Introduction

It is well known that bacteria have developed intrinsic and clever strategies to aid their survival and successful colonization in vivo. One strategy employed by particular groups of pathogenic bacteria is the production of proteolytic enzymes, which cleave particular proteins in order to destroy their function. One such group of enzymes are the bacterial IgA1 proteases.

IgA1 proteases are a group of proteolytic enzymes that have the characteristic ability to cleave human immunoglobulin A, subclass 1 (IgA1). They were originally named IgA1 proteases, as they did not have any other known substrates. IgA1 proteases are produced by several species of pathogenic bacteria that target mucosal surfaces, and IgA1 protease genes and proteins are absent from non-pathogenic members within a species. The earliest discoveries of IgA1 proteases were isolated from Neisseria gonorrhoeae, Neisseria meningitidis, Haemophilus influenzae and Streptococcus pneumoniae.

The IgA1 proteases are thought to produce a localized immune deficiency by cleaving IgA1 in the mucosal secretions into intact Fabβ and Fcα fragments. Thus IgA1 is unable to carry out its immunomodulatory clearance of the bacteria or other foreign substances at the mucosal surface. IgA1 proteases are therefore thought to be virulence factors for infection and colonization.

IgA1 proteases target the hinge region of human IgA1, which is composed of a primary sequence of proline (P), threonine (T) and serine (S) residues. These residues are arranged as a duplicated octapeptide sequence, T P P S T P P S S in the hinge region of the heavy chain of IgA1.1,11 (Fig. 1). The peptide bond cleaved by an IgA1 protease from any single species of bacteria is either a proline-serine bond, which is designated Type 1 cleavage or a proline-threonine bond, which is referred to as Type 2 cleavage.

The type of cleavage in Haemophilus influenzae has been localized to a 370 bp sequence in the N terminal region of the IgA1 protease gene, called the Cleavage Specificity Determinant (CSD).12 The inhibition of an IgA1 protease is affected by the cleavage specificity of the enzyme, as antibodies raised against the Type 1 IgA1 protease do not inhibit any Type 2 IgA1 proteases and vice versa.11

IgA1 proteases produced by non-typeable Haemophilus influenzae (NTHI) have shown the greatest variation in the pattern of cleavage of human IgA1. Thus far four different ‘cleavage types’ have been characterized according to the bonds cleaved within the hinge region sequence, which produce different sized Fabα and Fcα fragments.14-18

NTHI is a Gram-negative coccobacillus, which colonizes the human upper respiratory tract as part of the normal flora.19 However, NTHI can also chronically colonize the lower respiratory tract of patients with chronic bronchitis, bronchiectasis and chronic obstructive pulmonary disease.20-23 IgA1 protease activity was found to be higher among isolates of NTHI which were clinical strains involved in a disease compared to colonizing strains in asymptomatic carriers.24 Thus it is believed that the...
IgA1 proteases play a role in facilitating the infection and colonization of mucosal surfaces by NTHI.

Understanding the cleavage characteristics of IgA1 proteases from various bacteria should provide the basis for the development of specific agonists thereby reducing the pathogenic nature of the bacteria. The purpose of the current study was to characterize the cleavage characteristics of the IgA1 protease of an isolate of NTHI obtained from the respiratory tract of a bronchiectasis patient with chronic colonization.

**Results**

An IgA1 protease was isolated from a clinical isolate of non-typable *Haemophilus influenzae* (NTHI<sub>6988</sub>). It was shown to be a true IgA1 protease by incubations with human IgA1 and IgA2, which produced cleavage of human IgA1 only and not human IgA2 (Fig. 2).

However, the incubation with IgA1 during a 24 h time course experiment showed there were two IgA1 Fc fragments produced during the cleavage reaction and not one size of Fc fragment, as reported previously for other IgA1 proteases (Fig. 3).

The two Fc fragments were isolated and subjected to mass spectrometry and N terminal sequencing. The results of the mass spectrometry showed the fragments were those of the Fc portion of the human IgA1 heavy chain, and the N terminal sequencing found the N terminals of both the different sized fragments started with the sequence Thr-Pro-Ser-Pro-Ser. These results indicated that the IgA1 protease was cleaving proline-threonine bonds.

