The immunoglobulin A1 proteinase from *Streptococcus pneumoniae* is inhibited by tetracycline compounds

Stephen G. Walker[1], Oana I. Carnu[1], Gülay Tüter[2] & Maria E. Ryan[1]

[1]Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook, NY, USA; and [2]Department of Periodontology, Faculty of Dentistry, University of Gazi, Ankara, Turkey

Correspondence: Stephen G. Walker, Department of Oral Biology and Pathology, School of Dental Medicine, SUNY at Stony Brook, Stony Brook, NY, 11794-8702, USA. Tel.: +1 631 632 8916; fax: +1 631 632 9705; e-mail: stephen.walker@stonybrook.edu

Received 18 May 2006; revised 15 June 2006; accepted 28 July 2006.
First published online 21 September 2006.

DOI:10.1111/j.1574-695X.2006.00148.x

Editor: Patrick Brennan

Keywords
Streptococcus pneumoniae; IgA1 proteinase; chemically modified tetracycline; doxycycline; metalloproteinase.

Abstract

Streptococcus pneumoniae produces a zinc-dependent proteinase that cleaves human immunoglobulin (Ig) A1 in the hinge region. This metalloproteinase is hypothesized to act as a virulence factor by allowing *S. pneumoniae* to evade the protection provided by IgA1, thus enhancing its ability to colonize the human nasopharyngeal region. No biologically compatible inhibitors of this enzyme have been identified. We determined that doxycycline and a chemically modified tetracycline inhibit this enzyme *in vitro* at low micromolar concentrations.

Introduction

Streptococcus pneumoniae is an opportunistic human pathogen that continues to cause significant mortality and morbidity in spite of the success of an effective multivalent conjugate vaccine (Anonymous, 2003). This species can cause a range of respiratory tract and invasive infections that affect mainly infants, the elderly and those with immunodeficiencies (Anonymous, 1997). Protection of at-risk populations is currently hampered by antibiotic resistance strains, the limited range of serotypes covered by the vaccine and the emergence of non-vaccine serotypes (Karchmer, 2004; Bernatoniene & Finn, 2005; Byington *et al.*, 2005). Nasopharyngeal colonization by *S. pneumoniae* is a prerequisite for disease and so disruption of bacterial colonization is a potential target for drug development (Bogaert *et al.*, 2004).

Streptococcus pneumoniae produces a metalloproteinase that specifically cleaves human immunoglobulin A1 (IgA1) within the hinge region that links the Fab_{α} antigen-binding portion of the antibody to the Fc portion (Plaut & Wright, 1995). There is abundant circumstantial evidence that the IgA1 proteinase aids the species in the colonization of the nasopharynx (Kilian *et al.*, 1996; Weiser *et al.*, 2003). However, direct evidence for this function has not been obtained because experimental animals do not produce an IgA1 form that can serve as an equivalent substrate for the proteinase (Qiu *et al.*, 1996; Senior & Woof, 2005) and no inhibitors of these enzymes have been found which act in the submillimolar range and are safe to be administered to humans (Plaut, 2004). It is interesting to note that evidence has been mounting that the metallo-IgA1 proteinase of *S. pneumonia* may in fact have a substrate(s) other than human IgA1. Animal models have recently shown that *S. pneumonia* strains with an inactivated IgA1 proteinase gene are less virulent (Polissi *et al.*, 1998; Hava & Camilli, 2002; Chiavolini *et al.*, 2003). We report here that tetracycline derivatives inhibit the *S. pneumoniae* IgA1 proteinase *in vitro* at micromolar concentrations using a semi-quantitative assay.

Materials and methods

Streptococcus pneumoniae culture

Streptococcus pneumoniae R6x and P262 were obtained from J.N. Weiser (University of Pennsylvania School of Medicine). P262 is IgA1 proteinase negative due to the inser-

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femspd/article/48/2/218/594382 by Gazi University Library (GAZI) user on 10 December 2020

tion of *ermC* in *iga*, which encodes the IgA1 proteinase (Wani *et al*., 1996). Bacteria were grown on plates consisting of Todd–Hewitt Broth supplemented with 0.5% yeast extract and 1.5% agar in an atmosphere of 5% CO_2 at 37 °C. Erythromycin was added at a concentration of 1 μg mL^{-1} to the medium for growth of strain P262.

