-0.05% Tween 20 and were then incubated with 100 μl of a 1:1 mixture of solutions A and B from the ABTS peroxide substrate system (KPL) for 20 min at room temperature. The reaction was stopped using 100 μl of 1% SDS, and the absorbance was monitored at 405 nm with a spectrophotometer (Bio-Tek).

**Adherence to primary airway epithelial cells.** Human airway epithelial cells were obtained from human trachea and large bronchial airway samples from multiple individuals without lung disease under a protocol approved by the University of Iowa Institutional Review Board. Adherence of bacterial isolates to primary airway epithelial cells was measured as described previously (6). To ensure reproducible and generalizable results, paired bacterial isolates from the same patient were tested in the same assay at least three times, using cells from three different individuals. Results for a representative assay using paired isolates from each COPD patient are reported.

**Statistical analysis.** Differences in adherence between paired isolates from a given patient were analyzed for statistical significance using a one-way analysis of variance (ANOVA) for a factorial experimental design. The multicomparison significance level for the ANOVA was 0.05. When significance was achieved by one-way analysis, post-ANOVA comparison of means was performed using Bonferroni’s multiple-comparison test.

**RESULTS**

Levels of HMW1/HMW2 expression in *H. influenzae* strains isolated from patients with COPD. In the present study, we performed Western analysis on whole-cell sonicates of *H. influenzae* isolates recovered serially from 11 patients with COPD to assess the levels of HMW1 and HMW2 adhesins expressed by these strains. As controls for these blots, we used variants of strain Rd/HMW1 with 17, 20, or 27 tandem repeats upstream of the hmw1A ORF. In order to plot relative amounts of HMW1 and HMW2 protein expression, the intensities of the bands were quantified using Alphalager spot density tools. Because it is not possible to differentiate HMW1 from HMW2...
in a given strain by Western analysis, we considered the HMW1/HMW2 immunoreactive bands together for the purpose of quantification. The relative intensities of HMW1 plus HMW2 for serial isolates of a given strain were assigned values of \(+/-\), \(+\), \(;++\), or \(;+++\) (Table 1). The level of protein in the isolate with the highest expression level in the series from a particular patient was arbitrarily defined as \(;+++\) for the purpose of comparison with other isolates in the series. As summarized in Table 1, 9 of the 11 patients had isolates that displayed decreases in the amounts of HMW1 and HMW2 proteins over time. Figure 1 shows a representative Western blot on five separate sets of isolates, including two discrete sets that represent different strains from patient 56. These Western blot analyses indicate that strains of H. influenzae isolated from patients with COPD can express variable amounts of HMW1 and HMW2 and that the levels of these adhesins tend to gradually decrease over time.

**Table 1. Summary of HMW1/HMW2-like adhesin levels, hmw1A and hmw2A 7-bp repeat numbers, and HMW1/HMW2 antibody levels associated with serial isolates of Haemophilus influenzae from patients with COPD**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Visit no. a</th>
<th>Clinical status b</th>
<th>HMW protein level c</th>
<th>No. of 7-bp repeats</th>
<th>Serum antibody titer by ELISA</th>
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<tr>
<td>13</td>
<td>24</td>
<td>Exacerbation</td>
<td>++ ++</td>
<td>14, 16</td>
<td>1,557</td>
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<td>6,590</td>
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<td>36</td>
<td>Stable</td>
<td>+</td>
<td>19, 22</td>
<td></td>
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<tr>
<td>22</td>
<td>1</td>
<td>Exacerbation</td>
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<td>18, 18</td>
<td>13,170</td>
</tr>
<tr>
<td>3</td>
<td>Stable</td>
<td>++</td>
<td>19, 18</td>
<td></td>
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<tr>
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<td>Stable</td>
<td>+/-</td>
<td>21, 19</td>
<td></td>
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<tr>
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<td>17</td>
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<td>19, 16</td>
<td>21,024</td>
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<td></td>
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<tr>
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<td>++</td>
<td>20, 19</td>
<td></td>
<td>14,970</td>
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<td>7</td>
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<td>17, 19</td>
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<tr>
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<td></td>
<td>50,400</td>
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<td>28</td>
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<td>++ ++</td>
<td>17, 15</td>
<td>2,156</td>
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<tr>
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<td>Stable</td>
<td>++</td>
<td>16, 19</td>
<td></td>
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<td></td>
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<td>12, NA e</td>
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<td>Stable</td>
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<td>56 f</td>
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<td>5,689</td>
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<td>+</td>
<td>19, 20</td>
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<td>+</td>
<td>19, 18</td>
<td></td>
<td>11,746</td>
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<tr>
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<td>++ ++</td>
<td>17, 18</td>
<td>6,515</td>
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<tr>
<td>2</td>
<td>Exacerbation</td>
<td>+/-</td>
<td>17, 19</td>
<td></td>
<td>11,554</td>
</tr>
<tr>
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<td>17, 12</td>
<td>8,189</td>
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<tr>
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<td>Stable</td>
<td>++ ++</td>
<td>9, 16</td>
<td></td>
<td>10,504</td>
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<tr>
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<td>9</td>
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<td>17, 15</td>
<td>78,336</td>
</tr>
<tr>
<td>10</td>
<td>Stable</td>
<td>++ ++</td>
<td>19, 14</td>
<td></td>
<td>60,088</td>
</tr>
<tr>
<td>11</td>
<td>Stable</td>
<td>++ ++</td>
<td>18, 14</td>
<td></td>
<td>116,192</td>
</tr>
</tbody>
</table>

