Characterization of igaB, a Second Immunoglobulin A1 Protease Gene in Nontypeable Haemophilus influenzae

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Nontypeable Haemophilus influenzae is an important respiratory pathogen, causing otitis media in children and lower respiratory tract infection in adults with chronic obstructive pulmonary disease (COPD). Immunoglobulin A1 (IgA1) protease is a well-described protein and potential virulence factor in this organism as well as other respiratory pathogens. IgA1 proteases cleave human IgA1, are involved in invasion, and display immunomodulatory effects. We have identified a second IgA1 protease gene, igaB, in H. influenzae that is present in addition to the previously described IgA1 protease gene, iga. Reverse transcriptase PCR and IgA1 protease assays indicated that the gene is transcribed, expressed, and enzymatically active in H. influenzae. The product of this gene is a type 2 IgA1 protease with homology to the iga gene of Neisseria species. Mutants that were deficient in iga, igaB, and both genes were constructed in H. influenzae strain 11P6H, a strain isolated from a COPD patient who was experiencing an exacerbation. Analysis of these mutants indicated that igaB is the primary mediator of IgA1 protease activity in this strain. IgA1 protease activity assays on 20 clinical isolates indicated that the igaB gene is associated with increased levels of IgA1 protease activity. Approximately one-third of 297 strains of H. influenzae of diverse clinical and geographic origin contained igaB. Significant differences in the prevalence of igaB were observed among isolates from different sites of isolation (sputum > middle ear > nasopharynx). These data support the hypothesis that the newly discovered igaB gene is a potential virulence factor in nontypeable H. influenzae.

Nontypeable Haemophilus influenzae, a gram-negative cocccobacillus, is an important respiratory pathogen (36). This organism is the second most common cause of bacterial otitis media in children and the leading cause of recurrent otitis media (24, 36). Nontypeable H. influenzae also plays an important role in the course and pathogenesis of chronic obstructive pulmonary disease (COPD). It is the most commonly isolated organism from the lower airways of adults with COPD and is the most common bacterial cause of exacerbations or periodic worsening of the disease (57). Nontypeable H. influenzae is also a contributing etiologic agent in sinusitis in children and adults as well as in adult pneumonia (36).

A number of virulence factors have been identified in nontypeable H. influenzae, including (i) several adhesin molecules, which promote attachment to host structures; (ii) lipooligosaccharide, which promotes inflammation at the site of infection; and (iii) proteins involved in iron acquisition (49, 59, 60). Additionally, an enzyme which cleaves immunoglobulin A1 (IgA1) has been identified in H. influenzae (22, 32). This capability is advantageous to this organism because IgA is the predominant mucosal immunoglobulin, and most of the IgA in the respiratory mucosa is of the IgA1 subclass (34).

IgA1 proteases have been identified in several human mucosal pathogens, including H. influenzae, Streptococcus pneumoniae, Neisseria meningitidis, and Neisseria gonorrhoeae (22, 32, 41). The IgA1 protease genes of Haemophilus and Neisseria show a high degree of nucleotide homology by Southern blotting (25) and encode serine-type IgA1-specific endopeptidases which share approximately 50% amino acid homology with each other (40). These proteins cleave the IgA1 molecule at specific locations within the hinge region, releasing Fe and Fab fragments (34). The gram-negative IgA1 proteases are released from the bacterial cell by way of a type V secretion (autosecretory) mechanism (44) in an Omp85-dependent manner (64). The C-terminal autotransporter (β-core) domain is placed in the membrane and mediates the translocation of the proteolytic domain, which is cleaved from the precursor protein by an autoproteolytic mechanism. Previous studies have determined that almost all IgA1 protease activity from H. influenzae, N. meningitidis, and N. gonorrhoeae is found in the supernatant of broth cultures, indicating the secretion of these proteins (51).

All or nearly all strains of H. influenzae contain the iga gene that encodes an IgA1 protease (47, 54, 62). Sequence analysis of IgA1 protease genes from H. influenzae have demonstrated long stretches of amino acid homology punctuated by short spans of variable regions in the protease domain of the gene. The C-terminal autotransporter (β-core) domain is highly conserved (48). The H. influenzae IgA1 proteases are classified as type 1 or type 2 based on the specific site of cleavage in the hinge region of the IgA1 molecule (34, 35, 40). Vitovski et al. (62) demonstrated that the level of IgA1 protease activity was higher in invasive disease-causing strains than in strains isolated from throat swabs of asymptomatic people. However, the precise role of IgA1 protease in the pathogenesis of H. influenzae infection has not yet been elucidated.

In previous studies, we identified a second H. influenzae IgA1 protease gene, igaB, that is separate and distinct from the previously identified H. influenzae IgA1 protease gene, iga...
(12). The igaB gene displays a high degree of homology to neisserial IgA1 protease genes. In the present study, we characterize the structure and function of this second gene as well as its distribution among strains of *H. influenzae* isolated from patients with a variety of clinical infections.