Isolation of IgA1 proteinase

Growth from plate cultures was suspended in 100 mL of 50 mM Tri-HCl, pH 8.0, (Tris buffer) to an optical density at 600 nm of *c*. 4.0. Cells were removed by centrifugation at 3000 ***g*** for 10 min and the supernatant then was transferred to fresh tubes and centrifuged at 25 000 ***g*** for 30 min. Solid ammonium sulfate was slowly added to the final supernatant to obtain a saturation of 60%, then stirred slowly overnight. Precipitate was collected by centrifugation at 3000 ***g*** for 15 min, the supernatant discarded, then the solid material was suspended in a minimal volume (*c*. 1 mL) of Tris buffer. Salts were removed by exhaustive dialysis against Tris buffer and then the contents of the dialysis bag were centrifuged at 25 000 ***g*** for 10 min to remove any remaining precipitates. All manipulations were conducted at 4 °C. The final supernatant served as the isolated IgA1 proteinase and was stored at − 20 °C in aliquots of 50 μL until used.

Semi-quantitative IgA1 proteinase assay

Human IgA1 Kappa from Myeloma (Lampire Biological Laboratories, Pipersville, PA) was used as substrate and cleavage was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and proteins were visualized by staining with Coomassie Brilliant Blue R-250. Gels were photographed with a Kodak DC290 Digital Camera and band densities were quantified using Kodak 1D Image Analysis Software (Eastman Kodak Company, New Haven, CT). Enzyme reactions contained isolated IgA1 proteinase, 24 μg IgA1 substrate in 50 mM Tris-HCl, pH 8.0, in a final volume of 100 μL. Assays were incubated at 37 °C for 18 h, then 25 μL of 4 × SDS-PAGE sample buffer was added. The samples were heated at 100 °C for 5 min and 20 μL was added per gel lane. When required, inhibitors or calcium chloride were added but the final assay always contained the above concentrations of substrate and Tris buffer. Initial experiments were conducted to titrate the *S. pneumoniae* IgA1 proteinase such that under the standard condition of the assay 85–95% of the substrate was cleaved during the 18 h incubation. A positive control (no inhibitor added) and negative control (no IgA1 proteinase added) were used for each experiment and run on each gel. All assays were repeated three times and representative gels are shown in the results. The percentage inhibition of enzyme reactions in the presence of inhibitors did not vary by more than 5% between experiments. Unless stated otherwise, all reagents were obtained from Sigma-Aldrich (St Louis, MO). Chemically modified tetracyclines (CMTs) were a gift from CollaGenex Pharmaceuticals, Inc. (Newtown, PA). All inhibitors were dissolved in dimethylsulfoxide (DMSO; American Type Culture Collection, Manassas, VA) and the maximal concentration of DMSO in any of the experiments presented was 3%. Note that the assay described above is only semi-quantitative and not appropriate for enzyme kinetic analysis.

Results and discussion

Figure 1, lane 2, demonstrates the running positions of the heavy and light chain of IgA1 after separation by SDS-PAGE. When titrated IgA1 proteinase is present in the reaction, the heavy chain is cleaved, resulting in a band that runs between the heavy and light chain and a band that runs with an electrophoretic mobility slightly faster than the light chain (Fig. 1, lane 3). Inclusion of 100 μM (lane 4) or 500 μM (lane 5) 6 deoxy-6-demethyl-4-dedimethylaminotetracycline (CMT-3, also called COL-3 in some publications) in the proteinase assay resulted in a modest inhibition of proteinase activity. The estimated percent inhibition, relative to the no inhibitor control, is printed on all figures above the running position of the heavy chain. However, addition of 1.5 mM of $CaCl_2$ to the above mentioned reactions enhanced the inhibitory effect of CMT-3. The reactions run in lanes 4 and 7 are identical except that the reaction loaded in lane 7 was supplemented with 1.5 mM calcium. Lanes 5 and 8 also illustrated that inclusion of

