

Mary E. Marquart · Joseph J. Dajcs  
Armando R. Caballero · Brett A. Thibodeaux  
Richard J. O'Callaghan

## Calcium and magnesium enhance the production of *Pseudomonas aeruginosa* protease IV, a corneal virulence factor

Received: 11 August 2003 / Published online: 20 November 2003  
© Springer-Verlag 2003

**Abstract** The effect of calcium and magnesium on protease IV production during the growth of *Pseudomonas aeruginosa* was investigated. Strain PA103 was grown to stationary phase in medium containing various concentrations of either calcium or magnesium. Culture supernatants were concentrated, standardized relative to cell density, and the pyoverdine concentrations were measured. Overall extracellular protease activity and specific protease IV (lysine endoproteinase) activity were measured with or without TLCK, a serine protease inhibitor effective against protease IV activity. Protease IV activity was also observed by casein zymography. Calcium and magnesium were quantified in the corneas and aqueous humor of rabbits that were inoculated intrastromally with strain PA103. Pyoverdine production was not significantly different in cultures grown in medium with added calcium or magnesium, but extracellular caseinase activity increased in these cultures. Susceptibility of caseinase activity to TLCK inhibition and a specific assay for protease IV indicated that protease IV activity increased in cultures grown in calcium or magnesium. Casein zymography supported the observation that protease IV activity increased in the cultures with added calcium and magnesium. Addition of calcium or magnesium to the protease IV-specific assay had no effect on the catalytic activity of pure protease IV. Infection of rabbit corneas with PA103 did not change the magnesium concentration in either corneas or aqueous humor, but significantly increased the concentration of calcium in corneas. These results indicate that calcium and magnesium enhance the production of protease IV, but not pyoverdine production.

Calcium increases in the cornea following infection with *P. aeruginosa* could favor production of protease IV.

**Keywords** Calcium · Keratitis · Magnesium · Protease · *Pseudomonas aeruginosa*

### Introduction

As an opportunistic pathogen, *Pseudomonas aeruginosa* is toxic to, or can invade, a host cell [9]. Virulence is also related to the production of extracellular proteases. In the case of *Pseudomonas* keratitis, the leading type of contact lens-related keratitis [22], extracellular proteases are, in part, responsible for destruction of the corneal stroma. Elastase (LasB), alkaline protease, and protease IV are extracellular proteases that have been associated with corneal damage [2, 8, 10, 11, 16, 23, 31, 33].

Protease IV degrades the corneal epithelium and facilitates the establishment of keratitis by a *P. aeruginosa* mutant deficient in corneal virulence [8]. Expression of the cloned protease IV gene in *Pseudomonas putida* bestowed corneal virulence upon this species that normally lacks corneal virulence [31]. Protease IV, as analyzed in *P. aeruginosa* PA103, is a serine protease with the ability to cleave the C termini of lysine residues within peptides and proteins [3, 7]. Protease IV is also capable of self cleavage at a lysine residue, liberating mature protease IV from its propeptide domain [31]. Activity of this enzyme is dependent upon a catalytic triad consisting of histidine 72, aspartate 122, and serine 198 [32].

Extracellular enzyme production by *P. aeruginosa* is influenced by environmental factors. Changes in growth medium components, temperature, and aeration can greatly affect the amount of a given virulence factor produced. A 20-fold increase in calcium concentration can cause a 70% increase in exotoxin A production; however, concentrations higher than 500  $\mu$ M calcium are inhibitory [1]. Production of extracellular elastase A

M. E. Marquart · J. J. Dajcs · A. R. Caballero  
B. A. Thibodeaux · R. J. O'Callaghan (✉)  
Department of Microbiology, Immunology and Parasitology,  
Louisiana State University Health Sciences Center, 1901 Perdido  
Street, New Orleans, LA, 70112, USA  
E-mail: rocall@lsuhsc.edu  
Tel.: +1-504-5684072  
Fax: +1-504-5682918

(LasA) or LasB is enhanced by the addition of 2.5 mM calcium plus 0.1 mM zinc as compared to no added calcium plus zinc [27]. Inhibition of LasB production by a lysophospholipid can be reversed by addition of calcium, iron, magnesium, and zinc [20]. Thus, limitation or enhanced concentrations of any one of a number of nutrients or metals in the growth medium can cause a major change in the profile of extracellular enzyme production, and identical changes in the medium for two different strains can have different effects relative to the proteases produced [28].

Production of the lysine-specific protease of PAO1 was found to be co-regulated with pyoverdine production [34]. Wilderman et al. [34] designated this protease as PrpL to indicate that the sigma factor for pyoverdine production (PvdS) regulated the protease (PvdS regulated protease, lysyl). This designation, PrpL, is a potential renaming of protease IV based on the reported co-regulation of pyoverdine and this protease.

