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The cationic peptide magainin II is antimicrobial for *Burkholderia cepacia*-complex strains

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This study was undertaken to determine the antibacterial activity of eight cationic antimicrobial peptides towards strains of genomovars I–V of the *Burkholderia cepacia* complex (Bcc) in time–kill assays. All but one of the peptides failed to show activity against the panel of test strains. The exception was magainin II, a 23 aa peptide isolated from the epidermis of the African clawed frog, *Xenopus laevis*, which exhibited significant bactericidal activity for Bcc genomovars most frequently associated with lung infection of patients with cystic fibrosis. *In vitro* studies indicated that magainin II protected a human bronchial epithelial cell line (BEAS-2B) from killing by Bcc and suggest that this peptide may have therapeutic potential against these organisms.

INTRODUCTION

Organisms of the *Burkholderia cepacia* complex (Bcc) are associated with chronic opportunistic lung infections of patients suffering from cystic fibrosis (CF) and chronic granulomatous disease (Mahenthiralingam *et al.*, 2000). Infections with Bcc are often coupled with a particularly poor prognosis, resulting in a rapid and fatal decline in pulmonary function due to necrotizing pneumonia and sepsis (Isles *et al.*, 1984). This fatal decline in clinical condition has been termed 'cepacia syndrome' and has not been identified with any other CF-associated pathogen.

The Bcc comprises 15 distinct species formerly known as genomovars of *B. cepacia* (Vanlaere *et al.*, 2008). Of these, the majority of infections in CF are attributed to *Burkholderia multivorans* and *Burkholderia cenocepacia* in both the UK and the USA (Mahenthiralingam *et al.*, 2008; Reik *et al.*, 2005), although all genomovars have been recovered from CF patients (Ortega *et al.*, 2005). Eradication of an established Bcc infection is rarely achieved owing to the high intrinsic resistance of the genus to antimicrobial agents (Coenye *et al.*, 2001). Although preventative strategies are considered the principal approach for management of Bcc infections (Jones & Webb, 2003), combination therapy may be used, extending the spectrum of antimicrobial activity across multiple antibiotic classes (Bonacorsi *et al.*, 1999).

Novel antimicrobial therapeutic agents are urgently required due to the threat of naturally resistant and antibiotic-resistant strains. Cationic antimicrobial peptides (CAMPs) have potential, as they show broad-range activity against Gram-positive and Gram-negative bacteria, viruses and some fungal species (Hancock & Lehrer, 1998). Importantly, these peptides do not present the same issue of resistance observed with conventional antibiotics (Zasloff, 2002) and often have good activity against several multidrug-resistant bacterial species (Giacometti *et al.*, 2005a). There is also evidence that *in vitro* selection of CAMP-resistant mutants is difficult (Hancock & Lehrer, 1998).

CAMPs are short, amphipathic, positively charged peptides that occur naturally in a wide range of species as vital components of the innate immune system and are also implicated in the adaptive immune response. The effects of CAMPs include antimicrobial activity (Zasloff, 2002), mast-cell degranulation (Niyonsaba *et al.*, 2002), anti-endotoxin activity (Bowdish *et al.*, 2005) and enhanced pro-inflammatory responses (Lillard *et al.*, 1999).

As previous reports have described *B. cepacia* as being resistant to the action of CAMPs (Denyer & Maillard, 2002; Scott *et al.*, 1999), we aimed to determine the efficacy of a panel of antimicrobial peptides against *B. cepacia* from genomovars I–V.

METHODS

Bacterial strains, growth conditions and media. The bacterial strains used in this study included 18 Bcc strains from genomovars I–V, which were originally provided by Professor John Govan (University of Edinburgh, Edinburgh, UK) and obtained from the culture collection at the Defence Science and Technology Laboratory

Abbreviations: Bcc, *Burkholderia cepacia* complex; CAMP, cationic antimicrobial peptide; CF, cystic fibrosis; LDH, lactate dehydrogenase.

(Dstl, Porton Down, Salisbury, UK). Representative strains for each genomovar comprised *B. cepacia* J2540 (genomovar I), *B. multivorans* 7897 (genomovar II), *B. cenocepacia* J2956 (genomovar III), *Burkholderia stabilis* 7639 (genomovar IV) and *Burkholderia vietnamiensis* LMG 10929^T (genomovar V). All strains were handled in Advisory Committee for Dangerous Pathogens (ADCP) II containment facilities and were maintained on Luria–Bertoni (LB) agar plates or broth.

Antimicrobial agents. CAMPs were synthesized by Alta Biosciences (University of Birmingham, Birmingham, UK). The peptides tested comprised calcitermin (Cole *et al.*, 2001), cecropin A (Holak *et al.*, 1988), a granulysin fragment (Linde *et al.*, 2005), LL-37 (Agerberth *et al.*, 1995; Niyonsaba *et al.*, 2002), MUC7 (Bobek & Situ, 2003), P-113 (Giacometti *et al.*, 2005b), ovine polyaspartic acid (Brogden *et al.*, 1996) and magainin II (Giovannini *et al.*, 1987; Zasloff, 1987). All of these are α-helical peptides, with the exception of ovine polyaspartic acid, which is too small to determine any secondary structure (6 aa). Stock solutions were prepared by reconstituting each peptide in sterile PBS containing 0.02 % acetic acid and 0.4 % BSA (Sigma-Aldrich).

***In vitro* assays for antimicrobial activity.** The antimicrobial activity of each CAMP was determined relative to a control containing no peptide using a modified microtitre broth dilution method (Steinberg *et al.*, 1997). Liquid bacterial cultures were grown to mid-exponential phase and diluted to 1×10^5 c.f.u. ml^{-1}.

Antibacterial time–kill assays. Strains were grown to mid-exponential phase in LB broth at 37 °C. Aliquots of these cultures containing approximately 1×10^8 c.f.u. ml^{-1} were exposed separately to PBS (control) or 128 μg magainin II ml^{-1}. Cultures were maintained at 37 °C with shaking (180 r.p.m.) throughout the assay. Samples were taken at 0, 1, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, 180, 240 and 1440 min and then diluted serially in PBS and enumerated on LB agar. Viable counts (c.f.u. ml^{-1}) were obtained after 18 h incubation at 37 °C.

Magainin II stability assay. Aliquots of magainin II were prepared at 256 μg ml^{-1} in LB broth and incubated at 37 °C for 0, 2, 5, 24 or 48 h prior to inoculation with a culture of *B. cepacia* J2540 grown to mid-exponential phase and containing approximately 1×10^8 c.f.u. ml^{-1}. After inoculation, the concentration of magainin II in each sample was 128 μg ml^{-1}; the control contained an equal volume of PBS. Samples were taken from the culture prior to inoculation into LB broth containing magainin II, and then at 30 min and at 1, 2, 3 and 4 h post-inoculation. These samples were serially diluted, plated onto LB agar and incubated at 37 °C for 18 h for viable counts. The decrease in c.f.u. ml^{-1} for each of the magainin II samples was compared with that produced by the freshly prepared peptide (0 h) to give an indication of the activity of magainin II following incubation over time, whilst a PBS control was used to measure normal *B. cepacia* growth over the 4 h period.

Effects of proteases on magainin II activity. *B. cepacia* J2450 was grown to late-stationary phase in LB broth at 37 °C to allow maximal expression of extracellular proteases. Culture supernatant was passed through 0.45 and 0.2 μm filters (Whatman International) to remove all bacteria. PBS (control), filtered culture supernatant or filtered culture supernatant + Complete (1 tablet in 50 ml supernatant inhibits serine, metallo- and cysteine proteases; Roche Diagnostics) was added to magainin II at 102 μg ml^{-1} and incubated at 37 °C for 30 min. These pre-treated magainin II peptides were used in a standard time–kill assay, and samples were taken and enumerated at 0, 60, 240, 300, 360 and 1440 min. To visualize the susceptibility of magainin II to proteases, 500 μg magainin II ml^{-1} was incubated at 37 °C in supernatant from *B. cepacia* J2450 grown overnight in LB broth and filter-sterilized as above. Samples were removed at 0, 2, 5, 24 and 48 h and analysed by 4–20 % Tris/glycine SDS-PAGE (Invitrogen) before staining with Coomassie blue.

***In vitro* cell protection assay.** Apoptotic cell death was measured by the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH), which is released into the culture supernatant following damage to the cytoplasmic membrane and so is proportional to cell death. A LDH^{PLUS} assay (Roche Diagnostics) was used to quantify the amount of LDH present and was performed in accordance with the manufacturer's instructions. Briefly, BEAS-2B human bronchial epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10 % (v/v) fetal calf serum (FCS; Sigma-Aldrich) at 37 °C with 5 % CO_2. Cells were maintained as subconfluent monolayers, passaged by removal of the monolayer using DMEM culture medium and seeded into fresh polystyrene 96-well plates at 2×10^4 cells per well in a volume of 100 μl and incubated for 16 h. Cells were then incubated with magainin II across a concentration range of 25–200 μg ml^{-1} for 18 h at 37 °C with 5 % CO_2. As a control to quantify maximal LDH release, cells were lysed with 1 % (v/v) Triton X-100 (Sigma-Aldrich) and incubated for 10 min at room temperature. To quantify normal LDH release, cells were cultured and incubated with PBS. Spectrophotometric measurement of formazan dye at A_{450} was performed using a plate reader (EL800; BioTek Instruments). All tests were performed in triplicate with samples calculated as a percentage of the control (assuming 100 % cytotoxicity), giving an indication of the reduction of LDH release.

Statistical analysis of data. Two-way analysis of variance followed by Bonferroni's post-tests were carried out using Graphpad Prism version 4.0. P values $\leqslant 0.001$ were considered significant.

RESULTS

Antimicrobial activity of peptides against Bcc strains

Eight CAMPs were tested for inhibitory activity against 18 representative Bcc strains of genomovars I–V, and 50 and 90 % inhibitory concentration values (IC_{50} and IC_{90}, respectively) were determined (Table 1). Subsequent experiments used the following strains as representative of each genomovar: *B. cepacia* J2540, genomovar I; *B. multivorans* 7897, genomovar II; *B. cenocepacia* J2956, genomovar III; *B. stabilis* 7639, genomovar IV; and *B. vietnamiensis* LMG 10929^T, genomovar V. Polymyxin B was used as a control antibiotic to which widespread antimicrobial resistance has frequently been described in *B. cepacia* species. Calcitermin, cecropin A, granulysin, LL-37, MUC-7, P113 and poly-D-aspartic acid showed no growth inhibitory activity at concentrations up to 256 μg ml^{-1} towards any of the Bcc strains tested. However, magainin II exhibited variable growth inhibitory activity towards Bcc strains and within genomovars at concentrations of 128 and 256 μg ml^{-1}, causing a reduction in the c.f.u. ml^{-1} of most strains when compared with controls. A possible exception was with genomovar V (*B. vietnamiensis*) where all strains exhibited IC_{50} values of $\geqslant$128 μg ml^{-1}, suggesting marginally more magainin II resistance in this genomovar.

Table 1. IC values of magainin II against Bcc strains

Organism*	IC_{50} (μg ml^{-1})	IC_{90} (μg ml^{-1})
B. cepacia J2540	128	>256†
B. cepacia 25416	8	>128
B. cepacia C2970	32	>128
B. cepacia 9091	32	>128
B. cepacia C1964	128	>128
B. multivorans 7897	16	>256†
B. multivorans ATCC 17616	128	>128
B. multivorans 7732	>128	>128
B. cenocepacia J2956	>256†	>256
B. cenocepacia J415	>128	>128
B. cenocepacia C2836	32	>128
B. cenocepacia C1394	64	>128
B. stabilis 7639	32	64
B. stabilis 14294	128	>128
B. stabilis 8088	64	>128
B. vietnamiensis LMG 10929^T	>256†	>256†
B. vietnamiensis 549	128	>128
B. vietnamiensis C1709	128	>128

*Strains were tested in triplicate and analysed using a two-way analysis of variance.
†IC_{50} >256 μg ml^{-1} was considered to be resistant to magainin II.

Temporal effect of magainin II towards Bcc strains

In assays to evaluate the effect of magainin II over time, cultures of the five representative genomovar strains were incubated with 128 μg magainin II ml^{-1} and samples were collected at intervals between 0 and 1440 min. Fig. 1 shows that magainin II exerted strong antimicrobial activity against genomovars I (*B. cepacia* J2540) and V (*B. vietnamiensis* LMG 10929^T). In each case, a reduction in c.f.u. ml^{-1} occurred within minutes of introducing the peptide to the culture at a concentration of 128 μg ml^{-1}. Genomovar V was the most susceptible with complete killing after 10 min, whilst the count of genomovar I was reduced by 2 $\log_{10}$ within 5 min. After this initial decrease, magainin II appeared to exhibit a bacteriostatic effect, maintaining viable counts at approximately 1×10^6 c.f.u. ml^{-1}. By contrast, exposure of *B. multivorans* 7897 (genomovar II), *B. cenocepacia* J2956 (genomovar III) and *B. stabilis* 7639 (genomovar IV) to 128 μg magainin II ml^{-1} did not result in an immediate decrease in bacterial number (data not shown). Instead, bacteriostatic activity towards these strains became evident at 40–120 min post-exposure, by which time growth of the control cultures had increased by almost 1 $\log_{10}$, whilst the cultures exposed to peptide remained relatively constant. At 180 min post-exposure to magainin II, genomovars II, III and IV were reduced by 35, 20 and 85 %, respectively, of the initial c.f.u. ml^{-1} of the culture. All cultures, with the exception of *B. vietnamiensis* LMG 10929^T, exhibited regrowth to levels similar to that of controls after incubation at 37 °C for 24 h

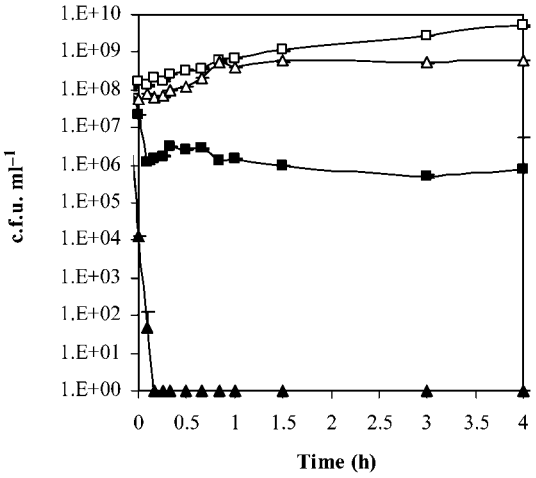

Fig. 1. Direct antimicrobial activity of 128 μg magainin II ml^{-1} against exponentially growing cultures of *B. cepacia* J2540 and *B. vietnamiensis* LMG 10929^T over time. ■, *B. cepacia* + 128 μg magainin II ml^{-1}; □, *B. cepacia* + PBS control; ▲, *B. vietnamiensis* + 128 μg magainin II ml^{-1}; △, *B. vietnamiensis* + PBS control. Error bars represent the SD of triplicate counts (c.f.u. ml^{-1}).

with magainin II. This regrowth at the 24 h time point may account for the apparently high IC_{50} values determined for some genomovars and may mask the initial antimicrobial activity; resumption of bacterial growth at 24 h may be due to degradation of the peptide. The IC_{50} values therefore possibly underestimated the activity of magainin, with cell-damaging effects being mediated at peptide concentrations lower than the IC_{50} concentrations.

Stability of magainin II

We hypothesized that the observed regrowth of bacteria after 24 h incubation with magainin II occurred because the peptide was unstable in solution, resulting in loss of antimicrobial activity. Thus 128 μg magainin II ml^{-1} was incubated in LB broth or bacterial culture supernatant for 0–48 h prior to exposure to *B. cepacia* J2540. Peptide activity was determined as a 2 $\log_{10}$ decrease in viable bacteria. These experiments showed that some antimicrobial activity towards *B. cepacia* J2540 was retained following incubation for up to 24 h in culture supernatant (Fig. 2). Although the activity was reduced compared with that of the freshly prepared peptide, magainin II incubated for 24 h in culture supernatant resulted in a 1 log decrease in viable bacteria compared with a 2 log decrease seen with freshly prepared magainin II. All antimicrobial activity was lost following incubation for 48 h in culture supernatant.

Effect of extracellular proteases on the antimicrobial activity of magainin II

Several reports have indicated that bacterial proteases can confer resistance to antimicrobial peptides (Guina *et al.*,

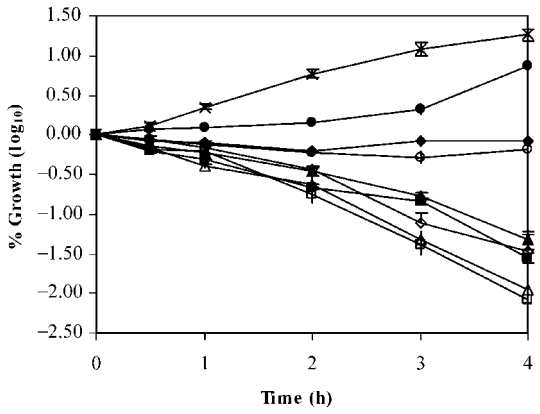

Fig. 2. Antimicrobial activity of 128 μg magainin II ml^{-1} against *B. cepacia* J2540 incubated in bacterial culture supernatant or LB broth. Magainin II was incubated in *B. cepacia* J2540 filter-sterilized culture supernatant or LB broth at 128 μg ml^{-1} for up to 48 h before use in antimicrobial assays for *B. cepacia* J2540. Results are shown for freshly prepared magainin II incubated in culture supernatant (filled symbols) or LB broth (unfilled symbols) at 0 (■, □), 2 (▲, △), 24 (◆, ◇) or 48 (●, ○) h prior to exposure with an exponentially growing culture of *B. cepacia* J2540. ×, PBS control. Error bars represent the SD from triplicate counts of c.f.u. ml^{-1}.

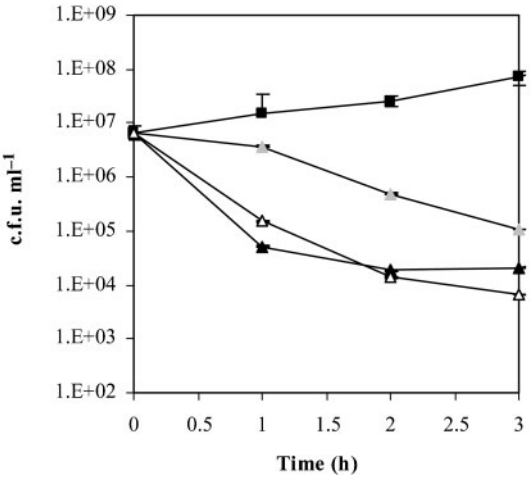

Fig. 3. Antimicrobial activity of magainin II against *B. cepacia* J2540 in the presence of protease. Magainin II was pre-treated in filtered *B. cepacia* J2540 culture supernatant (grey triangle) or in filtered *B. cepacia* J2540 culture supernatant + Complete protease inhibitor (△) or left untreated (▲) and then evaluated for its activity against *B. cepacia* J2540. ■, PBS control. The error bars represent the SD calculated from five individual samples.

2000; Park *et al.*, 2001; Schmidtchen *et al.*, 2002; Sieprawska-Lupa *et al.*, 2004; Thwaite *et al.*, 2006). To test whether this was true for Bcc strains, magainin II was incubated in the presence of filtered culture supernatant with and without a protease inhibitor for 30 min and then added to *B. cepacia* J2540 cells in broth. After incubation at 37 °C, the number of viable cells was determined at intervals up to 3 h. Initial studies confirmed that the protease inhibitor alone did not affect the growth of the test strain (data not shown). In the absence of magainin II, *B. cepacia* J2540 grew over the course of the experiment (Fig. 3), and in the presence of magainin II in PBS the number of viable bacterial cells decreased. The rate of decrease of viable cell numbers was slowed when magainin II was pre-incubated with culture supernatant, but pre-incubation of the peptide with culture supernatant containing protease inhibitors restored the killing activity of the peptide ($P < 0.0001$). To confirm the degradation of magainin II by proteases, samples of the peptide incubated in *B. cepacia* J2540 culture supernatant for up to 48 h were analysed by SDS-PAGE. At 48 h, magainin II could no longer be visualized by Coomassie blue staining (Fig. 4), indicating its digestion by proteases present in the supernatant.

Magainin II decreases LDH release from BEAS-2B cells in response to *B. cepacia* infection

As previous reports have shown that magainin II has substantial anti-cancer properties (Ohsaki *et al.*, 1992), the non-cancerous human bronchial epithelial cell line BEAS-2B was used to evaluate the antimicrobial effects of the peptide towards Bcc strains in cell culture. LDH release from infected cells was used as a measure of cell-membrane damage and loss of cell integrity. Exposure of BEAS-2B cells to the representative strain panel of the Bcc resulted in release of LDH into cell culture medium compared with control cells. The addition of magainin II to the cell culture to a concentration of 25–200 μg ml^{-1} and subsequent

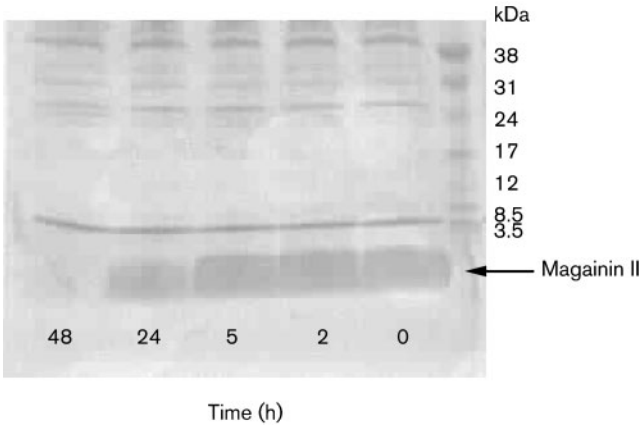

Fig. 4. Persistence of magainin II in *B. cepacia* culture supernatant. Magainin II (500 μg ml^{-1}) was incubated in *B. cepacia* J2540 culture supernatant for up to 48 h to determine its susceptibility to protease digestion. The presence of magainin II was visualized by SDS-PAGE and staining with Coomassie blue.

Table 2. Protection afforded to BEAS-2B cells by magainin II against Bcc infection

All values represent triplicate samples.

	LDH released (μg ml^{-1})	LDH released (%)* at a peptide concentration (μg ml^{-1}) of:					
		0	25	50	100	150	200
Background control†	2.88	0	0	3	9	15	33
Lysis control‡	41.50	100	100	100	100	100	100
B. cepacia J2540		100	100	99	58	0	0
B. multivorans 7897		100	100	100	86	6	0
B. cenocepacia J2956		100	100	93	86	78	80
B. stabilis 7639		100	84	83	72	3	0
B. vietnamiensis LMG 10929^{T}		100	14	1	0	0	0

*LDH released above the background control as a percentage of total cell lysis with Triton X-100.
†Background lysis control: no magainin II or bacteria.
‡Positive control: complete cell lysis with Triton X-100.

infection with the strains reduced the level of LDH release (Table 2). The degree of protection of BEAS-2B cells from lysis by *B. cepacia* was measured as the percentage of LDH released compared with the total LDH released after Triton X-100 lysis of the cells. This indicated that 200 μg magainin II ml^{-1} conferred complete protection to the cells from infection with *B. cepacia* J2540, but only 20 % protection against *B. cenocepacia* J2956. However, the peptide offered some protection against infection by all five genomovar strains.

