MAJOR ARTICLE

Expression of IgA Proteases by *Haemophilus influenzae* in the Respiratory Tract of Adults With Chronic Obstructive Pulmonary Disease

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Background. Immunoglobulin (Ig)A proteases of *Haemophilus influenzae* are highly specific endopeptidases that cleave the hinge region of human IgA1 and also mediate invasion and trafficking in human respiratory epithelial cells, facilitating persistence of *H. influenzae*. Little is known about the expression of IgA proteases in clinical settings of *H. influenzae* infection.

Methods. We identified and characterized IgA protease genes in *H. influenzae* and studied their expression and proteolytic specificity, in vitro and in vivo in 169 independent strains of *H. influenzae* collected longitudinally over 10 years from adults with chronic obstructive pulmonary disease.

Results. The *H. influenzae* pangenome has 2 alleles of IgA protease genes; all strains have *igaA*, and 40% of strains have *igaB*. Each allele has 2 variants with differing proteolytic specificities for human IgA1. A total of 88% of 169 strains express IgA protease activity. Expression of the 4 forms of IgA protease varies among strains. Based on the presence of IgA1 fragments in sputum samples, each of the different forms of IgA protease is selectively expressed in the human airways during infection.

Conclusions. Four variants of IgA proteases are variably expressed by *H. influenzae* during infection of the human airways.

Keywords. *Haemophilus influenzae*; IgA protease; chronic obstructive pulmonary disease; endopeptidase; airway infection; exacerbation of COPD; respiratory tract infection; respiratory tract colonization.

Selected human mucosal pathogens express immunoglobulin (Ig) A proteases, which are strongly associated with virulence. IgA proteases are a striking example of convergent evolution because 3 independent lines of these highly specific postproline endopeptidases (serine, metallo-, and cysteine types) are present in human bacterial pathogens, indicating a key role for IgA proteases in the host-pathogen interaction [1–3]. Discovered 30 years ago, IgA proteases cleave the hinge region of human IgA1, dissociating its antigen-binding Fab domains from the Fc domain, thus inhibiting functions of IgA, such as agglutination, inhibition of bacterial adhesion to epithelial cells, and opsonization [1, 2, 4]. IgA proteases were named because of their specificity for human IgA1. Since then, IgA proteases have been demonstrated to contribute to bacterial infection by several additional mechanisms, including stimulating production of proinflammatory cytokines, altering tumor necrosis factor α signaling, and mediating intracellular persistence [5–7].

After the discovery of IgA proteases, excellent work by several groups characterized the gene that encodes IgA protease and enzyme specificity in multiple strains [8–15]. Understanding the role of IgA protease in disease is challenging because the enzyme is highly specific for human IgA1, precluding studies in animal models.

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As a result, studies of IgA proteases of *Haemophilus influenzae* have stalled since the late 1990s. However, several recent compelling observations provide new evidence for IgA protease as a virulence factor for *H. influenzae* human respiratory tract infection, including (1) a strong association of IgA protease expression with disease [16–18]; (2) the discovery of a second distinct IgA protease gene that cleaves human lysosomal-associated membrane protein and is associated with infection in chronic obstructive pulmonary disease (COPD) [7, 17]; (3) the observation that IgA proteases partially mediate invasion and trafficking in human respiratory epithelial cells and are required for optimal intracellular persistence [7]; and (4) the up-regulation of IgA protease during persistence of *H. influenzae* in the nasopharynx in a human experimental challenge model, indicating selective pressure for expression of IgA protease during early colonization [19].

Several important questions regarding expression and cleavage specificity of IgA proteases require answers to elucidate its role in the pathogenesis of human respiratory tract infection. In particular, the variability of expression and of cleavage specificity of IgA proteases among clinical isolates of *H. influenzae* has been unexplained.

The goal of the present study is to elucidate the distribution of *iga* protease genes and assess the expression of IgA proteases in a rigorously characterized set of strains of *H. influenzae* collected longitudinally over 15 years from adults with COPD. We demonstrate that the *H. influenzae* genome has 2 alleles that encode IgA proteases (*igaA* and *igaB*); all strains have *igaA,* and 40% of strains have *igaB*. We further show that each allele expresses 2 variants of IgA proteases that cleave different sites of human IgA1 and that expression of these 4 forms of IgA protease varies among strains. Finally, we present direct evidence that each of the different forms of IgA protease is selectively expressed in the human airways during infection. These data clarify a robust and sometimes confusing literature on IgA proteases of *H. influenzae* of the past 3 decades, and they emphasize the importance of IgA proteases in the pathogenesis of human airway infection by *H. influenzae*.

MATERIALS AND METHODS

The study was approved by the institutional review boards of the University at Buffalo and the Veterans Affairs Western New York Healthcare System. Written, informed consent was provided by participants.