Growth medium effects on the production of extracellular *P. aeruginosa* protease IV have not been determined, aside from the finding that iron is inhibitory [34]. Divalent cation content of the bacterial environment could have an effect on the amount of protease IV produced by *P. aeruginosa*, and, thus, could relate to the pathogenicity of *Pseudomonas keratitis*. This study was undertaken to examine the effects of calcium and magnesium on protease IV production relative to pyoverdine and, more importantly, to determine if the in vitro findings for divalent cations could relate to the corneal virulence of *Pseudomonas keratitis*.

## Materials and methods

### Reagents

All reagents were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

### Bacterial strains and growth conditions

*P. aeruginosa* strain PA103, reported to be deficient in elastase and alkaline protease [26], was obtained from the American Type Culture Collection (Manassas, VA). Bacterial colonies were isolated on tryptic soy agar (TSA, Becton Dickinson Diagnostic Systems, Sparks, MD). Three isolated colonies of *P. aeruginosa* PA103 were suspended together in 1 ml tryptic soy broth (TSB, Becton Dickinson Diagnostic Systems) that had been dialyzed to remove high molecular mass molecules, was chelated by mixing with Chelex 100 (Bio-Rad Laboratories, Hercules, CA) for 1 h, and was then supplemented with 50 mM monosodium glutamate and 1% glycerol. This suspension was used to inoculate several flasks containing 10 ml of medium with or without CaCl<sub>2</sub> or MgSO<sub>4</sub> at a final concentration of 0, 0.1, 1, or 10 mM. The bacteria were cultured at 37°C with aeration (170 rpm in beveled culture flasks) for 17 h to achieve stationary phase. The cell density was determined by measuring the optical density at 600 nm. To obtain culture supernatants free of bacteria, stationary phase cultures were centrifuged at 8,000 g for 10 min, and any remaining bacteria in the extracellular material were eliminated by filtration through 0.45-µm porosity filters. Each 10-ml sample of extracellular material was concentrated to 1 ml using an Amicon stirred ultrafiltration cell

(Millipore, Billerica, MA) equipped with a 3-kDa molecular mass cutoff filter. Culture supernatants were adjusted according to cell density for all determinations in this study; cell densities varied less than twofold among the cultures analyzed.

The same procedure was done with cultures of PA103 containing additional NaCl or Na<sub>2</sub>SO<sub>4</sub> to test for possible effects of the anion portions of the metal salts. NaCl was the control for CaCl<sub>2</sub>, and Na<sub>2</sub>SO<sub>4</sub> was the control for Mg<sub>2</sub>SO<sub>4</sub>. The concentrations of NaCl or Na<sub>2</sub>SO<sub>4</sub> were added to generate final concentrations of 0.1, 1, or 10 mM chloride or sulfate in the medium.

### Pyoverdine production

Pyoverdine in the culture supernatant (before tenfold concentration) from each stationary phase culture was measured using the spectrophotometric method of Shen et al. [29].

### Protease assays

Protease activities of concentrated culture supernatants were determined by microplate assays of fluorescent casein hydrolysis (BODIPY-casein, EnzChek Protease Assay Kit, Molecular Probes, Eugene, OR). For inhibitor measurements, samples were incubated with the inhibitor [2 mM tosyl-L-lysine chloromethylketone (TLCK)], for 30 min at 37°C prior to addition of substrate. Samples were incubated with the substrate for 24 h at 37°C. Fluorescence was measured with excitation and emission wavelengths of 485 nm and 520 nm, respectively.

### Protease IV assay

Protease IV activity was specifically measured using the chromogenic substrate tosyl-Gly-Pro-Lys-*p*-nitroanilide as previously described [7]. For inhibitor assays, samples were incubated with 2 mM TLCK for 30 min at 37°C prior to addition of substrate. Samples were incubated with the chromogenic substrate for 24 hours at 37°C. For assays using pure protease IV only, the enzyme was incubated with substrate for 2 h. Release of *p*-nitroanilide due to cleavage at lysine was measured spectrophotometrically at A<sub>410</sub>.

### Zymography

Zymography was performed using modifications of the method of Twining et al. [33]. A 30-µl aliquot of each concentrated culture supernatant was electrophoresed at 4°C and 20 mAmp through a 10% SDS-polyacrylamide gel containing 0.1% casein. SDS was removed by washing twice for 20 min with 100 ml 2.5% Triton X-100. Gels were washed and incubated for proteolysis in 50 mM TRIS-HCl pH 8.0 and 150 mM NaCl at 37°C for 24 h. Gels were stained for 20 min in 0.5% Coomassie brilliant blue R-250 in glacial acetic acid:isopropanol:dH<sub>2</sub>O (1:3:6), and then destained in dH<sub>2</sub>O.

