Burkholderia cenocepacia zinc metalloproteases influence resistance to antimicrobial peptides

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Burkholderia cenocepacia secretes two zinc-dependent metalloproteases, designated ZmpA and ZmpB. Previously, ZmpA and ZmpB have been shown to cleave several proteins important in host defence. In this study, the ability of ZmpA and ZmpB to digest and inactivate antimicrobial peptides involved in innate immunity was examined. ZmpB but not ZmpA cleaved β -defensin-1. ZmpA but not ZmpB cleaved the cathelicidin LL-37. Both enzymes cleaved elafin and secretory leukocyte inhibitor, which are antimicrobial peptides as well as neutrophil elastase inhibitors. Both ZmpA and ZmpB cleaved protamine, a fish antimicrobial peptide, and a *zmpA zmpB* mutant was more sensitive to protamine killing than the parental strain. ZmpA or ZmpB cleavage of elafin inactivated its anti-protease activity. The effect of ZmpA and ZmpB on the neutrophil proteases elastase and cathepsin G was also examined but neither enzyme was active against these host proteases. These studies suggest that ZmpA and ZmpB may influence the resistance of *B. cenocepacia* to host antimicrobial peptides as well as alter the host protease/anti-protease balance in chronic respiratory infections.

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INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) consists of at least 17 different species based on 16S RNA sequences, DNA–DNA homology, lipid and fatty acid analysis and phenotypic characteristics (Coenye *et al.*, 2001, 2003; Vandamme *et al.*, 1997; Vanlaere *et al.*, 2008, 2009). The Bcc are important pathogens in people with cystic fibrosis (CF) as well as emerging opportunistic pathogens in non-CF patients (Govan *et al.*, 2007; Mahenthiralingam *et al.*, 2005, 2008). Some Bcc strains are transmitted between patients and have been referred to as epidemic strains (Chen *et al.*, 2001; Coenye & LiPuma, 2003; Govan *et al.*, 1993; LiPuma *et al.*, 1990; Speert *et al.*, 2002). Although strict infection control measures have reduced Bcc infections to only 4–7% of CF patients (Govan *et al.*, 2007; Mahenthiralingam *et al.*, 2005), these infections can result in necrotizing pneumonia and sepsis that can lead to the rapid deterioration of the patient in what has been termed ‘cepacia syndrome’ (Isles *et al.*, 1984). In patients without ‘cepacia syndrome’, Bcc infections are also associated with increased morbidity and mortality (Corey & Farewell, 1996; Murray *et al.*, 2008). Bcc strains are emerging as nosocomial pathogens in patients without CF, and outbreaks resulting in bacteraemia have been reported (Bressler *et al.*, 2007). Bcc infections are difficult to treat

due to these species’ inherent resistance to antibiotics (Mahenthiralingam *et al.*, 2005; Nair *et al.*, 2004, 2005).

B. cenocepacia and *Burkholderia multivorans* are the most frequently isolated Bcc species from CF patients (Govan *et al.*, 2007; LiPuma, 2005; Reik *et al.*, 2005). *B. cenocepacia* accounts for most transmissible strains, although transmissible strains of *B. multivorans* and *Burkholderia dolosa* have also been reported in some CF centres (Biddick *et al.*, 2003; LiPuma *et al.*, 2001; McDowell *et al.*, 2004; Speert *et al.*, 2002). CF patients infected with *B. cenocepacia* have a much lower success rate after lung transplantation compared to CF patients infected with other Bcc species or not infected with Bcc (Alexander *et al.*, 2008; Murray *et al.*, 2008).

Several potential virulence factors have been characterized and implicated in the pathogenesis of Bcc lung infections (Mahenthiralingam *et al.*, 2005; Vial *et al.*, 2007). *B. cenocepacia* and at least four other Bcc species contain two distinct zinc metalloproteases, designated ZmpA and ZmpB. The *zmpA* and *zmpB* genes are always present in the same Bcc species but are not genetically linked. Species that lack both these genes have no detectable protease activity on skim milk agar, suggesting that these are the only extracellular proteases with casein activity (Corbett *et al.*, 2003; Ginges *et al.*, 2005; Kooi *et al.*, 2006). Both ZmpA and ZmpB have pre-proenzyme structures and are autoproteolytically converted into a mature protease and a propeptide (Kooi *et al.*, 2005, 2006). Recombinant forms of both proteases have been expressed and purified from

Abbreviations: Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; h β D-1, human beta defensin-1; HNP-1, human neutrophil peptide-1; SLPI, secretory leukocyte protease inhibitor.