COPD Study Clinic

The COPD study clinic, described elsewhere [20], is a prospective study started in 1994. Subjects were seen monthly and at suspected exacerbations. Rigorous clinical criteria determined whether patients were experiencing an exacerbation. At each visit expectorated sputum samples were collected. An exacerbation caused by *H. influenzae* was defined by the onset of clinical symptoms simultaneous with the acquisition of a new strain [20].

Strains

H. influenzae were identified using standard techniques and were further distinguished from *Haemophilus haemolyticus* using monoclonal antibody 7F3. which recognizes an epitope on the P6 protein [21] (see Supplement for details). Strains used in this study were passaged twice from the original isolation from sputum. From April 1994 through March 2009, a total of 169 independent isolates were identified based on multilocus sequence typing [22]. Each isolate is the first acquisition of that strain for each patient. The duration of carriage for each strain was determined by subjecting strains recovered from monthly sputum cultures to multilocus sequence typing.

Determination of *iga* Genes in Strains

IgA protease genes were identified by polymerase chain reaction (PCR) using oligonucleotide primers specific for the variants of *iga* genes (see Supplement for details).

Determination of Expression of IgA Proteases

To assess in vitro expression of IgA proteases, the cleavage pattern of each strain was determined by immunoblot assays after incubation of bacterial culture supernatant with human IgA, as described elsewhere [13, 17].

Analysis of Sputum Samples

Samples from subjects in the COPD Study Clinic were processed as described elsewhere [20, 23]. Fragments of IgA were purified from sputum supernatants by affinity chromatography and subjected to immunoblot assay with anti–human IgA–conjugated antibodies [24, 25]. The IgA fragments were further characterized by determining N-terminal sequences.

Real-time PCR

Real-time quantitative PCR to assess *iga* gene transcription was performed as described elsewhere [7].

Statistical Analyses

Analyses were done with SAS software, version 9.3 (SAS Institute). *H. influenzae* strains were classified as persistent if an individual carried the same strain for ≥6 months. We examined the association between presence or expression of a given *iga* gene with carriage for ≥6 months and exacerbation of COPD, using χ^2 test or Fisher exact tests, as appropriate.

RESULTS

Identification of Variants of the *igaA* Allele

To assess in vitro expression of IgA proteases, 169 independent, minimally passaged isolates of nontypeable *H. influenzae* cultured from the sputum of 78 adults with COPD longitudinally

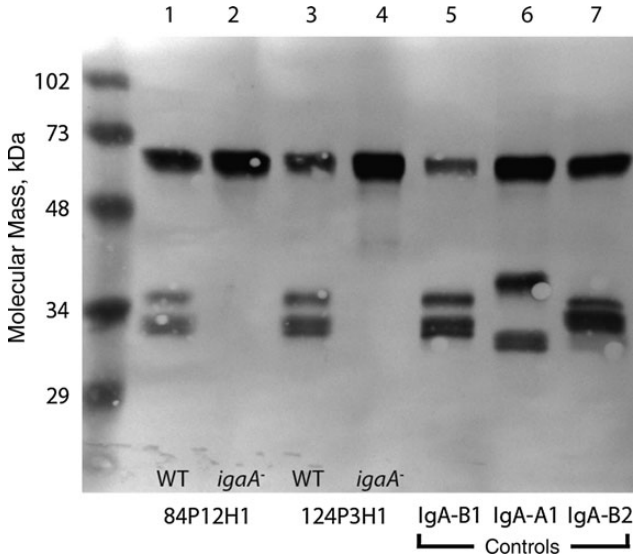

Figure 1. Immunoblot assay probed with peroxidase-conjugated anti human immunoglobulin (Ig) A. Purified human IgA1 was incubated with broth culture supernatants of *Haemophilus influenzae* strains: 84P12H1 (lane 1), 84P12H1 *igaA* knockout mutant (lane 2), 124P3H1(lane 3), 124P3H1 *igaA* knockout mutant (lane 4), 11P6H (IgA protease B1 control) (lane 5), Rd (IgA protease A1 control) (lane 6), and 2019 (IgA protease B2 control) (lane 7). Abbreviation: WT, wild type.

over 15 years were studied by determining the cleavage patterns by immunoblot assays after incubation of bacterial culture supernatant with human IgA1. Previous work showed that IgA proteases A and B each produced characteristic banding patterns of cleavage products of human IgA [17]. However, in assays to characterize the IgA proteolysis patterns of 169 strains, we observed unexpected patterns. Specifically, 17 strains that contained the *igaA* gene, but lacked the *igaB* gene, showed a proteolytic cleavage pattern that is characteristic of the IgA B protease (Figure 1, lanes 1 and 3). We considered 2 possibilities to explain this result. The first was that the strains might contain the *igaB* gene but its presence was missed by the primers used to probe genomic DNA. The second was that the *igaA* gene encoded an IgA protease that had a cleavage pattern indistinguishable from that of the IgA protease encoded by the *igaB* gene.