**MATERIALS AND METHODS**

**Bacterial strains and growth.** *H. influenzae* strain 11P6H was initially isolated from the sputum of an adult during an acute exacerbation of COPD (56, 58, 65). Sources and clinical characteristics of other strains are listed in Table 1.

<table>
<thead>
<tr>
<th>Strain collection</th>
<th>Clinical source of isolates</th>
<th>No. of isolates</th>
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<tbody>
<tr>
<td>National</td>
<td>Blood, cerebrospinal fluid, middle ear fluid</td>
<td>54</td>
<td>33, 37</td>
</tr>
<tr>
<td>Buffalo, NY</td>
<td>Sputum of adults with COPD</td>
<td>132</td>
<td>56</td>
</tr>
<tr>
<td>Michigan day care centers</td>
<td>Nasopharynx of children</td>
<td>33</td>
<td>11</td>
</tr>
<tr>
<td>Otitis media (various centers)</td>
<td>Middle ear fluid obtained by tympanocentesis</td>
<td>78</td>
<td>7, 10</td>
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Sources and clinical characteristics of other strains are listed in Table 1.

Strains of *H. influenzae* were grown on chocolate agar, in brain heart infusion (BHI) broth supplemented with hemin and isovitalex, or in chemically defined media (CDM) as described previously (53).

**Sequence analysis.** DNA sequences, obtained by standard methods, were aligned using Sequencher (Version 4.5; Gene Code Company). Comparisons to known genes were performed with BLASTN, BLASTP, and BLASTX analysis (http://www.ncbi.nlm.nih.gov/BLAST/).

**Reverse transcriptase PCR (RT-PCR).** All experiments were carried out with RNA isolated from strain 11P6H or the mutant strains using the QIAGEN RNaseasy kit and the Quareshidder kit, followed by 30 min of treatment with DNase I (Promega, Madison, WI) at 37°C and 10 min of DNase stop solution (Promega) at 65°C. RT-PCR was performed on these samples using the QIAGEN OneStep RT-PCR kit, using the optional Q solution and RNaseOut RNase inhibitor (Invitrogen, Carlsbad, Calif.) according to the manufacturer’s instructions. Primers were designed based on strain 11P6H sequence to amplify fragments approximately 500 bp in size. For every amplification, additional reactions were performed using primers to amplify a fragment of the constitutively expressed P2 outer membrane protein as an RNA control. Every reaction performed had a parallel reaction performed using the same primers and template to test for contaminating DNA in the samples. Following amplification, samples were electrophoresed on 1.5% agarose gels.

**Construction of mutants.** Two 1-kb regions were amplified by PCR using primers (Table 2) specific to the areas immediately upstream and downstream of the igaB gene in strain 11P6H. The primers also contained *H. influenzae* uptake sequences and appropriate restriction enzyme sites. These fragments were sequentially cloned into pGEM-3Zf (Promega). Similar amplifications and cloning of the 5’ 1 kb and 3’ 1 kb of the iga gene (the previously described igaA1 protease gene [6, 48]) were performed. The chloramphenicol resistance cassette from pACYC184 (New England Biolabs, Beverly, MA) was amplified by PCR with specific primers containing appropriate restriction enzyme sites. The two plasmids were linearized by restriction enzyme digestion between the two genomic fragments, and the resistance cassette was ligated to the upstream and downstream regions. These constructs were then linearized by appropriate restriction endonuclease digestion and transformed individually into strain 11P6H, which was made competent by the method of Herriott et al. (17) using the transformation protocol of Poje and Redfield (46). This resulted in two mutant strains, Δiga and ΔigaB. To construct a double mutant, the procedure for the ΔigaB mutant was repeated using a spectinomycin cassette from pSpecl (2). The Δiga strain was then transformed with this construct to create strain Δiga ΔigaB. Confirmation of homologous recombination was verified by PCR and sequencing of the regions surrounding the antibiotic resistance cassettes in all three mutants.

**Southern blot analysis of mutants.** Genomic DNA was purified from strain 11P6H and the three mutant strains using the Wizard genomic DNA purification kit (Promega). Approximately 20 µg of DNA was digested with BglII and EcoRI simultaneously. Five-microgram samples of each digest were subjected to electrophoresis on a 0.8% agarose gel. The DNA was transferred to a Hybond-XL membrane (Amersham Biosciences, Buckinghamshire, United Kingdom) using a Hoefer TransVac vacuum blotting unit (San Francisco, CA) following the manufacturer’s instructions. The membrane was cross-linked by UV light exposure and cut into four pieces, each containing one sample from each of the four strains. Probes ranging from 200 bp to 350 bp in size, corresponding to the iga and igaB genes as well as the chloramphenicol and spectinomycin resistance cassettes, were amplified by PCR from strain 11P6H, pACYC184, or pSpecl. Probes were labeled with the NEBlot Phototube kit (New England Biolabs) according to the manufacturer’s instructions so as to produce approximately 1000-bp gene probes.