a Visit number corresponds to the number of months after the initial visit to the COPD Clinic.
b “Exacerbation” refers to clinical exacerbation, and “stable” refers to stable clinical status. The criteria for exacerbation included an increase in symptoms compared to baseline levels, and clinical status was considered stable when the symptoms were no different from baseline symptoms.
c Protein levels were based on quantification of bands from Western blot analysis by densitometry and represent total HMW1/HMW2 protein. In clinical isolates, HMW1 and HMW2 cannot be distinguished from each other by Western analysis.
d Isolates on visits 10, 12, and 16 were distinct from isolates on visits 34, 40, and 41.
e NA, not applicable.
f ND, not determined.
in the serial isolates, we performed colony PCR using primers that distinguished *hmw1A* repeats from *hmw2A* repeats (referred to as 1679 repeats and 1598 repeats, based on the 5' primer and the adjacent ORF). In all cases where the quantities of HMW1 and HMW2 decreased over time, the decreases in levels of HMW1 and HMW2 correlated with increases in numbers of 7-bp repeats (Table 1). These data indicate that *H. influenzae* isolates recovered from the sputum samples of patients with COPD contain variable numbers of 7-bp repeats found upstream of *hmw1A* and *hmw2A* and that these numbers tend to increase over time as the levels of HMW1 and HMW2 proteins decrease.

**Levels of HMW-specific antibodies present in the serum samples of patients with COPD.** The HMW1 and HMW2 adhesins are known to be the predominant target of the serum antibody response in patients with acute *H. influenzae* infection (2), suggesting the hypothesis that bacteria expressing low levels of HMW1 and HMW2 correlated with increases in numbers of 7-bp repeats (Table 1). These data indicate that *H. influenzae* isolates recovered from the sputum samples of patients with COPD contain variable numbers of 7-bp repeats found upstream of *hmw1A* and *hmw2A* and that these numbers tend to increase over time as the levels of HMW1 and HMW2 proteins decrease.

We found that HMW1/HMW2-specific antibody titers were high, with no particular relationship between antibody titers and levels of HMW1/HMW2 expression in the corresponding isolate (Table 1). Antibody titers were high even in patients who had isolates with relatively stable total levels of HMW1 and HMW2 over time.

**Relationship between levels of HMW1/HMW2 expression and adherence to primary airway epithelial cells by *H. influenzae* strains isolated from patients with COPD.** To determine the relationship between levels of HMW1 and HMW2 and capacity to adhere to airway epithelium, we performed adherence assays with primary airway epithelial cells and the first and last isolates from 10 serial sets, including 9 sets with decreases in levels of HMW1 and HMW2 over time and 1 set with no change in levels of HMW1 and HMW2. As shown in Fig. 2, in general there was little change in adherence between the first and last isolates, even when the levels of HMW1 and HMW2 decreased markedly, as highlighted with the isolates from patients 22, 31, and 57 and the first set of isolates from patient 56. In contrast, adherence decreased significantly in association with reduction in levels of HMW1 and HMW2 in isolates from patients 13 and 48 and the second set of isolates from patient 56, all examples of isolates where the levels of
HMW1 and HMW2 decreased from +++ to either + or +/-.
Interestingly, there was considerable strain-to-strain variation in levels of adherence, independent of levels of HMW1 and HMW2.

