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Drug susceptibility and biofilm formation of *Burkholderia pseudomallei* in nutrient-limited condition

Anutrakunchai, C.[1,2,3], Sermswan, R.W.[1,3], Wongratanacheewin, S.[1,4], Puknun, A.[1,2,5] and Taweechaisupapong, S.[1,2*]
[1]Melioidosis Research Center, Khon Kaen University, Thailand
[2]Biofilm Research Group, Faculty of Dentistry, Khon Kaen University, Khon Kaen, Thailand
[3]Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Thailand
[4]Department of Microbiology, Faculty of Medicine, Khon Kaen University, Thailand
[5]School of Medical Science, University of Phayao, Thailand
[*]Corresponding author email: suvi_taw@kku.ac.th
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Abstract. *Burkholderia pseudomallei* is the causative agent of melioidosis, which can form biofilms and microcolonies *in vivo* and *in vitro*. One of the hallmark characteristics of the biofilm-forming bacteria is that they can be up to 1,000 times more resistant to antibiotics than their free-living counterpart. Bacteria also become highly tolerant to antibiotics when nutrients are limited. One of the most important causes of starvation induced tolerance *in vivo* is biofilm growth. However, the effect of nutritional stress on biofilm formation and drug tolerance of *B. pseudomallei* has never been reported. Therefore, this study aims to determine the effect of nutrient-limited and enriched conditions on drug susceptibility of *B. pseudomallei* in both planktonic and biofilm forms *in vitro* using broth microdilution method and Calgary biofilm device, respectively. The biofilm formation of *B. pseudomallei* in nutrient-limited and enriched conditions was also evaluated by a modified microtiter-plate test. Six isolates of ceftazidime (CAZ)-susceptible and four isolates of CAZ-resistant *B. pseudomallei* were used. The results showed that the minimum bactericidal concentrations of CAZ against *B. pseudomallei* in nutrient-limited condition were higher than those in enriched condition. The drug susceptibilities of *B. pseudomallei* biofilm in both enriched and nutrient-limited conditions were more tolerant than those of planktonic cells. Moreover, the quantification of biofilm formation by *B. pseudomallei* in nutrient-limited condition was significantly higher than that in enriched condition. These data indicate that nutrient-limited condition could induce biofilm formation and drug tolerance of *B. pseudomallei*.

INTRODUCTION

Burkholderia pseudomallei is recognized as an emerging pathogen, responsible for melioidosis. Infections have been increasingly reported in many countries in tropical and subtropical regions of the world (Currie *et al.*, 2008; Rolim *et al.*, 2009; Mukhopadhyay *et al.*, 2011). *Burkholderia pseudomallei* is an intrinsically resistant to many of the currently marketed antibiotics, including third-generation cephalosporins, penicillins, rifamycins, aminoglycosides (White, 2003; Cheng & Currie, 2005), quinolones and macrolides which limit therapeutic options for the treatment of melioidosis (Thibault *et al.*, 2004). Ceftazidime (CAZ) is the current drug of choice of severe melioidosis, but the mortality rate in treated patients is greater than 40% (White, 2003). In addition, the emergence of *B. pseudomallei* strains that are resistant to CAZ and trimethoprim have been reported (Dance *et al.*, 1989; Wuthiekanun *et al.*, 2005). Several antibiotic resistance mechanisms have been documented in *B. pseudomallei*. These mechanisms range from exclusion from the cell due to permeability issue (Siritapetawee *et al.*, 2004; Novem *et al.*, 2009), efflux from

the cell (Mima *et al.*, 2011; Schweizer, 2012), enzymatic inactivation (Rholl *et al.*, 2011; Sarovich *et al.*, 2012) and altered target sites (Chantratita *et al.*, 2011). However, several other factors have been demonstrated to affect the antimicrobial susceptibility of *B. pseudomallei*. These include: the biofilm mode of growth known to substantially lower antimicrobial susceptibility (Vorachit *et al.*, 1993; Sawasdidoln *et al.*, 2010; Pibalpakdee *et al.*, 2012); growth under stress conditions that may induce antimicrobial resistance mechanisms (Pumirat *et al.*, 2009); and the chronic or latent infection state in which bacteria presumably reside intracellularly in a nonreplicating altered metabolic state where they are less susceptible to conventional antibiotics (Hamad *et al.*, 2011). Some of these mechanisms may not be regarded as true resistance, but rather tolerance mechanisms.

Recently starvation induces stringent response has been shown to mediate antibiotic tolerance in nutrient-limited and biofilm cells of *Pseudomonas aeruginosa* (Nguyen *et al.*, 2011). Although growth of *B. pseudomallei* under stress condition has been reported to induce a beta-lactamase-like protein that was accompanied by increased CAZ resistance (Pumirat *et al.*, 2009), the antibiotic tolerance of *B. pseudomallei* in nutrient-limited condition has not been reported yet. Moreover, no experimental evidence was presented to evaluate biofilm formation of *B. pseudomallei* in nutrient-limited condition. Therefore, the objective of the present study was to determine the effect of nutrient-limited and enriched conditions on drug susceptibility and biofilm formation of *B. pseudomallei*.