### Ocular calcium and magnesium concentrations during infection

New Zealand white rabbits were used for measurements of calcium and magnesium in the cornea and the aqueous humor during *P. aeruginosa* keratitis. These experiments adhered to the protocols written in accordance with established guidelines for the use of animals in research. A subcutaneous injection of ketamine:xylazine (5:1; Ketaset, Fort Dodge Animal Health, Fort Dodge, IA; Xylazine-100 Injectable, The Butler Company, Columbus, OH) was used to systemically anesthetize rabbits for infection, extraction of aqueous humor, and sacrifice. Topical drops of proparacaine hydrochloride ophthalmic solution (The Butler Company) were administered to the eyes prior to infection and extraction of

aqueous humor. Infection consisted of an intrastromal injection of 1,000 log-phase colony-forming units (CFU) of *P. aeruginosa* strain PA103. Aqueous humor (0.1 ml) was extracted with a tuberculin syringe and 30-gauge needle. Rabbits were killed with an overdose of intravenous sodium pentobarbital.

In one experiment, rabbits were killed and their corneas taken at 0, 17, 22, and 31 h post infection (p.i.). Each cornea was dissected into several pieces, placed in 3 ml PBS, and homogenized with an Ultra-Turrax T25 Basic homogenizer (Janke and Kunkel, IKA-Labortechnik, Staufen, Germany). Dilutions of the corneal homogenates were plated on TSA to determine the CFU. Hexadecyltrimethylammonium bromide (CTAB, 0.1 ml of 0.5%) was added to each corneal homogenate and the homogenate was freeze-thawed once. The concentrations of calcium or magnesium in each cornea were determined using calcium (Arsenazo III) reagent or magnesium (Magon) reagent, respectively, according to the manufacturer's instructions (Sigma). PBS containing CTAB was used as the negative control. Standard curves were generated from known concentrations of calcium and magnesium.

In a second experiment, the aqueous humor was taken from three rabbits several hours before infection, and subsequently collected at 15 and 25 h p.i. These rabbits were killed at 25 h p.i. and corneas were assayed for calcium or magnesium concentrations as described above.

#### Statistics

The Student's *t*-test (two-tailed) was used to analyze data on pyoverdine concentrations, enzyme assays, and calcium or magnesium concentrations in vivo. The Least Squares Means test was used to analyze data on bacterial CFU in infected corneas. A *P* value of less than 0.05 was considered significant for all statistical analyses.

## Results

### Pyoverdine production

The amount of pyoverdine relative to the cell number was equivalent in all of the cultures (Table 1, *P*=0.15).

### Total extracellular protease activity

Relative to cell number, extracellular caseinolytic activity increased significantly in cultures grown in 0.1–

**Table 1** Pyoverdine concentration in PA103 culture supernatants

Metal concentration (mM)	Relative pyoverdine concentration <sup>a</sup>
0	1.24 ± 0.07
CaCl <sub>2</sub>	
0.1	1.25 ± 0.04
1	1.28 ± 0.08
10	1.17 ± 0.01
MgSO <sub>4</sub>	
0.1	1.25 ± 0.06
1	1.28 ± 0.01
10	1.39 ± 0.02

<sup>a</sup>Culture supernatants were adjusted with dialyzed and chelated tryptic soy broth to account for minor differences in cell density. The values listed are the averages of the two experiments ± standard deviations. Not shown are pyoverdine concentrations of cultures grown in NaCl or Na<sub>2</sub>SO<sub>4</sub>, which were not statistically different from any of the concentrations shown above

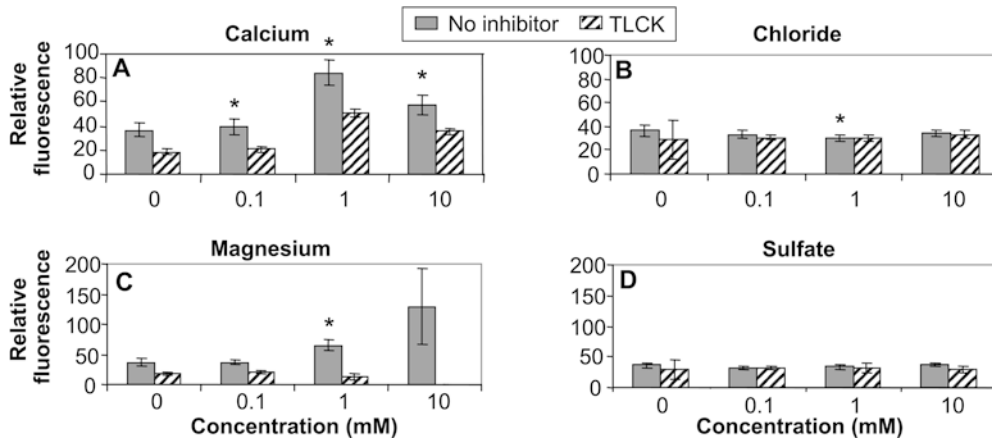
10 mM calcium compared to cultures grown in unmodified medium (*P* ≤ 0.02, Fig. 1A). Cultures with 1 mM calcium produced maximal activity. Cultures grown in 1 mM magnesium caused a significant increase in caseinase activity compared to cultures in unmodified medium (*P*=0.01, Fig. 1C). The caseinase activities of supernatants from cultures grown in equivalent concentrations of NaCl or Na<sub>2</sub>SO<sub>4</sub> had the same caseinase activity as cultures grown in unmodified medium (*P* ≥ 0.06, Fig. 1B, D), with the exception of 1 mM chloride, which was statistically lower than unmodified medium (*P*=0.03).