The cleavage pattern of this IgA1 protease could be the result of the NTHI isolate (NTHI<sub>6988</sub>) producing two IgA1 proteases or the one isolate producing one enzyme with capabilities of cleaving two bonds within the specific octapeptide sequence.
In order to investigate this further, PCR was carried out with IgA1 protease sequence specific primers to find the number of iga sequences within the NTHI6988 genome. Sequence specific primers used to amplify the gene sequence of the protease domain, the beta domain and also the complete iga gene. The PCR reactions produced one protease domain fragment (approximately 3 kbp), one beta domain fragment (approximately 2 kbp) and one fragment of the whole iga sequence (5 kbp) (Fig. 4A and B). The PCR products were cloned and sequenced to confirm the gene sequences were those of an IgA1 protease. The protease domain sequence can be accessed with Genbank Accession Number DQ167218.1. The beta domain sequence can be found with Genbank Accession Number DQ167219.1.

The gene sequence of the CSD region of the IgA1 protease gene was compared with the Type 1, Type 2 and the Type 3 CSD sequences of other IgA1 proteases and is shown in Figure 5.

Each PCR reaction yielded one PCR product indicating that there is only one iga sequence within the NTHI genome of the isolate that is producing the IgA1 protease with an unusual pattern of cleavage for human IgA1.

The NTHI genomic DNA was also screened with the igaB primers to detect the igaB gene. The PCR was negative, as there were no 1,650 bp fragment produced.

In addition, the IgA1 protease was incubated with human IgA1 and different classes and types of inhibitors to see if these inhibitors could eliminate the proteolytic activity of this protease. Thus far, there are no naturally occurring inhibitors of IgA1 proteases therefore if there was no inhibition by the inhibitors (natural and synthetic) then it would also indicate that the proteolytic enzyme that has been isolated is an IgA1 protease. The inhibition experiment showed that the IgA1 protease was still able to cleave the human IgA1 and the natural protease inhibitors (SLPI, alpha-1-antitrypsin, cathepsin G and cystatin C) and synthetic protease inhibitors (Tos-Lys-chloromethylketone.HCl, ZD0892) were unable to inhibit its proteolytic activity. These results show the IgA1 protease isolated from the NTHI has two proteolytic activities, whereby one or both of the cleavage activities could not be eliminated by the different types of protease inhibitors (Gel not shown).

**Discussion**

IgA1 proteases are known to cleave post-proline peptide bonds within a duplicated octapeptide sequence within the hinge region of IgA1. IgA1 proteases cleave either a proline-serine bond or a proline-threonine bond within this specific sequence. Recently, Johnson et al. have reported the structure of the *Haemophilus influenzae* IgA1 protease after carrying out crystallographic studies and molecular modeling. The proposed model of cleavage is in the context of binding to IgA1, and the IgA1 protease
In this investigation, a patient isolate of non-typable Haemophilus influenzae was found to produce an IgA1 protease that cleaved human IgA1 to produce two different sized Fc fragments. If the digested fragments of human IgA1 were a result of true IgA1 protease activity, the digested fragments were expected to begin with a serine residue (cleavage of a Pro-Ser bond) or a threonine residue (cleavage of a proline-threonine bond). In this study we were able to confirm the sites of cleavage by the IgA1 protease, by carrying out N terminal sequencing of the two Fc fragments of human IgA1. The N terminus sequences of both the cleavage fragments were the sequence Thr-Pro-Ser-Pro-Ser. As both the fragments began with the sequence Thr-Pro-Ser-Pro-Ser, N terminal sequence contains a chymotrypsin-like fold, which acts as a lid to the active site. This active site becomes available only after binding to human IgA1, and regions of the Fc portion of the molecule are essential for cleavage, thus explaining the specificity for the hinge sequence of human IgA1.