MATERIALS AND METHODS

Bacteria and growth condition

Ten isolates of *B. pseudomallei* (Table 1) and one isolate of *B. thailandensis* UE5 which was the environmental isolate from soil of northeastern endemic region of Thailand (Taweechaisupapong *et al.*, 2005), were used in this study. The bacteria were grown in enriched and nutrient-limited conditions. Luria-Bertani (LB; 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) was used as an enriched medium while a modified Vogel and Bonner's medium (Lam *et al.*, 1980) with 2 g/l D-glucose (0.2G MVBM; 0.2 g/l $MgSO_4.7H_2O$, 2 g/l citric acid (anhydrous), 3.5 g/l $NaNH_4HPO_4$, 10 g/l K_2HPO_4, 0.36 g/l $CaCl_2.2H_2O$, 2 g/l D-glucose [pH 7.2]) was used as a nutrient-limited medium.

Burkholderia pseudomallei and *B. thailandensis* UE5 were maintained on nutrient agar (NA) (Criterion™; Hardy Diagnostics) except for *B. pseudomallei* M10 mutants which was grown on LB agar (Criterion™; Hardy Diagnostics) containing 15 µg/ml tetracycline. A single colony of each bacteria initially grown on NA or LB agar containing 15 µg/ml tetracycline (for mutant) was inoculated into 2 ml of LB or 0.2G MVBM, incubated overnight at 37°C with shaking at 200 rpm and used as inoculum for all experiments.

Growth rate measurement

Burkholderia pseudomallei K96243 was selected as a representative isolate to determine the growth rates using a computerized spectrophotometric incubator (Varioskan Flash, Thermo Fisher Scientific, USA). The bacteria in LB and 0.2G MVBM from an overnight culture were adjusted to an optical density (OD) at 540 nm of 0.1. Two percent of inocula (v/v) from each medium was inoculated into fresh media. Then 200 µl of each bacterial suspension was added into 6 wells of a sterile 96-well round-bottomed plastic tissue culture plate. The wells contained only the medium served as a negative control. The microtiter plate was placed in a computerized spectrophotometric incubator and incubated at 37°C. Growth of bacterial cells was automatically monitored by the computerized instrument in terms of the change in the turbidity (absorbance at 540 nm), at 60-min intervals, for a period of 72 hours.

Antimicrobial susceptibility testing of planktonic *B. pseudomallei*

To study effect of nutrient-limited condition on drug tolerance of *B. pseudomallei*, the

broth microdilution method (National Committee for Clinical Laboratory Standards, 2002) was used. Ten milliliters of LB or 0.2G MVBM were inoculated with 2% inocula (v/v) from an overnight culture, incubated at 37ºC with shaking at 200 rpm and growth until stationary phase. Cultures of each bacterial strain were adjusted to ~1×10^6 CFU/ml in each medium, which was verified by the total viable count. The antibiotic used in this study was CAZ, drug of choice for melioidosis treatment. CAZ was 2-fold serially diluted in LB or 0.2G MVBM with the final volumes of 50 µl in each well of 96-well microtiter plates. Then the bacterial suspension (50 µl) was added in each well. The final concentrations of CAZ were ranging from 0.5-2048 µg/ml. Wells containing only media and only the bacteria without CAZ were included as negative controls. The plates were incubated at 37ºC for 24 hours. Then the bacterial growth was examined and the lowest concentration of CAZ which inhibit the visible growth of the bacteria were recorded as the minimum growth inhibitory concentration (MIC). Aliquots of the mixture of CAZ and the bacteria which showed negative-visible growth after the first 24 hours of incubation were inoculated onto the surface of NA. The lowest concentration of CAZ showed negative growth of the bacteria was recorded as the minimum bactericidal concentration (MBC). All experiments were repeated on three separate occasions, with duplicate determinations on each occasion.

Antimicrobial susceptibility testing of *B. pseudomallei* biofilm

To study effect of nutrient-limited condition on drug tolerance of *B. pseudomallei* biofilm, the Calgary biofilm device (CBD) were used as previously described (Sawasdidoln *et al.*, 2010) with slight modification. The CBD consists of 2 components where the top component forms a lid that has 96 pegs. The pegs are designed to sit in the channels of the bottom component of a standard 96-well plate. Each peg will form the equivalent biofilms. The bacterial biofilm were formed on each pegs in the culture prepared in fresh media with the initial cell concentration of 10^8 CFU/ml. A final volume (150 µl) of each bacterial culture was placed in each well of 96-well plate. Medium alone were served as the negative control. The plates were incubated on the rocking platform (Shaker SK-101, HL instruments, Thailand) at 37ºC at approximately 100 rpm for 24 hours.

Biofilms formed on the pegs of the CBD were transferred to a standard 96-well plate in which CAZ dilution in Muller-Hinton (MH) were prepared. Antibiotic-free wells were also included for growth control by adding only the media. Antimicrobial plates were incubated overnight at 37ºC for 24 hours. Then the lids were removed and the antimicrobial plates were checked for turbidity in the wells on the microplate reader at 620 nm to determine planktonic MIC (PMIC) values. Then aliquots of the mixture of CAZ and the bacteria in each well were inoculated onto the surface of NA. The lowest concentration of CAZ giving negative growth of the bacteria were recorded as the planktonic MBC (PMBC).

To determine minimum biofilm elimination concentration (MBEC), the pegs were then rinse in PBS and placed in a second 96-well plate containing MH. The biofilm were removed from the CBD pegs by sonication for 5 min. A new plate cover were added, and the viability of the biofilm was determined after 24 hours of incubation at 37ºC by reading the turbidity at 620 nm in a 96-well plate reader. The PMIC is defined as the minimum inhibitory concentration of CAZ against the planktonic bacteria shed from the biofilm during the challenge incubation while PMBC is defined as minimum bactericidal concentrations of CAZ against those planktonic bacteria. The MBEC is defined as the minimum concentration of antibiotic that inhibits regrowth of biofilm bacteria in the recovery media. Clear wells ($OD_{620} < 0.1$) were evidence of inhibition.

Biofilm formation quantification

The quantitative estimation of the biofilm produced was determined according to the method as described previously (Taweechaisupapong *et al.*, 2005). Briefly, a single bacterial colony was inoculated into 2 ml of LB and incubated overnight at 37ºC with shaking at 200 rpm. Then 2% inocula (v/v)

were inoculated into 10 ml of fresh-media and incubated overnight at 37°C with shaking at 200 rpm. After incubation, bacteria were adjusted to OD at 540 nm of 0.8-0.9 in fresh medium. The 200 µl of each bacterial suspension were added into 96-well flat-bottomed plate. Wells contained only the medium served as a negative control. The plates were incubated aerobically at 37°C for 3 hours to allow bacteria adhesion to the wells. Thereafter, the supernatant fluid of each well were aspirated gently to remove non-adherent bacteria, and replaced with 200 µl of fresh medium. After incubation at 37°C for an additional 21 hours, the non-adherent bacteria were again removed and the wells containing adherent bacteria were washed with 200 µl of sterilized deionized water and fresh medium were added once more. After incubation for an additional 24 hours, the supernatant were again removed and the wells were finally washed three more times with 200 µl of sterilized deionized water. The attached bacteria, representing a 2-day biofilm culture, were fixed with 200 µl of 99% methanol for 15 minutes and the plates were dried at room temperature. The plates were stained for 5 minutes with 200 µl of 2% Hucker crystal violet. Excess stains were removed with running tap water. The plates were air dried and the dye bound to the adherent cells was solubilized with 200 µl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 620 nm using a microplate reader. The ability of each isolate to produce biofilm was determined in 2 independent experiments and the results reported were the average value from these two independent experiments. To compare the relative capacity of different isolates in producing biofilm, their OD values were normalized against that the OD value produced by a strain of *B. thailandensis* UE5. *Burkholderia thailandensis* UE5 was added into every 96-well plates and used as a reference in all experiments (Taweechaisupapong *et al.*, 2005). Under the enriched condition, the OD_{620} value (mean±SD) of *B. thailandensis* UE5 was 0.451±0.238. Biofilm-forming capacity (corrected OD_{620}) values were calculated by a following formula :

Corrected OD_{620} = OD_{620} of tested *B. pseudomallei* x (0.451 / OD_{620} of *B. thailandensis* UE5 of each plate)

Biofilm formation in nutrient-limited condition by 0.2G MVBM was determined according to the method described as above. The OD_{620} value (mean±SD) of *B. thailandensis* UE5 in nutrient-limited condition was 1.152±0.360.

Statistical analysis

Comparison of biofilm-forming capacity of each isolate between the enriched and nutrient-limited conditions was evaluated using Student's *t* test. A P value of 0.05 was considered statistically significant.

RESULTS

The growth curves of *B. pseudomallei* K96243 grown on enriched (LB) and nutrient-limited media (0.2G MVBM) are shown in Fig. 1. The bacteria grown on 0.2G MVBM showed a longer lag phase and lower OD level at 72 h compared to those grown on LB. The MIC and MBC of CAZ for planktonic *B. pseudomallei* isolates in LB and 0.2G MVBM are shown in Table 1. Based on our previous study, the cut-off MIC values for CAZ-resistant *B. pseudomallei* was 32 µg/ml (Sawasdidoln *et al.*, 2010). Among 6 isolates of CAZ-susceptible *B. pseudomallei*, the MIC of CAZ against 4 isolates in 0.2G MVBM were less than those in LB while isolate 1026b in 0.2G MVBM showed higher MIC. In contrast, the MIC against most CAZ-resistant isolates in 0.2G MVBM were higher than those in LB, with the exception of isolate 316c. For the MBC, all isolates in 0.2G MVBM showed higher MBC than those in LB.

Interestingly, when these bacteria were induced to form biofilm using CBD, the PMBC of CAZ in each media against shedding planktonic cells were much higher than the MBC values although the PMIC values were

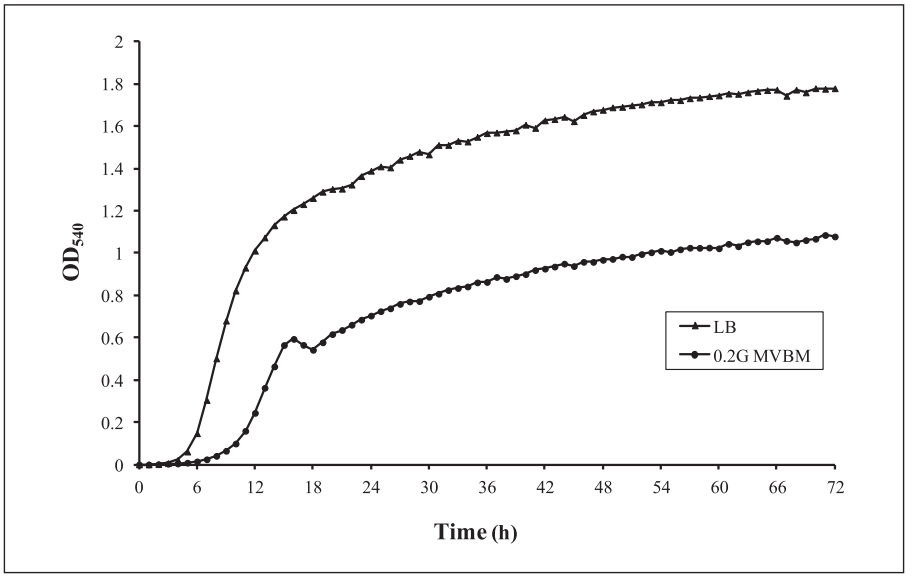

Figure 1. Growth rate of *B. pseudomallei* K96243 in Luria-Bertani broth (LB) and modified Vogel and Bonner's medium containing 2g/L glucose (0.2G MVBM).

Table 1. Minimum inhibitory concentration and minimum bactericidal concentration of ceftazidime for planktonic *B. pseudomallei* isolates in enriched (LB) and nutrient-limited conditions (0.2G MVBM)

Isolate	Relevant characteristics	LB		0.2G MVBM	
		MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
1026b	Clinical isolate from blood, susceptible to CAZ (DeShazer *et al.*, 1997)	4	8	32	256
K96243	Clinical isolate, susceptible to CAZ (Holden *et al.*, 2004)	4	8	1	512
NF10/38	Clinical isolate from blood, susceptible to CAZ (Tunpiboonsak *et al.*, 2010)	2	4	1	256
H777	Wild type, clinical isolate from blood, susceptible to CAZ (Taweechaisupapong *et al.*, 2005)	2	4	2	256
M10	Biofilm-defective mutant from a clinical isolated H777 wild type, susceptible to CAZ (Taweechaisupapong *et al.*, 2005)	4	4	1	256
EPMK31	Clinical isolate from pus, susceptible to CAZ	4	8	1	32
316c	Clinical isolate from blood, CAZ resistant (Kanthawong *et al.*, 2009)	128	128	128	1024
979b	Clinical isolate from blood, CAZ resistant (Kanthawong *et al.*, 2009)	64	128	128	1024
EPMN34	Clinical isolate from pus, CAZ resistant	64	128	512	2048
EPMN159	Clinical isolate from blood, CAZ resistant	64	128	256	>2048

LB, Luria-Bertani broth; 0.2G MVBM, modified Vogel and Bonner's medium containing 2g/L glucose; MIC, Minimum inhibitory concentration; MBC, minimum bactericidal concentration; CAZ, ceftazidime

Table 2. Susceptibility of *B. pseudomallei* isolates in planktonic and biofilm forms to ceftazidime determined by Calgary biofilm device

Isolate	LB			0.2G MVBM		
	PMIC (µg/ml)	PMBC (µg/ml)	MBEC (µg/ml)	PMIC (µg/ml)	PMBC (µg/ml)	MBEC (µg/ml)
1026b	2	256	2048	2	1024	>2048
K96243	2	512	2048	2	512	>2048
NF10/38	1	256	512	1	512	>2048
H777	4	512	2048	2	512	1024
M10	128	256	>2048	2	512	>2048
EPMK31	4	256	>2048	2	4	1024
316c	32	>2048	>2048	64	>2048	>2048
979b	128	2048	>2048	256	>2048	>2048
EPMN34	64	1024	2048	64	>2048	>2048
EPMN159	512	>2048	>2048	128	>2048	>2048

LB, Luria-Bertani broth; 0.2G MVBM, modified Vogel and Bonner's medium containing 2g/L glucose; PMIC, planktonic minimum inhibitory concentration; PMBC, planktonic minimum bactericidal concentration; MBEC, minimum biofilm elimination concentration.

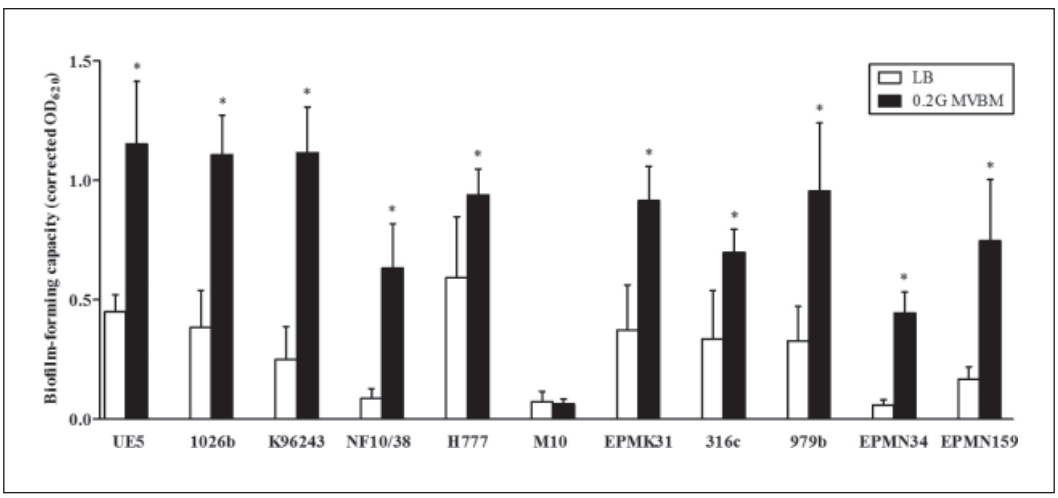

Figure 2. Relative biofilm-forming capacity of *B. pseudomallei* isolates in enriched (LB) and nutrient-limited conditions (0.2G MVBM). UE5 is the *B. thailandensis* isolate used as reference for the calculation of biofilm-forming capacity. Data are presented as the mean and standard deviation of two independent experiments performed in eightplicate.
* $P < 0.05$ compared to enriched (LB) condition

not differ much from the MIC (Table 1 and 2). Moreover, the drug susceptibilities of *B. pseudomallei* biofilm (MBEC results) in both enriched and nutrient-limited conditions were much higher than those of planktonic cells (both MBC and PMBC results).

The quantitative results of the capacity to produce biofilm showed that all isolates of *B. pseudomallei* in this study produced biofilm varied in quantity in each isolate. It is clear that all isolates of *B. pseudomallei* in the nutrient-limited condition (0.2G MVBM) produced statistically ($P < 0.05$) higher levels of biofilm than those in enriched condition (LB) except *B. pseudomallei* M10 which is the biofilm-defective mutant (Fig. 2).

DISCUSSION

Our results showed that the nutrient-limited condition contributed to antibiotic tolerance of planktonic *B. pseudomallei* (Table 1). Interestingly, not only 6 isolates of CAZ susceptible *B. pseudomallei*, but 4 CAZ resistant isolates also showed higher MBC values in the nutrient-limited condition compared to those in enriched condition. We hypothesized that the formation of antibiotic tolerance during nutrient depletion (0.2G MVBM), at least partially due to reduce growth and metabolic activity of bacteria (Fig. 1). Our results are in accordance with several previous reports which showed decreased potency of many antibiotics against slowly growing or persister cells (Brown *et al.*, 1990; Gilbert *et al.*, 1990; Lewis, 2010; Baek *et al.*, 2011). To prove our hypothesis, the stationary phase planktonic *B. pseudomallei* in 0.2G MVBM was regrown in LB and the MIC and MBC of CAZ were determined again. We found that the new population of *B. pseudomallei* become susceptible to CAZ and the MIC and MBC of CAZ against these new population were equivalent to those cultured in LB at first time (unpublished data). This is in agreement with the phenomenon, termed bacterial persistence (Balaban *et al.*, 2004; Lewis, 2010). Persister cells is dormant and non-dividing cells during nutrient depletion, could spontaneously switch to fast growth and generate a population that is sensitive to the antibiotic when inoculated into fresh enriched medium. Similar to our study, a study of the effects of nutrient deprivation on antibiotic tolerance in *Escherichia coli* showed that phosphate starvation promoted a transient increase in tolerance to the fluoroquinolone ofloxacin (Fung *et al.*, 2010). In addition, deprivation of amino acids was linked to short-term tolerance to ofloxacin and ampicillin. Moreover, highly sustainable tolerance of ampicillin and/or ofloxacin could be produced by simultaneous depletion of amino acids and glucose (Fung *et al.*, 2010).