To distinguish between these possibilities, we engineered mutants by knocking out the *igaA* gene of strains 84P12H1 and 124P3H1, which both lacked a detectable *igaB* gene but showed an apparent IgA B protease cleavage pattern. The result in Figure 1 shows that knocking out the *igaA* gene eliminated all IgA protease activity in these 2 strains. The N-terminal amino acid sequence of the protein band that resulted from cleavage of IgA by strain 124P3H1 was identical to that of the IgA B protease of strain 11P6H (Table 1). We conclude that the *igaA* gene in these strains encodes an IgA protease that has a proteolytic cleavage pattern that is identical to IgA B protease. We propose to name this gene *igaA2* to distinguish it from the previously identified

Table 1. Results of N-terminal Amino Acid Sequences of Cleavage Products of the 4 IgA Proteases of *H. influenzae*

Strain or Sputum[a]	Gene	Amino Terminal Sequence of IgA Protease Cleavage Product
Rd	*igaA1*	S-T-P-P-T-P-S-P-S-C-C
Sputum 84PS8	*igA1*	S-T-P-X-T-P-S-P-S[b]
Strain 124P3H1	*igaA2*	T-P-S-P-S-C-C-H-P-R
Sputum 124PS3	*igaA2*	T-P-S-P-S-C-C-H-P-R-L
Strain 11P6H	*igaB1*	T-P-S-P-S-C-C-H-P-R-L
Sputum 87PS37	*igaB1*	T-P-S-P-S-C-C-H-P-R
Strain 2019	*igaB2*	S-P-S-C-C-H-P-R-L-S

Abbreviations: *H. influenzae, Haemophilus influenzae*; IgA, immunoglobulin A.

[a] For each strain, the N-terminal amino acid sequence of the fragment from the in vitro assay of IgA protease is shown. Each sputum sample result represents the fragment recovered directly from a sputum sample from which *H. influenzae* was isolated.

[b] *X* indicates an ambiguous residue.

iga gene in strain Rd (GenBank accession No. X59800) [26], which we propose to name *igaA1* (Figure 2).

Identification of Variants of the *igaB* Allele

We observed 2 proteolytic cleavage patterns among strains with the *igaB* gene. Figure 3 shows the proteolytic cleavage patterns of prototype strains 11P6H and 2019, along with mutants in which the *igaA* and *igaB* genes in these strains were individually knocked out. The N-terminal amino acid sequence of the protein band that results from cleavage by the *igaB* gene in strain 11P6H differs from that of strain 2019 (Table 1). We propose to name the *igaB* gene of strain 11P6H *igaB1* (GenBank accession DQ423203), previously identified as *igaB* [17]. We propose to name the *igaB* gene, of which strain 2019 is the prototype, *igaB2* (GenBank accession No. KC607498). Figure 2 shows the proteolytic cleavage sites in the hinge region of human IgA1 of the IgA proteases encoded by *igaA* (*igaA1* and *igaA2*) and *igaB* (*igaB1* and *igaB2*). The *igaA* and *igaB* genes are located in separate regions of the *H. influenzae* genome, as reported elsewhere [17, 18].

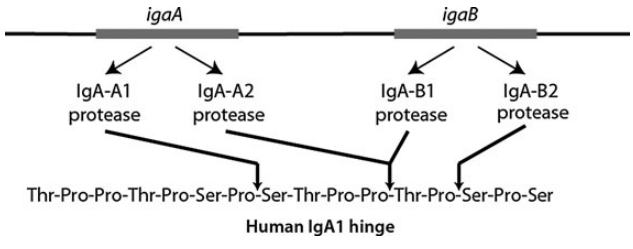

Figure 2. Diagram showing the amino acid sequence of the hinge region of the α chain of human immunoglobulin (Ig) A1 and the sites at which the 4 IgA proteases of *Haemophilus influenzae* cleave.

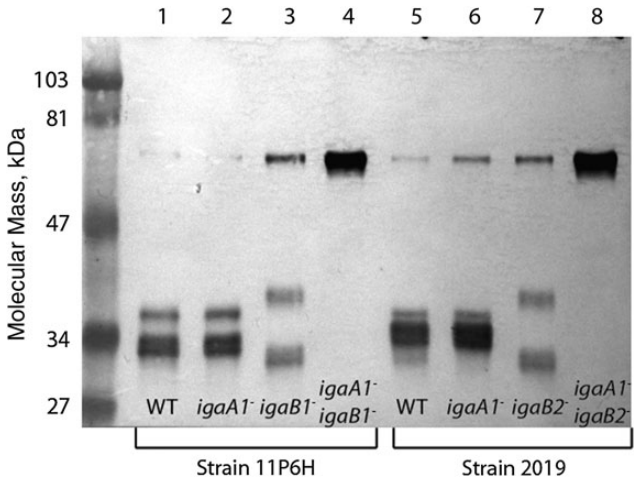

Figure 3. Immunoblot assay probed with peroxidase-conjugated anti–human immunoglobulin (Ig) A. Purified human IgA1 was incubated with broth culture supernatants of *Haemophilus influenzae* strains: 11P6H (lane 1), 11P6H *igaA1* knockout mutant (lane 2), 11P6H *igaB1* knockout mutant (lane 3), 11P6H *igaA1, igaB1* double-knockout mutant (lane 4), 2019 (lane 5), 2019 *igaA1* knockout mutant (lane 6), 2019 *igaB2* knockout mutant (lane 7), and 2019 *igaA1 igaB2* double-knockout mutant (lane 8). Abbreviation: WT, wild type.