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**Table 1. Sources of Haemophilus influenzae strains**

<table>
<thead>
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<th>5’ Primer sequence</th>
<th>3’ Primer sequence</th>
<th>Purpose of primers</th>
</tr>
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<tr>
<td>igaBscreen</td>
<td>TGCCCGTTACCGGATAAATG</td>
<td>AACCCACGGGCAACAAACAGCC</td>
<td>Amplify 1,011-bp region of igaB in screen of 298 strains</td>
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<tr>
<td>igaB2screen</td>
<td>TTTTTCGCAAAAGAAACCGC</td>
<td>ATCTATAAAAAAGAAATTGC</td>
<td>Amplify 1,011-bp region of igaB in screen of 298 strains</td>
</tr>
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<td>igaup</td>
<td>AAAGGATTCGCGGTTAT</td>
<td>AAAGGATTCGCGGCGGCTG</td>
<td>Amplify and clone 5’ 1 kb of iga gene</td>
</tr>
<tr>
<td>igadown</td>
<td>TGCCCGTTACCGGATAAATG</td>
<td>AACCCACGGGCAACAAACAGCC</td>
<td>Amplify and clone 5’ 1 kb of iga gene</td>
</tr>
<tr>
<td>igauB</td>
<td>AAAGGATTCGCGGTTAT</td>
<td>AAAGGATTCGCGGCGGCTG</td>
<td>Amplify and clone 3’ 1 kb of iga gene</td>
</tr>
<tr>
<td>igaBdown</td>
<td>AAAGGATTCGCGGTTAT</td>
<td>AAAGGATTCGCGGCGGCTG</td>
<td>Amplify and clone 1 kb upstream of igaB gene</td>
</tr>
<tr>
<td>igaprobe</td>
<td>AAAGGATTCGCGGTTAT</td>
<td>AAAGGATTCGCGGCGGCTG</td>
<td>Amplify and clone 1 kb downstream of igaB gene</td>
</tr>
<tr>
<td>igaBprobe</td>
<td>AAAGGATTCGCGGTTAT</td>
<td>AAAGGATTCGCGGCGGCTG</td>
<td>Amplify and clone 1 kb downstream of igaB gene</td>
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<tr>
<td>specprobe</td>
<td>AAAGGATTCGCGGTTAT</td>
<td>AAAGGATTCGCGGCGGCTG</td>
<td>Amplify and clone 1 kb downstream of igaB gene</td>
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<tr>
<td>catprobe</td>
<td>AAAGGATTCGCGGTTAT</td>
<td>AAAGGATTCGCGGCGGCTG</td>
<td>Amplify and clone 1 kb downstream of igaB gene</td>
</tr>
</tbody>
</table>

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PBS and blocking with 2% nonfat dry milk for 90 min at room temperature. (ThermoLabsystems, Franklin, MA) for 2 h at 37°C, followed by washing with 1:2,000 rabbit anti-mouse Ig (Z0259; DakoCode) to Immulon 4HBX plates. IgA1 protease reactions were stopped by adding 200 μl of a 1:1,000 anti-lambda light-chain horseradish peroxidase conjugate (code P0130; Dako) was added to the wells and incubated at room temperature for 2 h. After washing, color development was accomplished using tetramethyl benzidine. Absorbance readings were normalized to controls for that supernatant sample to allow for comparisons between assays.

Screening of strains for igaB. The sequence of the igaB gene was used in a PCR-based screening method as previously described (12). Whole bacterial cell lysates were made from strains of H. influenzae using previously described methods (19). Briefly, bacteria grown overnight on chocolate agar plates were harvested with a sterile loop and suspended in 100 μl sterile water by vortexing. The bacteria were incubated at 100°C in a heat block for 5 min, resuspended by vortexing, and incubated at 100°C for an additional 5 min. The samples were centrifuged at 1 min at 16,000 x g, and the supernatants were saved for use as the templates in PCR. Two sets of internal primers (Table 2) were designed for igaB based on the nucleotide sequence of strain 11P6H in this region. PCRs were carried out under the following conditions: 10 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C; followed by 3 min at 72°C. Samples prepared from the following strains were used as positive and negative controls in the PCR screening experiments: H. influenzae strain 11P6H (positive control), the sequenced H. influenzae strain KW20 Rd (negative control), and Moraxella catarrhalis strain 43617 (negative control). Water controls were also included in the analysis. Univariate analyses to identify associations between the presence of igaB and a variety of clinical syndromes were performed with chi-square analysis.