The nutrient-limited condition in this study also contributed to higher levels of biofilm production in *B. pseudomallei*. More biofilm formation may contribute to the antibiotic tolerance of *B. pseudomallei* observed in the present study. It is well accepted that bacteria growing in a biofilm are more recalcitrant to the action of antibiotics than cells growing in a planktonic state (Hoiby *et al.*, 2010). *Burkholderia pseudomallei* has been reported to form biofilm both *in vitro* and *in vivo* (Vorachit *et al.*, 1995). We have demonstrated that pre-grown *B. pseudomallei* biofilm were markedly resistant to all antimicrobial agents tested, i.e., CAZ, doxycycline, imipenem, and trimethoprim/sulfamethoxazole when compared to the corresponding planktonic cells of the same isolates (Sawasdidoln *et al.*, 2010). Drug tolerance of *B. pseudomallei* biofilm in Table 2 of this study is in accordance with our previous study (Sawasdidoln *et al.*, 2010). However, drug tolerance may not depend on levels of biofilm production per se because biofilm-defective mutant *B. pseudomallei* M10 produced less biofilm than another isolates also showed higher PMBC and MBEC (Table 2) than MBC of its planktonic state (Table 1). These results demonstrated that the biofilm bacteria and planktonic bacteria shed from the biofilm had different antibiotic tolerance phenotypes from their planktonic counterparts. Since biofilm growth is one of the most important factors of starvation induced tolerance, it is possible that several bacterial stress defense mechanisms in mediating formation of the nutrient-sensitive antibiotic tolerance phenotypes may be involved. These include the stringent (Rodionov & Ishiguro, 1995; Magnusson *et al.*, 2005; Potrykus & Cashel, 2008), SOS (Miller *et al.*, 2004; Janion, 2008), and RpoS (Klauck *et al.*, 2007) mediated responses, which may be elicited when bacteria encounter nutrient starvation, physiological stress, or cellular damages. Recently starvation induces stringent response has been shown to mediate antibiotic tolerance in nutrient-limited and biofilm cells of *P. aeruginosa* (Nguyen *et al.*, 2011). Inactivation of the stringent response in *P. aeruginosa* enhanced susceptibility to several anti-microbials and, as well as enhanced the survival of antimicrobial-treated animals in *P. aeruginosa* animal

infection models (Nguyen *et al.*, 2011). Therefore, the role of stringent response may be involved in *B. pseudomallei* drug tolerance during nutrient-limited condition. Further studies are needed to elucidate these mechanisms in *B. pseudomallei*.

In conclusion, the findings of this study indicated that nutrient-limited condition induced biofilm formation and drug tolerance of *B. pseudomallei*. Interestingly, knowing where and when a particular tolerance mechanism might be recruited *in vivo* could inform an appropriate choice of therapeutic options and develop the possible new strategies for the prevention in melioidosis.

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REFERENCES

Baek, S.H., Li, A.H. & Sassetti, C.M. (2011). Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PLoS Biology* **9**: e1001065.

Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science* **305**: 1622-1625.

Brown, M.R., Collier, P.J. & Gilbert, P. (1990). Influence of growth rate on susceptibility to antimicrobial agents: modification of the cell envelope and batch and continuous culture studies. *Antimicrobial Agents and Chemotherapy* **34**: 1623-1628.

Chantratita, N., Rholl, D.A., Sim, B., Wuthiekanun, V., Limmathurotsakul, D., Amornchai, P., Thanwisai, A., Chua, H.H., Ooi, W.F., Holden, M.T., Day, N.P., Tan, P., Schweizer, H.P. & Peacock, S.J. (2011). Antimicrobial resistance to ceftazidime involving loss of penicillin-binding protein 3 in *Burkholderia pseudomallei*. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 17165-17170.

Cheng, A.C. & Currie, B.J. (2005). Melioidosis: epidemiology, pathophysiology, and management. *Clinical Microbiology Reviews* **18**: 383-416.

Currie, B.J., Dance, D.A. & Cheng, A.C. (2008). The global distribution of *Burkholderia pseudomallei* and melioidosis: an update. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **102 Suppl 1**: S1-4.

Dance, D.A., Wuthiekanun, V., Chaowagul, W. & White, N.J. (1989). The antimicrobial susceptibility of *Pseudomonas pseudomallei*. Emergence of resistance in vitro and during treatment. *Journal of Antimicrobial Chemotherapy* **24**: 295-309.

DeShazer, D., Brett, P.J., Carlyon, R. & Woods, D.E. (1997). Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. *Journal of Bacteriology* **179**: 2116-2125.

Fung, D.K., Chan, E.W., Chin, M.L. & Chan, R.C. (2010). Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. *Antimicrobial Agents and Chemotherapy* **54**: 1082-1093.

Gilbert, P., Collier, P.J. & Brown, M.R. (1990). Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. *Antimicrobial Agents and Chemotherapy* **34**: 1865-1868.

Hamad, M.A., Austin, C.R., Stewart, A.L., Higgins, M., Vazquez-Torres, A. & Voskuil, M.I. (2011). Adaptation and antibiotic tolerance of anaerobic *Burkholderia pseudomallei*. *Antimicrobial Agents and Chemotherapy* **55**: 3313-3323.

Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S. & Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents* **35**: 322-332.

Holden, M.T., Titball, R.W., Peacock, S.J., Cerdeno-Tarraga, A.M., Atkins, T., Crossman, L.C., Pitt, T., Churcher, C., Mungall, K., Bentley, S.D., Sebaihia, M.,

Thomson, N.R., Bason, N., Beacham, I.R., Brooks, K., Brown, K.A., Brown, N.F., Challis, G.L., Cherevach, I., Chillingworth, T., Cronin, A., Crossett, B., Davis, P., DeShazer, D., Feltwell, T., Fraser, A., Hance, Z., Hauser, H., Holroyd, S., Jagels, K., Keith, K.E., Maddison, M., Moule, S., Price, C., Quail, M.A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Simmonds, M., Songsivilai, S., Stevens, K., Tumapa, S., Vesaratchavest, M., Whitehead, S., Yeats, C., Barrell, B.G., Oyston, P.C. & Parkhill, J. (2004). Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 14240-14245.

Janion, C. (2008). Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*. *International Journal of Biological Sciences* **4**: 338-344.

Kanthawong, S., Nazmi, K., Wongratanacheewin, S., Bolscher, J.G., Wuthiekanun, V. & Taweechaisupapong, S. (2009). *In vitro* susceptibility of *Burkholderia pseudomallei* to antimicrobial peptides. *International Journal of Antimicrobial Agents* **34**: 309-314.

Klauck, E., Typas, A. & Hengge, R. (2007). The sigmaS subunit of RNA polymerase as a signal integrator and network master regulator in the general stress response in *Escherichia coli*. *Science Progress* **90**: 103-127.

Lam, J., Chan, R., Lam, K. & Costerton, J.W. (1980). Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infection and Immunity* **28**: 546-556.

Lewis, K. (2010). Persister cells. *Annual Review of Microbiology* **64**: 357-372.

Magnusson, L.U., Farewell, A. & Nystrom, T. (2005). ppGpp: a global regulator in *Escherichia coli*. *Trends in Microbiology* **13**: 236-242.

Miller, C., Thomsen, L.E., Gaggero, C., Mosseri, R., Ingmer, H. & Cohen, S.N. (2004). SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. *Science* **305**: 1629-1631.

Mima, T., Schweizer, H.P. & Xu, Z.Q. (2011). *In vitro* activity of cethromycin against *Burkholderia pseudomallei* and investigation of mechanism of resistance. *Journal of Antimicrobial Chemotherapy* **66**: 73-78.

Mukhopadhyay, C., Kaestli, M., Vandana, K.E., Sushma, K., Mayo, M., Richardson, L., Tuanyok, A., Keim, P., Godoy, D., Spratt, B.G. & Currie, B.J. (2011). Molecular characterization of clinical *Burkholderia pseudomallei* isolates from India. *American Journal of Tropical Medicine and Hygiene* **85**: 121-123.

National Committee for Clinical Laboratory Standards. (2002). Performance standards for antimicrobial susceptibility testing: *Twelfth informational supplement M100-S12*. Wayne, PA.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y., Britigan, B.E. & Singh, P.K. (2011). Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* **334**: 982-986.

Novem, V., Shui, G., Wang, D., Bendt, A.K., Sim, S.H., Liu, Y., Thong, T.W., Sivalingam, S.P., Ooi, E.E., Wenk, M.R. & Tan, G. (2009). Structural and biological diversity of lipopolysaccharides from *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *Clinical and Vaccine Immunology* **16**: 1420-1428.

Pibalpakdee, P., Wongratanacheewin, S., Taweechaisupapong, S. & Niumsup, P.R. (2012). Diffusion and activity of antibiotics against *Burkholderia pseudomallei* biofilms. *International Journal of Antimicrobial Agents* **39**: 356-359.