Escherichia coli (Kooi *et al.*, 2005, 2006). Although ZmpA and ZmpB are functionally similar, they share little sequence similarity with the exception of the conserved active site and zinc ligand motifs characteristic of zinc metalloproteases.

ZmpA and ZmpB have broad-spectrum activity and degrade substrates involved in tissue integrity or host defence, including type IV collagen, fibronectin, neutrophil α_1 -proteinase inhibitor and α_2 -macroglobulin. ZmpA cleaves gamma interferon and ZmpB degrades transferrin, lactoferrin and human immunoglobulins IgA, IgG and IgM. The specificity of each enzyme is clearly different in that ZmpA generally cleaves its substrates into two to three peptides whereas ZmpB cleaves substrates into a large number of small peptides (Kooi *et al.*, 2005, 2006). We have shown that both proteases contribute to virulence in a chronic respiratory infection model. A mutation in either gene in *B. cenocepacia* strain K56-2 results in decreased lung pathology compared to the parent strain; however, a mutation in *zmpA* also results in reduced ability to survive *in vivo* since *zmpA* mutants are cleared from the lungs whereas the parent strain maintains a persistent chronic infection (Corbett *et al.*, 2003; Kooi *et al.*, 2006).

Burkholderia spp. are highly resistant to antimicrobial cationic peptides that typically kill bacteria by membrane disruption. LPS structure has been shown to play an important role in resistance to polymyxins and other peptides in both *B. cenocepacia* (Loutet *et al.*, 2006) and *Burkholderia pseudomallei* (Burtneck & Woods, 1999). Other mechanisms of bacterial resistance to antimicrobial peptides have been described, including alteration of surface charges, changes in membrane proteins, efflux pumps/transporters and proteolytic enzymes (Brogden, 2005; Hiemstra *et al.*, 2004; Potempa *et al.*, 2009; Yount & Yeaman, 2005). It is likely that *Burkholderia* spp. employ multiple mechanisms to achieve their high degree of resistance to antimicrobial peptides. Since ZmpA and ZmpB are both broad-spectrum proteases, it is possible that one or both may be able to inactivate antimicrobial peptides by proteolytic cleavage. In this study, we determined the ability of purified ZmpA and ZmpB to degrade representative α - and β -defensins, LL-37, elafin and secretory leukocyte protease inhibitor (SLPI). We also compared the resistance of *B. cenocepacia* wild-type and a *zmpA zmpB* mutant to antimicrobial peptides to determine if the absence of ZmpA and ZmpB renders *B. cenocepacia* more sensitive to microbial killing.

METHODS

Strains. *B. cenocepacia* K56-2 is a CF respiratory isolate (Mahenthalingam *et al.*, 2000). K56-2 *zmpA zmpB* has been previously described (Kooi *et al.*, 2006). This mutant has a trimethoprim resistance cassette inserted into the *Bsi*WI site of *zmpA* and a tetracycline resistance cassette inserted into the remaining *Sall* site in *zmpB* after deletion of the internal 1.4 kb *Sall* fragment. K56-2 *zmpA zmpB* was grown in medium supplemented with 200 μ g tetracycline (Tc) ml^{-1} and 100 μ g trimethoprim (Tp) ml^{-1} .

Substrates. Human neutrophil peptide-1 (HNP-1), human beta defensin-1 (h β D-1) and LL-37 were obtained from AnaSpec. Recombinant h β D-1 was obtained from Cell Sciences. Recombinant human SLPI was obtained from R&D Systems. Protamine sulfate salt from salmon, cathepsin G from human sputum, elastase from human leukocytes (HLE), lysozyme from human neutrophils, recombinant human elafin and succinyl-Ala-Ala-Ala-p-nitroanilide were purchased from Sigma.

Protease digestions. Recombinant ZmpA and ZmpB were purified as previously described (Kooi *et al.*, 2005, 2006). Using hide powder azure as a substrate (Rinderknecht *et al.*, 1968), one unit of activity was defined as the amount of protease that produced a change of 0.1 A_{595} per hour at 37 °C. Protease digestions of antimicrobial peptides were performed essentially as previously described (Kooi *et al.*, 2005, 2006). One microgram of peptide was incubated at 37 °C with 2 U ZmpB in 25 mM MES pH 5.6, 1 mM CaCl_2 or ZmpA in 10 mM Tris pH 7.2 in a final volume of 50 μ l for 16 h unless otherwise indicated. Recombinant ZmpB, recombinant ZmpA and substrate-only controls were included. Products of digested peptides were separated by Tricine/SDS-16% PAGE (Schagger & von Jagow, 1987) and were visualized by Coomassie blue R staining.

Antimicrobial peptide killing assays. Liquid protamine killing assays were performed essentially as described previously (Banemann *et al.*, 1998), with minor modifications. Briefly, overnight cultures were grown for 16 h at 37 °C in L broth, subcultured at 100-fold dilution in L broth without antibiotics and grown to mid-exponential phase. Dilutions of the cultures were made in L broth or PBS. Serial twofold dilutions of the antimicrobial peptide were performed in water (pH 5.0). Fifty microlitres of each peptide dilution was mixed with an equal volume of bacterial suspension (10^5 c.f.u. ml^{-1}). A PBS/bacteria mix was used as a reference control to determine a 100% survival rate. After 1 h incubation at 37 °C, serial dilutions of the bacteria/peptide mixture were plated on LB agar plates. Results were recorded as percentage survival. Elafin (Simpson *et al.*, 1999) and h β D-1 (Morrison *et al.*, 1998) killing assays were performed as previously described except the h β D-1 bacteria/peptide mixtures were incubated for 2.5 h.

Inactivation of antimicrobial peptide activity. Protamine (125 μ g ml^{-1}) was treated with ZmpA (2 U) in 10 mM Tris pH 7.2 or ZmpB (2 U) in 25 mM MES pH 5.6, 1 mM CaCl_2 in a final volume of 50 μ l for 16 h at 37 °C. Protamine (125 μ g ml^{-1})-only (no protease) and buffer-only controls were included. After incubation, the samples were serially diluted twofold (125 to 0.11 μ g ml^{-1}) and 10^5 c.f.u. *E. coli* DH5 α were added, followed by incubation for 1 h at 37 °C with shaking. Samples were serially diluted and plated on L-agar plates to obtain surviving bacterial numbers.

Inactivation of elafin anti-protease activity. Recombinant ZmpA or ZmpB (4 U) in a final volume of 100 μ l or buffer controls were incubated with elafin (5 μ g) for 16 h at 37 °C. Half of the samples were removed to confirm digestion by Tricine/SDS-PAGE and the other half were retained for elastase inhibition assays based on Cooley *et al.* (2001) with some modifications. Briefly, serial dilutions of treated elafin were incubated with porcine pancreatic elastase (1.25 μ M) at final elafin:elastase molar ratios of 1:4 to 1:128 in PBS, 0.05% Tween-20. A porcine pancreatic elastase only (no elafin, no protease) reference control was set up in parallel. After 30 min incubation at 37 °C, 77 μ l of 20 mM Tris/HCl, pH 7.4, 0.5 M NaCl, 0.1% PEG and 3 μ l 50 mM substrate succinyl-Ala-Ala-Ala-p-nitroanilide was added. Changes in A_{415} were monitored. The A_{415} of the elastase-only control was set at 100% and the % elastase activity of the samples were determined as follows: (A_{415} of the sample/ A_{415} of the elastase-only control) $\times$ 100.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test or Student's *t*-test. A *P*-value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Ability of ZmpA and ZmpB to use antimicrobial peptides as substrates

Defensins are important components of lung innate immunity. HNP-1 is expressed predominantly by neutrophils and h β D-1 is constitutively expressed by mucosal epithelial cells (Brogden, 2005; Brown & Hancock, 2006; Mookherjee & Hancock, 2007). LL-37 is a cathelicidin expressed by both neutrophils and epithelial cells. To determine if ZmpA and ZmpB were able to use these antimicrobial peptides as substrates, recombinant forms of ZmpA and ZmpB were incubated with the peptides, after which the incubation mixtures were separated by Tricine/SDS-PAGE. Neither ZmpB nor ZmpA cleaved HNP-1 (Fig. 1a). Interestingly, ZmpB but not ZmpA degraded h β D-1 (Fig. 1b), and ZmpA but not ZmpB degraded LL-37 (Fig. 1c). Due to the small size of h β D-1 and LL-37 it was not possible to determine by Tricine/SDS-PAGE if these peptides were cleaved more than once by the proteases. Elafin and SLPI are peptides with antimicrobial activity, with the ability to eliminate pulmonary pathogens, as well as inhibitors of neutrophil elastase (Doumas *et al.*, 2005; Hiemstra *et al.*, 2004; Sallenave, 2000). Both ZmpB and ZmpA digested elafin and SLPI (Fig. 1d, e). As with h β D-1 and LL-37, the elafin cleavage products were not visible on the gel. The SLPI cleavage products were, however, clearly resolved by Tricine/SDS-PAGE. The masses of the peptides following incubation with ZmpA or ZmpB were different (Fig. 1e). Similar results have been observed with other host protein substrates, confirming that these proteases have different specificities (Kooi *et al.*, 2005, 2006). Protamine is a fish antimicrobial peptide that is similar in size and charge to the defensins. Salmon protamine was digested by both ZmpB and ZmpA into different-size fragments (Fig. 1f). Intact protamine was not visible on the gel, possibly due to aggregation, which may have prevented migration into the gel. Lysozyme is a naturally occurring antibacterial protein that is found in saliva, mucus, nasal secretions, serum and the lysosomes of neutrophils and macrophages. Neither ZmpB nor ZmpA was active against human neutrophil lysozyme under the conditions employed (Fig. 1g). The protease digestion assays were performed for 16 h for maximum sensitivity; however, digestion of LL-37 by ZmpB and ZmpA, respectively, was determined to occur in less than 1 h (data not shown).

Sensitivity of K56-2 and K56-2 *zmpA zmpB* to antimicrobial peptides

Since at least one of the *B. cenocepacia* zinc metalloproteases could digest h β D-1, LL-37, SLPI, elafin and

Fig. 1. Ability of *B. cenocepacia* ZmpA and ZmpB to digest antimicrobial peptides. One microgram of peptide was incubated with 2 U ZmpA or ZmpB in a final volume of 50 μ l and products were separated by Tricine/SDS-16% PAGE. (a) HNP-1 (3.4 kDa), (b) h β D-1 (3.9 kDa), (c) LL-37 (4.5 kDa), (d) elafin (6.0 kDa), (e) SLPI (11.7 kDa), (f) protamine (major component is 5.1 kDa) and (g) lysozyme (14.7 kDa). Lanes: 1, ZmpB only; 2, ZmpB and antimicrobial peptide; 3, antimicrobial peptide only; 4, ZmpA and antimicrobial peptide; 5, ZmpA only. Gels were scanned with a Umax scanner.

protamine, we next determined if there was a difference in susceptibility to killing by these antimicrobial peptides between a protease-negative mutant (K56-2 *zmpA zmpB*) and the wild-type strain (K56-2). The *zmpA zmpB* double mutant was used in most of these assays since one protease with activity against the antimicrobial peptide could render it inactive. K56-2 and K56-2 *zmpA zmpB* were equally resistant to h β D-1 (50 μ g ml⁻¹) (data not shown). Elafin and SLPI at concentrations up to 2 μ M had no effect on the viability of either strain (data not shown). Although K56-2 was consistently more resistant to LL-37 than K56-2 *zmpA zmpB*, no significant difference was found (data not shown). A difference was observed in protamine suscepti-

ibility, however, in that K56-2 was significantly more resistant to protamine than was K56-2 *zmpA zmpB* (Fig. 2) (ANOVA, $P<0.05$). Similar results were observed with a K56-2 *zmpB* mutant that still produces active ZmpA.