RESULTS

Sequence analysis of the igaB locus. In previous work, we identified igaB, a gene in H. influenzae strain 11P6H whose product has high homology to neisserial IgA1 proteases, that is separate and distinct from the previously identified IgA1 protease encoded by iga (6, 48). To confirm the sequence of the igaB locus, a series of overlapping fragments that span the entire region was amplified by PCR from the genomic DNA of strain 11P6H, and the sequences of the fragments were determined (GenBank accession no. DQ423203). The igaB open reading frame is 5,664 nucleotides (1,887 amino acids) in length (Fig. 1). Blastx analysis determined that the igaB gene is
homologous to the IgA1 protease genes of Neisseria gonorrhoeae and Neisseria meningitidis. The degree of homology of igaB to neisserial iga genes is greater than the homology of igaB to Haemophilus iga genes. The majority of the IgaB protein homology to neisserial Iga proteins occurred in amino acids 1 to 1020 (92% identity, 95% similarity; corresponding to the secreted protease domain) and amino acids 1529 to 1887 (53% identity, 71% similarity; corresponding primarily to the auto-

Both the igaB gene and the previously identified IgA1 protease gene (known as iga or H10990 in strain KW20 Rd) are present in strain 11P6H. The iga gene was amplified by PCR from strain 11P6H using previously described primers (62), and the sequence was determined. The sequence was highly similar to H. influenzae iga genes in the National Center for Biotechnology Information database. Whereas iga is present in the genomes of all four sequenced strains of H. influenzae (9, 13, 15), igaB is absent from the four strains. Furthermore, igaB is distinct from other known variants of the iga gene in this organism (48).

The igaB gene is 885 to 1,068 nucleotides longer than IgA1 protease genes with the highest BLAST matches. This size differential and the low level of sequence match in the third quarter of the gene are due to the presence of a repeated element in this section of the gene. A 423-bp section is present three times in succession in this region (Fig. 1, Repeats). The first 13 bp of these repeats (noted by asterisks in Fig. 1) are repeated a fourth time at the 3’ end of this region. Blast analysis of the 423-bp sequence showed small stretches of nucleotide match to Neisseria IgA1 protease genes but yielded no amino acid match.

Analysis of the deduced amino acid sequence of the igaB gene reveals a primary structure similar to that of known IgA1 proteases. Based on homology to other IgA1 proteases, the first 25 amino acids are a signal sequence capable of targeting translocation to the periplasm via the Sec pathway (38). Autocatalytic cleavage sites that produce the 961-amino-acid mature secreted enzyme and a short peptide (~32 amino acids) 3’ to the secreted domain are present in the N-terminal half of the igaB gene (Fig. 1). Conserved sequences predicted to encode the catalytically active site of IgA1 proteases (30, 44, 48) are found in the igaB gene. Further analysis of the amino acid sequence of the region that determines cleavage specificity (14) of the igaB gene indicates that the gene likely encodes a type 2 IgA1 protease. There is no apparent cleavage site in IgaB that would yield an α-protein as described for N. gonorrhoeae strain MS11 (44); however, there is a proline-rich region closer to the conserved β-core that, if cleaved, would yield a protein approximately 476 amino acids in length. This proline-rich region is homologous to a similar region in N. gonorrhoeae strain FA 1090 (GenBank accession no. YP_207437). The C-terminal 414 amino acids of IgaB, primarily corresponding to the β-core autotransporter domain, are conserved among neisserial IgA1 proteases. This region of the gene encodes the portion of IgA1 proteases that forms a β-barrel in the outer membrane, facilitating export of the mature protein.

**Transcription of IgA1 proteases in strain 11P6H.** To determine whether the iga and igaB genes are transcribed in H. influenzae strain 11P6H, reverse transcriptase PCR (RT-PCR) was performed. RNA was isolated from this strain and treated with DNase I to eliminate contaminating DNA. RT-PCR was performed using primers designed to amplify 500-bp fragments of iga, igaB, and the constitutively expressed OMP P2 gene as a positive control. RT-PCR analysis demonstrated transcription of both the iga and igaB genes in strain 11P6H (Fig. 2). Simultaneous parallel reactions performed using the same primers and template but with Taq polymerase and without reverse transcriptase were all negative, indicating the absence of contaminating DNA.

**Construction and characterization of IgA1 protease mutants.** To analyze the role of the two IgA1 proteases in the phenotype of strain 11P6H, three mutants were constructed. A Δiga mutant was constructed by replacing ~3 kb of the gene with a chloramphenicol resistance cassette. A ΔigaB mutant was constructed by replacing the entire gene with the chloramphenicol resistance cassette. A Δiga ΔigaB double mutant was constructed by replacing the igaB gene with a spectinomycin resistance cassette in the Δiga mutant. To confirm putative mutants, transformants that grew on appropriate selective media were tested by PCR for the presence and size of the construct, the presence of the resistance cassette, and the absence of the deleted gene. The three mutant strains were further confirmed by PCR and sequencing using one primer from the sequence within the resistance cassettes and one primer from the genomic sequence located outside of the sequence contained on the plasmid used to create the mutant. These reactions showed amplification of the appropriate-sized amplicons.
Sequencing across the regions of homologous recombination confirmed the presence of the cassette in the proper location.