Potrykus, K. & Cashel, M. (2008). (p)ppGpp: still magical? *Annual Review of Microbiology* **62**: 35-51.

Pumirat, P., Saetun, P., Sinchaikul, S., Chen, S.T., Korbsrisate, S. & Thongboonkerd, V. (2009). Altered secretome of *Burkholderia pseudomallei* induced by

salt stress. *Biochimica et Biophysica Acta* **1794**: 898-904.

Rholl, D.A., Papp-Wallace, K.M., Tomaras, A.P., Vasil, M.L., Bonomo, R.A. & Schweizer, H.P. (2011). Molecular Investigations of PenA-mediated beta-lactam Resistance in *Burkholderia pseudomallei*. *Frontiers in Microbiology* **2**: 139.

Rodionov, D.G. & Ishiguro, E.E. (1995). Direct correlation between overproduction of guanosine 3',5'-bispyrophosphate (ppGpp) and penicillin tolerance in *Escherichia coli*. *Journal of Bacteriology* **177**: 4224-4229.

Rolim, D.B., Rocha, M.F., Brilhante, R.S., Cordeiro, R.A., Leitao, N.P., Jr., Inglis, T.J. & Sidrim, J.J. (2009). Environmental isolates of *Burkholderia pseudomallei* in Ceara State, northeastern Brazil. *Applied and Environmental Microbiology* **75**: 1215-1218.

Sarovich, D.S., Price, E.P., Von Schulze, A.T., Cook, J.M., Mayo, M., Watson, L.M., Richardson, L., Seymour, M.L., Tuanyok, A., Engelthaler, D.M., Pearson, T., Peacock, S.J., Currie, B.J., Keim, P. & Wagner, D.M. (2012). Characterization of ceftazidime resistance mechanisms in clinical isolates of *Burkholderia pseudomallei* from Australia. *PLoS ONE* **7**: e30789.

Sawasdidoln, C., Taweechaisupapong, S., Sermswan, R.W., Tattawasart, U., Tungpradabkul, S. & Wongratanacheewin, S. (2010). Growing *Burkholderia pseudomallei* in biofilm stimulating conditions significantly induces antimicrobial resistance. *PLoS One* **5**: e9196.

Schweizer, H.P. (2012). When it comes to drug discovery not all Gram-negative bacterial biodefence pathogens are created equal: *Burkholderia pseudomallei* is different. *Microbial Biotechnology* **5**: 581-583.

Siritapetawee, J., Prinz, H., Samosornsuk, W., Ashley, R.H. & Suginta, W. (2004). Functional reconstitution, gene isolation and topology modelling of porins from *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *Biochemical Journal* **377**: 579-587.

Taweechaisupapong, S., Kaewpa, C., Arunyanart, C., Kanla, P., Homchampa, P., Sirisinha, S., Proungvitaya, T. & Wongratanacheewin, S. (2005). Virulence of *Burkholderia pseudomallei* does not correlate with biofilm formation. *Microbial Pathogenesis* **39**: 77-85.

Thibault, F.M., Hernandez, E., Vidal, D.R., Girardet, M. & Cavallo, J.D. (2004). Antibiotic susceptibility of 65 isolates of *Burkholderia pseudomallei* and *Burkholderia mallei* to 35 antimicrobial agents. *Journal of Antimicrobial Chemotherapy* **54**: 1134-1138.

Tunpiboonsak, S., Mongkolrob, R., Kitudomsub, K., Thanwatanaying, P., Kiettipirodom, W., Tungboontina, Y. & Tungpradabkul, S. (2010). Role of a *Burkholderia pseudomallei* polyphosphate kinase in an oxidative stress response, motilities, and biofilm formation. *Journal of Microbiology* **48**: 63-70.

Vorachit, M., Lam, K., Jayanetra, P. & Costerton, J.W. (1993). Resistance of *Pseudomonas pseudomallei* growing as a biofilm on silastic discs to ceftazidime and co-trimoxazole. *Antimicrobial Agents and Chemotherapy* **37**: 2000-2002.

Vorachit, M., Lam, K., Jayanetra, P. & Costerton, J.W. (1995). Electron microscopy study of the mode of growth of *Pseudomonas pseudomallei in vitro* and *in vivo*. *Journal of Tropical Medicine and Hygiene* **98**: 379-391.

White, N.J. (2003). Melioidosis. *Lancet* **361**: 1715-1722.

Wuthiekanun, V., Cheng, A.C., Chierakul, W., Amornchai, P., Limmathurotsakul, D., Chaowagul, W., Simpson, A.J., Short, J.M., Wongsuvan, G., Maharjan, B., White, N.J. & Peacock, S.J. (2005). Trimethoprim/sulfamethoxazole resistance in clinical isolates of *Burkholderia pseudomallei*. *Journal of Antimicrobial Chemotherapy* **55**: 1029-1031.