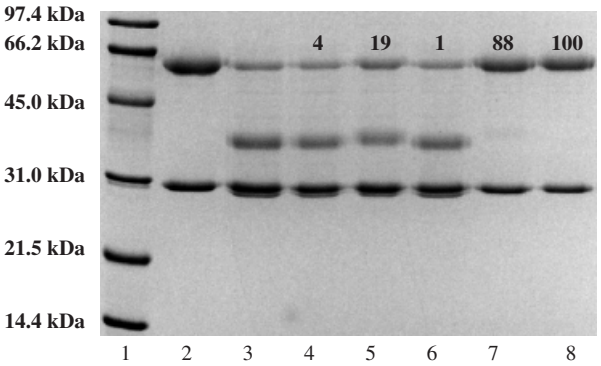


Fig. 1. Polyacrylamide gel, stained with Coomassie Brilliant Blue R250, depicting the action of IgA1 proteinase activity on human IgA1 in the presence or absence of CMT-3. The enzyme reactions were carried out as described in the Materials and methods. Lane 1, molecular mass standard; lane 2, no enzyme control; lane 3, no inhibitor control; lanes 4–8, standard reaction containing 100 μM CMT-3 (4); 500 μM CMT-3 (5); 1.5 mM $CaCl_2$ (6); 1.5 mM $CaCl_2$+100 μM CMT-3 (7); 1.5 mM $CaCl_2$+500 μM CMT-3 (8). The numbers written on polyacrylamide gels in all figures, above the running position of the heavy chain of IgA1, are the calculated percentage inhibition of the IgA1 proteinase with respect to the 'no inhibitor' control.

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femspd/article/48/2/218/594382 by Gazi University Library (GAZI) user on 10 December 2020

1.5 mM calcium (lane 8) enhances the inhibitory action of CMT-3. Lane 6 is a control to show that 1.5 mM $CaCl_2$ does not inhibit the action of the proteinase towards the substrate. A mock IgA1 proteinase preparation, using *S. pneumoniae* P262, did not cleave the heavy chain of IgA1, in agreement with Wani *et al.* (1996) (data not shown). This confirms that the IgA1 proteinase is responsible for cleavage of the heavy chain of IgA1.

The concentration of tetracycline compound required to inhibit the enzymatic activity by *c.* 50% (IC_{50}) was estimated. Figure 2a demonstrates that the IC_{50} for CMT-3 is between 10 and 50 μM. Lane 2 is the negative control in which IgA1 was incubated in buffer alone, and the sample in lane 3 was incubated in buffer containing 1.5 mM $CaCl_2$ demonstrating that calcium does not alter the mobility of the heavy or light chains of the IgA1 substrate. Lanes 4 and 5 are controls that demonstrate that 1.5 mM $CaCl_2$ does not inhibit the activity of the enzyme. The samples in lanes 6–10 contained increasing concentrations of CMT-3. Figure 2b demonstrates that the estimated IC_{50} for doxycycline is *c.* 15 μM. The reaction loaded into lanes 4–9 contained decreasing concentrations of doxycycline. Note that lane 10 is a control for the effect of DMSO on the activity of the IgA1 proteinase. Samples in lane 3 and 10 were incubated under identical conditions except the sample in lane 10 contained 3% DMSO and this concentration inhibited the proteinase activity by *c.* 20%. A correction for the presence of DMSO was made in all of the calculations and the maximal concentration of DMSO in any of the reactions

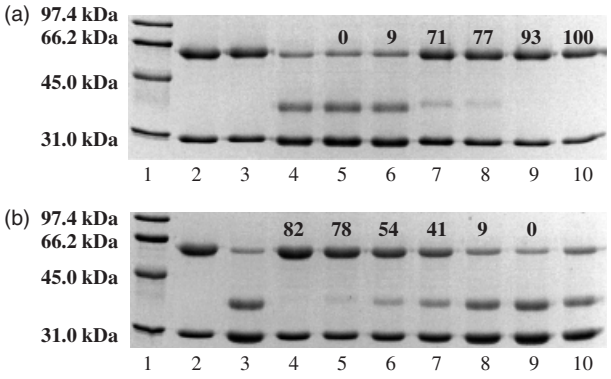


Fig. 2. Polyacrylamide gel, stained with Coomassie Brilliant Blue R250, used to estimate the IC_{50} of CMT-3 and doxycycline against IgA1 proteinase. a)Lane 1, molecular mass standard; lane 2, no enzyme control; lane 3, no enzyme control+1.5 mm $CaCl_2$; lane 4, no inhibitor control; lane 5, no inhibitor control+1.5 mM $CaCl_2$; lanes 6–10 reactions contain 1.5 mM $CaCl_2$ and CMT-3 at a concentration of 10 μM (6); 50 μM (7); 100 μM (8); 250 μM (9); 500 μM (10). b) Lane 1, molecular mass standard; lane 2, no enzyme control; lane 3, no inhibitor control; lanes 4–9 reactions contain 1.5 mM $CaCl_2$ and doxycycline at a concentration of 30 μM (4); 20 μM (5); 15 μM (6); 10 μM (7); 5 μM (8); 1 μM (9); lane 10, standard reaction plus 3% DMSO.