### Analysis of the caseinase activity

The recognized caseinases of *P. aeruginosa* are protease IV, which is a serine protease susceptible to the inhibitor TLCK, and LasB and alkaline protease, which are metalloproteases that are not susceptible to TLCK [3]. Figure 1A, C shows that the total caseinase activity present in cultures grown with 1 or 10 mM calcium or magnesium is inhibited by TLCK, indicating that protease IV is responsible for about 40–100% of the total caseinase activity.

To further support the finding that the increased caseinase activity was due, in part, to enhanced production of protease IV, culture supernatants were assayed for protease IV using a colorimetric assay specific for this protease [3]. Proteolysis of the chromogenic substrate significantly increased due to the presence of 0.1–10 mM calcium or magnesium in the growth medium as compared to the activity of cultures grown in unmodified medium (*P* ≤ 0.05, Fig. 2A, C). TLCK almost completely inhibited the proteolytic activity in the colorimetric assay of the supernatants from cultures grown in the presence of any tested concentration of calcium (Fig. 2A). For supernatants of cultures supplemented with magnesium, TLCK inhibited approximately 82% of the activity in the colorimetric assay for cultures grown in 1 mM magnesium and had about a 43% inhibitory effect on cultures grown in 10 mM magnesium (Fig. 2C).

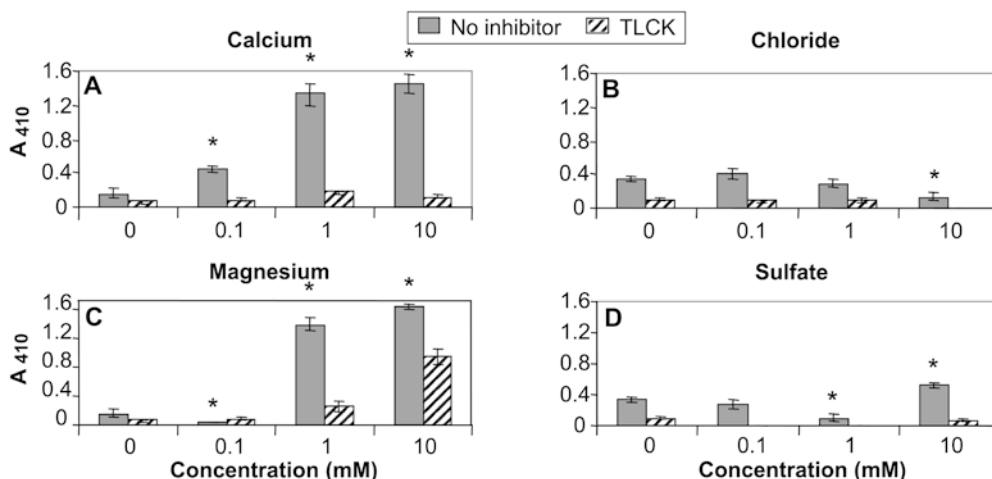
The cleavage of the chromogenic substrate by protease in supernatants from cultures grown in increased NaCl concentrations were equivalent to that of cultures grown in unmodified medium (Fig. 2B, *P* ≥ 0.3), except for cultures grown in 10 mM chloride, which had statistically less activity than unmodified medium (*P*=0.03). Cultures supplemented with 0.1 mM sulfate had the same activity as supernatants from unmodified medium (*P*=0.40); however, cultures grown in 1 mM sulfate had less activity (*P*=0.03), and 10 mM sulfate had more activity (*P*=0.03), than unmodified (Fig. 2D). Since cultures supplemented with 10 mM sulfate had more protease IV-specific activity than cultures grown in unmodified medium, a statistical comparison of protease IV activity from cultures supplemented with 10 mM sulfate and those supplemented with 10 mM magnesium



**Fig. 1** Caseinase activity of PA103 concentrated supernatants from stationary phase cultures grown in the presence of different concentrations of  $\text{CaCl}_2$  (A),  $\text{NaCl}$  (B),  $\text{MgSO}_4$  (C), or  $\text{Na}_2\text{SO}_4$  (D). The caseinase activity was tested in the presence and absence of the serine protease inhibitor, TLCK. The positive control for the assay was 1  $\mu\text{g}$  pure *P. aeruginosa* LasB (emission of  $541 \pm 41.58$ ) and was not inhibited by TLCK (emission  $556.67 \pm 34.93$ ). Error bars represent the standard deviation of three samples, and asterisks indicate values that were significantly different from values for cultures grown in unmodified medium

was done. Cultures supplemented with 10 mM magnesium had significantly more protease IV-specific activity than cultures supplemented with 10 mM sulfate ( $P=0.0007$ ), indicating that the increase in protease IV activity of cultures grown in 10 mM magnesium was significant regardless of the presence of sulfate.