The IgA1 proteases of Haemophilus influenzae have been found to have the greatest diversity in cleavage specificities for human IgA1. The Type 1 IgA1 proteases cleave the Pro231-Ser232 bond and the Type 2 proteases attack the Pro235-Thr236 bond. A third IgA1 protease hydrolyses the Pro237-Ser238 bond and other IgA1 proteases have been isolated that produce a double cleavage pattern although the specific bonds have yet to be identified.14-18,30

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the results indicate that this IgA1 protease specifically cleaves proline-threonine bonds. This composition of residues is found twice in the duplicated octapeptide sequence of the human IgA1 hinge region, at positions Thr228-Pro229-Ser230-Pro231-Ser232 and Thr236-Pro237-Ser238-Pro239-Ser240. However, human IgA1 also contains variable O-glycan side chains within the hinge region, which could cause variation in the size of the heavy chain fragments due to deglycosylation.31 In order to eliminate this possibility, the IgA1 protease (NTHI6988) was also incubated with deglycosylated human IgA1. This still resulted in two Fc fragments, which did not degrade further. If the two Fc fragments had been different sized fragments due to glycosylation, then incubation of the deglycosylated IgA1 should only produce one fragment with a lower molecular weight.31

As the IgA1 protease does not cleave human IgA2 and prolonged incubation of the IgA1 protease with human IgA1 does not result in degradation of the Fc fragments, these results indicate that the IgA1 protease is not cleaving or degrading the Fc fragment at the C terminus end of the IgA1 protein. The results however indicate that the IgA1 protease is cleaving two bonds in the IgA1 sequence.

The double cleavage could be due to one IgA1 protease being able to cleave more than one bond within the hinge region or NTHI6988 is able to produce two types of IgA1 proteases simultaneously.32 The iga gene normally exists as a single copy in the chromosomal DNA of pathogenic bacteria.33 However, recent investigations have discovered another iga gene, called igaB, which has also been found to be present in NTHI at the same time as the iga gene.28

To investigate this further, the gene(s) for the IgA1 protease and its two subunits (the protease and beta domains) were amplified with sequence specific primers for iga. Primers corresponding to a 1,650 bp fragment within the igaB were used to screen for the igaB gene.

The results showed one 3 Kbp PCR product for the protease domain, one 2 Kbp beta domain fragment and one 5 Kbp fragment of the whole iga gene product. Our screening of the isolate NTHI6988 with iga Bscreen primers did not produce a PCR product for the igaB gene. Our results therefore indicate there is one IgA1 protease sequence from our Haemophilus influenzae isolate. These results do not concur with those of Fernaays et al. who reported a second IgA1 protease, igaB from the one bacterial isolate.

The protease and beta domain fragments were cloned and fully sequenced and the sequences confirmed their identities as the gene of an IgA1 protease. The whole iga gene PCR product was partially sequenced and was also confirmed to be the sequence of an IgA1 protease. The iga gene sequence isolated from NTHI6988 was not homologous with the igaB gene of Fernaays et al. These results indicate that the isolate of NTHI under investigation contains one gene for the IgA1 protease that produces an unusual pattern of cleavage of IgA1.

The sequence of the IgA1 protease was analyzed further in the N terminus of the protein sequence, the CSD, by comparing it with the Type 1 and Type 2 IgA1 protease sequences. The IgA1 protease sequence under investigation was also compared to an additional iga gene sequence33 from a strain of Haemophilus influenzae that produces an IgA1 protease of a novel cleavage type (which will be referred to as Type 3). Lomholt et al.32,33 observed that this particular IgA1 protease also produced more than two cleavage products of human IgA1 and hypothesized that there appeared to be cleavage occurring at more than one site; however they had not identified the positions of cleavage.

The sequence in the N terminus of the IgA1 protease is a region called the cleavage specificity determinant (CSD). Grundy et al.12 found that the ‘type’ of IgA1 protease was determined by the CSD, which is a 370 base pair sequence in the 5’ portion of the iga gene. The sequences of the CSD regions of a type 1 and type 2 protease contain high degrees of non-homology. The CSD sequence of this IgA1 protease was compared to those of the Type 1, 2 and 3 IgA1 protease CSD sequences (Fig. 5). The sequence of this IgA1 protease showed a higher degree of homology to the Type 1 IgA1 protease sequence, similar to the Type 3 IgA1 protease sequence;33 however there were several residues that were not identical and in addition there was an insertion in the sequence (TEK DKE GNL LR), which is of similar length as an insertion found in the CSD sequence of the Type 3 protease. This insertion may be of importance as the size of the CSD is thought to act as a spacer and therefore any insertions or deletions may alter the cleavage specificity of the IgA1 protease. Lomholt et al.33 found that there was a proportional relationship between the length of the CSD and the distance from the interchain disulphide bridge that marks the end of the IgA1 hinge region. Therefore the length and amino acid composition of the CSD could determine which bond is accessible in the hinge region.

