ANTIBIOSIS OF *BURKHOLDERIA UBONENSIS* AGAINIST *BURKHOLDERIA PSEUDOMALLEI*, THE CAUSATIVE AGENT FOR MELIOIDOSIS

Kristy Marshall[1], Sabina Shakya[1], Andrew R Greenhill[1,2], Gabriel Padilla[1], Anthony Baker[1] and Jeffrey M Warner[1]

[1]Environmental and Public Health Microbiology Research Group, Microbiology and Immunology, James Cook University, Townsville, QLD, Australia; [2]Infection and Immunity, Institute of Medical Research, Goroka, EHP, Papua New Guinea

Abstract. Melioidosis, caused by *Burkholderia pseudomallei*, is an enigmatic infectious disease that afflicts individuals in many tropical and developing regions. Treatment is hampered by the organism's innate antibiotic resistance and the disease's non-pathognomic presentation. Recently, added attention has been given to this organism due to its classification as a potential biowarfare agent. Therefore, methods of preventing acquisition of infection are needed. We investigated antagonism between *Burkholderia* spp and *B. pseudomallei* derived from the same ecological niche in a melioidosis endemic region in Papua New Guinea. Isolates of environmentally derived non-pseudomallei *Burkholderia* spp (n=16) were screened for antibiosis against 27 *B. pseudomallei* isolates. Three isolates subsequently identified as *B. ubonensis* produced specific antagonistic activity against all *B. pseudomallei* isolates tested. The antagonistic compound in a cell-free state was obtained from a representative producing strain, with subsequent biological characterization revealing a pepsin sensitive peptide moiety consistent with a bacteriocin-like compound. To our knowledge, this is the first report of antagonistic activity demonstrated by near-neighbor Burkholderia against *B. pseudomallei* . This antagonism may be important in the micro-ecology of *B. pseudomallei*, and could also have application in the biocontrol of this pathogen.

Key words: *Burkholderia pseudomallei, B. ubonensis,* antibiosis, melioidosis

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis, an often fatal disease in endemic tropical and developing countries. The presentation of melioidosis is frequently ambiguous, thus hampering targeted therapeutic treatment. Even with optimal targeted therapy, mortality rates approach 25% (Leelarasamee and Bovornkitti, 1989). *B. pseudomallei* is known to exist in the environment in moist, clay soils, in association with plant rhizospheres but also in articulated and stored water for human and animal use (Currie *et al*, 2000; Inglis *et al*, 2001). The environmental prevalence of the organism is linked to clinical incidence of the disease and considerable spatial clustering of the organism may impact upon localized

Correspondence: Dr Jeffrey M Warner, Microbiology and Immunology, School of Veterinary and Biomedical Sciences, James Cook University, Townsville, QLD, 4811, Australia.
Tel: +61 7 4781 6375; Fax: +61 7 4779 1526
E-mail: jeffrey.warner@jcu.edu.au

disease prevalence (Trakulsomboon *et al*, 1999). However, little is known of factors that influence the organism's persistence and dispersal in the environment. Melioidosis is endemic in low-income tropical regions of the world where accurate clinical diagnosis is unlikely due to poor laboratory facilities or a lack of clinical awareness (Currie *et al*, 2000; Inglis *et al*, 2001). A better understanding of factors influencing local distribution of *B. pseudomallei* could improve clinical outcomes.

Studies within melioidosis endemic regions have noted that in environments where *B. thailandensis* populations outnumber *B. pseudomallei*, the incidence of melioidosis is considerably lower than in environments where *B. pseudomallei* outnumbers *B. thailandensis* (Trakulsomboon *et al*, 1999; Warner *et al*, 2008). While other environmental factors influence microbial diversity, antibiosis mediated microbial antagonism may also play a role in the survival and persistence of *Burkholderia* spp. Antibiosis has been described in the *Burkholderia* genus, such as pyrrolnitrin in the *B. cepacia* complex (el-Banna and Winkelmann, 1998; Cain *et al*, 2000), but production of an antagonistic agent by *Burkholderia* spp against *B. pseudomallei* has not been previously described. This study investigates antagonism between *Burkholderia* spp and *B. pseudomallei* derived from the same environmental niche in a melioidosis endemic region in Papua New Guinea (PNG). Preliminary studies into the nature of the microbial antagonism by biological characterization of the antagonistic compound are also documented.

MATERIALS AND METHODS

Bacteria used in this study

Isolates of *B. thailandensis* (*n*=4) and *B. ubonensis* (n=11) collected in the Balimo region of PNG (Warner *et al*, 2008) were presumptively identified using typical biochemsitry and sequencing of the *recA* gene as described by Payne *et al* (2005) (data not shown).

Of the panel of *B. pseudomallei* isolates used as targets, 17 were clinical or environmental isolates from PNG, and 9 were clinical isolates from northern Australia. Control type strains of *B. pseudomallei* and *B. thailandensis* were also included. All the *Burkholderia* isolates were taken from storage in the James Cook University culture collection and recovered onto Ashdown selective (ASH) agar (Ashdown, 1979). A variety of clinical or taxonomic related gram-positive and gram-negative organisms were also taken from the James Cook University culture collection and used for specificity screening. These organisms (Table 1) were recovered onto nutrient agar or blood agar.

Antagonism screening

Two antagonism screening techniques were used based on modifications of Govan and Harrris (1985). Fresh cultures of the *Burkholderia* spp were grown on ASH agar at 30ºC for 48 hours. The organisms used for specificity testing were grown on nutrient agar at 35ºC for 24 hours prior to use in the antagonism assay. Saline suspensions of all isolates used were prepared to 0.5 MacFarland using physiological saline.

Spot on lawn screening. A 1 µl suspension of each "producer" *Burkholderia* spp isolate was spot inoculated onto the surface of a 10 ml (half depth) Mueller Hinton (MH) agar plate (8 per plate), then incubated at 30ºC for 24 hours. Suspensions of each "target" *B. pseudomallei* isolate and each isolate used for specificity testing

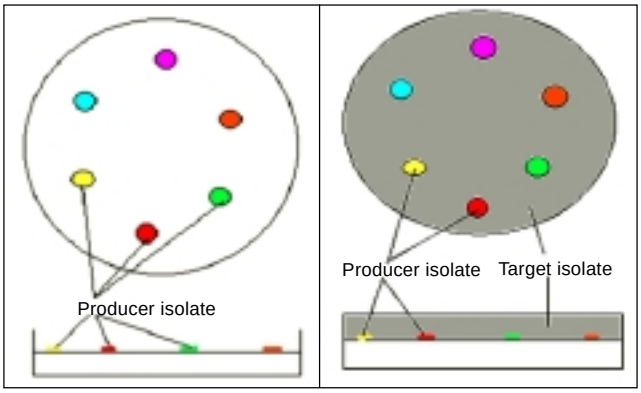

Fig 1a–Direct spot on lawn method.

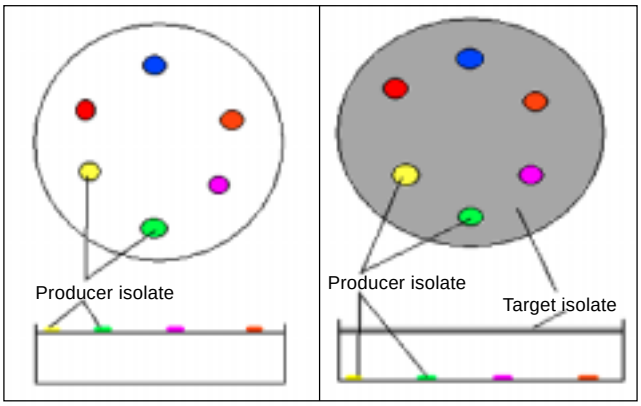

Fig 1b–Flip streak method.

were prepared and 100 µl inoculated into 10 ml aliquots of molten (44ºC) MH agar. The inoculated molten agar was poured over the surface of the agar plates previously incubated with "producer" *Burkholderia* spp. Plates were incubated at 37ºC for 5 days and observed daily for zones of inhibition (ZOI) in the overlay (Fig 1a). Results were recorded qualitatively (zone/no zone).

Flip streak screening. Spot inoculums (1 µl) of "producer" *Burkholderia* spp were dispensed onto 20 ml MH plates (8 per plate, on duplicate plates) and incubated at 30ºC for 24 hours. The agar was aseptically detached from the Petri dish, inverted, and placed back into the dish. Suspensions of each *B. pseudomallei* isolate were prepared and aliquots of 10 ml of molten (44ºC) MH agar were inoculated with 100 µl of the suspension. The molten agar was poured over the surface of the agar plates previously incubated with "producer" *Burkholderia* spp. Plates were incubated at 37ºC for 5 days, and observed daily for ZOI in the *B. pseudomallei* lawn (Fig 1b). Results were recorded qualitatively (zone/no zone).

Specificity testing was conducted using the flip streak method. Suspensions for each organism were made and inoculated, and plates incubated and interpreted as for the *B. pseudomallei* isolates, above.

Obtaining the antagonistic compound in a cell free state

One *B. ubonensis* isolate (A21) demonstrating specific antagonism towards *B. pseudomallei* was selected for further study. The isolate was grown in LB broth at 30ºC and aliquots taken from culture at time points reflecting lag, exponential, stationary and decline phase (data not shown). Samples were centrifuged at 10,000*g* for 30 minutes and filter sterilized to remove cells. Antagonistic activity was measured in the cell free supernatant (CFS) using a well diffusion assay. A suspension of *B. pseudomallei* was inoculated onto the entire surface of an MH plate. After drying, wells 6 mm in diameter were cut into the agar and 100 µl of CFS was dispensed into the wells. Plates were incubated at 30ºC for 48 hours and ZOI measured. The ZOI measurements include the diameter of the well (6 mm). As such, a recorded zone of 6 mm corresponds with no antagonistic activity, and zones >6 mm are indicative of increasing antagonistic activity.

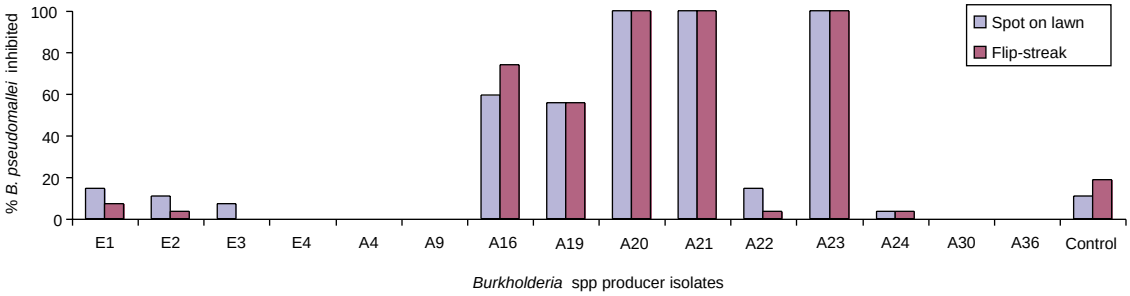

Fig 2–Spectrum of antagonistic activity by *B. thailandensis* (E1 – E4; $n = 4$) and *B. ubonensis* (A4 – A38; $n = 11$) against *B. pseudomallei* ($n = 27$). Control *B. thailandensis*. Means value of three experiments.

Characterization of antagonistic compound

The antagonistic compound was characterized in terms of its heat stability, pH sensitivity, and susceptibility to proteolytic enzymes and catalase. The effect of each treatment was determined using the well diffusion assay and measuring zones of inhibition (as above) of triplicate samples.

To determine heat stability aliquots of CFS were heated to three different temperatures (50, 70 and 100ºC) for three different durations (10, 30 and 60 minutes), resulting in nine different heat treatments.

To determine pH stability two aliquots of CFS were adjusted to pH 4.0 by adding 6M HCl and incubated at room temperature for 30 minutes, then one aliquot was readjusted back to pH 7.0 using 1M NaOH. Two CFS aliquots were adjusted to pH 9.0 using 1M NaOH and incubated at room temperature for 30 minutes, and then one aliquot was readjusted back to pH 7.0 with 6M HCl. The pH of the uninoculated LB broth was adjusted in parallel as a control.

For determination of proteolytic enzyme and catalase susceptibility, aliquots of normal, heat treated (70ºC for 10 minutes) and pH adjusted (pH 4.0 and 9.0) CFS were prepared. Pepsin, trypsin, papain and catalase were added to CFS to a final concentration of 2 mg/ml and proteinase K to a final concentration of 0.5 mg/ml. Samples were incubated at 37ºC for 2 hours prior to inoculation o the well diffusion assay (as above).

RESULTS

Antagonism screening

Ten of the 15 test isolates and the control strain exhibited antagonistic activity against one or more *B. pseudomallei* isolates (Fig 2). Five of the 11 *B. ubonensis* isolates inhibited over half the *B. pseudomallei* isolates, whereas the *B. thailandensis* isolates that were inhibitory were active against less than 20% of isolates. Of particular interest were three *B. ubonensis* isolates (A20, A21 and A23) which exhibited antagonism against all 27 *B. pseudomallei* isolates in both the spot on lawn and flip streak assays. Moreover, the antagonism exhibited by these three isolates was specific, with none exhibiting antagonism against any of the taxonomically and clinically related organisms (Table 1). In contrast, broad spectrum inhibitory activity was demonstrated by the *B. thailandensis* isolates including the control *B. thailandensis* (Table 1).

Characterization of antagonistic activity in the CFS

The onset of antagonistic activity in CFS occurred at between 21 and 24 hours, consistent with late log phase (data not

Table 1
Inhibition of selected bacteria (taxonomically and clinically related to *B. pseudomallei*) by isolates of *B. thailandensis* and *B. ubonensis* using the flip streak screening method.

Species	*B. thailandensis*				*B. ubonensis*											
Target organism	E1	E2	E3	E4	A4	A9	A16	A19	A20	A21	A22	A23	A24	A30	A36	Control
Burkholderia cepacia	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Bacillus cereus	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+
Streptococcus pyogenes	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+
Streptococcus pneumoniae	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Enterococcus faecalis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VRE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+
MRSA	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+
Escherichia coli	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+
Proteus vulgaris	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacter aerogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Control, *B. thailandensis* wild strain donated from The Townsville Hospital culture collection
VRE, vancomycin resistant *Enterococcus faecalis*
MRSA, methicillin resistant *Staphylococcus aureus*
+, inhibition; -, no inhibition

Table 2
The effect of heat and pH treatments on the antagonistic activity of *B. thailandensis* cell free supernatant against *B. pseudomallei* A78 using the well diffusion assay. ZOI values represent the mean of triplicate samples.

Treatment	ZOI (mm)	Interpretation
Temperature		
Control	10	Inhibition
50ºC, 10 minutes	21	Enhanced activity
50ºC, 30 minutes	23	Enhanced activity
50ºC, 60 minutes	24	Enhanced activity
70ºC, 10 minutes	24	Enhanced activity
70ºC, 30 minutes	24	Enhanced activity
70ºC, 60 minutes	24	Enhanced activity
100ºC, 10 minutes	6	No inhibition
100ºC, 30 minutes	6	No inhibition
100ºC, 60 minutes	6	No inhibition
pH		
Control (no treatment)	10	Inhibition
Control (water added)	10	No effect
pH 4.0	16	Enhanced activity
pH 7.0 ↔ pH 4.0	16	Enhanced activity
pH 9.0	13	Enhanced activity
pH 7.0↔ pH 9.0	17	Enhanced activity

ZOI, Zone of inhibition, including the diameter of the well (6 mm)
Control (water added), an equivalent volume of water was added as the volume of acid/base required to adjust pH to 4.0/9.0, to account for dilution effect.
↔ , pH of CFS was taken from original value (~ 7.0) to acid or alkaline, and then re-adjusted back to neutral.

shown). Heating the CFS to 50 and 70ºC enhanced antagonistic activity but antagonistic activity was completely lost when heated to 100ºC. Adjustment of pH to either 4.0 or 9.0 also enhanced antagonistic activity (Table 2). The antagonistic compound was susceptible only to pepsin, the other proteases did not inhibit the antagonistic activity of the CFS. Exposure of the CFS to heat and alkaline conditions prior to pepsin treatment rendered the antagonistic compound resistant to pepsin, but exposure to acidic conditions had no such effect. None of the pre-treatments impacted upon the effect of the proteases to which CFS was not susceptible. Catalase did not inhibit the antagonistic effect of the CSF per se, however CFS that were pH adjusted to either 4.0 or 9.0 were susceptible to catalase.

DISCUSSION

We report for the first time *Burkholderia* spp capable of producing antagonistic activity against *B. pseudomallei*. This could be an important determinant in the microbial ecology of *B. pseudomallei* in terms of its survival and persistence in its environmental niche, and it also raises the potential of antibiosis as a biocontrol option for melioidosis and perhaps as a therapeutic agent.

Our findings suggest the antagonism is due to the production of a bacteriocin or bacteriocin-like inhibitory substance (BLIS). Bacteriocins, both those naturally produced by endogenous flora and as additives, are commonly used to control the growth of target bacteria in foods (Cleveland *et al*, 2001; Galvez *et al*, 2007). Recent investigations have shown a potential role for bacteriocins in the control of plant pathogens (Hammami *et al*, 2009; Hert *et al*, 2009). Much effort has been spent on the environmental control of other human pathogens, either directly or through vector control measures, but to date no studies have comprehensively addressed the possibility of using bacteriocins for the biocontrol of naturally occurring pathogens in the environment. This is despite the evolution of bacteriocins to offer producer strains a competitive advantage in natural environments (Czaran *et al*, 2002). Limiting the reservoir of *B. pseudomallei* in clinically relevant environments, and thus the exposure to the organism, may decrease the incidence of melioidosis. Removal of *B. pseudomallei* has been attempted using quicklime to alter soil pH (Na-ngam *et al*, 2004), however large amounts of lime were required, and such a control measure may negatively impact on other macro- and micro-biota. Much future work is required, including virulence studies of the producer isolates in animal models, as *Burkholderia* spp of the cepacia complex have the potential to cause opportunistic infection in immunocompromised people (Glass *et al*, 2006), but bacteriocin-mediated environmental control warrants further investigation, particularly given its specific nature.

Two broad groups of *Burkholderia* spp isolates screened demonstrated antibiosis. Isolates of *B. ubonensis* that were inhibitory to *B. pseudomallei* generally produced specific, narrow spectrum activity with little or no activity against the taxonomically and clinically related isolates in the specificity group. Isolates A20, A21 and A23 demonstrated diffusible activity against all *B. pseudomallei* tested. In contrast, the inhibitory *B. thailandensis* isolates (E1, E2 and E3) inhibited less than 20% of the *B. pseudomallei* isolates, yet produced a broad spectrum activity against almost 50% of the isolates in the specificity group. This is in agreement with the control *B. thailandensis* which produced similar activity.

Inhibition using the flip streak method is indicative of a diffusible compound, and rules out bacteriophage activity through the physical separation of the producer organism and the target organism. *B. thailandensis* is known to harbor a bacteriophage specific for *B. mallei* that has demonstrated no cross-reactivity with *B. pseudomallei* (Woods *et al*, 2002). The findings of our study do not rule out a role for bacteriophages produced by *B. thailandensis* being active against *B. pseudomallei*, and this line of investigation warrants further attention. However, our results are indicative of bacteriocin or BLIS production, as similar levels of antagonistic activity were observed using both assay methods. As such, efforts were made to preliminarily characterize the active component of the CFS of a representative isolate (A21). Analysis revealed that the antagonistic compound contains a peptide moiety which is responsible for antibacterial activity. Due to its narrow spectrum of activity and resistance to heat shock, pH and various proteases, yet susceptibility to pepsin, it appears that the antagonistic compound is a bacteriocin or a BLIS.

Characterization studies revealed that activity of the CFS was enhanced following moderate heat treatment (50°C and 70°C, but not 100°C). Perhaps heat promoted conformational change, allowing

for new intermolecular interactions which henceforth enhanced the antagonistic activity of the protein (Pretzer *et al*, 1991; Rondeau *et al*, 2007). Conformational changes are also suspected following pH adjustment. Enhancement of antagonistic activity, particularly bacteriocin activity, has been demonstrated when the pH is lowered (Blom *et al*, 1997; Houlihan *et al*, 2004).

Enzyme studies provide further insight into the nature of the inhibitory compound. Pepsin preferentially cleaves aromatic amino acids, particularly phenylalanine and tyrosine, and has no effect on valine, alanine or glycine (Silver and Stoddard, 1972). This suggests that phenylalanine or tyrosine form part of the active site of the *B. ubonensis* BLIS, due to the loss of activity following pepsin treatment. Pepsin is irreversibly inactivated above pH 6.0 which accounts for the loss of sensitivity when the pH of the supernatant was adjusted to 9.0. Heat treatment, promoting structural changes as described above, is suspected responsible for the pepsin resistance observed in heat treated supernatant in our study.

Further characterization of the BLIS and its expression will assist to determine if it can be exploited in therapy or as a biocontrol agent. Also, evidence that *Burkholderia* spp can inhibit the survival and persistence of *B. pseudomallei* through antibiosis in situ will aid our understanding of the ecology of melioidosis.

REFERENCES

Ashdown LR. An improved screening technique for isolation of *Pseudomonas pseudomallei* from clinical specimens. *Pathology* 1979; 11: 293-7.

Blom H, Katla T, Hagen BF, Axelsson L. A model assay to demonstrate how intrinsic factors affect diffusion of bacteriocins. *Int J Food Microbiol* 1997; 38: 103-9.