Southern blots were also performed to confirm the absence of the IgA1 protease genes and the presence of the resistance cassettes in the appropriate knockouts. Genomic DNA from the parent strain and the three mutants was digested, electrophoresed, transferred, and hybridized with Phototope-Star-labeled probes that were amplified from the iga gene, the igaB gene, the chloramphenicol resistance cassette, and the spectinomycin resistance cassette. The patterns of signal confirmed the presence of iga in the parent and the ΔigaB mutant, the presence of igaB in the parent and the Δiga mutant, the presence of the chloramphenicol cassette in all three mutants, and the presence of the spectinomycin cassette in the double mutant only (Fig. 3).

To further confirm that the iga and igaB genes were not transcribed in the mutants, RT-PCR was performed (Fig. 4). As expected, the Δiga mutant showed only igaB transcription, the ΔigaB mutant showed only iga transcription, and the Δiga ΔigaB mutant showed transcription of neither.

The ΔigaB and Δiga ΔigaB mutants were further analyzed for transcription of the upstream (HI0185 and HI0184) and downstream (HI0164 and HI0166) genes. Primers were designed to amplify approximately 500-bp fragments from each of these genes. RT-PCRs were performed as described above, with strain 11P6H RNA used as a positive control. Both mutants showed transcription of the upstream and downstream genes (Fig. 5). The RT-PCR amplification reactions of the downstream genes in the ΔigaB strain showed less product than those from the parent strain. This is likely due to encroachment on the promoter region of those genes by the chloramphenicol resistance cassette in the construct that was used to create the ΔigaB mutant. These genes encode subunits of a Na⁺-translocating NADH-quinone reductase, thus, reduction in transcription of these genes is not expected to affect IgA1 protease activity of this strain. The gene downstream of the iga gene is convergently expressed, so polar effects of mutations in the iga gene are not expected. Further, this mutant was constructed so as to homologically recombine within the 5' 1 kb and 3' 1 kb of the iga gene, thus, adverse effects on regulatory regions of adjacent genes are not expected.

Outer membrane proteins were isolated from strain 11P6H and the three mutant strains to analyze any effects of deleting IgA1 protease genes on other membrane protein patterns. All strains had the same outer membrane protein pattern on SDS-PAGE (data not shown).

Identification of secreted IgA1 protease encoded by igaB.

Strain 11P6H and the three mutant strains were grown in chemically defined media to late logarithmic phase. The cells were removed by centrifugation, and the supernatants were concentrated approximately 100-fold using the Amicon ultrafiltration system and electrophoresed on SDS-PAGE (Fig. 6).
A band was present in both strain 11P6H (lane A) and the Δiga mutant (lane B) at the expected position of ~106 kDa, which corresponds to the predicted secreted portion of IgaB protein. If the iga gene expressed a stable product in this experiment, we would expect to see a second band in strain 11P6H (lane A) and a corresponding band in the ΔigaB mutant (lane C) only, or a band at the same level as IgaB in the ΔigaB mutant that was still not seen in the double mutant (lane D). No such band that would correspond to the product of the iga gene was noted. This result indicates that (i) strain 11P6H secretes IgaB, and (ii) although transcription of the iga gene was noted by RT-PCR, final expression of the gene was significantly lower than that of igaB, or the protein is labile in the culture supernatant.

Cleavage specificity of IgA1 proteases in strain 11P6H. IgA1 proteases are classified as type 1 or type 2 based on the specific site of enzymatic cleavage in the hinge region of the IgA1 molecule. To characterize the enzymatic specificity of the IgA1 proteases of strain 11P6H, digests were performed with IgA1 molecule. To characterize the enzymatic specificity of the IgA1 proteases of strain 11P6H, digests were performed with IgA1 protease activity of strain 11P6H and the three mutant strains using a total of four aliquots of culture supernatant from three different cultures (with each aliquot assayed in duplicate, this resulted in eight data points for each dilution for each strain). Each data point was normalized by converting the absolute value to a percentage of the controls for that strain in that assay (absent IgA means 0% signal, thus, maximum protease activity; IgA and sterile broth means 100% signal, thus, zero protease activity). Curves for each strain were generated using the LOESS (locally weighted scatterplot smoothing; SAS v9.1; SAS Institute, Inc.) technique. The curve for strain 11P6H indicates that this strain has IgA1 protease activity. The curves for 11P6H and the Δiga mutant were very similar, indicating that the majority of the IgA1 protease activity for this strain comes from igaB. The curve for the double mutant shows no degradation of IgA1, confirming previous observations that this strain has no detectable IgA1 protease activity. The curve for the ΔigaB mutant shows a low level of activity at maximal concentration that is quickly diluted out to form a curve that is similar to that of the double mutant, indicating that iga contributes minimally to the IgA1 protease activity of this strain.

A similar experiment performed with myeloma IgA2 showed no digestion by strain 11P6H or the mutant strains, indicating that neither IgA1 protease in this strain is active against IgA2 (Fig. 7). This is consistent with previous reports that most IgA1 proteases do not digest IgA2 (34, 40, 43).