The results of several studies, including this investigation, show that the IgA1 proteases produced by different strains of Haemophilus influenzae show a wide range of diversity, resulting in different cleavage specificities of human IgA1.

The significance of the cleavage type of the IgA1 protease is related to the inhibition of the IgA1 proteases. DeVenyi et al. found that neutralizing antibodies were specific to the cleavage type of the enzyme. They found that the antibodies raised against the Type 1 IgA1 protease did not inhibit any Type 2 IgA1 proteases and vice versa. From their investigations they concluded that a specific epitope recognized by the neutralizing antibodies may be situated within or near the cleavage specificity determinant region.

Zakrzewski et al. hypothesized that the successful colonization and infection of the pathogen may be dependent upon the disruption of a delicate balance between the IgA1 proteases and the neutralizing antibodies at the mucosal surface. Therefore the antigenic diversity demonstrated by the IgA1 proteases suggests that this may be a mechanism to avoid the host immune system by changing surface epitopes that could be recognized by neutralizing antibodies remaining after a previous infection. Furthermore, IgA1 protease gene sequences reveal highly heterogeneous nucleotide sequences that would result in antigenic variance of the IgA1 proteases.

Therefore, IgA1 proteases of different cleavage specificities are not necessarily going to be inhibited by the same antibodies or inhibitors. This has major implications upon the production of specific antibodies or the design of synthetic inhibitors, which would be required to eradicate IgA1 protease activity. Thus far,
lactoferrin has been found to inhibit IgA1 protease by cleaving it; however there are no other physiological inhibitors of IgA1 protease and there have been limited studies on the production of synthetic inhibitors, which have not been investigated further in order to be suitable for use in vivo.

As IgA1 protease is thought to act as a virulence factor for successful bacterial colonization and infection, it would be advantageous to have a specific inhibitor that could target a specific part of the IgA1 protease, since the IgA1 proteases, particularly of *Haemophilus influenzae* species, show such a high degree of heterogeneity. Therefore it is necessary to investigate the discoveries of new cleavage specificities of IgA1 proteases, as this may have implications on the design of inhibitors for this diverse group of enzymes.

**Methods**

**Bacterial culture.** An isolate of NTHI (NTHI<sub>6988</sub>) from a patient with bronchiectasis who was persistently colonized with *Haemophilus influenzae* was provided by The Respiratory Research Microbiology Laboratory, Queen Elizabeth Hospital, Birmingham. The isolate was cultured, examined and reported by an experienced microbiologist and confirmed by X and V factor requirements. The isolate required haemin and NAD (nicotinamide adenine dinucleotide) growth factors and did not grow on blood agar plates. The isolate of NTHI was not, therefore, a nonhemolytic *Haemophilus haemolyticus*. H. haemolyticus are not producers of IgA1 protease.

A primary plate of NTHI<sub>6988</sub> was cultured on chocolate agar (Biomerieux) from a frozen glycerol stock. A 5 ml inoculum was grown from a single colony of the NTHI isolate, and this was added to 485 ml brain heart infusion broth (Oxoid Ltd., Basingstoke, Hampshire) supplemented with 500 μg β-nicotinamide adenine dinucleotide (0.5 mg/ml; Sigma-Aldrich Co., Ltd., Poole, Dorset) and 10 ml hemin (10 mg/ml; Sigma-Aldrich Co., Ltd.). The culture was incubated overnight at 37°C with gentle agitation.