Cain CC, Henry AT, Waldo RH, Casida LJ, Falkinham JO. Identification and characteristics of a novel *Burkholderia* strain with broad-spectrum antimicrobial activity. *Appl Environ Microbiol* 2000; 66: 4139-41.

Cleveland J, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: safe, natural antimicrobials for food preservation. *Int J Food Microbiol* 2001; 71: 1-20.

Currie BJ, Fisher DA, Howard DM, *et al*. The epidemiology of melioidosis in Australia and Papua New Guinea. *Acta Trop* 2000; 74: 121-7.

Czaran TL, Hoekstra RF, Pagie L. Chemical warfare between microbes promotes biodiversity. *Proc Natl Acad Sci USA* 2002; 99: 786-90.

el-Banna N, Winkelmann G. Pyrrolnitrin from *Burkholderia cepacia*: antibiotic activity against fungi and novel activities against streptomycetes. *J Appl Microbiol* 1998; 85: 69-78.

Galvez A, Abriouel H, Lopez RL, Ben Omar N. Bacteriocin-based strategies for food biopreservation. *Int J Food Microbiol* 2007; 120: 51-70.

Glass MB, Gee JE, Steigerwalt AG, *et al*. Pneumonia and septicemia caused by *Burkholderia thailandensis* in the United States. *J Clin Microbiol* 2006; 44: 4601-4.

Govan JR, Harris G. Typing of *Pseudomonas cepacia* by bacteriocin susceptibility and production. *J Clin Microbiol* 1985; 22: 490-4.

Hammami I, Rhouma A, Jaouadi B, Rebai A, Nesme X. Optimization and biochemical characterization of a bacteriocin from a newly isolated *Bacillus subtilis* strain 14B for biocontrol of *Agrobacterium* spp strains. *Lett Appl Microbiol* 2009; 48: 253-60.

Hert AP, Marutani M, Momol MT, Roberts PD, Olson SM, Jones JB. Suppression of the bacterial spot pathogen *Xanthomonas euvesicatoria* on tomato leaves by an

attenuated mutant of *Xanthomonas perforans*. *Appl Environ Microbiol* 2009; 75: 3323-30.

Houlihan AJ, Mantovani HC, Russell JB. Effect of pH on the activity of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. *FEMS Microbiol Lett* 2004; 231: 27-32.

Inglis TJ, Mee BJ, Chang BJ. The environmental microbiology of melioidosis. *Rev Medic Microbiol* 2001; 12: 13-20.

Leelarasamee A, Bovornkitti S. Melioidosis - review and update. *Rev Infect Dis* 1989; 11: 413-25.

Na-ngam N, Angkititakul S, Noimay P, Thamlikitkul V. The effect of quicklime (calcium oxide) as an inhibitor of *Burkholderia pseudomallei*. *Trans R Soc Trop Med Hyg* 2004; 98: 337-41.

Payne GW, Vandamme P, Morgan SH, *et al*. Development of a recA gene-based identification approach for the entire *Burkholderia* genus. *Appl Environ Microbiol* 2005; 71: 3917-27.

Pretzer D, Schulteis BS, Smith CD, Vander Velde DG, Mitchell JW, Manning MC. Stability of the thrombolytic protein fibrolase: effect of temperature and pH on activity and conformation. *Pharm Res* 1991; 8: 1103-12.

Rondeau P, Armenta S, Caillens H, Chesne S, Bourdon E. Assessment of temperature effects on beta-aggregation of native and glycated albumin by FTIR spectroscopy and PAGE: relations between structural changes and antioxidant properties. *Arch Biochem Biophys* 2007; 460: 141-50.

Silver MS, Stoddard M. Amino-enzyme intermediates in pepsin-catalyzed reactions. *Biochemistry* 1972; 11: 191-200.

Trakulsomboon S, Vuddhakul V, Tharavichitkul P, Na-Gnam N, Suputtamongkol Y, Thamlikitkul V. Epidemiology of arabinose assimilation in *Burkholderia pseudomallei* isolated from patients and soil in Thailand. *Southeast Asian J Trop Med Public Health* 1999; 30: 756-9.

Warner JM, Pelowa DB, Gal D, *et al*. The epidemiology of melioidosis in the Balimo region of Papua New Guinea. *Epidemiol Infect* 2008; 136: 965-71.

Woods DE, Jeddeloh JA, Fritz DL, DeShazer D. *Burkholderia thailandensis* E125 harbors a temperate bacteriophage specific for *Burkholderia mallei*. *J Bacteriol* 2002; 184: 4003-17.