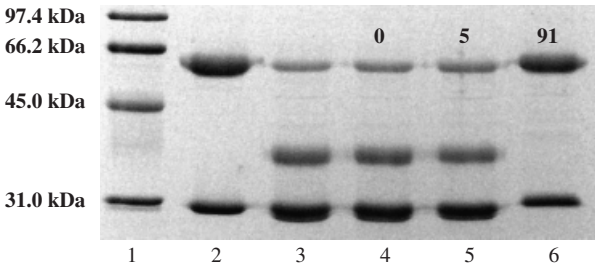


Fig. 3. Polyacrylamide gel, stained with Coomassie Brilliant Blue R250, depicting the inability of CMT-5 to inhibit IgA1 proteinase activity at μM concentrations. Lane 1, molecular mass standard; lane 2, no enzyme control; lane 3, no inhibitor control; lane 4, 100 μM CMT-5; lane 5, 500 μM CMT-5; lane 6, 5 mM CMT-5. All enzyme reactions contained 1.5 mM $CaCl_2$.

was 3%. Like CMT-3, doxycycline was a much more potent inhibitor in the presence of 1.5 mM $CaCl_2$ (not shown).

Tetracyclinpyrazole (CMT-5) was synthesized such that the metal binding functional groups, present in CMT-3, are absent and thus the compound has very low inhibitory potency when used to inhibit zinc-dependent metalloproteinases (Golub *et al.*, 1991). Figure 3 demonstrates that CMT-5 is a poor inhibitor of IgA1 proteinase activity even in the presence of 1.5 mM $CaCl_2$. Note that all reactions shown in Fig. 3 contained calcium. A concentration of 500 μM CMT-5 inhibited IgA1 proteinase activity by only 5% (lane 5). Note that 50 μM CMT-3 inhibited the proteinase activity by *c.* 71% (Fig. 2a, lane 7), suggesting that the metal-binding functional groups of CMT-3 play a role in the inhibition of IgA1 proteinase activity. However, if the concentration of CMT-5 is increased to 5 mM, a 91% inhibition of IgA1 proteinase activity is noted (Fig. 3, lane 6), indicating that inhibition of IgA1 proteinase activity may be mediated by other mechanisms than metal-binding alone.

The metal-ion chelators EDTA and 1,10-phenanthroline were used to investigate the role of calcium in the IgA1 proteinase assay (Fig. 4). In the presence of 500 μM EDTA (lane 4) and 1000 μM EDTA (lane 6) the proteinase is inhibited by *c.* 55% and 75%, respectively, in the absence of calcium. The addition of 1.5 mM $CaCl_2$ decreases the inhibitory effect of the EDTA dramatically. The assays in lanes 4 and 5 were identical except that the assay in lane 5 contained 1.5 mM calcium. The assay conditions of lanes 6 and 7 were also identical but lane 7 in addition contained 1.5 mM $CaCl_2$. Lanes 8 and 9 of Fig. 4 illustrate that 1,10-phenanthroline, like the tetracycline compounds, is a more potent inhibitor of the IgA1 proteinase in the presence of 1.5 mM $CaCl_2$. The reaction loaded in lane 9 contained 500 μM 1,10-phenanthroline and the reaction loaded in lane 8 contained 500 μM 1,10-phenanthroline plus 1.5 mM $CaCl_2$. Thus the tetracyclines behaved more like 1, 10-phenanthroline than EDTA. This can be explained by the

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femspd/article/48/2/218/594382 by Gazi University Library (GAZI) user on 10 December 2020

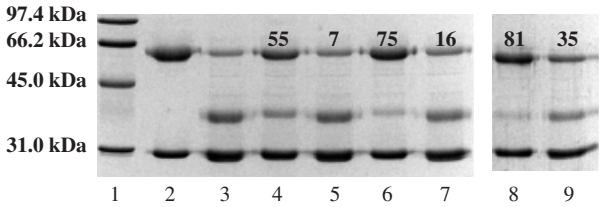


Fig. 4. Polyacrylamide gel, stained with Coomassie Brilliant Blue R250, depicting inhibition of the IgA1 proteinase by EDTA and 1,10-phenanthroline in the presence and absence of calcium. Lane 1, molecular mass standard; lane 2, no enzyme control; lane 3, no inhibitor control; lane 4, 100 μM EDTA; lane 5, 100 μM EDTA+1.5 mM $CaCl_2$; lane 6, 1000 μM EDTA; lane 7, 1000 μM EDTA+1.5 mM $CaCl_2$; lane 8, 500 μM 1,10-phenanthroline+1.5 mM $CaCl_2$; lane 9, 500 μM 1,10-phenanthroline.