**Partial purification of the IgA1 protease.** The partial purification of IgA1 protease was carried out using a method described by Mulks et al. A 500 ml overnight culture of NTHI<sub>6988</sub> was centrifuged to remove the bacterial cells, and the supernatant was retained. Approximately 300 g ammonium sulphate (Sigma-Aldrich Co., Ltd.) was added to the culture supernatant until the protein precipitated from the solution. The precipitate was collected by centrifugation and the pellet was resuspended in 20 ml 0.015 M Tris phosphate buffer (pH 8.0) (Sigma-Aldrich Co., Ltd.). The protein solution was subsequently dialysed against 4 liters of 0.015 M Tris phosphate buffer (pH 8.0) for 24 h at 4°C with constant stirring and dialysed for a further 24 h with fresh 4 liters of 0.015 M tris phosphate buffer (pH 8.0). The dialysed protein solution was placed through a DE52 anion exchange column (Whatman International Ltd.) equilibrated with 0.015 M tris phosphate buffer (pH 8.0) and the flowthrough containing the IgA1 protease was collected as 1 ml fractions.

**Genomic DNA.** The genomic DNA of NTHI<sub>6988</sub> was extracted by a method described by Allsubel et al. A 3 ml overnight bacterial culture of NTHI<sub>6988</sub> was centrifuged to retain the bacterial cells and the supernatant was discarded. The cell pellet was resuspended in 567 μl 10 mM Tris/HCl, 1 mM EDTA, pH 8 buffer and 30 μl 10% SDS (Sigma-Aldrich Co., Ltd.) and 3 μl proteasein K (20 mg/ml; Sigma-Aldrich Co., Ltd.) and incubated at 37°C for 60 min. Following the incubation, 100 μl 5 M sodium chloride (BDH Chemicals Ltd., Poole, UK) was added to the resuspension and the tube was mixed. A volume of 80 μl of CTAB solution (Sigma-Aldrich Co., Ltd.) (preheated to 65°C) was added and the tube was incubated at 65°C for 10 min. On completion of the incubation, 800 μl chloroform/isoamyl alcohol (Sigma-Aldrich Co., Ltd.) was added and the mixture was centrifuged for 10 min at 10,666 g. The aqueous phase was removed into a clean eppendorf and an equal volume of phenol/chloroform/isoamyl alcohol (Sigma-Aldrich Co., Ltd.) was added and the tube was shaken vigorously. The tube was centrifuged for 10 min at 10,666 g and the supernatant was discarded. The DNA pellet was washed with 700 μl 70% ethanol by centrifugation at 10,666 g for 5 min. The ethanol supernatant was discarded and the pellet was air-dried at room temperature. The pellet was subsequently re-suspended in 20 μl sterile water and RNase A (Sigma-Aldrich Co., Ltd.) was added to a final concentration of 50 μl/ml to the DNA preparation and the reaction was incubated at 37°C for 30 min. The treated DNA was stored at -20°C until required.

**Cloning.** The primers used for the amplification of the protease domain sequence were 5'-GAT GGA TCC GCC TGG GTA CAA TAT-3' and 3'-TTA TTA TAA GGT CGA GTA AAC ATG TTC GGA ACA-5'.

The sequences of the beta domain primers were 5'-GAT GGA TCC TCA GAG ACA ACT GAA ACA GTG GCT GAA-3' and 3'-CTT AAT TTT GAT TCA AAA TCA AAA ATT TTC GGA ACA-5'.

An optimized PCR amplification contained 100 ng genomic DNA (pretreated with RNaseA), 100 pmoles of each primer (Alta Bioscience, University of Birmingham, UK), 1 μl 10 mM dNTPs (Amersham Biosciences UK Ltd.), 5 μl 10x *Taq* polymerase buffer (Promega Corporation, Madison, USA). The total volume was made up to 49.5 μl sterile water and sealed with a drop of mineral oil. The PCR (Omnigene, Hybaid Ltd.) was started by preheating the reaction to 99°C for 5 min before the addition of 0.5 μl *Taq* polymerase (Promega). The program continued for 35 cycles with denaturation at 95°C for 30 s, annealing at 53°C for 1 min and extension at 72°C for 3 min (protease domain) or 2 min (beta domain).

The PCR products were cloned into the pGem T Easy T-A vector (Promega) according to the manufacturer’s recommendations.

