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

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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 (Siripatapawee 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 MgSO₄·7H₂O, 2 g/l citric acid (anhydrous), 3.5 g/l NaNH₄HPO₄, 10 g/l K₂HPO₄, 0.36 g/l CaCl₂·2H₂O, 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 37ºC 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 37ºC. 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 37°C with shaking at 200 rpm and growth until stationary phase. Cultures of each bacterial strain were adjusted to ~1×10⁶ 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 37°C 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).

**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 10⁸ 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 37°C 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 37°C 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 37°C 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 (OD₆₂₀ < 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 37°C 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 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
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)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Relevant characteristics</th>
<th>LB</th>
<th></th>
<th></th>
<th></th>
<th>0.2G MVBM</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC (µg/ml)</td>
<td>MBC (µg/ml)</td>
<td>MIC (µg/ml)</td>
<td>MBC (µg/ml)</td>
<td>MIC (µg/ml)</td>
<td>MBC (µg/ml)</td>
<td></td>
</tr>
<tr>
<td>1026b</td>
<td>Clinical isolate from blood, susceptible to CAZ</td>
<td>4</td>
<td>8</td>
<td>32</td>
<td>256</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K96243</td>
<td>Clinical isolate, susceptible to CAZ</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>512</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF10/38</td>
<td>Clinical isolate from blood, susceptible to CAZ</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>256</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>H777</td>
<td>Wild type, clinical isolate from blood, susceptible to CAZ</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>256</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M10</td>
<td>Biofilm-defective mutant from a clinical isolated H777 wild type, susceptible to CAZ</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>256</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPMK31</td>
<td>Clinical isolate from pus, susceptible to CAZ</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>32</td>
<td></td>
<td></td>
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<td>316c</td>
<td>Clinical isolate from blood, CAZ resistant</td>
<td>128</td>
<td>128</td>
<td>128</td>
<td>1024</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>979b</td>
<td>Clinical isolate from blood, CAZ resistant</td>
<td>64</td>
<td>128</td>
<td>128</td>
<td>1024</td>
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<td>EPMN34</td>
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<td>128</td>
<td>512</td>
<td>2048</td>
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<tr>
<td>EPMN159</td>
<td>Clinical isolate from blood, CAZ resistant</td>
<td>64</td>
<td>128</td>
<td>256</td>
<td>&gt;2048</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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).
not differ much from the MIC (Table 1 and 2). Moreover, the drug susceptibilities of \textit{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 \textit{B. pseudomallei} in this study produced biofilm varied in quantity in each isolate. It is clear that all isolates of \textit{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 \textit{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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