IgA1 protease activity in strain 11P6H. To quantify the relative contributions of the products of iga and igaB in the IgA1 protease activity of strain 11P6H, assays were performed as previously described on the parent strain and the three mutant strains (50, 51). In this assay, serial dilutions of broth culture supernatants are incubated with IgA1, and the resulting reactions are analyzed for intact IgA1 in ELISA. Therefore, a greater signal indicates more intact IgA, thus, less IgA1 protease activity. Data from the dilutions of supernatant yield roughly sigmoidal curves (Fig. 8). More concentrated samples have more IgA1 protease activity, thus, less intact IgA and lower values. More dilute samples have less IgA1 protease activity, thus, more intact IgA and higher values. Taken as a whole, curves shifted to the left indicate that the strains in question have less IgA1 protease activity, while curves shifted to the right indicate strains with more activity.

IgA1 protease activity ELISAs were performed on strain 11P6H and the three mutant strains using a total of four aliquots of culture supernatant from three different cultures (with each aliquot assayed in duplicate, this resulted in eight data points for each dilution for each strain). Each data point was normalized by converting the absolute value to a percentage of the controls for that strain in that assay (absent IgA means 0% signal, thus, maximum protease activity; IgA and sterile broth means 100% signal, thus, zero protease activity). Curves for each strain were generated using the LOESS (locally weighted scatterplot smoothing; SAS v9.1; SAS Institute, Inc.) technique. The curve for strain 11P6H indicates that this strain has IgA1 protease activity. The curves for 11P6H and the Δiga mutant were very similar, indicating that the majority of the IgA1 protease activity for this strain comes from igaB. The curve for the double mutant shows no degradation of IgA1, confirming previous observations that this strain has no detectable IgA1 protease activity. The curve for the ΔigaB mutant shows a low level of activity at maximal concentration that is quickly diluted out to form a curve that is similar to that of the double mutant, indicating that iga contributes minimally to the IgA1 protease activity of this strain.

Distribution of igaB among clinical isolates of H. influenzae. A total of 297 well-characterized clinical isolates of nontypeable H. influenzae collected from a variety of sources (Table 1) were screened by PCR for the presence of igaB as described...
previously (12). Two sets of internal primers were designed to amplify fragments of the secreted portion of the igaB gene. Each assay contained control amplification reactions using template DNA from strain 11P6H (positive), the sequenced strain KW20 Rd (negative), Moraxella catarrhalis strain 43617 (negative), and water (negative). PCRs were performed in duplicate on separate days, with all strains scored for presence or absence of amplification of the predicted size fragments detected by agarose gel electrophoresis. When replicates of a strain-primer combination did not agree (less than 1% of all reactions), additional reactions were carried out with either purified genomic DNA or a second lysate preparation. The final determination was made based on these repeated reactions. The specificity of the igaB primers was confirmed using the IgA1 protease mutant strains. PCRs were performed with both igaB primer sets on strain 11P6H as well as the mutant strains. These reactions showed amplification from the parent and the igaB mutant but not the igaB mutant or the double mutant (Fig. 9). These results indicate that the igaB primers amplify a portion of the igaB gene but not the iga gene.

Among the 297 strains of nontypeable H. influenzae, 101 (or 34%) were positive for amplification of the igaB gene (Fig. 10). Analysis of the presence of igaB related to the site of isolation (sputum, middle ear, nasopharynx, and invasive disease [blood and cerebrospinal fluid {CSF}]) demonstrated a significant distribution (P < 0.001; chi square). Significant differences
between individual sites of infection were also seen by chi square (Fig. 10). The proportion of sputum isolates from adults with COPD with igaB was significantly greater than the proportion of middle ear isolates from children with igaB (P = 0.027; chi square). Further, nasopharyngeal colonizing strains had significantly lower prevalence of igaB than sputum isolates, middle ear isolates, and all disease isolates combined (P < 0.001, P = 0.011, and P = 0.0014, respectively).

IgA1 protease activity of strains of *H. influenzae* with and without igaB. To assess the IgA1 protease activity in strains of *H. influenzae* that contain igaB compared with those that do not, the IgA1 protease activity assay was performed on 10 clinical isolates that contain the igaB gene and 10 that do not. All strains were isolated from the sputum of adults with COPD. The assays were carried out on two aliquots of culture supernatant, each from different cultures (each aliquot was assayed in duplicate for a total of four data points at each dilution). The data were normalized relative to the absent IgA and sterile broth controls as previously described, and LOESS plots were generated. Based on these plots, the dilution that resulted in 50% IgA1 protease activity for each strain was determined. A higher value indicates more IgA1 protease activity, while lower values indicate less IgA1 protease activity. For those strains that had undiluted supernatants that did not produce 50% activity, theoretical values were determined based on extrapolation of the LOESS curves into negative values. The results of this analysis are summarized in Fig. 11. The mean value of the 10 strains that contain the igaB gene (2.57) was significantly greater than the mean value of the 10 strains that lacked the igaB gene (0.17; P = 0.0411; unpaired t test). These data indicate that those strains that contain igaB exhibit more IgA1 protease activity than those that do not. We conclude that the total IgA1 protease activity of strains of *H. influenzae* results from the combination of the activities of the IgA1 proteases encoded by iga and igaB.

