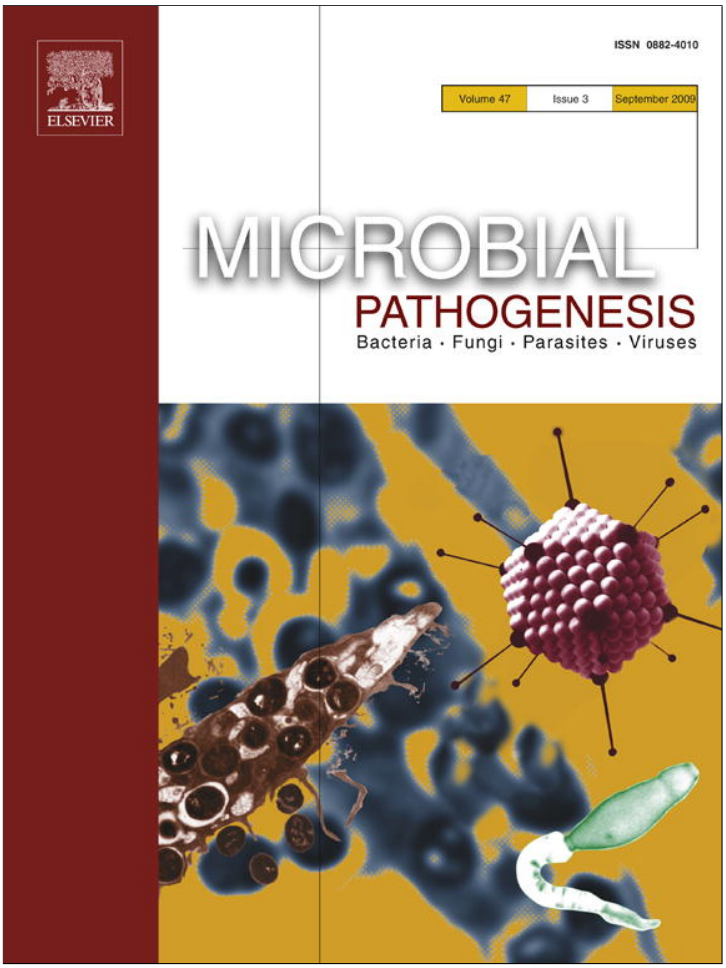

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Comparative analysis of extracellular enzymes and virulence exhibited by *Burkholderia pseudomallei* from different sources

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ABSTRACT

To evaluate the potential role of extracellular proteins in the pathogenicity and virulence of *Burkholderia pseudomallei*, the activities of several enzymes in the culture filtrates of nine clinical and six environmental isolates were investigated *in vitro* and *in vivo* in ICR strain of mice. The production of protease, phosphatase, phospholipase C, superoxide dismutase, catalase and peroxidase were detected in the culture filtrates of all the 15 isolates at different time points of growth 4–24 h. Over time, activity of each enzyme at each time point varied. Profile of secretion was similar among the 15 isolates irrespective of source, that is clinical or environmental. Catalase, phosphatase and phospholipase C were found to be increased in 60–100% of the isolates post-passage in mice. *In vivo* inoculation studies in ICR mice demonstrated a wide difference in their ability to cause bacteraemia, splenic or external abscesses and mortality rate ranged from few days to several weeks.

1. Introduction

Burkholderia pseudomallei, a gram-negative, motile bacillus is the causative agent of melioidosis. Melioidosis is a fatal disease that can manifest as an acute, subacute or chronic infection. Despite adequate treatment, mortality rate in patients with septic shock caused by melioidosis are approximately 80–95% [1]. The severe course of infection, aerosol infectivity and worldwide availability of *B. pseudomallei* has raised concerns that it may be used as a bioterror agent [2]. Additionally the organism may remain latent or dormant in the host for prolonged periods of time giving rise to relapse and recurrence especially in immunocompromised patients [3]. US Centres for Disease Control and Prevention included *B. pseudomallei* in the category B list of critical agents because it was considered to have potential in germ warfare and regarded as a potential bioterrorist weapon [4].

The factors involved in the mechanisms and pathogenicity of *B. pseudomallei* are not thoroughly understood. The organism is known to produce several virulence factors, including endotoxin, exotoxin, and protease, and others such as lecithinase, catalase, peroxidase, superoxide dismutase, cytotoxic exolipid, lipase, hemolysin, and water-soluble siderophore for iron acquisition from the host, which contributes to its survival and maintenance [4–6]. Flagella, type II and type III protein secretion system are also known virulence determinants with potential virulence factors including polysaccharides, exoproteins, fimbriae, pili and putative adhesions [7–11]. Apart from that, *B. pseudomallei* is also known to be a facultative bacterial intracellular pathogen. It can invade both phagocytic and nonphagocytic cells followed by intracellular multiplication and intercellular spread [12–14].

Many workers have reported the possible role of housekeeping extracellular enzymes such as proteases [15], acid phosphatase and lecithinase [16,17] in the virulence and pathogenesis of bacterial diseases. Anti-oxidant enzymes such as catalase, peroxidase and superoxide dismutase have also been reported to play important role in virulence [18]. Although many studies have suggested the possible role of extracellular enzymes in virulence, little attention has been paid to examine the effect of varying levels secreted and the difference in virulence exhibited by different isolates of the same species.

The objectives of this study were to investigate the serial production of certain key enzymes and also to study the modification in the production of these enzymes after a single passage in mice and if time taken to kill coincided with high, low and medium producers.

2. Results

All 16 isolates used in this study demonstrated similar growth profiles irrespective of source of isolate. Following the initial lag

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phase of approximately four hours the cell density increased rapidly during the log phase and reached stationary phase in 16 h (Fig. 1). There was also good correlation (>95%) observed between the optical density value of the bacterial culture and the viable count of the 24-h culture.

Enzymatic activities were studied every two hours and it was found that the first detection of any activity was only detected in the culture medium at the four-h sampling (log phase) of the growth cycle. The secretion of protease, phosphatase and phospholipase C activities increased with bacterial growth rate and there was no further increase during the stationary phase. However, levels of anti-oxidant enzymes, such as, catalase, peroxidase and SOD were low at the log phase but increased substantially following eight hours of growth. Overall the amount was significantly lower as compared to other enzymes. Quantitative analysis of the amount of enzymes produced by each of the strains included in the study demonstrated wide variation amongst the different isolates. There was no correlation of amount of enzyme produced to source of isolate, that is clinical or environmental, except for one isolate from pus, Bp51674, which was phenotypically mucoid and exhibited very low levels of all enzymes (Fig. 2(a)).

All the isolates were found to secrete all six enzymes and phospholipase C was found to be the most abundant of the enzymes secreted (4.3–29.0 U ml^{-1}) followed by catalase (1.2–12.2 U ml^{-1}). Phospholipase C activity was high among the clinical isolates, of which 60% of the isolates were from blood. In contrast all pus isolates demonstrated lower activity of this enzyme. *B. pseudomallei* strain 23343 (ATCC) and all environmental isolates also exhibited high phospholipase C activity. Phosphatase (4.5–15.9 U ml^{-1}) and protease (3.5–11.0 U ml^{-1}) were released at similar amounts at intermediary levels and peroxidase (0.6–4.0 U ml^{-1}) and superoxide dismutase (0.3–5.2 U ml^{-1}) were detected at lower levels. Inter-isolate variations in the level of enzymes released were also observed. The largest variations were observed in the amount of catalase (1.2–12.5 U ml^{-1}), and phospholipase C activities (4.3–29 U ml^{-1}), whereas smaller variations were observed with phosphatase (5–16 U ml^{-1}) and protease activities (3.5–11 U ml^{-1}).

The effect on production of the extracellular enzymes following a single passage in mice demonstrated that among the eight clinical isolates, increased activity was observed for phospholipase C (87.5% isolates), catalase (75% isolates), phosphatase (62.5% isolates), peroxidase (50% isolates), superoxide dismutase (50% isolates) and protease (25% isolates) (Fig. 2). Among the six environmental isolates, increased activity was similarly observed for phospholipase C, phosphatase and peroxidase (66.7% isolates), superoxide dismutase and protease (50% isolates) and catalase (33.3% isolates) (Fig. 3). The *B. pseudomallei* strain 23343 (ATCC) showed an increase in all the enzyme activities except for phospholipase C and protease (Fig. 2).

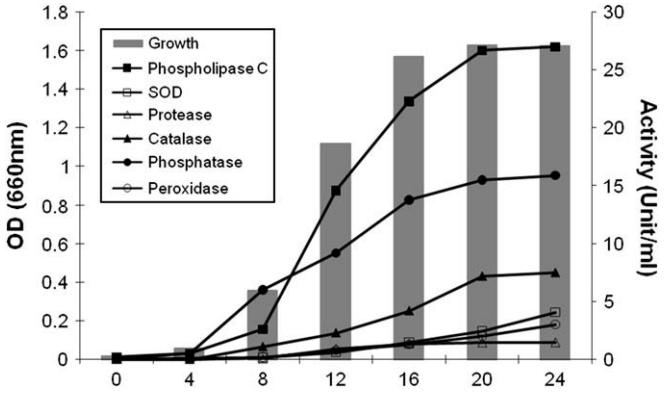

Fig. 1. Serial production of protease (a), phosphatase (b), phospholipase C (c), catalase (d), peroxidase (e) and superoixde dismutase (f) by a representative isolate of *B. pseudomallei* (Bp84881) at different phases of growth. Assays for enzymes were performed in triplicate and the mean value was used. Unit enzyme activity was calculated for each enzyme as described in Materials and methods.

Mice mortality rate of each of the isolates was investigated using three mice per group with an infective dose of 10^6 organisms, subcutaneously. Comparison between the clinical and environmental isolates demonstrated that 37.5% clinical isolates induced early mortality (up to 2 days), as opposed to only 16.6% of the environmental isolates (2 days); intermediate mortality was induced by 12.5% clinical isolates (8 days) and similarly by 16.6% environmental isolates (10 days); and late mortality was induced by 50% of the clinical isolates (15–23 days) whereas amongst environmental isolates 66.6% (15–28 days) demonstrated late mortality. *B. pseudomallei* strain 23343 (ATCC) induced early mortality (3 days) (Table 1).

Overall, 100% of the isolates that induced early mortality were found to have an increase in catalase activity following a single passage in mice as compared to only 37.5% isolates that induced late mortality. Increased phosphatase activity was also observed in 80% of the isolates that induced early mortality as compared to 62.5% isolates that induce late mortality. However increased phospholipase C activity was observed in 75% isolates that induced late mortality as opposed to 60% of the isolates that induced early mortality. Increased activity of protease, peroxidase and superoxide dismutase was observed in similar number of isolates that induced early and late mortality.

Amongst mice infected with *B. pseudomallei* strain 23343 (ATCC), it was found that 100% mice developed bacteraemia and 25% developed splenic abscesses (Table 1). None of the mice developed external abscesses. Amongst the eight clinical isolates, bacteraemia occurred in all mice for five of the isolates, 75% of the mice for one isolate from blood and 50% of the mice in two isolates from blood as well. Splenic abscesses were present in all mice due to four isolates (Bp69425, Bp03611, Bp29564, and Bp12237) and in 75% of the mice due to two isolates (Bp57325, Bp51674). However, two of the clinical isolates did not cause any splenic abscesses. Bacteraemia was also present in all mice infected by the six environmental isolates. Isolate E79/76 caused bacteraemia in 75% of the mice and isolates E77/96 and B7-13 in 50% of the mice. All mice infected with three of the environmental isolates, E958, E79/76 and B7-15 developed splenic abscesses. Amongst the remaining isolates 75% of mice infected with E77/96 and 50% of the mice infected with B7-13 demonstrated splenic abscesses. However there was one isolate that did not cause any splenic abscess formation.

In general external abscesses were only visible in the mice that demonstrated late mortality.

3. Discussion

Various studies have demonstrated the presence of secreted proteins and their potential role in virulence of *B. pseudomallei* [26–28]. Concentration and effect of different secreted proteins and extent of disease exhibited by *B. pseudomallei* isolates from different sources have not yet been reported.

Six housekeeping enzymes, which included phospholipase C, catalase, phosphatase, protease, superoxide dismutase and peroxidase were selected for assay to determine the concentration of enzyme secreted and the time-point in the growth phase for their secretion in order to determine if isolates from different sources had different patterns of secretion, thus differences in virulence. These six enzymes were chosen due to their potential role in virulence of *B. pseudomallei* [29]. Proteases have been found to digest biologically important proteins involved in invasion such as

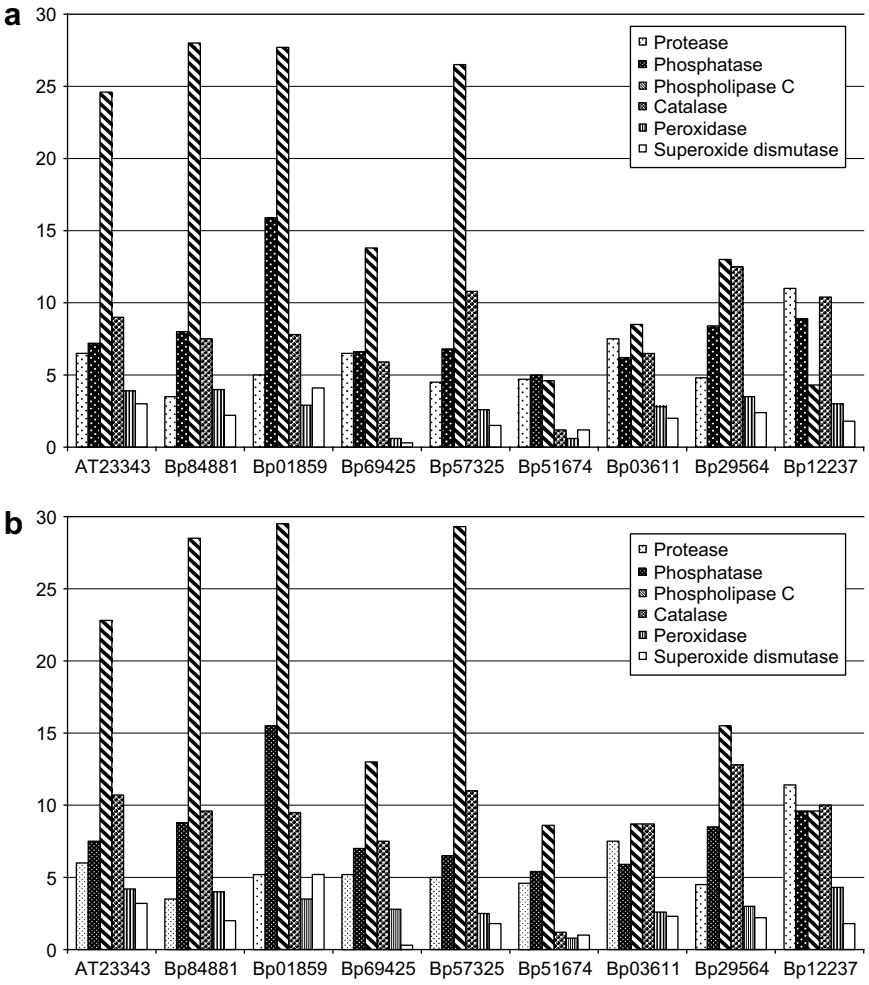

Fig. 2. Enzyme activity of clinical isolates of *B. pseudomallei* before (a) and after (b) a single passage in mice. Assays for enzymes were performed in triplicate and the mean value was used. Unit enzyme activity was calculated for each enzyme as described in Materials and methods.

collagen and elastin [30] and modulate immune response by digesting the cell surface markers, receptors, complements and immunoglobulins [31]. Acid phosphatases are a ubiquitous class of enzymes that catalyze the hydrolysis of phosphomonoesters at an acidic pH. In addition to mobilization of phosphate, some members of this class of enzymes perform many essential biological functions including regulation of metabolism, energy conversion, and signal transduction [32]. Phospholipase C is known to cleave the phosphodiester bond of phospholipids to yield diglycerol and water-soluble phosphate ester [33]. This can facilitate host cell lysis because phospholipids such as phosphatidylcholine are primarily found in eukaryotic cell membranes and lung surfactans [34]. This enzyme was also found to play a role in escape of the pathogen from the phagosome membrane and invasion of adjacent cells [35]. Catalase, peroxidase and superoxide dismutase are known to be putative candidates that resist toxic oxygen intermediates such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) produced by host phagocytes [18]. Catalase negative mutants of *Mycobacterium tuberculosis* have also been found to exhibit lower virulence in guinea pigs [36].

In this study, all the six enzymes assayed *in vitro* were found to be present in the culture supernatant of all the isolates. No correlation was observed between the amount of these enzymes in the culture supernatant and the source of isolate, that is clinical or environmental. Phospholipase C, catalase, and phosphatase activities contributed to the major enzymes present in the culture filtrate of the isolates whereas protease, superoxide dismutase and peroxidase activities were detected at lower concentrations among the different isolates. All enzymatic activities were detected in the culture medium of the isolates after the first four hours of growth indicating that the enzymes were secreted while the cells are actively growing in the exponential phase. Therefore, the enzymes detected in the culture supernatant were those that were released from intact viable cells and not as a result of release due to cell death and lysis.

Catalase and superoxide dismutase were found to be released at lower levels during the log phase but increased during the early stationary phase. This might be important *in vivo* as these enzymes have been reported to provide a higher protection to *Burkholderia cepacia* to establish a chronic infection where the bacteria will be facing a physiological situation similar to the stationary phase of growth. Other conditions of stress such as tissue inflammation may also stimulate a bacterial response similar to that of stationary phase and induce activity of these enzymes [37].

B. pseudomallei isolates used in this study were also subjected to a single passage in mice in order to evaluate the modification in the production of the six enzymes. Increased activity of all the enzymes was observed in 46–73% of the isolates. Nevertheless, this data has to be treated with caution because we do not know if the same condition is observed in the *in vivo* situation.

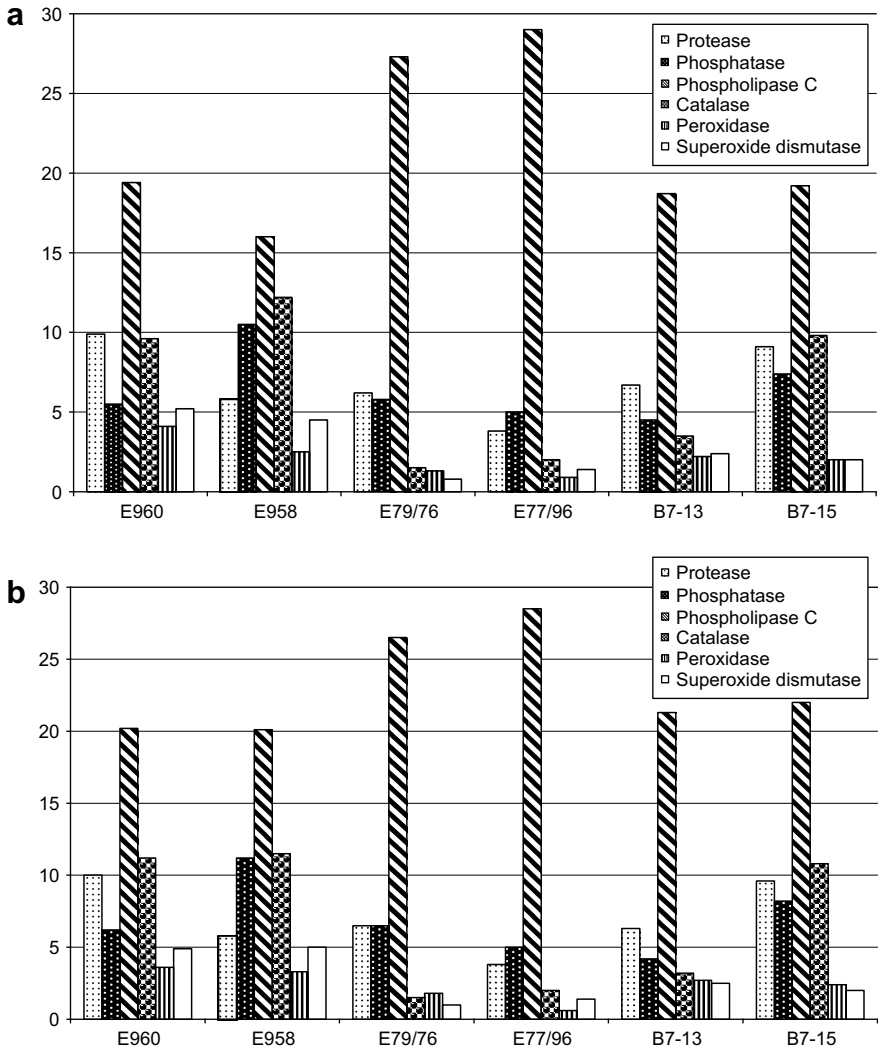

Fig. 3. Enzyme activity of environmental isolates of *B. pseudomallei* before (a) and after (b) a single passage in mice. Assays for enzymes were performed in triplicate and the mean value was used. Unit enzyme activity was calculated for each enzyme as described in Materials and methods.

Infection studies in ICR mice also demonstrated varying degree of virulence induced by the clinical as well as environmental isolates. These findings are consistent with those of Ulett et al. [38], who demonstrated that there was no association between virulence and isolate origin (i.e. clinical vs environmental). Amongst the isolates that induced early mortality, 100% showed an increase in catalase activity and 80% showed an increase in phosphatase activity. Once inside the mice, the bacteria may be exposed to a variety of host killing mechanisms, including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) generated by the respiratory burst as the bacterium remains bound within an endosome [39]. Hydrogen peroxide has bacteriocidal activity. In addition, interaction of H_2O_2 with myeloperoxidase, reduced iron, or products of nitric oxide synthase may lead to formation of more toxic intermediates [40]. It has been postulated that bacterial factors that inactivate H_2O_2, such as catalase, may interrupt production of these toxic species and aid persistence and survival within host cells and tissues [41]. Acid phosphatases have been also predicted to play a role in virulence, most often in intracellular pathogens, by the inhibition of respiratory burst [42,32]. Therefore, these enzymes could be important in persistance of a pathogen. This could also be one of the mechanisms used by *B. pseudomallei* to interrupt or modify pathways involved in disease process. Other workers have also reported the correlation between virulence and increased activity of catalase in *Neisseria meningitides* [43], *Legionella pneumophila* [44], *M. tuberculosis* [45] and phosphatases in *M. tuberculosis* [46], *Yersinia pseudotuberculosis* [47] and *Salmonella typhimurium* [48].

Phospholipase C activity was found to be produced in the highest amount in 87% of the isolates before and post-passage in mice. Furthermore, 75% of the isolates that induced late mortality also showed an increase in the activity of this enzyme. External abscesses were visible in these mice. Phospholipase C is known to contribute to cytotoxicity [49]. Disruption of the host cell membrane can take place and facilitate entry of the bacteria into the bloodstream resulting in systemic spread. Macrophage lysis might also increase inflammation and the release of enzymes contributing to local tissue destruction. Previous studies have also implicated phospholipase C as virulence factor involved in infection of pathogenic bacteria including *B. pseudomallei* [50], *Listeria Monocytogenes* [35], *M. tuberculosis* [51] and *Pseudomonas aeruginosa* [52].

The six enzymes assayed may not necessarily be the only enzymes responsible for bacterial virulence. Presence or absence of other extracellular enzymes such as alanine dehydrogenase, glutamine synthetase, nicotinamidase, alcohol dehydrogenase have also

Table 1
Pathology induced by different isolates in ICR mice.

Isolate (Source)	Early mortality[a]	Intermediate mortality[a]	Late mortality[a]	Bacteraemia (%)[b]	Splenic abscess (%)[b]	External abscess (%)[b]
ATCC						
AT23343 (Blood)	3.25			100	25	0
Clinical isolates						
Bp84881 (Blood)	1.75			100	0	0
Bp01859 (Blood)	2.00			100	0	0
Bp69425 (Blood)	2.50			50	100	0
Bp57325 (Blood)		8.50		100	75	0
Bp51674 (Blood)			23.00	75	75	50
Bp03611 (Blood)			15.00	100	100	25
Bp29564 (Pus)			19.25	100	100	75
Bp12237 (Pus)			20.50	50	100	50
Environmental isolates						
E960 (Soil)	2.29			100	0	0
E958 (Soil)		10.00		100	100	0
E79/76 (Soil)			19.25	75	100	75
E77/96 (Soil)			24.00	50	75	25
B7-13 (Soil)			28.25	50	50	25
B7-15 (Soil)			15.50	100	100	0

[a] Mean number of day survival. The mice were monitored for 30 days and the surviving animals were sacrificed on day 30.
[b] Percentage was calculated from the total number of animals demonstrated bacteraemia/abscess.

been reported to be correlated with the virulence [53]. Further studies using microarray and proteomics approach to map the extracellular proteome of *B. pseudomallei* in order to understand other extracellular proteins that might be involved in pathogenesis and virulence could be carried out to complement the present study.

4. Materials and methods

4.1. Bacterial isolates and culture conditions

Nine clinical and six environmental isolates of *B. pseudomallei* were used in this study. The clinical isolates were from haemoculture or pus of patients with either septicemic or localised melioidosis from the University of Malaya Medical Center and the environmental isolates were kindly provided by Dr. E.H. Yap, Defense, Medical & Environmental Research Institute, DSO National Laboratories, Republic of Singapore. These isolates were confirmed biochemically using the API 20NE system (Biomerieux, France) and characterised as arabinose-negative (ara−) biotypes. In addition all 15 isolates were also examined for siderophore production in a previous study using the Chrome azurol S (CAS) method described by Schwyn & Neilands [19] and were found to be positive for siderophore production.

4.2. Viable count

A single colony of bacterial culture on nutrient agar was inoculated into 500 ml LB broth in a 2-l conical flask and incubated at 37 °C for 24 h with constant agitation at 180 rpm. Samples were taken out at 4, 8, 12, 16, 20 and 24 h and bacterial density was read at 660 nm. Viable count was also determined by plating serial dilutions of culture onto nutrient agar plates.

4.3. Secretory enzyme assays

A single colony of each culture on nutrient agar was inoculated into 500 ml LB broth and incubated at 37 °C for 24 h with constant agitation at 180 rpm after which the bacterial density was read at 660 nm. The culture was then centrifuged at 20,000 *g* and the supernatant collected and concentrated 50-fold by ultrafiltration employing 10 kDa ultra-free centrifugal filters (Millipore, USA). The final volume was adjusted according to the bacterial count (OD at 660 nm) of the culture. In order to investigate the level of extracellular enzymes produced over time the isolates were also grown in 50 ml LB broth for 4, 8, 12, 16, 20 and 24 h and tested. All enzymatic activities were expressed as unit activity per milliliter of culture filtrate supernatant.

4.3.1. Protease assay

The protease assay in aqueous solution was performed as described by [20] with minor modifications. Briefly, varying concentrations of extracellular secretory products were incubated with 0.5 ml of 0.05 M phosphate buffer (pH, 7.5) containing 5 mg ml^{-1} azocoll, overnight at 37 °C. The microcentrifuge tubes were then spun at 200 *g* for 5 min, and the absorbance of the supernatant measured at 540 nm. One unit of activity was calculated as the amount of protease needed to increase the absorbance per hour from 0.05 to 0.1.

4.3.2. Acid phosphatase assay

Detection of acid phosphatase activity was assayed by measuring the release of p-nitrophenol (p-NP) from p-nitrophenyl phosphate (p-NPP) at a wavelength of 405 nm [21]. One ml of 100 mM sodium acetate buffer (pH 5.0) and 0.1 ml of 250 mM p-NPP were added to 0.1 ml of the extracellular secretory products to initiate the reaction. The reaction was terminated by the addition of 2 ml of 0.4 N NaOH. One unit of phosphatase activity was defined as the amount needed to release 1 μmol of p-nitrophenol per min.

4.3.3. Catalase assay

Catalase activity was determined by the decrease in the A_{240} of H_2O_2 [22]. The catalase activity of the extracellular secretory products was assayed by adding 0.05–0.7 ml of freshly prepared 13.2 mM H_2O_2 in 0.05 M potassium phosphate buffer (pH 7.0). The solution was mixed, and a loss of absorbance was determined at 230 nm for 1–3 min. One unit of catalase activity was defined as 1 μmol of decomposed H_2O_2 per min.

4.3.4. Peroxidase assay

Peroxidase activity was determined by using o-dianisidine [23]. Briefly, 0.05 ml of the extracellular secretory product was added to 0.75 ml of 0.01 M phosphate buffer (pH 6.0) containing o-dianisidine (10 mg ml^{-1}), then 0.05 ml of 0.3% freshly prepared H_2O_2 in distilled water was added to the reaction mixture and the change

of absorbance recorded at 460 nm for 3–5 min. One unit of activity was defined as 1 μmol of decomposed H_2O_2 per min.

4.3.5. Superoxide dismutase assay (SOD)

The SOD activity was assayed by monitoring the inhibition of pyrogallol auto-oxidation at pH 8.0 [24]. The change in optical density during the SOD mediated inhibition of oxygen free radicals was measured at 420 nm. One unit of activity was defined as the amount of SOD needed to reduce the absorbance per minute from 0.02 to 0.01.

4.3.6. Phospholipase C assay

Phospholipase C activity was assayed using p-nitrophenyl phosphorylcholine (p-NPPC) as the substrate [25]. In brief, a 20 mM solution of p-NPPC was prepared in 0.25 M Tris–HCl buffer pH 7.0 containing 60% glycerol (v/v) and 1 mM $ZnCl_2$. The reaction was started by the addition of 50 μl of the extracellular secretory products into a total of 1 ml reaction mixture and incubated at 37 °C after which the absorbance was read periodically at 405 nm. One unit of enzyme activity was calculated as the amount required for the release of 1 μmol p-nitrophenol per min.

4.4. Animal passage studies

Two-month-old female ICR mice were inoculated subcutaneously with 1×10^3 CFU (in 0.01 M PBS, pH 7.2) of each isolate of *B. pseudomallei*. Four days later, the mice were sacrificed and the spleens macerated using a syringe piston after which 10 μl of the suspension were transferred to 5 ml LB broth. Following incubation at 37 °C for 2 h, with agitation at 180 rpm, 10 μl was added to freshly prepared 500 ml LB broth, incubated at 37 °C for 24 h and the extracellular products were processed as described above.

4.5. Infection studies

Two-month-old female ICR mice were inoculated subcutaneously with 1×10^6 organisms (in 0.01 M PBS, pH 7.2), in groups of four mice per isolate, and the mortality was monitored for up to 30 days post-inoculation. On day 30 or on mortality, the mice were dissected and examined for the presence of splenic abscesses as confirmation of infection. Further bacteriological confirmation was based on culture and identification for the bacteria isolated from the spleen. Mortality rates were classified as early mortality (<4 days), intermediate mortality (4–14 days) and late mortality (15 days or more).

Acknowledgements

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