Since previous studies have demonstrated that *Burkholderia* species are very resistant to β -defensins and other antimicrobial peptides (Baird *et al.*, 1999; Burtneck & Woods, 1999; Loutet *et al.*, 2006; Sahly *et al.*, 2003), it is possible that mutations in *zmpA* and/or *zmpB* are insufficient to result in increased susceptibility to the antimicrobial activity of these defensins, due to myriad resistance mechanisms. It is also possible that the cleavage products generated by ZmpA or ZmpB retain antimicrobial activity. To test this hypothesis, we attempted to perform killing assays with peptides following incubation with either ZmpA or ZmpB using *E. coli* DH5 α to test for killing. Treatment of protamine with either ZmpA or ZmpB decreased its ability to kill *E. coli* (Fig. 3). There was a significant difference between the antimicrobial activity of protamine and that of protamine treated with ZmpB, at concentrations of protamine ranging from 0.9 to 3.6 $\mu\text{g ml}^{-1}$, and protamine treated with ZmpA at 0.9 $\mu\text{g ml}^{-1}$ (Fig. 3). No differences in killing were observed at concentrations above 3.6 $\mu\text{g ml}^{-1}$ or below 0.9 $\mu\text{g ml}^{-1}$ (data not shown).

Similar experiments were performed with h β D-1 (up to 50 $\mu\text{g ml}^{-1}$), elafin (up to 15 $\mu\text{g ml}^{-1}$) and SLPI (up to 30 $\mu\text{g ml}^{-1}$), but the commercial preparations used of

Fig. 2. Protamine liquid killing assay. K56-2, K56-2 *zmpA*, K56-2 *zmpB*, K56-2 *zmpA zmpB* and *E. coli* DH5 α were incubated with various concentrations of protamine and the percentage of surviving cells was determined; 100 % survival corresponds to 0 mg protamine ml⁻¹. K56-2 killing by protamine was significantly different from K56-2 *zmpB* and K56-2 *zmpA zmpB* ($P<0.01$, ANOVA, Dunnett's multiple comparisons test). The results represent the mean $\pm$ SEM of four independent assays, each performed in duplicate.

Fig. 3. Ability of ZmpA and ZmpB treatment to decrease protamine antimicrobial activity. Protamine was incubated with 2 U ZmpA or ZmpB, or buffer controls. The residual antimicrobial activity against *E. coli* DH5 α was determined using a liquid killing assay. Survival of the control without protamine was set at 100 %. ZmpB-treated protamine and buffer-treated protamine were significantly different at 0.9, 1.8 and 3.6 $\mu\text{g ml}^{-1}$, and significantly different between ZmpA-treated protamine and buffer-treated protamine at 0.9 $\mu\text{g ml}^{-1}$ (ANOVA, $P<0.05$). Values represent the mean $\pm$ SD of triplicate assays.

these peptides had no antimicrobial activity against *E. coli* (data not shown). Elafin and h β D-1 have previously been shown to be active against *Pseudomonas aeruginosa* (Morrison *et al.*, 1998; Simpson *et al.*, 1999). Inactivation of the antimicrobial activity of either elafin or h β D-1 could be relevant in *B. cenocepacia* and *Ps. aeruginosa* co-infections. Therefore, we tested the antimicrobial activity of elafin digested with ZmpA or ZmpB, and h β D-1 digested with ZmpB, against *Ps. aeruginosa* strain PAO1 in comparison to the intact peptides. Although recombinant elafin was employed in this assay, we did not observe killing of PAO1 (data not shown). It has previously been demonstrated that the C-terminal domain of elafin contains the antimicrobial activity (Simpson *et al.*, 1999). Since elafin is degraded into very small peptides by ZmpA and ZmpB, it is unlikely that these peptides would be of sufficient size to retain activity even if a preparation of elafin with antimicrobial activity was used in the assay. We were able to demonstrate killing of PAO1 by h β D-1; however, no difference in killing was observed between intact peptide and that previously incubated with ZmpB at the 50 $\mu\text{g ml}^{-1}$ concentration tested (data not shown). These data suggest that the proteolytic fragments generated by ZmpB do retain some antimicrobial activity.

ZmpA and ZmpB inactivate the anti-elastase activity of elafin

In addition to their antimicrobial properties, elafin and SLPI are inhibitors of host proteases, particularly human neutrophil elastase. To determine if cleavage by ZmpA or ZmpB reduced the ability of elafin to inhibit elastase, twofold serial dilutions of elafin, pre-incubated with ZmpA

or ZmpB, were incubated with porcine pancreatic elastase (1.25 μ M) at final elafin:elastase molar ratios of 1:4 to 1:128. Both ZmpA and ZmpB cleavage of elafin significantly decreased its anti-elastase activity (Table 1). Neither ZmpA nor ZmpB had any activity against the elastase substrate, succinyl-Ala-Ala-Ala-*p*-nitroanilide. Complete elafin cleavage in these assays was confirmed by Tricine/SDS-PAGE (data not shown). At higher elafin concentrations (1:4 elafin:elastase molar ratio) elafin inactivation of elastase was observed likely due to the presence of residual uncleaved elafin. The commercial SLPI preparation was found to be inactive since a SLPI:elastase molar ratio of 1:1 resulted in no significant decrease in elastase activity (data not shown).

Since inactivation of elafin could alter the protease/antiprotease balance in the lung, the ability of ZmpA and ZmpB to cleave neutrophil proteases was also examined. Cathepsin G is a chymotrypsin-like protease that is a major constituent of human neutrophil granulocytes. Both neutrophil elastase and cathepsin G have been found to be essential for neutrophil killing of bacteria (Reeves *et al.*, 2002). Both cathepsin G and neutrophil elastase are present in the CF lung (Rees & Brain, 1995) and can cause tissue destruction. Under the digestion conditions employed, neither ZmpB nor ZmpA degraded cathepsin G or neutrophil elastase (data not shown).

SUMMARY AND CONCLUSIONS

Bacterial proteases are an important mechanism of resistance to host defences and are increasingly being recognized as factors that contribute to antimicrobial peptide resistance. Many proteases have been reported to degrade LL-37, including *Proteus mirabilis* ZapA (Belas *et al.*, 2004), *Staphylococcus aureus* aureolysin (Sieprawska-

Lupa *et al.*, 2004), *Ps. aeruginosa* LasB, *Enterococcus faecalis* gelatinase, *Streptococcus pyogenes* SpeB (Schmidtchen *et al.*, 2002), and a *Bacillus anthracis* metalloprotease (Thwaite *et al.*, 2006). *E. coli* OmpT and OmpP proteases degrade protamine (Hwang *et al.*, 2007; Stumpe *et al.*, 1998). Fewer studies have examined the activity of bacterial proteases against other antimicrobial peptides but *Pr. mirabilis* ZapA has been shown to degrade h β D-1 (Belas *et al.*, 2004) and LasB has been shown to cleave SLPI (Sponer *et al.*, 1991). Our studies have shown that at least one of the *B. cenocepacia* ZmpA and ZmpB proteases had activity against h β D-1, LL-37, elafin and SLPI. Inactivation of these peptides by ZmpA or ZmpB could contribute to the inherent resistance of *B. cenocepacia*.

Proteases have been reported to enhance resistance of other bacterial pathogens to antimicrobial peptides. *S. aureus* strains that produce aureolysin are more resistant to LL-37 than strains that express no aureolysin activity (Sieprawska-Lupa *et al.*, 2004). *Clostridium botulinum* protease-positive strains are resistant to protamine whereas protease-negative strains are susceptible (Hansen *et al.*, 2001). We did demonstrate increased protamine susceptibility of a *zmpA zmpB* mutant compared to the *B. cenocepacia* wild-type strain, suggesting that these proteases could contribute to resistance. The level of susceptibility of the *zmpB* mutant and the *zmpA zmpB* mutant was similar to that of the *E. coli* control, whereas the *zmpA* mutant was intermediate between the double mutant and the wild-type strain. We also demonstrated that protamine treated with either ZmpB or ZmpA had decreased antimicrobial activity against *E. coli*.

Unfortunately, commercial preparations of h β D-1, elafin and SLPI did not have antimicrobial activity against *B. cenocepacia*; therefore, we were not able to determine if the *zmpA zmpB* mutant was more susceptible than the wild-type to these peptides or if cleavage of these peptides with ZmpA or ZmpB influenced their antimicrobial activity. Some increased susceptibility of the *zmpA zmpB* mutant to LL-37 was consistently observed; however, the differences were not statistically significant. It may be difficult to demonstrate large differences in susceptibility in the assay employed since dilution of mid-exponential-phase cultures for the killing assay would result in very low amounts of protease being present.

Although proteolytic enzymes may contribute to the resistance of *B. cenocepacia* to antimicrobial peptides, it is recognized that other factors, particularly LPS, likely play a greater role. The presence of 4-amino-4-deoxyarabinose (Ara-4N) moieties attached to phosphate residues in the lipid A backbone is believed to be involved in the resistance of *B. cepacia* to antimicrobial peptides (Cox & Wilkinson, 1991; Shimomura *et al.*, 2003). A complete lipid A-core oligosaccharide was recently shown to be required for resistance to several unrelated antimicrobial peptides and for survival of *B. cenocepacia* K56-2 in a rat chronic infection model (Loutet *et al.*, 2006).

Table 1. ZmpA and ZmpB inactivation of elafin elastase inhibition activity

Elafin (μ g)	Elastase activity (%)*		
	Buffer only	ZmpB	ZmpA
0	100 $\pm$ 4.2	124.4 $\pm$ 15.6	102.1 $\pm$ 26.4
0.375	0.7 $\pm$ 1.2	4.9 $\pm$ 4.8	10.7 $\pm$ 3.6†
0.188	3.1 $\pm$ 2.8	6.1 $\pm$ 4.6	14.4 $\pm$ 4.5†
0.094	4.5 $\pm$ 3.0	10.0 $\pm$ 2.4	20.9 $\pm$ 4.0†
0.047	9.4 $\pm$ 7.5	11.0 $\pm$ 4.3	29.8 $\pm$ 6.9†
0.023	13.1 $\pm$ 0.9	48.4 $\pm$ 9.1†	60.8 $\pm$ 18.9†
0.012	27.6 $\pm$ 1.3	82.6 $\pm$ 5.8†	96.3 $\pm$ 12.3†

*Recombinant ZmpA or ZmpB (4 U/50 μ l) or buffer controls were incubated with elafin (5 μ g) for 16 h at 37 °C. Treated elafin was incubated with porcine pancreatic elastase (1.25 μ M) at final elafin:elastase molar ratios of 1:4 to 1:128.

†Significantly different from no-protease control ($P < 0.05$, ANOVA, Dunnett's multiple comparisons test).

Previously we have demonstrated that ZmpA and ZmpB cleave neutrophil α_1 -proteinase inhibitor and α_2 -macroglobulin (Kooi *et al.*, 2005, 2006). In this study, we demonstrate that these proteases also have activity against elafin and SLPI. We determined that ZmpA or ZmpB cleavage of elafin reduced its anti-elastase inhibitory activity. A great deal of CF lung damage is thought to be due to increased neutrophil elastase levels. Inactivation of elastase inhibitors such as α_1 -proteinase inhibitor, SLPI and elafin by ZmpA and ZmpB could disrupt the balance of neutrophil elastase and its inhibitors, contributing to the elevated neutrophil elastase levels in the lung. Since ZmpA and ZmpB did not have any direct activity against neutrophil elastase or cathepsin G, ZmpA or ZmpB produced by *B. cenocepacia* in the lung would primarily influence the levels of the inhibitors of the neutrophil proteases. Neutrophil elastase and cathepsin G expressed by neutrophils and monocytes are implicated in antimicrobial defence by degrading engulfed micro-organisms inside the neutrophil phagolysosomes. Mice lacking neutrophil elastase (Belaouaj *et al.*, 1998, 2000) and cathepsin G (Reeves *et al.*, 2002) are deficient in bacterial killing. Elevated levels of neutrophil elastase that occur in CF inflammatory lung disease, however, also have the ability to digest structural elastin, collagen and fibronectin, resulting in extensive tissue damage (Shapiro, 2002). This protease can also degrade the chemokine receptor CXCR1 [interleukin-8RA (IL-8RA)] on neutrophils, which in turn can disable the bacterial-killing capacity of these cells (Hartl *et al.*, 2007).

Recently, *B. cenocepacia* *zmpA* expression was shown to be upregulated sixfold by growth in medium containing CF sputum, suggesting that ZmpA would likely be present in lungs of CF patients infected with *B. cenocepacia* (Drevinek *et al.*, 2008). Although *zmpB* expression was not reported to be elevated in this study, expression analysis was performed on mid-exponential-phase cultures and *zmpB* is poorly expressed during this phase of growth. Previously, we have reported that both ZmpA and ZmpB contribute to the lung pathology observed in a chronic respiratory infection model. It is likely that ZmpA and ZmpB enhance the virulence of *B. cenocepacia* by a variety of mechanisms including degradation of antimicrobial peptides, resulting in decreased susceptibility to their killing activity, degradation of neutrophil protease inhibitors, resulting in increased inflammation, as well as direct destruction of tissue and other host defence proteins such as immunoglobulins, transferrin and lactoferrin.

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