![Graph showing IgA1 protease activity](image1)

**FIG. 8.** IgA1 protease activity assays. Lines indicate LOESS plots of the IgA1 protease activity as a percentage of control of culture supernatants of *H. influenzae* strain 11P6H and the mutant strains as noted. **x** axis, the degree of twofold dilution of the original samples; **y** axis, activity as a percentage of maximal activity based on positive (IgA with sterile BHI broth) and negative (undiluted culture supernatants absent IgA) controls. Each data point is an average of eight replicates. Error bars represent the standard error for each dilution.

![Western blot assays of IgA digests](image2)

**FIG. 7.** Western blot assays of IgA digests. (A) Lanes contain purified human IgA1 digested with culture supernatants of *H. influenzae* or PBS in place of the supernatant, as follows: strain 11P6H (lane 1); Δiga mutant (lane 2); ΔigaB mutant (lane 3); Δiga ΔigaB double mutant (lane 4); type 1 IgA1 protease control, *H. influenzae* strain KW20 Rd (lane 5); type 2 IgA1 protease control, *H. influenzae* biogroup aegyptius strain BPF 17 (lane 6); sterile BHI broth (lane 7); and PBS (lane 8). (B) Lanes contain purified human IgA2 digested with culture supernatants of *H. influenzae* or broth or PBS in place of the supernatant, as follows: strain 11P6H (lane 1); Δiga mutant (lane 2); ΔigaB mutant (lane 3); Δiga ΔigaB double mutant (lane 4); sterile BHI broth (lane 5); PBS (lane 6); and PBS (unincubated) (lane 7). Molecular mass markers are labeled in kilodaltons.

![Agarose gel showing specificity of primers for PCR amplification](image3)

**FIG. 9.** Agarose gel showing specificity of primers for PCR amplification of IgA1 protease genes. DNA from strain 11P6H and the mutant strains were used as the templates for PCRs as noted. Primers used in each PCR are noted at the top of the gel. Primers used for the amplification of iga were the “P” primers used by Vitovski et al. (62).
DISCUSSION

In this study, we have characterized a second IgA1 protease gene, igaB, and have demonstrated that the gene is present in one-third of strains of *H. influenzae* isolated from a wide range of clinical and geographic sources. The igaB gene is separate and distinct from the well-characterized IgA1 protease encoded by iga that is present in all strains of *H. influenzae* studied thus far. The IgA1 protease encoded by igaB shows greater homology to neisserial IgA1 proteases than to the IgA1 protease encoded by the *H. influenzae* iga gene. Detailed analysis of a strain of *H. influenzae* that was isolated from an adult with COPD at the time of an exacerbation revealed that the igaB gene is transcribed, and the protein is expressed and secreted in its active form with type 2 IgA1 protease activity.

IgA1 proteases cleave the hinge region of IgA1, which is the predominant immunoglobulin on the human respiratory tract mucosal surface. The potential significance of IgA1 protease in the pathogenesis of *H. influenzae* infection has been difficult to elucidate in part because the IgA1 subclass molecule is unique to humans and some higher primates, limiting the use of animal models to study IgA1 protease. However, several lines of evidence suggest that IgA1 protease is a virulence factor. Among gram-negative bacteria, IgA1 protease is present predominantly in pathogenic species. *Neisseria meningitidis*, *N. gonorrhoeae*, and *H. influenzae* have IgA1 proteases, while the commensals *N. mucosa*, *N. lactamica*, *N. flavescens*, *N. cinerea*, *N. subflava*, *N. flava*, *N. perflava*, *N. sicca*, *N. elongata*, *H. parainfluenzae*, *H. aphrophilus*, *H. paraphrophilus*, *H. segnis*, *H. paraphrophaeomlyticus*, *H. hemoglobinophilus*, and *H. haemolyticus* do not (20, 39, 54). In addition, studies of *N. meningitidis* showed significantly increased levels of IgA1 protease activity among invasive isolates compared to isolates from asymptomatic carriers (63). A similar finding was reported in a study of nontypeable *H. influenzae*. Strains isolated from sputum, blood, CSF, and other disease-associated sites had higher IgA1 protease levels than nasopharyngeal isolates from healthy volunteers (62), suggesting that IgA1 protease is a virulence factor. The present study contributes another indirect line of evidence suggesting that IgA1 protease is a potential virulence factor in human infection. The proportion of *H. influenzae* strains isolated from middle ear fluid of children with otitis media with the iga gene (31%) was significantly greater than the proportion of nasopharyngeal strains with igaB (9%; P = 0.013). Further studies of the prevalence of igaB in well-characterized clinical isolates are needed to test this hypothesis further.