**DISCUSSION**

In the present study, we examined serial isolates of *H. influenzae* recovered from the sputum samples of patients with COPD to assess changes in levels of the HMW1 and HMW2 adhesins over time. We found that levels of HMW1 and HMW2 typically decreased from high to low in association with persistence. In all cases, the decreases in protein levels were associated with progressive increases in the numbers of 7-bp repeats upstream of the HMW1 and HMW2 structural genes, consistent with earlier work demonstrating an inverse correlation between the levels of HMW1 and HMW2 expression and the numbers of 7-bp repeats found upstream of the *hmw1A* and *hmw24* genes (7). All patients had appreciable titers of antibodies against the HMW1 and HMW2 proteins, as assessed by ELISA using purified HMW1 from prototype *H. influenzae* strain 12 as the antigen.

The HMW1 and HMW2 adhesins play an important role in mediating adherence to host epithelium (15, 16). At the same time, these proteins are highly immunogenic and are major targets of the antibody response to infection (2). In earlier work, we examined paired nasopharyngeal and middle ear isolates of *H. influenzae* from patients with acute otitis media and observed high levels of HMW1 and HMW2 in the nasopharyngeal isolates and low levels of HMW1 and HMW2 in the middle ear isolates (7), suggesting that phase variation of the HMW1 and HMW2 adhesins allows the organism to survive in diverse environments in the human host, including the nasopharynx, where HMW1 and HMW2 facilitate colonization, and middle ear fluid, where HMW1 and HMW2 may enhance antibody-mediated clearance. Our results in the current study argue that phase variation of HMW1 and HMW2 is also important in allowing *H. influenzae* to persist in the lower respiratory tract in patients with COPD. The fact that adults generally have high titers of antibodies against the HMW1/HMW2 proteins (2) suggests that serum antibody represents a selective pressure against organisms with high levels of HMW1 and HMW2. However, since high antibody titers are present even at the time of initial infection of the lower respiratory tract in patients with COPD, presumably other selective pressures against HMW1 and HMW2 also exist and play an important role when infection persists. It is possible that antibody titers in respiratory mucosal secretions increase over the course of infection and are a more important selective pressure.

In comparing levels of adherence to airway epithelial cells by serial isolates expressing variable levels of HMW1 and HMW2, in most cases we observed similar levels of adherence, independent of levels of HMW1 and HMW2. At the same time, in three cases we found that lower levels of HMW1 and HMW2 resulted in reduced levels of adherence. These results suggest that there may be some strain-to-strain variation in the quantities of HMW1 and HMW2 required for full-level adherence, perhaps reflecting the presence of other adhesive proteins or other factors that modulate the adhesive activity and affinity of HMW1 and HMW2. At this point, it is difficult to know what selective advantage high levels of HMW1 and HMW2 confer at early stages of infection of the lower respiratory tract.

In considering our adherence results with primary airway epithelial cells, it is important to recognize that the airway cells were grown immersed in tissue culture medium rather than an air-liquid interface. As a consequence, the cells were relatively less differentiated and may have provided an imperfect representation of epithelial cell-bacterium interactions in the airway. Similarly, the characteristics of the airway cells were likely distinct from the characteristics of nasopharyngeal cells.

It is interesting to compare the numbers of 7-bp repeats that are associated with high, medium, and low levels of HMW1 and HMW2 expression among different strains. As an example, in the strain from patient 22, 18 1679 repeats and 18 1598 repeats were associated with high levels of HMW1/HMW2, whereas in the strain from patient 57, 18 1679 repeats and 19 1598 repeats were associated with low levels of HMW1/HMW2. It is also interesting that with some strains, minimal changes in repeat number result in a dramatic change in protein level, as highlighted by the strain from patient 74. In this strain, 17 1679 repeats and 18 1598 repeats were associated with high levels of HMW1 and HMW2 (isolate at visit 1), while 17 1679 repeats and 19 1598 repeats were associated with negligible levels of HMW1 and HMW2 (isolate at visit 2). Thus, an increase of one repeat unit resulted in marked decreases in HMW1 and HMW2 expression. Together, these observations suggest that other factors influence the impact of the *hmw1A* and *hmw2A* repeats on *hmw1A* and *hmw2A* gene expression. Alternatively, independent of the repeats and lev-
els of gene expression, there may be factors that influence HMW1 and HMW2 production and stability.

The human host possesses a sophisticated immune system for combating microbial intruders and protecting against disease. At the same time, bacterial pathogens have evolved creative mechanisms for evading host immunity, with phase variation representing one important example. In this study, we demonstrated that nontypeable H. influenzae undergoes phase-variable expression of the HMW1 and HMW2 adhesins in the lower respiratory tracts of patients with COPD, resulting in frequent selection against organisms expressing high levels of HMW1 and HMW2. We speculate that phase variation of HMW1 and HMW2 allows H. influenzae to persist in the lower respiratory tract in patients with COPD.

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