**The screening for the igaB gene.** The genomic DNA from NTHI<sub>6988</sub> was screened for the igaB gene using the method by Fernaays et al. The primers used were 5'-TGC CCG TTA CCG GAC GTG TGG TAT-3' and 5'-AAC CAC GGG CAC AAA CAG CC-3'. PCR was 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. The results were visualized with agarose gel electrophoresis.

**Antibodies/western blotting.** Human IgA1 (Calbiochem-Novabiochem Ltd.) and IgA1 cleavage products were separated...
by SDS polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Following wet-blotting onto Hybond-C nitrocellulose membrane (Amersham Biosciences), the membranes were immunostained with 1:2,000 rabbit anti-human IgA1 (Biogenesis Ltd.) and 1:2,000 anti-rabbit HRP conjugate (DAKO Ltd.). The results were visualized with the ECL system (Amersham Biosciences).

DNA sequencing. DNA sequencing of the protease and beta domain clones were carried out by Alta Bioscience (The University of Birmingham).

N terminal sequencing. Sequencing was carried out on two protein fragments generated from the cleavage of human IgA1 (Calbiochem-Novabiochem Ltd.) and the partially purified IgA1 protease. The reaction was incubated for 2 h at 37°C and the cleavage products were separated by polyacrylamide gel electrophoresis under reducing conditions (Invitrogen Life Technologies). The protein was transferred onto PVDF membrane, and the appropriate protein bands were cut out on the PVDF. The proteins were N terminally sequenced by The Biomolecular Analysis Facility, The University of Leeds.

Deglycosylation of human IgA1. A 20 μl sample of human IgA1 in plasma (500 μg/ml; Calbiochem-Novabiochem Ltd.) was treated with Protein Deglycosylation Mix (New England Biolabs Inc.) containing PNGase F, Endo-0-N-Acetylgalactosaminidase, -Neuraminidase, β-1-4 Galactosidase and β-N-Acetylgalactosaminidase to remove all N-linked and O-linked glycans. The reaction mixture was incubated at 37°C for 4 h as recommended by the manufacturer’s instructions. Results were visualized by mobility shifts of the treated protein on polyacrylamide gels.

Proteolytic assays. Human IgA1 from plasma (500 μg/ml; Calbiochem-Novabiochem Ltd.) was dialysed overnight at 4°C in 500 ml 50 mM Tris buffer (pH 7.66).

Single reactions of human IgA1 or human IgA2 from myeloma (Calbiochem-Novabiochem Ltd.) and IgA1 protease were carried out with 5 μl of the IgA1 protease preparation and 2 μl human IgA1 (500 μg/ml). The reaction was incubated overnight at 37°C and subsequently the reaction mix was electrophoresed on an SDS-PAGE gel to visualize the cleavage products.

The time course experiment was carried out with 6 μl deglycosylated human IgA1 (500 μg/ml) and 20 μl IgA1 protease preparation in 0.015 M Tris phosphate buffer (pH 8). The reaction was incubated at 37°C for 24 h. A 5 μl aliquot was removed from the reaction mixture at time 0 min, 30 min, 90 min, 3 h, 6 h and 24 h. The time course samples were electrophoresed on PAGE and the results were visualized by western blotting with the anti-IgA1 antibody (AbD Serotec).

**Inhibition of IgA1 protease.** The IgA1 protease of NTHI was tested with different classes of protease inhibitors, which included the serine protease inhibitors secretory leukoprotease inhibitor (SLPI), alpha-1-antitrypsin (Binding Site Ltd.), and the synthetic inhibitors ZD0892 and Z-Gly-Leu-Phe-CH2I-L (Car G inhibitor; Enzyme Systems Products). The IgA1 protease was also incubated with recombinant cystatin C (cysteine proteinase inhibitor) and Tos-Lys-chloromethylketone.HCl (caspase inhibitor; Bachem).

The reactions were carried out with 5 μl IgA1, 5 μl IgA1 protease preparation (NTHI) and 100 μM of the appropriate inhibitor and incubated overnight at 37°C. The results were visualized by polyacrylamide gel electrophoresis.

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**References**

1. Plaut AG, Genco RJ, Tomasi TB. Isolation of an enzyme from *Streptococcus sanguis* which specifically cleaves IgA. J Immunol 1974; 113:289-91.
2. Plaut AG, Gilbert JV, Arnott MS, Capra JD. *Neisseria gonorrhoeae* and *Neisseria meningitidis*: extracellular enzyme cleaves human immunoglobulin A. Science 1975; 190:1103-5.
3. Mulks MH, Plaut AG. IgA proteinase production as a characteristic distinguishing pathogenic from harmless neisseriae. N Engl J Med 1978; 299:973-6.
4. Senior BW, Ip CL. An investigation into the influences of species and biotype on the type of IgA protease produced by isolates of Haemophilus. J Med Microbiol 1999; 48:389-94.
5. Kilian M, Mestrey J, Schroenholzer RE. Infect Immun 1979; 26:143-9.
6. Male CJ. Immunoglobulin A1 protease production by *Haemophilus influenzae* and *Streptococcus pneumoniae*. Infect Immun 1979; 26:254-61.
7. Mulks MH, Kornfeld SJ, Plaut AG. Specific proteolysis of human IgA by *Streptococcus pneumoniae* and *Haemophilus influenzae*. J Infect Dis 1980; 141:450-6.
8. Aht T, Reinholdt J. Detection of immunoglobulin A1 protease-induced Fab alpha fragments on dental plaque bacteria. Infect Immun 1991; 59:569-9.
9. Munas B, Kiliän M. Retained antigen-binding activity of Fab alpha fragments of human monoclonal Immunoglobulin A1 (IgA1) cleaved by IgA1 protease. Infect Immun 1986; 52:171-4.
10. Tramont EC. Inhibition of adherence of *Neisseria gonorrhoeae* by human genital secretions. J Clin Invest 1977; 59:117-24.
11. Frangione B, Wolfenstein-Todel C. Partial duplication in the “hinge” region of IgA1 myeloma proteins. Proc Natl Acad Sci USA 1972; 69:3673-6.
12. Grundy FJ, Plaut AG, Wright A. Localization of the cleavage site specificity determinant of *Haemophilus influenzae* immunoglobulin A protease genes. Infect Immun 1990; 58:320-31.
13. Devenyi AG, Plaut AG, Grundy FJ, Wright A. Post-infectious human serum antibodies inhibit IgA proteinases by interaction with the cleavage site specificity determinant. Mol Immunol 1993; 30:1243-8.
14. Bricker J, Mulks MH, Plaut AG, Monon ER, Wright A. IgA proteinases of *Haemophilus influenzae* cloning, characterization and expression in *Escherichia coli* K-12. Proc Natl Acad Sci USA 1983; 80:2681-5.
15. Insel RA, Allen FZ, Berkwits ID. Types and frequency of *Haemophilus influenzae* IgA1 proteases. In: Robbins JB, Hill JC, Sadoff JC, Eds. Bacterial Vaccines. New York: Thieme-Stratton 1982; 225-31.
16. Bautz AG, Gilbert JV, Artenstein MS, Capra JD. Immunoglobulin A1 protease production by *Neisseria meningitidis*: cloning and characterization in *Escherichia coli* K-12. Proc Natl Acad Sci USA 1972; 69:3673-6.
17. Kilian M, Thomsen B. Antigenic heterogeneity of Immunoglobulin A1 proteinases from encapsulated and non-encapsulated *Haemophilus influenzae*. Infect Immun 1983; 42:626-32.
18. Plaut AG. The IgA proteases of pathogenic bacteria. Ann Rev Microbiol 1983; 37:603-22.
19. Turk DC. The pathogenicity of *Haemophilus influenzae*. J Med Microbiol 1984; 18:1-16.
20. Bandi V, Apicella MA, Mason E, Murphy TF, Siddiqi A, Armar RL, et al. Nontypeable *Haemophilus influenzae* in the lower respiratory tract of patients with chronic bronchitis. Am J Respir Crit Care Med 2001; 164:2114-9.
21. Cole P. Bronchiectasis. In: Brewis RAL, Gibson GJ, Geddes DM, Eds. Respiratory Medicine. London: Bailliere Tindall 1990; 726-59.
22. Lieberman D, Lieberman D, Ben-Yakov M, Lazovich Z, Hoffman S, Ohana B, et al. Infectious etiologies in acute exacerbation of COPD. Diag Microbiol Infect Dis 2001; 40:95-102.
23. Murphy TF, Brauer AL, Schüffner M, Sethi S. Persistent colonization by *Haemophilus influenzae* in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2004; 170:666-72.
24. Vinovsk S, Dunkin KT, Howard AJ, Sayers JR. Nontypeable *Haemophilus influenzae* in carriage and disease: A difference in IgA protease activity levels. JAMA 2002; 287:1699-705.
25. Norskov-Lauritsen N, Overhale MD, Kilian M. Delineation of the species *Haemophilus influenzae* by phenotype, multilocus sequence phylogeny and detection of marker genes. J Bacteriol 2009; 191:822-31.
26. Poulsen K, Reinholdt J, Kilian M. A comparative genetic study of serologically distinct \textit{Haemophilus influenzae} type 1 immunoglobulin A1 proteases. J Bacteriol 1992; 174:2913-21.

27. Allsubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current Protocols in Molecular Biology. Hoboken: John Wiley and Sons, Inc., 1999.

28. Fernaays MM, Lese AJ, Cai X, Murphy TF. Characterisation of igaB, a second immunoglobulin A1 protease gene in nontypable \textit{Haemophilus influenzae}. Infect Immun 2006; 74:5860-70.

29. Johnson TA, Qiu J, Plaut AG, Holyoak T. Active-site gating regulates substrate selectivity in a chymotrypsin-like serine protease: The structure of \textit{Haemophilus influenzae} immunoglobulin A1 protease. J Mol Biol 2009; 389:559-74.

30. Kilian M, Thomsen B, Petersen TE, Bleeg H. Molecular biology of \textit{Haemophilus influenzae} IgA1 proteases. Mol Immunol 1983; 20:1051-8.

31. Martu TS, Pleas RJ, Willis AG, Kilian M, Wiemold MR, Lellouch LC, et al. The glycosylation and structure of human serum IgA1, Fab and Fc regions and the role of N-glycosylation on Fc receptor interactions. J Biol Chem 1998; 273:2260-72.

32. Lomholt H, van Alphen L, Kilian M. Antigenic variation of immunoglobulin A1 proteases among sequential isolates of \textit{Haemophilus influenzae} from healthy children and patients with chronic obstructive pulmonary disease. Infect Immun 1993; 61:4575-81.

33. Lomholt H, Poulsen K, Kilian M. Comparative characterization of the iga gene encoding IgA1 protease in \textit{Neisseria meningitidis}, \textit{Neisseria gonorrhoeae} and \textit{Haemophilus influenzae}. Mol Microbiol 1995; 15:495-506.

34. Zakerzowski J, Bechert T, Guggerbichler JP. IgA1 protease production by bacteria colonizing the upper respiratory tract. Infection 1998; 26:116-9.

35. Qiu J, Hendrixson DR, Baker EN, Murphy TF, St. Geme JW, 3rd, Plaut AG. Human milk lactoferrin inactivates two putative colonization factors expressed by \textit{Haemophilus influenzae}. Proc Natl Acad Sci USA 1998; 95:12641-6.

36. Plaut AG, Qiu J, St. Geme JW, 3rd. Human lactoferrin proteolytic activity: analysis of the cleaved region in the IgA protease of \textit{Haemophilus influenzae}. Vaccine 2000; 19:48-52.

37. Bachovchin WW, Plaut AG, Flentke GR, Lynch M, Kermner CA. Inhibition of IgA1 proteinases from \textit{Neisseria meningitidis}, \textit{Neisseria gonorrhoeae} and \textit{Haemophilus influenzae} by peptide prolyl boronic acids. J Biol Chem 1990; 265:3738-43.

38. Burton J, Wood SG, Lynch M, Plaut AG. Substrate analogue inhibitors of the IgA proteases from \textit{Neisseria gonorrhoeae}. J Med Chem 1988; 31:1647-51.