The present study indicates that genes iga and igaB are transcribed in strain 11P6H. The study further showed that igaB is directly responsible for a substantial amount of IgA1 protease activity in strain 11P6H. The observation that strains of *H. influenzae* that have both IgA1 protease genes express higher levels of total IgA1 protease activity than strains that have iga exclusively suggests that igaB contributes to the IgA1 protease activity in other strains as well (Fig. 11). However, additional study is needed to establish this observation with certainty in other strains. Overall, these observations support the conclusion that the IgA1 protease activity measured in assays represents the cumulative IgA1 protease activity of both iga and igaB genes. The observation of the contribution of a second IgA1 protease in *H. influenzae* has implications in the interpretation of previous studies involving measurements of IgA1 protease activities of clinical isolates. Several groups have identified strains that have two protease activity specificity types (23, 28, 29, 35, 39, 54). Lomholt and Kilian (28) identified a strain of nontypeable *H. influenzae* with two different protease types that had inhibition of IgA1 protease activity by using antisera raised to neisserial IgA1 proteases. Also, antisera raised against secreted proteases from that strain inhibited IgA1 proteases from *N. meningitidis and N. gonorrhoeae*, suggesting that this strain encodes a neisserial iga-like IgA1 protease (i.e., igaB). Further, this strain showed Southern hybridization of probes derived from iga to two portions of the genome (47). We speculate that these strains have both iga and igaB genes expressing IgA1 proteases.

In addition, the varying levels of IgA1 protease activity...
among clinical isolates observed by Vitovski et al. (62) is likely a result of the IgA1 proteases encoded by iga in all strains and igaB in some strains. In that study, PCR was used to confirm the presence of the iga gene in virtually all strains of *H. influenzae* isolates examined. We confirmed that the primers used in the Vitovski study (62) did not amplify the igaB gene. Further, the primers we used to screen for igaB did not amplify the iga gene (Fig. 9). These data confirm the results of epidemiological studies performed by Vitovski et al. that showed iga in all strains of *H. influenzae*, while we showed that igaB is present in approximately one-third of strains.

In view of the high degree of homology between igaB and the iga genes from both *N. meningitidis* and *N. gonorrhoeae*, we speculate that igaB was acquired by horizontal transfer from *Neisseria*. The major difference between igaB and the neisserial iga genes is the presence of 423-bp direct repeats between base pairs 5498 and 6779. It appears that this sequence originally appeared as a single copy that was expanded by a mechanism possibly mediated by 13-bp direct repeats on both ends of the sequence. In addition, the G+C content of this gene is greater than the average for sequenced *H. influenzae* strains, approaching the G+C contents of neisserial iga genes and neisserial genomes (12). Transfer from *Haemophilus* to *Neisseria* has been suggested in a number of studies (8, 26, 52, 55), but evidence of transfer from *Neisseria* to *Haemophilus* has not been established. Analysis of the igaB locus suggests that this gene was acquired and integrated into the *H. influenzae* genome as part of a large genomic inversion event, as the genes upstream and downstream of igaB in strain 11P6H are not present in such close proximity nor in the same orientation in the genomes of the four sequenced strains of this organism. IgA1 proteases may play a role in pathogenesis by a mechanism independent of their ability to cleave IgA1. IgA1 was the only known substrate of IgA1 protease until recently. LAMP1, a membrane component of late endosomes and lysosomes, is also cleaved by IgA1 proteases, resulting in destabilized lysosomes and increased invasion of human cells by the bacterium (1, 16, 18, 27). Further, the α-protein, which may be a component of the igaB protein, induces human T-cell responses and translocates to human nuclei, potentially altering host gene transcription (21, 45). IgA1 proteases also induce a number of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-8 independent of proteolytic activity (31), as well as a specific T-cell response in humans (61). In addition, IgA1 proteases inhibit TNF-α-mediated apoptosis of human monocytes by cleavage of the TNF receptor II (3). These examples illustrate contributions that one or both of the IgA1 proteases may make to the pathogenesis of *H. influenzae*, thus, the relative importance of each of these genes goes well beyond a discussion of IgA1 protease activity levels. It will be important to determine the relative contributions of both IgA1 proteases in these other functions.

In summary, this study characterized the second IgA1 protease gene, igaB, which is separate and distinct from the well-characterized IgA1 protease encoded by iga, which is present in all strains of *H. influenzae*. The igaB gene is transcribed and expressed in *H. influenzae* strain 11P6H and is present in approximately one-third of strains. The IgA1 protease activity of strains of *H. influenzae* results from IgA1 proteases encoded by the iga and igaB genes. Future studies will examine the significance of these genes in the pathogenesis of *H. influenzae* in the context of human infection.

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**REFERENCES**