equilibrium constants for the binding of calcium to the chelators (Auld, 1995). 1,10-Phenanthroline has a weak binding constant for calcium but a high binding constant for zinc, whereas EDTA has a high binding constant for both calcium and zinc. In the absence of calcium, 100 μM EDTA inhibited the IgA1 proteinase activity by *c.* 55%, whereas in the presence of both 100 μM EDTA and 1.5 mM calcium the activity was inhibited by only 7%. This is most likely due to the EDTA binding the free calcium to such an extent that the metal binding groups became saturated with calcium. However, when 1,10-phenanthroline is used, in the presence (lane 8) or absence (lane 9) of calcium, we noted that, like doxycycline and CMT-3, the calcium augments the inhibitory potential of the chelator. Like 1,10-phenanthroline, doxycycline and CMT-3 have a much higher binding constant for zinc than for calcium (Auld, 1995; Nelson, 1998). The mechanism for this enhanced inhibition by tetracyclines and 1,10-phenanthroline in the presence of calcium is not known but may be due to the binding of calcium to the IgA1 proteinase. This binding of calcium may alter the conformation of the enzymes such that the inhibitors had greater access to the zinc-binding site of the enzyme. It is known that the binding of calcium can alter the structure of another bacterial zinc-dependent enzyme. The binding of calcium to the zinc-dependent collagenase of *Clostridium histolyticum* alters the conformation and function of the enzyme (Wilson *et al.*, 2003). Mammalian matrix metalloproteinases also bind calcium and the catalytic domain contains two zinc ions and at least one calcium ion (Massova *et al.*, 1998). However, the calcium effect could also be due to the ion binding to the tetracycline compounds. It is known that tetracyclines bind divalent cations and the binding alters the interaction of the molecules with bacterial ribosomes and membranes (Nelson, 1998).

In conclusion, we have demonstrated using a semi-quantitative assay that tetracycline compounds, which can be administered safely to humans (Dezube *et al.*, 2006), inhibit the action of the IgA1 proteinase of *S. pneumoniae*. CMT-3 is a modified form of tetracycline in which the C4 dimethylamino group has been removed and thus lacks the functional groups that interact with the bacterial ribosome (Nelson, 1998). Therefore, the use of CMT-3 *in vivo* does not produce a traditional antibiotic effect on the bacterial flora and should not select for the development of antibiotic resistance strains. This is the first report of a biologically compatible inhibitor of a metallo-IgA1 proteinase that acts in the low micromolar concentration range. If tetracyclines are capable of inhibiting the proteinase *in vivo*, then these compounds could have the capacity to interfere with colonization of the host by *S. pneumoniae*. This could represent a new strategy for preventing infections in targeted at-risk populations. Currently, there is a need for new strategies to deal with *S. pneumoniae* in long-term-care facilities (Bradley, 2005). A drug that inhibited colonization of *S. pneumonia* could be an additional tool, along with isolation of carriers, vaccination, and prophylactic antibiotics, to prevent transmission of the pathogen between patients and staff (Carter *et al.*, 2005; Fry *et al.*, 2005).

Acknowledgements

We are grateful to J.N. Weiser for providing the bacterial strains and Collagenex Pharmaceuticals, Inc., for supplying CMT-3 and CMT-5.

References

Anonymous (2003) Pneumococcal vaccines. *Wkly Epidemiol Rec* **78**: 110–119.

Anonymous (1997) Prevention of pneumonococcal disease: recommendations of the advisory committee on immunization practices (ACIP). *Morb Mortal Wkly Rep* **46**: 1–24.

Auld DS (1995) Removal and replacement of metal ions in metallopeptidases. *Methods Enzymol* **248**: 228–242.

Bernatoniene J & Finn A (2005) Advances in pneumococcal vaccines: advantages for infants and children. *Drugs* **65**: 229–255.

Bogaert D, de Groot R & Hermans PWM (2004) *Streptococcus pneumoniae* colonization: the key to pneumococcal disease. *Lancet Infect Dis* **4**: 144–154.

Bradley SF (2005) Double, double, toil and trouble: infections still spreading in long-term-care facilities. *Infect Control Hosp Epidemiol* **26**: 227–230.