DISCUSSION

This study describes an evaluation of the antimicrobial potential of several CAMPs against strains of the *B. cepacia* complex, with the ultimate aim of developing a more efficacious treatment for infections with these organisms. From the panel of antimicrobial peptides analysed, we have reported the novel finding that only magainin II showed moderate activity against Bcc genomovars I–V.

Magainins are broad-spectrum, positively charged, antimicrobial peptides that are secreted constitutively from the skin of the African clawed frog (*Xenopus laevis*) onto the epithelial surface (Zasloff, 1987). These 23 aa peptides exhibit antibacterial, antiviral, antifungal and tumoricidal properties and are considered an essential facet of the innate immune system in preventing infection. Magainin II has previously been described to exert antimicrobial activity against multidrug-resistant bacteria such as meticillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* (Giacometti *et al.*, 2005a) and *Stenotrophomonas maltophilia* (Giacometti *et al.*, 2000) and against some viruses including herpes simplex virus types 1 and 2 (Albiol Matanic & Castilla, 2004). We determined that magainin II was the only antimicrobial peptide tested in this study that exerted activity against selected strains of the Bcc. It was bacteriostatic for representative strains of genomovars I, II, III and IV and bactericidal for genomovar V. The strain-to-strain differences observed in IC_{50} values for magainin II within the Bcc may be a consequence of the extreme genetic diversity within the Bcc group. The large (~8 Mb) genome is carried on multiple replicons, which may add greater flexibility in the acquisition, loss and expression of genes (Lessie *et al.*, 1996; Mahenthiralingam & Drevinek, 2007).

Owing to the direct antimicrobial activity of CAMPs on the bacterial membrane, acquisition of resistance to these peptides is unlikely considering the highly conserved structure of the target molecule (Hancock & Lehrer, 1998; Matsuzaki *et al.*, 1995). Furthermore, the selection of resistant organisms within a bacterial population would be unlikely because of the rapid and broad-spectrum activity of the peptides and the high metabolic burden of membrane alteration (Hancock & Scott, 2000; Zasloff, 2002). Pathogens exhibiting natural resistance against antimicrobial peptides utilize a range of strategies to render the peptides less effective (Ganz, 2001). Here, we showed that Bcc species produce extracellular proteases that are capable of degrading antimicrobial peptides, a characteristic also described in a number of other pathogens (Schmidtchen *et al.*, 2002; Thwaite *et al.*, 2006). We found that magainin II was unstable at 37 °C in LB broth or culture supernatant, leading to compromised antimicrobial activity. This problem may be ameliorated by the synthesis of magainin II derivatives containing D-amino acids (Bessalle *et al.*, 1990) or β-peptides (Frackenpohl *et al.*, 2001) or the use of non-peptidic antibiotic CAMP mimics (Tew *et al.*, 2006) to provide more stable antimicrobial compounds. Stabilization of magainin II may therefore reduce enzymic degradation and increase the potency of the peptide. Indeed, novel therapeutic compounds based on magainin II are currently under development to maximize activity and decrease proteolytic sensitivity (Fuchs *et al.*, 1998;

Gottler & Ramamoorthy, 2008). Magainin II can be used for topical application (Chopra, 1993), but to date has not been evaluated as a nebulized agent for delivery into the lungs of CF patients.

Recent observations have indicated that magainin II acts synergistically with several antibiotics including piperacillin, ceftazidime, imipenem, meropenem, clarithromycin and polymyxin E (Giacometti *et al.*, 2000). A synergistic action with ceftazidime, one of the few antibiotics of choice for treatment of Bcc infections (Rajyaguru & Muszynski, 1997), may be advantageous for a combinational treatment, with the peptide reducing the initial bacterial load and perhaps providing an extended window for antibiotic treatment or possibly enhancing the efficacy of the antibiotic.

Despite the range of virulence factors conferring resistance to antimicrobial peptides, we have shown that magainin II retains moderate antimicrobial activity against Bcc strains *in vitro*. Further studies are required to determine the utility of the peptide *in vivo*. However, this peptide has potential advantages over classical antibiotics as an antimicrobial with rapid, broad-spectrum activity and potentially fewer problems associated with resistance.

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