**Fig. 2** Lysine-specific chromogen cleavage assay of PA103 concentrated supernatants from stationary-phase cultures grown in the presence of different concentrations of  $\text{CaCl}_2$  (A),  $\text{NaCl}$  (B),  $\text{MgSO}_4$  (C), or  $\text{Na}_2\text{SO}_4$  (D), with or without TLCK. The control was 2  $\mu\text{g}$  pure protease IV ( $A_{410} 1.44 \pm 0.04$ ) and was fully inhibited by TLCK ( $A_{410} 0.01 \pm 0$ ). Error bars represent the standard deviation of three samples, and asterisks indicate values that were significantly different from values for cultures grown in unmodified medium

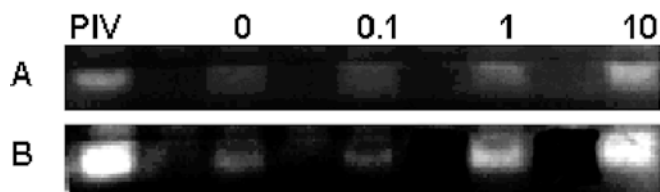


### Zymography of protease IV activity

Casein zymography of the concentrated culture supernatants, as in the case of the protease IV-specific chromogen assay, showed an increase in protease IV activity for cultures grown in medium supplemented with calcium compared to cultures grown without calcium (Fig. 3). Likewise, protease IV activity increased when cultures were grown in medium supplemented with magnesium. The difference in band intensity observed for purified protease IV between the two zymograms is probably due to differences in enzyme units per mg protein, as enzyme activity can degrade upon temperature changes.

### The effect of calcium and magnesium on protease IV catalytic activity

The increased protease activity demonstrated in cultures with higher concentrations of calcium and magnesium could be due to the increased production of the protease IV enzyme or to enhanced catalytic activity of an unchanging amount of the enzyme produced. To address this issue, protease IV was incubated with the chromogenic substrate in the presence of different concentrations of calcium or magnesium. The enzyme activity



**Fig. 3** Casein zymograms of concentrated culture supernatants from stationary phase PA103 grown in the presence of 0, 0.1, 1, or 10 mM  $\text{CaCl}_2$  (A) or  $\text{MgSO}_4$  (B). The region of each zymogram with protease IV activity (at  $>200$  kDa) is shown. The positive control for each zymogram was  $2 \mu\text{g}$  of pure protease IV (PIV). Millimolar concentrations of  $\text{CaCl}_2$  or  $\text{MgSO}_4$  are indicated. A Culture supernatants supplemented with  $\text{CaCl}_2$ , B culture supernatants supplemented with  $\text{MgSO}_4$

remained constant despite 100-fold increases (0.1–10 mM) in the concentration of divalent cations (not shown). The use of a low concentration (1 ng) of protease IV in the assay proved that the metal salts did not enhance protease IV activity, and the use of a high concentration ( $2 \mu\text{g}$ ) of protease IV in the assay proved that the metal salts did not inhibit the activity of the protease.

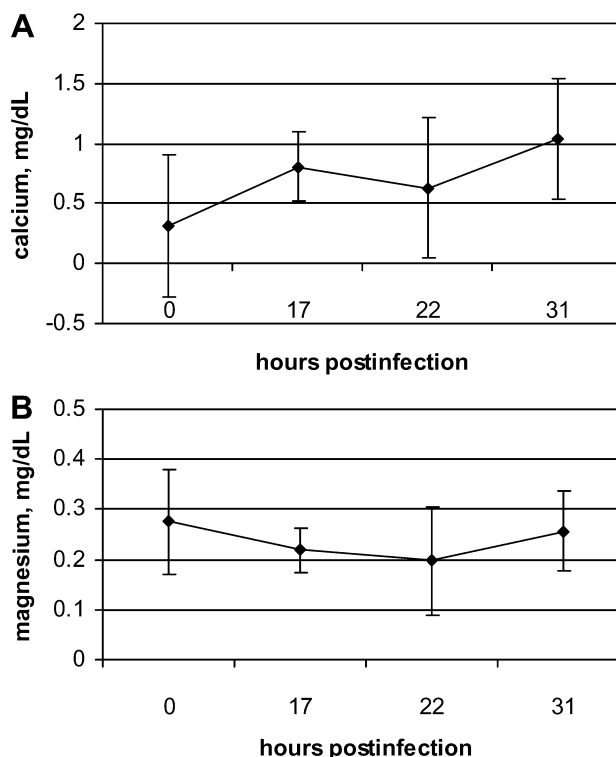
#### Calcium and magnesium concentrations in vivo