Byington CL, Samore MH, Stoddard GJ *et al.* (2005) Temporal trends in invasive disease due to *Streptococcus pneumoniae* among children in the intermountain west: emergence of nonvaccine serogroups. *Clin Infect Dis* **41**: 21–29.

Carter RJ, Sorenson G, Heffernan R, Kiehlbauch JA, Kornblum JS, Leggiadro RJ, Nixon LJ, Wertheim WA, Whitney CG & Layton M (2005) Failure to control an outbreak of multidrug-resistant *Streptococcus pneumoniae* in a long-term-care facility:

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femspd/article/48/2/218/594382 by Gazi University Library (GAZI) user on 10 December 2020

emergence and ongoing transmission of a fluoroquinolone-resistant strain. *Infect Control Hosp Epidemiol* **26**: 248–255.

Chiavolini D, Memmi G, Maggi T, Lannelli F, Pozzi G & Oggioni MR (2003) The three extra-cellular zinc metalloproteinases of *Streptococcus pneumoniae* have different impact on virulence in mice. *BMC Microbiol* **3**: 14.

Dezube BJ, Krown SE, Lee JY, Bauer KS & Aboulafia DM (2006) Random phase II trial of matrix metalloproteinase inhibitor COL-3 in AIDS-related Kaposi's sarcoma: an AIDS malignancy consortium study. *J Clin Oncology* **24**: 1389–1394.

Fry AM, Udeagu CCN, Soriano-Gabarro M *et al.* (2005) Persistence of fluoroquinolone-resistant, multidrug-resistant *Streptococcus pneumoniae* in a long-term-care facility: efforts to reduce intrafacility transmission. *Infect Control Hosp Epidemiol* **26**: 239–247.

Golub LM, Ramamurthy NS, McNamara TF, Greenwald RA & Rifkin BR (1991) Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. *Crit Rev Oral Biol Med* **2**: 297–321.

Hava DL & Camilli A (2002) Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Mol Microbiol* **45**: 389–1405.

Karchmer AW (2004) Increased antibiotic resistance in respiratory tract pathogens: PROTEKT US – an update. *Clin Infect Dis* **39** (Suppl. 3), S142–S150.

Kilian M, Reinholdt J, Lomholt H, Poulsen K & Frandsen EVG (1996) Biological significance of IgA1 proteases in bacterial colonization and pathogenesis: critical evaluation of experimental evidence. *APMIS* **104**: 321–338.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Massova I, Kotra LP, Fridman R & Mobashery S (1998) Matrix metalloproteinases: structures, evolution, and diversification. *FASED J* **12**: 1075–1095.

Nelson ML (1998) Chemical and biological dynamics of tetracyclines. *Adv Dent Res* **12**: 5–11.

Plaut AG (2004) IgA-specific metalloendopeptidase. *Handbook of Proteolytic Enzymes*, 2nd edn (Barrett AJ, Rawlings ND & Woessner JF, eds), pp. 772–775. Academic Press, London.

Plaut AG & Wright A (1995) Immunoglobulin A-metallo-type specific prolyl endopeptidases. *Methods Enzymol* **248**: 634–642.

Polissi A, Pontiggi A, Feger G, Altier M, Mottl H, Ferrari L & Simon D (1998) Large-scale identification of virulence genes from *Streptococcus pneumoniae*. *Infect Immun* **66**: 5620–5629.

Qiu J, Brackee GP & Plaut AG (1996) Analysis of the specificity of bacterial IgA proteases by a comparative study of ape serum IgA as substrates. *Infect Immun* **64**: 933–937.

Senior BW & Woof JM (2005) The influences of hinge length and composition of the susceptibility of human IgA to cleavage by diverse bacterial IgA1 proteases. *J Immunol* **174**: 7792–7799.

Wani JH, Gilbert JV, Plaut AG & Weiser JN (1996) Identification, cloning, and sequencing of the immunoglobulin A1 protease gene of *Streptococcus pneumoniae*. *Infect Immun* **64**: 3967–3974.

Weiser JN, Bae D, Fasching C, Scamurra RW, Ratner AJ & Janoff EN (2003) Antibody-enhanced pneumococcal adherence requires IgA1 protease. *Proc Nat Acad Sci USA* **100**: 4215–4220.

Wilson JJ, Matsushita O, Okabe A & Sakon J (2003) A bacterial collagen-binding domain with novel calcium-binding motif controls domain orientation. *EMBO J* **22**: 1743–1752.

Downloaded from https://academic.oup.com/femspd/article/48/2/218/594382 by Gazi University Library (GAZI) user on 10 December 2020

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved