Tropical Biomedicine 29(1): 160–168 (2012)

Enzymatic profiling of clinical and environmental isolates of *Burkholderia pseudomallei*

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Received 9 June 2011, received in revised form 29 November 2011; accepted 1 December 2011

Abstract. Melioidosis has been recognized as an important cause of sepsis in the tropics. The disease caused by an environmental saprophyte *Burkholderia pseudomallei*, affects mostly adults with underlying immunocompromised conditions. In this study, the enzymatic profiles of 91 clinical and 9 environmental isolates of *B. pseudomallei* were evaluated using the APIZYM system, in addition to assessment of protease, phospholipase C and sialidase activities using agar plate methods and other assays. The activity of 10 enzymes - alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase were detected in >75% of the clinical isolates. The majority of *B. pseudomallei* isolates in this study exhibited protease and phospholipase activities. No sialidase activity was detected. Five *Burkholderia thailandensis* isolates had similar APIZYM profiles as *B. pseudomallei* clinical isolates except for the lower detection rate for N-acetyl-β-glucosaminidase. The subtle differences in the number of enzymes secreted and the levels of enzymatic activities of phenotypically identical clinical and environmental strains of *B. pseudomallei* give weight to the fact that the causative agent of melioidodis originates from the environment.

INTRODUCTION

Melioidosis which is caused by an environmental saprophyte, *Burkholderia pseudomallei*, affects mostly adults with underlying immunocompromised conditions. The intracellular persistence of the organism in the host may lead to latency, relapse and recurrence in melioidosis (Cheng & Currie, 2005). Another *Burkholderia* species, *Burkholderia thailandensis* is genetically and immunologically related to *B. pseudomallei*, but relatively avirulent and non-pathogenic or weakly pathogenic in humans (Wiersinga *et al*., 2008). Analytical Profile Index (API) 20NE system (Dance *et al.*, 1989) and API 50CH system (Wuthiekanun *et al*., 1996) have been used in differentiating *B. pseudomallei* and *B. thailandensis*, in which both isolates show similar biochemical profiles except for the significant difference in the ability to assimilate L-arabinose by *B. thailandensis*.

APIZYM (bioMérieux, Marcy l'Etoile, France) is a semi-quantitative micromethod for analysing enzymatic activities of microorganisms, which has been used to characterize many microorganisms (Tharagonnet *et al*., 1977; Poh & Loh, 1988; Chan & Tay, 2008). In a study by Leone and co-workers (1998), the APIZYM system revealed significant difference in enzyme production in *Cryptococcus neoformans* strains isolated from immunocompromised patients and the environment. However, there are no published reports on the enzymatic characterization of *B. pseudomallei* and *B. thailandensis* using the APIZYM system.

Enzymes are believed to play important roles in the pathogenesis of bacterial diseases. The production of proteases (Sexton *et al.*, 1994), acid phosphatase and phospholipase C (Berka *et al.*, 1981; Ostanin *et al.*, 1992; Korbsrisate *et al.*, 2007) have been reported to be involved in the virulence and pathogenesis of melioidosis. A previous study on the production of protease, phosphatase, phospholipase C, superoxide dismutase, catalase and peroxidase exhibited no difference in enzyme production by clinical and environmental isolates of *B. pseudomallei* (Vellasamy *et al.*, 2009). However, very little is known about the other enzymes present in *B. pseudomallei* of clinical and environmental origin.

The objectives of this study were to screen clinical and environmental isolates of *B. pseudomallei* for the production and quantitation of 19 enzymes using the APIZYM system, in addition to assessment of protease, phospholipase C and sialidase activities using agar plate methods and other assays and to elucidate if there is/are any differences in enzyme production between organisms of different origins. This work would give us a comprehensive picture of the presence or absence of a number of secreted enzymes of *B. pseudomallei* and an insight into its possible virulence mechanisms.

MATERIALS AND METHODS

Bacterial strains

Ninety-one randomly selected clinical isolates of *B. pseudomallei* from melioidosis patients seen at various hospitals in Malaysia, and 9 environmental isolates - 4 from Singapore and 5 from Thailand, and 5 isolates of *B. thailandensis* were included. The organisms were identified, confirmed by routine laboratory methods and stored in 20% glycerol stock solution at – 80ºC. Working cultures were maintained in Luria-Bertani (LB) agar.

Analytical Profile Index (API) ZYM analysis

B. pseudomallei isolates cultured on LB medium were inoculated into 2 ml of API suspension medium and adjusted to McFarland 5. The enzymatic analysis was performed in accordance to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). Briefly, 65 microliters of bacterial suspension were dispensed into each cupule and incubated at 37ºC for 4 h. A drop of Zym-A and Zym-B reagents were added and the strips were placed under a powerful light source for 5 min so that the negative reactions became colourless. Values ranging from 0 (negative reaction) to 5 (maximum reaction) were assigned to each test reaction corresponding to the colour intensity, using the scale given by the manufacturer. A reading of one to 5 was equivalent to 5, 10, 20, 30 and 40 nmols respectively of the APIZYM substrate being metabolized by the bacterial cultures (Leone *et al.*, 1998). Reactions giving a reading of $\geq$3 (20 nmols) were considered strongly positive; one and 2 were considered weakly positive; and 0 was considered as negative/no activity. Minimal medium (Cowan, 1974) composed of 10% sterile glucose solution and trace elements, was used to culture 23 clinical and 9 environmental isolates of *B. pseudomallei* and 5 *B. thailandensis* isolates to determine whether enzyme production was affected when isolates were grown on minimal medium.

Protease activity

Skim milk agar (3%) prepared by dissolving 30 g of skim milk powder (Difco) in 500 ml of distilled water was autoclaved at 115ºC for 10 min and cooled to 50ºC before mixing with an equal volume of double strength nutrient agar (Cowan, 1974). Two microliters of a 24-h bacterial suspension in Brain Heart Infusion (BHI) broth (McFarland 3) were inoculated into a small well (2 mm diameter) in the agar plate and incubated at 37ºC for

2 d. Protease activity was determined by the ratio of the diameter of the clear zone to the colony diameter, as described by Dogan & Boor (2003). *B. pseudomallei* ATCC 23343 was used as positive control while an uninoculated well in the agar plate represented the negative control.

Phospholipase C (PLC) activity

Egg yolk agar (2%) was prepared by adding 20 ml of egg yolk emulsion to one liter of molten nutrient agar containing 1% sodium chloride (Bridson, 1998). Two microliters of a 24-h bacterial suspension in BHI (McFarland 3) were inoculated into a small well in the agar plate and incubated at 37°C for 7 d. Phospholipase C activity was determined by the ratio of the diameter of opaque zone to the colony diameter, as described by Dogan & Boor (2003). *Staphylococcus aureus* ATCC 25423 and *Escherichia coli* ATCC 25922 strains were used as positive and negative controls, respectively.

Sialidase activity

Sialidase activity of *B. pseudomallei* was determined by a filter paper spot test (Braham & Moncla, 1992) and a microplate assay method (Beighton & Whiley, 1990). For the spot test, a stock solution of the substrate was prepared by dissolving 2'-4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (Sigma) in distilled water at a concentration of 100 µg/ml and stored in 180 µl volumes at – 20°C. Working solutions were prepared by adding 20 µl of 1.0 M sodium acetate buffer (pH 4.6) to the thawed substrate. A Whatman no.2 filter paper strip (0.5 cm x 6 cm) was immersed into the substrate solution. One to 2 colonies of a 24-h bacterial culture were spotted onto the strip, incubated at 37°C for 30 min and examined using a hand-held UV lamp (long wavelength, 365 nm). A fluorescent blue spot observed on the filter paper was considered as a positive reaction. For the microplate assay, 20 µl of the substrate solution was mixed with 50 µl of bacterial suspension (McFarland 3) in a flat-bottomed microplate well, incubated at 37°C for 30 min and observed under a UV transilluminator. Cell-free supernatants of 10 isolates of *B. pseudomallei* were also tested using the microplate assay. *Streptococcus pneumoniae* (a clinical isolate) and *Candida albicans* (a clinical isolate) were used as positive and negative controls, respectively.

RESULTS

Results of the APIZYM tests (Table 1) revealed the activity of 10 enzymes - alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase were detected in >75% of the clinical isolates. Of these enzymes, high activities of alkaline phosphatase, esterase lipase, lipase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase were detected (Table 2). Cystine arylamidase was detected in 75.8% of the isolates. Trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase were not detected.

All the environmental isolates of *B. pseudomallei* had similar profiles as the clinical isolates, N-acetyl-β-glucosaminidase was detected in only 6 (66.7 %) of the 9 enviornmental isolates. Five *B. thailandensis* isolates had similar profiles as *B. pseudomallei* clinical isolates except for the low detection rate for N-acetyl-β-glucosaminidase (only one was weakly positive) (Table 1). In general, only small difference was noted in the amounts of substrates metabolized by clinical and environmental isolates of *B. pseudomallei* and *B. thailandensis* (Tables 1 and 2).

The use of either rich LB medium or minimal medium did not make any difference to the APIZYM results except for N-acetyl-β-glucosaminidase. Higher levels of N-acetyl-β-glucosaminidase activity were detected in the minimal medium in comparison to the LB medium, for 13 of 23 clinical isolates, 4 of 9 environmental isolates and one of 5 *B. thailandensis* isolates tested in this study (data not shown).

Table 1. Enzyme production of *B. pseudomallei* and *B. thailandensis*

Enzymes	*B. pseudomallei*								*B. thailandensis* (*n*=5)			
	Clinical (*n*=91)				Environmental (*n*=9)							
	negative	weakly positive	strongly positive	% positive isolates	negative	weakly positive	strongly positive	% positive isolates	negative	weakly positive	strongly positive	% positive isolates/ average substrate metabolised
Alkaline phosphatase	0	0	91	100	0	0	9	100	0	0	5	100/36
Esterase	0	55	36	100	0	5	4	100	0	4	1	100/12
Esterase lipase	0	12	79	100	0	1	8	100	0	0	5	100/34
Lipase	1	34	56	98.9	0	3	6	100	0	0	5	100/36
Leucine arylamidase	0	0	91	100	0	0	9	100	0	0	5	100/40
Valine arylamidase	2	89	0	97.8	0	9	0	100	0	5	0	100/9
Cystine arylamidase	22	66	3	75.8	0	9	0	100	0	5	0	100/7
Acid phosphatase	0	0	91	100	0	0	9	100	0	0	5	100/40
Naphthol-AS-BI-phosphohydrolase	0	3	88	100	0	0	9	100	0	0	5	100/26
N-acetyl-β-glucosaminidase	4	65	22	95.6	3	6	0	66.7	4	1	0	20/2

Table 2. Average amounts of APIZYM substrates metabolized by clinical and environmental isolates of *B. pseudomallei* and *B. thailandensis*

	B. pseudomallei isolates			
	clinical (*n*=91)		environmental (*n*=9)	
Enzymes	nmol	s.d.	nmol	s.d.
Alkaline phosphatase	32	8	31	8
Esterase	14	6	14	5
Esterase lipase	22	7	27	9
Lipase	21	11	28	14
Leucine arylamidase	37	6	38	7
Valine arylamidase	5	2	8	3
Cystine arylamidase	5	4	8	3
Trypsin	0	0	0	0
α-chymotrypsin	0	0	0	0
Acid phosphatase	40	1	40	0
Naphthol-AS-BI-phosphohydrolase	30	9	27	9
α-galactosidase	0	0	0	0
β-galactosidase	0	0	0	0
β-glucuronidase	0	0	0	0
α-glucosidase	0	0	0	0
β-glucosidase	0	0	0	0
N-acetyl-β-glucosaminidase	10	7	5	4
α-mannosidase	0	0	0	0
α-fucosidase	0	0	0	0

s.d. = standard deviation

All *B. pseudomallei* isolates produced protease. Higher protease activity were demonstrated in the environmental isolates, as indicated by the higher ratio of the diameter of the clearing zone to the colony diameter on skim milk agar (average ratio = 3.301 ± 0.503), as compared to those of the clinical isolates (average ratio = 2.764 ± 0.592). Phospholipase C was detected in 89 (97.8%) *B. pseudomallei* clinical isolates with average ratio of the diameter of the opaque zone to the colony diameter on egg yolk agar being 2.518 ± 1.043, as compared to those of the environmental isolates (average ratio = 2.023 ± 0.763). No sialidase activity was detected from the bacterial suspensions or cell free supernatants of the isolates (data not shown).

DISCUSSION

Enzymatic profiling using the APIZYM system is rapid, simple, specific, sensitive and has been useful in the identification of many fermentative and non-fermentative bacteria. The APIZYM results obtained in this study were consistent, reproducible and able to determine the level of enzyme production semi-quantitatively. We observed that the APIZYM profiles of *B. thailandensis* isolates were almost similar to those of *B. pseudomallei*, except for the low N-acetyl-β-glucosaminidase detection rate in *B. thailandendis* (weakly positive in one of five isolates). Hence it is not possible to differentiate the two *Burkholderia* species based on enzymatic profiling. Our findings

showed that a wide range of enzymes were produced by *B. pseudomallei*. However, it is not clear to what extent these enzymes are involved in biological events *in vivo*. Elevated productions of alkaline phosphatase, esterase lipase, lipase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase by *B. pseudomallei* and *B. thailandensis* are an indication that these may be housekeeping enzymes which are required for the survival of the bacteria.

Alkaline phosphatase which cleaves orthophosphoric monoesters to orthophosphates and alcohols in alkaline conditions, has essential biological functions such as regulation of metabolism, energy conversion and signal transduction in microorganisms (Reilly *et al.*, 1996). This enzyme is normally cell wall-associated but in some cases, it can be detected in the periplasmic space (Kushnarev & Smirnova, 1966; Cheng *et al.*, 1970; Lindsay *et al.*, 1973) and cell surface (Nisonson *et al.*, 1969). Acid phosphatase has been predicted to play a role in virulence, most often in intracellular pathogens by suppressing the neutrophil respiratory burst and the production of superoxide anion, thus enhancing microbial survival and persistence in the host (Baca *et al.*, 1993).

Secreted esterase of Group A *Streptococcus* has been implicated in subcutaneous infections and dissemination in mice (Zhu *et al.*, 2009). Esterase cleaves short-chain fatty acids at ester linkages (Chahinian *et al.*, 2002). Our isolates exhibited high activities of esterase lipase and lipase compared to esterase. Lipase catalyzes hydrolysis and the synthesis of triglycerides and other water insoluble esters to glycerol and fatty acids (Schmid & Verger, 1998).

Arylamidase, found in gram negative organisms catalyzes the hydrolysis of N-terminal amino acid from peptides, amides or arylamides. Pathogenic strains of *Leptospira* demonstrated 10 to 20 times higher arylamidase activity than saprophytic strains (Burton *et al.*, 1970). *Burkholderia pseudomallei* isolates exhibited stronger leucine arylamidase activity compared to valine and cystine arylamidases.

N-acetyl-β-glucosaminidase hydrolyses chitobiose (product of chitin degradation) to produce N-acetylglucosamine as a nutrient for some marine chitinolytic bacteria (Lin *et al.*, 2006). *Burkholderia pseudomallei* has been found to invade spores of arbuscular mycorrhizal fungi (Levy *et al.*, 2003). We postulate that the production of N-acetyl-β-glucosaminidase as detected in our study may assist bacterial entry into fungal hyphae by degradation of the chitinous cell wall.

The ability of *B. pseudomallei* to produce protease and phospholipase C was demonstrated for almost all isolates in this study. A zinc metalloprotease (Sexton *et al.*, 1994), serine metalloprotease (Lee & Liu, 2000) and a calcium-dependent serine protease (Ling *et al.*, 2001) have been described in *B. pseudomallei*. These proteases have been found to digest biologically important proteins involved in invasion such as collagen and elastin (Rechnitzer *et al.*, 1992) and modulate the immune response by digesting the cell surface markers, receptors, complements and immunoglobulins (Mintz *et al.*, 1993). These proteases are capable of causing soft tissue necrosis and promoting localized lesions during infection which is in keeping with the non-septicaemic presentation of melioidosis (Puthucheary, 2009). Although the environmental isolates appear to produce high levels of protease in this study, these results need to be validated using more isolates.

Two phospholipases C have been characterized in *B. pseudomallei* (Korbsrisate *et al.*, 2007). These enzymes are believed to cleave the phosphodiester bond of phospholipids such as phosphatidylcholine, phosphatidylinositol or sphingomyelin, contributing to eukaryotic signaling events, inflammatory processes as well as eukaryotic cell apoptosis (Titball, 1998). They also assist in the escape of pathogens from phagosomes into adjacent cells (Smith *et al.*, 1995). The majority of our *B. pseudomallei* isolates exhibited phospholipase activity and we believe this enzyme plays a major role in the pathogenesis of melioidosis.

The gene encoding the enzyme sialidase (neuraminidase) has been annotated in

the *B. pseudomallei* genome. Sialidase hydrolyzes the terminal sialic acid residues found on glycolipids, oligosaccharides and glycoproteins of cell surfaces in animals and some microorganisms (Corfield & Schauer, 1982). However, sialidase activity was not detected from our isolates. It is probable that our assays were not sensitive enough for its detection.

In a previous study on the production of 6 enzymes including protease, phosphatase, phospholipase C, superoxide dismutase, catalase and peroxidase, no difference was found among 15 isolates irrespective of the source of *B. pseudomallei* (Vellasamy *et al.*, 2009). The high similarity found in the enzymatic profiles of clinical and environmental isolates of *B. pseudomallei* in our study gives credibility to the established belief that the environment is the source of the organisms responsible for infections in humans and animals. Significant differences in enzymatic profiles of *B. thailandensis* were not seen as only a few isolates were available. There are no published reports on the enzymatic characterization of *B. pseudomallei* using the APIZYM system, and we found small difference in the types and levels of enzymatic activities in phenotypically identical strains (Table 1). These enzymes may be responsible for the many forms of clinical manifestations in melioidosis, depending on which genes are being upregulated. However, little is known on how enzyme production is regulated in *B. pseudomallei*. For instance, some *B. pseudomallei* isolates may be triggered to produce N-acetyl-β-glucosaminidase in conditions that lack nutrients (as observed in minimal media in this study), which is believed to be the bacterial strategy to survive harsh environmental conditions. Collectively, the findings of this study provide information about the enzymatic profiles of clinical and environmental isolates of *B. pseudomallei* as well as *B. thailandensis*.

Acknowledgements. This research was supported by the grants from the Short Term Research Fund (Vote-F) (FS198/2008B) and the Postgraduate Research Fund (PS164/2009B) from the University of Malaya. We wish to thank Dr. E.H. Yap, Defense, Medical and Environmental Research Institute, DSO National Laboratories, Republic of Singapore for providing environmental isolates of *B. pseudomallei*, and Mr. Chong Miow Lim @ Xavier for technical assistance.

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