Calcium and magnesium concentrations were measured in the corneas and aqueous humor of uninfected and infected rabbits. The concentration of calcium in uninfected corneas was significantly lower than the concentration in infected corneas at 31 h ( $P=0.009$ ), but not at 17 or 22 h p.i. ( $P>0.1$ , Fig. 4A). The concentration of magnesium in infected corneas at 17, 22, or 31 h p.i. was not significantly different than the concentration in uninfected corneas (Fig. 4B,  $P\geq 0.2$ ). The log CFU per cornea at 31 h p.i. ( $7.84 \pm 0.06$ ) was significantly greater than the log CFU per cornea at 22 h ( $7.31 \pm 0.06$ ) or 17 h ( $7.29 \pm 0.08$ ) p.i. ( $P<0.001$ ).

The concentration of calcium in the aqueous humor was  $10.17 \pm 1.88$  mg/100 ml prior to infection and  $8.46 \pm 1.35$  mg/100 ml 25 h p.i., and these concentrations were not significantly different ( $P=0.15$ ,  $n=6$  per group). There was no difference in the magnesium concentration in the aqueous humor prior to infection ( $2.97 \pm 0.75$  mg/100 ml) compared to 25 h p.i. ( $2.63 \pm 0.23$  mg/100 ml,  $P=0.29$ ,  $n=6$  per group). The log CFU  $\pm$  SEM for the corneas of these rabbits ( $n=6$ ) at 25 h p.i. was  $7.63 \pm 0.06$ .

#### Discussion

This study showed that protease IV production was enhanced by the addition of calcium or magnesium to the growth medium of *P. aeruginosa*. This increase in protease IV production was not accompanied by an increase in pyoverdine, suggesting that their regulation is not fully linked. More importantly, infected rabbit cor-



**Fig. 4** Calcium (A) and magnesium (B) concentrations were determined in uninfected and infected rabbit corneas. The concentration of each metal in the cornea was determined from a 3-ml suspension of homogenized cornea. For all times except 17 h p.i.,  $n=6$ . For 17 h p.i.,  $n=4$ . Error bars represent the standard deviation (p.i. post infection)

neas at 31 h p.i. contained an approximate threefold higher concentration of calcium (an average of 1.04 mg/100 ml, or 0.25 mM) than uninfected corneas (0.31 mg/100 ml, or 0.08 mM), which coincides with the calcium concentrations having an effect on protease IV production in vitro.

An interesting observation from this study was that protease IV production was enhanced by the addition of either calcium or magnesium to the *P. aeruginosa* growth medium, but pyoverdine production was not increased. Increased protease production and unaffected pyoverdine production has also been shown for stationary-phase *P. fluorescens* grown with additional 1 mM  $\text{CaCl}_2$  [24]. These findings suggest that protease IV production and pyoverdine production are not necessarily linked. Previous studies [18, 34] proposed that protease IV production involves an alternative sigma factor, PvdS. PvdS is reported to increase when pyoverdine binds iron (ferripyoverdine) and then binds a cellular receptor (FpvA) that activates PvdS. Our findings indicate that protease IV activity is produced in a manner unrelated to pyoverdine and is responsive to calcium or magnesium.

An unexpected finding was that the caseinase activity from the supernatants of stationary phase bacteria grown in 1 mM chloride (Fig. 1B) proved to be statistically lower than from those grown in unmodified

medium. Although the appearance of the figure indicates no obvious differences, analysis by the 2-tailed Student's *t*-test did indicate a significant difference.

Production of *P. aeruginosa* extracellular products other than protease IV has been reported to change upon the addition of metals to the growth medium. Olson and Ohman [27] showed that LasB production increased following supplementation of modified TSB with 2.5 mM CaCl<sub>2</sub>. Magnesium limitation has been shown to decrease total protease production by mucoid and non-mucoid strains, and elastase production by non-mucoid strains, of *P. aeruginosa* [28]. Magnesium limitation also decreases extracellular polysaccharide production by mucoid *P. aeruginosa* [6, 28]. Exotoxin A is another secreted virulence factor that has enhanced production due to calcium and magnesium addition to the growth medium [1]. Calcium-binding proteins are required for secretion of phospholipase C [5], indicating a role for calcium in this virulence factor.