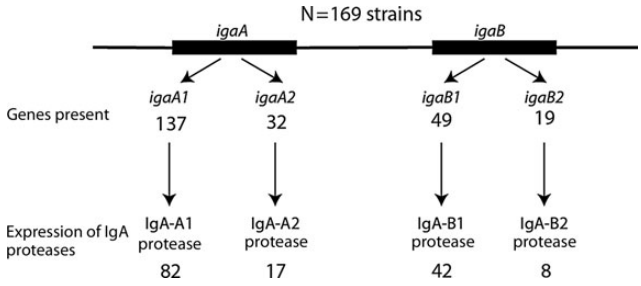

Figure 4. Diagram showing distribution of *igaA* and *igaB* genes in 169 longitudinally collected strains of *Haemophilus influenzae* from sputum samples of adults with chronic obstructive pulmonary and the expression of immunoglobulin (Ig) A proteases among strains. Numbers under genes and proteases are numbers of strains.

Distribution of *iga* Genes in COPD Strains of *H. influenzae*

Having identified 4 variants of IgA proteases encoded by 2 *iga* alleles, we designed primers to distinguish each of the variants of *igaA* and *igaB* by PCR in our set of 169 strains (see Supplement for details). We identified primers that distinguish among the variants, except we could not identify primers that reliably distinguished *igaB1* and *igaB2* by PCR. To distinguish *igaB1* and *igaB2* from each other, we used the observation that the region immediately upstream of the start codon of *igaB2* has repeats not present upstream of *igaB1*. A 687 bp fragment that spans the start codon of *igaB* genes was amplified and sequenced. A varying number of TCAAAAT repeats was present upstream of *igaB2*, whereas *igaB1* lacked upstream repeats. Therefore, to distinguish *igaB1* and *igaB2*, the fragment upstream of *igaB* was amplified by PCR, and the sequence was determined.

An *igaA* gene is present in every strain. Of the 169 strains, 137 (81%) have *igaA1* and 31 (19%) have *igaA2* (Figure 4). Of the 169 strains, 68 (40%) have an *igaB* gene, 49 (29%) have *igaB1,* and 19 (11%) have *igaB2* (Figure 4).

Expression of IgA Proteases

To assess expression of IgA proteases in vitro, we incubated broth culture supernatants of each of the 169 strains with human IgA1, and the resulting reactions were subjected to immunoblot assays, which were probed with anti human IgA. A total of 148 strains (88%) had IgA protease activity; thus, 21 strains did not express IgA protease under these conditions. One strain expressed 2 IgA proteases simultaneously (IgA protease A1 and B2).

To assess the effect of in vitro passage on expression of IgA proteases, we passaged selected strains with each of the 4 *iga* genes in vitro 5 times and tested them again for expression of IgA protease. In all cases, IgA protease expression patterns remained unchanged with in vitro passage (see Supplement for details).

The observation that *igaA2* and *igaB1* encode IgA proteases that cleave at the same site in the human IgA1 hinge region, and

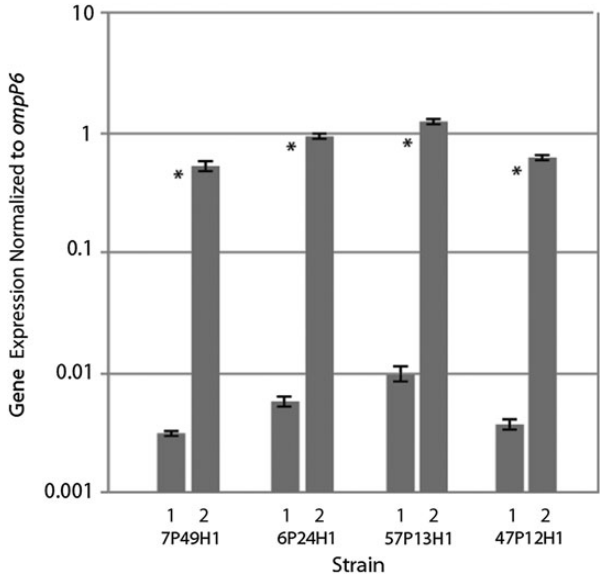

Figure 5. Quantitative real-time polymerase chain reaction results. Immunoglobulin (Ig) A protease gene expression in 4 strains of nontypeable *Haemophilus influenzae* showing expression of *igaA2* (lanes 1) and *igaB1* (lanes 2) relative to expression of *ompP6*. Error bars represent standard deviations of experiments in triplicate wells. **P* < .001 (*igaA2* vs *igaB1* for each strain; paired *t* test).

thus produce identical cleavage patterns, complicates determination of IgA protease expression in strains that express *igaA2* and *igaB1*. For 9 strains with both genes and that expressed the IgA-A2/IgA-B1 cleavage pattern, we performed quantitative real-time PCR to assess transcription of the genes. In all 9 strains, the *igaB1* gene transcription was approximately 100-fold greater than that of the *igaA2* gene (Figure 5).

Figure 4 summarizes the distribution of the 4 IgA protease genes and their expression in 169 consecutive, independent strains isolated longitudinally from adults with COPD. All strains contain an *igaA* gene, but only 59% of strains express an IgA protease A. An *igaB* gene is present in 68 strains (40%) (49 *igaB1* and 19 *igaB2*). Of strains with *igaB1*, 42 of 49 (86%) express IgA protease B1, whereas only 8 of 19 (42%) with *igaB2* express IgA protease B2.

Expression of IgA Proteases in Vivo

To assess expression of IgA proteases during human infection, IgA fragments from sputum supernatants were affinity purified, subjected to immunoblot assays, and probed directly with anti human IgA to detect fragments of IgA. Thus, human IgA fragments detected in this assay represent cleavage that occurred in the human airways. A total of 81 sputum supernatants from samples that grew *H. influenzae* were studied; 49 samples lacked detectable IgA cleavage fragments. Others produced a variety of patterns in immunoblot assays, precluding reliance on banding patterns of immunoblot assays to identify specific IgA proteases. Thus, to identify the specificity of IgA proteases that cleaved IgA during human airway infection, we excised bands in the predicted molecular mass range of IgA fragments from blots of 20 sputum samples and subjected them to N-terminal amino acid sequence determination (Figure 6).

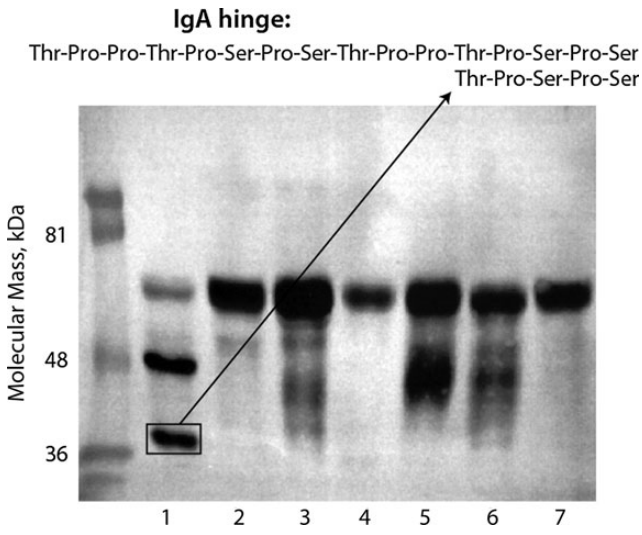

Figure 6. Immunoblot assay of sputum supernatants from adults with chronic obstructive pulmonary disease, whose sputum cultures grew *Haemophilus influenzae,* probed with peroxidase-conjugated anti human immunoglobulin (Ig) A. Lanes contain sputum samples: 54PS24 (lane 1), 56PS34 (lane 2), 56PS41 (lane 3), 57PS13 (lane 4), 59PS3 (lane 5), 59PS7 (lane 6), and 63PS35 (lane 7). The N-terminal sequence of the band noted by the box is shown aligned with the amino acid sequence of the hinge region of the α chain of human IgA1; the sequence corresponds to the cleavage pattern of IgA protease B1, which is expressed by strain 54P24H1 which was isolated from the sputum sample 54PS24.

Ten bands showed sequences consistent with known cleavage sites of oral streptococci, 3 yielded no clear sequence, 1 yielded IgA protease A1, and 6 yielded a sequence consistent with IgA protease B1 or A2 (B1 and A2 have identical cleavage sites). Analysis of the *iga* genes in the corresponding strains isolated from the sputum samples allowed us to conclude that 4 of these 6 strains expressed IgAB1 protease (the strains had the *igaA1* variant), 1 expressed IgA protease A2 (the strain had no *igaB* gene), and 1 could be either IgA protease B1 or A2 because the strain had both genes. We conclude from these results that IgA proteases A1, A2, and B1 are expressed in the human airways of adults with COPD.

Association of Genes and Expression of Genes With Exacerbation and Duration of Carriage

Of the 169 strains, 75 were acquired simultaneous with the onset of symptoms of an exacerbation (exacerbation strain), and 94 were acquired with no change in symptoms (colonization strain). The *igaB1* gene was more prevalent among exacerbation strains (28 of the 75 [37%]) compared with colonization strains (21 of 94 [22%]) ($P = .03$). None of the other *iga* alleles were associated with exacerbation or colonization. Expression of *igaA1* was less common in exacerbation strains. Of the 137 strains with *igaA1*, 61 were exacerbation strains and 76 were colonization strains; *igaA1* was expressed in 29 (48%) and 53 (70%) of exacerbation and colonization strains, respectively ($P = .008$). Expression of *igA2*, *igaB1,* or *igaB2* did not significantly differ among exacerbation and colonization strains.

After eliminating 14 strains that were cultured at a patient's first visit or near the patient's last visit in the study, the duration of carriage was available for 155 of the 169 strains (see Supplement for details). Of 155 strains, 49 (32%) were carried for ≥6 months. The prevalence and expression of *igaA1*, *igA2*, *igaB1,* and *igaB2* did not significantly differ by duration of carriage.

DISCUSSION

In this study, we demonstrate that the genomes of strains of *H. influenzae* have 2 alleles that encode IgA proteases (*igaA* and *igaB*) and that each allele has 2 variants of IgA proteases that cleave different sites of human IgA1. The work further shows that expression of these 4 forms of IgA protease varies among strains. Finally, we present direct evidence that each of the different forms of IgA protease is selectively expressed in the

human airways during infection. Although the results reveal a more complex picture of *H. influenzae* IgA proteases than previously understood, these data bring clarity to the literature on IgA proteases of *H. influenzae* of the past 3 decades. The observation that each of the variants is expressed during human infection emphasizes the importance of IgA proteases in the pathogenesis of human airway infection by *H. influenzae*.

The elucidation of expression of variants of IgA protease with differing proteolytic specificities explains the observation in several previous studies of IgA protease that show differing levels of IgA protease activity and differing IgA protease specificities among strains, including some strains that express >1 specificity [13–15]. Elucidating the basis of variability in IgA protease expression and cleavage specificity among isolates, particularly in view of the strong association of IgA protease with virulence [16] and the strong selective pressure for *H. influenzae* to express IgA protease [19], provides a foundation for investigating the roles of distinct IgA proteases of *H. influenzae* in human infection.

Our present findings establish that the *H. influenzae* pangenome includes 2 alleles (*igaA* and *igaB*) that encode distinct IgA proteases. Each allele has 2 variants that encode IgA proteases with characteristic cleavage patterns (*igaA1, igaA2, igaB1,* and *igaB2*) (Figure 4). Superimposed on the presence of 4 gene variants is the observation that expression of the IgA proteases also seems to be regulated. Of the 169 strains, 147 expressed a single IgA protease, 21 did not express IgA protease, and 1 expressed 2 IgA proteases simultaneously.

A variable number of repeats were identified upstream of the start codon of *igaB2*, providing evidence that expression *igaB2* is regulated by slipped-strand mispairing. No repeats or obvious regulatory sequences are present upstream of the *igaA1, igaA2* or *igaB1* genes. Expression of these genes may be constitutive or may be regulated by other mechanisms. Previous work with our prototype strain, 11P6H, shows constitutive expression of *igaA1* and *igaB1* with approximately 10-fold higher level of transcription of *igaB1* compared with *igaA1* [7]. The present study demonstrated that in the case of strains that have both *igaA2* and *igaB1*, *igaB1* is expressed at an approximately 100-fold higher level than *igaA2*. These results suggest higher baseline expression of *igaB* compared with *igaA*. Alternatively, point mutations could place selected genes out of frame and interrupt expression. Finally, expression may be regulated by phase variable restriction modification systems, such as DNA methyltransferase [27, 28]. It is important to recognize that our results reflect expression under in vitro conditions, which differ from in vivo conditions.

To assess expression in vivo, we identified IgA protease cleavage fragments in sputum samples from adults with COPD, providing direct evidence for expression of IgA proteases A1, A2, and B1 in the human respiratory tract. The majority of the 81 sputum supernatant samples that we studied had no detectable IgA fragments by immunoblot assay. One must be cautious in interpreting these results to assess the frequency with which IgA proteases are expressed in the airways, because multiple factors other than lack of expression can explain an absence of cleavage fragments in expectorated sputum samples. These include binding of fragments to bacteria and/or mucin removed by centrifugation during processing of sputum supernatants, degradation by nonspecific proteases in sputum, and the low sensitivity of immunoblot assays. The importance of this novel observation is the demonstration of expression of each of the variants of IgA proteases in the airways during human infection.

We showed expression of 3 of 4 variants of IgA proteases (IgA1, A2 and B1) in human airways by demonstrating cleavage fragments of human IgA in sputum. The study of Poole et al [19], who challenged human volunteers with a strain of *H. influenzae* that has the *igaB2* gene, showed that persistence in the human upper airway resulted in up-regulation of *igaB2*, which shifted from phase-off expression in the challenge inoculum to phase-on during colonization of the human nasopharynx over 6 days. Thus, all 4 variants of IgA proteases of *H. influenzae* are expressed in the human respiratory tract.

The acquisition of a new strain of *H. influenzae* is associated with the onset of an exacerbation [20, 21]. We tested the hypothesis that specific *iga* genes and expression of specific *iga* genes were associated with exacerbation. The presence of the *igaB1* gene was associated with the propensity to cause exacerbations, and expression of IgA protease A1 was associated with colonization. The duration of persistence of nontypable *H. influenzae* in the airways of adults with COPD shows substantial variation among strains [29]. We saw no association between expression of specific IgA proteases and duration of carriage of individual strains.

Multiple determinants account for a strain's capacity to cause exacerbations and persist in COPD airways, including host factors, particularly because COPD involves multiple pathological processes whose effects are modified by varying host susceptibilities [30]. Assessing the role of individual determinants of infection and carriage is further confounded by the enormous genetic heterogeneity among strains of *H. influenzae* [31–35]. We speculate that in view of the role of IgA proteases in invasion of host cells and levels of intracellular persistence, the variable expression patterns of IgA proteases influences the propensity of strains to cause exacerbations and to persist in respiratory epithelial cells, contributing to the marked differences in these characteristics among strains of *H. influenzae* in the setting of COPD.

Discovered 30 years ago by virtue of their capacity to cleave the hinge region of human IgA1 with high specificity, IgA proteases have more recently been recognized as having a broader range of activity in mediating pathogenesis of human respiratory tract infection, particularly intracellular persistence. The present study establishes that (1) the *H. influenzae* genome

contains 2 *iga* alleles, (2) each allele expresses 2 variants with specific proteolytic degradation specificities, (3) strains with the *igaB1* variant gene are associated with the propensity of a strain to cause an exacerbation of COPD, (4) strains of *H. influenzae* show variable expression of IgA proteases, and (5) variants of IgA protease are expressed in the human respiratory tract during infection. These observations will be important in understanding the role of IgA proteases in the pathogenesis of *H. influenzae* respiratory tract infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Kilian M, Reinholdt J, Lomholt H, Poulsen K, Frandsen EVG. Biological significance of IgA1 proteases in bacterial colonization and pathogenesis: critical evaluation of experimental evidence. APMIS **1996**; 104: 321–8.
2. Kilian M, Russell MW. Microbial evasion of IgA functions. In: Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, Mayer LW, eds. Mucosal immunology. 3rd ed. Burlington, MA: Elsevier-Academic Press, **2005**:291–303.
3. Mistry D, Stockley RA. IgA1 protease. Int J Biochem Cell Biol **2006**; 38:1244–8.
4. Kilian M, Mestecky J, Russell MW. Defense mechanisms involving Fc-dependent functions of immunoglobulin A and their subversion by bacterial immunoglobulin A proteases. Microbiol Rev **1988**; 52: 296–303.
5. Lorenzen DR, Dux F, Wolk U, Tsirpouchtsidis A, Haas G, Meyer TF. Immunoglobulin A1 protease, an exoenzyme of pathogenic *Neisseriae*, is a potent inducer of proinflammatory cytokines. J Exp Med **1999**; 190:1049–58.
6. Beck SC, Meyer TF. IgA1 protease from *Neisseria gonorrhoeae* inhibits TNFalpha-mediated apoptosis of human monocytic cells. FEBS Lett **2000**; 472:287–92.
7. Clementi CF, Hakansson AP, Murphy TF. Internalization and trafficking of nontypeable *Haemophilus influenzae* in human respiratory epithelial cells and roles of IgA1 proteases for optimal invasion and persistence. Infect Immun **2014**; 82:433–44.
8. Male CJ. Immunoglobulin A1 protease production by *Haemophilus influenzae* and *Streptococcus pneumoniae*. Infect Immun **1979**; 26:254–61.
9. Kilian M, Mestecky J, Schrohenloher RE. Pathogenic species of the genus *Haemophilus* and *Streptococcus pneumoniae* produce immunoglobulin A1 protease. Infect Immun **1979**; 26:143–9.
10. Mulks MH, Kornfeld SJ, Frangione B, Plaut AG. Relationship between the specificity of IgA proteases and serotypes in *Haemophilus influenzae*. J Infect Dis **1982**; 146:266–74.
11. Poulsen K, Hjorth JP, Kilian M. Limited diversity of the immunoglobulin A1 protease gene *(iga)* among *Haemophilus influenzae* serotype b strains. Infect Immun **1988**; 56:987–92.
12. Kilian M, Poulsen K. Enzymatic, serologic, and genetic polymorphism of *Haemophilus influenzae* IgA1 proteases. J Infect Dis **1992**; 165: S192–3.
13. Reinholdt J, Kilian M. Comparative analysis of immunoglobulin A1 protease activity among bacteria representing different genera, species, and strains. Infect Immun **1997**; 65:4452–9.
14. Senior BW, Ip CL. An investigation into the influences of species and biotype on the type of IgA1 protease produced by isolates of *Haemophilus*. J Med Microbiol **1999**; 48:389–94.
15. Mistry DV, Stockley RA. The cleavage specificity of an IgA1 protease from *Haemophilus influenzae*. Virulence **2011**; 2:103–10.
16. Vitovski S, Dunkin KT, Howard AJ, Sayers JR. Nontypeable *Haemophilus influenzae* in carriage and disease: a difference in IgA1 protease activity levels. JAMA **2002**; 287:1699–705.
17. Fernaays MM, Lesse AJ, Cai X, Murphy TF. Characterization of *igaB*, a second immunoglobulin A1 protease gene in nontypeable *Haemophilus influenzae*. Infect Immun **2006**; 74:5860–70.
18. Murphy TF, Lesse AJ, Kirkham C, Zhong H, Sethi S, Munson RS Jr. A clonal group of nontypeable *Haemophilus influenzae* with two IgA proteases is adapted to infection in chronic obstructive pulmonary disease. PLoS One **2011**; 6:e25923.
19. Poole J, Foster E, Chaloner K, et al. Analysis of nontypeable *Haemophilus influenzae* phase-variable genes during experimental human nasopharyngeal colonization. J Infect Dis **2013**; 208:720–7.
20. Sethi S, Evans N, Grant BJB, Murphy TF. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. N Engl J Med **2002**; 347:465–71.
21. Murphy TF, Brauer AL, Sethi S, Kilian M, Cai X, Lesse AJ. *Haemophilus haemolyticus*: a human respiratory tract commensal to be distinguished from *Haemophilus influenzae*. J Infect Dis **2007**; 195:81–9.
22. Meats E, Feil EJ, Stringer S, et al. Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol **2003**; 41:1623–36.
23. Sethi S, Muscarella K, Evans N, Klingman KL, Grant BJB, Murphy TF. Airway inflammation and etiology of acute exacerbations of chronic bronchitis. Chest **2000**; 118:1557–65.
24. Sandin C, Linse S, Areschoug T, Woof JM, Reinholdt J, Lindahl G. Isolation and detection of human IgA using a streptococcal IgA-binding peptide. J Immunol **2002**; 169:1357–64.
25. Murphy TF, Brauer AL, Aebi C, Sethi S. Antigenic specificity of the mucosal antibody response to *Moraxella catarrhalis* in chronic obstructive pulmonary disease. Infect Immun **2005**; 73:8161–6.
26. Bricker J, Mulks MH, Plaut AG, Moxon ER, Wright A. IgA1 proteases of *Haemophilus influenzae*: cloning and characterization in *Escherichia coli* K-12. Proc Natl Acad Sci U S A **1983**; 80:2681–5.
27. Gawthorne JA, Beatson SA, Srikhanta YN, Fox KL, Jennings MP. Origin of the diversity in DNA recognition domains in phasevarion associated *modA* genes of pathogenic *Neisseria* and *Haemophilus influenzae*. PLoS One **2012**; 7:e32337.
28. Srikhanta YN, Fox KL, Jennings MP. The phasevarion: phase variation of type III DNA methyltransferases controls coordinated switching in multiple genes. Nat Rev Microbiol **2010**; 8:196–206.
29. Murphy TF, Brauer AL, Schiffmacher AT, Sethi S. Persistent colonization by *Haemophilus influenzae* in chronic obstructive pulmonary disease. Am J Respir Crit Care Med **2004**; 170:266–72.
30. Wedzicha JA. The heterogeneity of chronic obstructive pulmonary disease. Thorax **2000**; 55:631–2.
31. Fernaays MM, Lesse AJ, Sethi S, Cai X, Murphy TF. Differential genome contents of nontypeable *Haemophilus influenzae* strains from adults

with chronic obstructive pulmonary disease. Infect Immun **2006**; 74: 3366–74.
32. Sacchi CT, Alber D, Dull P, et al. High level of sequence diversity in the 16S rRNA genes of *Haemophilus influenzae* isolates is useful for molecular subtyping. J Clin Microbiol **2005**; 43:3734–42.
33. Erwin AL, Sandstedt SA, Bonthuis PJ, et al. Analysis of genetic relatedness of *Haemophilus influenzae* isolates by multilocus sequence typing. J Bacteriol **2008**; 190:1473–83.
34. Hogg JS, Hu FZ, Janto B, et al. Characterization and modeling of the *Haemophilus influenzae* core and supragenomes based on the complete genomic sequences of Rd and 12 clinical nontypeable strains. Genome Biol **2007**; 8:R103.
35. Power PM, Bentley SD, Parkhill J, Moxon ER, Hood DW. Investigations into genome diversity of *Haemophilus influenzae* using whole genome sequencing of clinical isolates and laboratory transformants. BMC Microbiol **2012**; 12:273.