The concentration of calcium in rabbit corneas 31 h p.i. with strain PA103 was threefold higher than in uninfected corneas. The aqueous humor was also tested because it could have been a potential source of extracellular calcium or magnesium for the cornea; however, no significant differences in the metal concentrations were found when comparing uninfected animals to infected animals. The increase in calcium in corneas during the later and more severe stage of infection (31 h p.i.) was expected because calcium has been shown to be increased in cells during inflammation and wound repair [17, 19, 30]. At this stage of infection in the cornea, proteases have caused destruction of the corneal epithelium and stroma [8, 13, 21, 25, 31, 33], and an intense influx of polymorphonuclear cells into the anterior chamber has occurred [4, 12, 14, 15]. The increase in calcium during the inflammatory response in *Pseudomonas* keratitis could be detrimental to the host in that calcium also enhances the production of protease IV. A more detailed analysis of the environmental regulation of protease IV and other *P. aeruginosa* proteases, and how this regulation is involved in keratitis, is underway.

**Acknowledgements** The authors wish to acknowledge Dalia O. Girgis for discussions and critical review of this research and Julian M. Reed for technical assistance. This research was funded by Public Health Service grants R01EY12961 (R.J.O.) and F32EY13651 (M.E.M.), National Eye Institute, National Institutes of Health (Bethesda, MD).

## References

- Blumentals II, Skaja AK, Kelly RM, Clem TR, Shiloach J (1987) Effect of culturing conditions on the production of exotoxin A by *Pseudomonas aeruginosa*. *Ann N Y Acad Sci* 506:663–668
- Burns FR, Paterson CA, Gray RD, Wells JT (1990) Inhibition of *Pseudomonas aeruginosa* elastase and *Pseudomonas* keratitis using a thiol-based peptide. *Antimicrob Agents Chemother* 34:2065–2069
- Caballero AR, Moreau JM, Engel LS, Marquart ME, Hill JM, O'Callaghan RJ (2001) *Pseudomonas aeruginosa* protease IV enzyme assays and comparison to other *Pseudomonas* proteases. *Anal Biochem* 290:330–337
- Cole N, Willcox MD, Fleiszig SM, Stapleton F, Bao B, Tout S, Husband A (1998) Different strains of *Pseudomonas aeruginosa* isolated from ocular infections or inflammation display distinct corneal pathologies in an animal model. *Curr Eye Res* 17:730–735
- Cota-Gomez A, Vasil AI, Kadurugamuwa J, Beveridge TJ, Schweizer HP, Vasil ML (1997) PlcR1 and PlcR2 are putative calcium-binding proteins required for secretion of the hemolytic phospholipase C of *Pseudomonas aeruginosa*. *Infect Immun* 65:2904–2913
- Dunne WMJ, Buckmire FL (1985) Effects of divalent cations on the synthesis of alginic acid-like exopolysaccharide from mucoid *Pseudomonas aeruginosa*. *Microbios* 43:193–216
- Engel LS, Hill JM, Caballero AR, Green LC, O'Callaghan RJ (1998) Protease IV, a unique extracellular protease and virulence factor from *Pseudomonas aeruginosa*. *J Biol Chem* 273:16792–16797
- Engel LS, Hill JM, Moreau JM, Green LC, Hobden JA, O'Callaghan RJ (1998) *Pseudomonas aeruginosa* protease IV produces corneal damage and contributes to bacterial virulence. *Invest Ophthalmol Vis Sci* 39:662–665
- Evans DJ, Frank DW, Finck-Barbancon V, Wu C, Fleiszig SM (1998) *Pseudomonas aeruginosa* invasion and cytotoxicity are independent events, both of which involve protein tyrosine kinase activity. *Infect Immun* 66:1453–1459
- Gupta SK, Masinick SA, Hobden JA, Berk RS, Hazlett LD (1996) Bacterial proteases and adherence of *Pseudomonas aeruginosa* to mouse cornea. *Exp Eye Res* 62:641–650
- Hobden JA (2002) *Pseudomonas aeruginosa* proteases and corneal virulence. *DNA Cell Biol* 21:391–396
- Kernacki KA, Berk RS (1995) Characterization of arachidonic acid metabolism and the polymorphonuclear leukocyte response in mice infected intracorneally with *Pseudomonas aeruginosa*. *Invest Ophthalmol Vis Sci* 36:16–23
- Kernacki KA, Fridman R, Hazlett LD, Lande MA, Berk RS (1997) In vivo characterization of host and bacterial protease expression during *Pseudomonas aeruginosa* corneal infections in naive and immunized mice. *Curr Eye Res* 16:289–297
- Kernacki KA, Barrett RP, Hobden JA, Hazlett LD (2000) Macrophage inflammatory protein-2 is a mediator of polymorphonuclear neutrophil influx in ocular bacterial infection. *J Immunol* 164:1037–1045
- Kessler E, Mondino BJ, Brown SI (1977) The corneal response to *Pseudomonas aeruginosa*: histopathological and enzymatic characterization. *Invest Ophthalmol Vis Sci* 16:116–125
- Kessler E, Spierer A, Blumberg S (1983) Specific inhibition of *Pseudomonas aeruginosa* elastase injected intracorneally in rabbit eyes. *Invest Ophthalmol Vis Sci* 24:1093–1097
- Kraus D, Kaiserman I, Frucht-Pery J, Rahamimoff R (2001) Calcium—an “all-round player” in the cornea. *Isr Med Assoc J* 3:269–274
- Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML (2002) Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 99:7072–7077
- Lansdown AB, Sampson B, Rowe A (1999) Sequential changes in trace metal, metallothionein and calmodulin concentrations in healing skin wounds. *J Anat* 195:375–386
- Laux DC, Corson JM, Givskov M, Hentzer M, Moller A, Wosencroft KA, Olson JC, Krogfelt KA, Goldberg JB, Cohen PS (2002) Lysophosphatidic acid inhibition of the accumulation of *Pseudomonas aeruginosa* PAO1 alginate, pyoverdinin, elastase and LasA. *Microbiology* 148:1709–1723
- Lomholt JA, Poulsen K, Kilian M (2001) Epidemic population structure of *Pseudomonas aeruginosa*: evidence for a clone that is pathogenic to the eye and that has a distinct combination of virulence factors. *Infect Immun* 69:6284–6295

22. Marquart ME, Hume EBH, Zheng X, et al (2002) Bacterial keratitis. In: Agarwal S, Apple DJ, Agarwal A, et al (eds) Textbook of ophthalmology. Jaypee Brothers Medical Publishers, New Delhi, pp 991–1019
23. Matsumoto K, Shams NB, Hanninen LA, Kenyon KR (1993) Cleavage and activation of corneal matrix metalloproteases by *Pseudomonas aeruginosa* proteases. Invest Ophthalmol Vis Sci 34:1945–1953
24. McKellar RC, Cholette H (1986) Possible role of calcium in the formation of active extracellular proteinase by *Pseudomonas fluorescens*. J Appl Bacteriol 60:37–44
25. Miyajima S, Akaike T, Matsumoto K, Okamoto T, Yoshitake J, Hayashida K, Negi A, Maeda H (2001) Matrix metalloproteinases induction by pseudomonal virulence factors and inflammatory cytokines in vitro. Microb Pathog 31:271–281
26. Ohman DE, Sadoff JC, Iglewski BH (1980) Toxin A-deficient mutants of *Pseudomonas aeruginosa* PA103: isolation and characterization. Infect Immun 28:899–908
27. Olson JC, Ohman DE (1992) Efficient production and processing of elastase and LasA by *Pseudomonas aeruginosa* require zinc and calcium ions. J Bacteriol 174:4140–4147
28. Ombaka EA, Cozens RM, Brown MR (1983) Influence of nutrient limitation of growth on stability and production of virulence factors of mucoid and nonmucoid strains of *Pseudomonas aeruginosa*. Rev Infect Dis 5 Suppl 5:S880–S888
29. Shen J, Meldrum A, Poole K (2002) FpvA receptor involvement in pyoverdine biosynthesis in *Pseudomonas aeruginosa*. J Bacteriol 184:3268–3275
30. Thorey IS, Roth J, Regenbogen J, Halle JP, Bittner M, Vogl T, Kaesler S, Bugnon P, Reitmaier B, Durka S, Graf A, Wockner M, Rieger N, Konstantinow A, Wolf E, Goppelt A, Werner S (2001) The Ca<sup>2+</sup>-binding proteins S100A8 and S100A9 are encoded by novel injury-regulated genes. J Biol Chem 276:35818–35825
31. Traidej M, Caballero AR, Marquart ME, Thibodeaux BA, O'Callaghan RJ (2003) Molecular analysis of *Pseudomonas aeruginosa* protease IV expressed in *Pseudomonas putida*. Invest Ophthalmol Vis Sci 44:190–196
32. Traidej M, Marquart ME, Caballero AR, Thibodeaux BA, O'Callaghan RJ (2003) Identification of the active site residues of *Pseudomonas aeruginosa* protease IV. Importance of enzyme activity in autoprocessing and activation. J Biol Chem 278:2549–2553
33. Twining SS, Kirschner SE, Mahnke LA, Frank DW (1993) Effect of *Pseudomonas aeruginosa* elastase, alkaline protease, and exotoxin A on corneal proteinases and proteins. Invest Ophthalmol Vis Sci 34:2699–2712
34. Wilderman PJ, Vasil AI, Johnson Z, Wilson MJ, Cunliffe HE, Lamont IL, Vasil ML (2001) Characterization of an endoprotease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa*. Infect Immun 69:5385–5394