ENVIRONMENTAL FACTORS AFFECTING *BURKHOLDERIA PSEUDOMALLEI* BIOFILM FORMATION

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Abstract. Melioidosis is highly prevalent in Northeast Thailand which is associated with high incidence of *Burkholderia pseudomallei* present in the soil of this region. *B. pseudomallei* when present in biofilm becomes resistant to numerous environmental factors and also to certain antibiotics. In this study, we examined the effects of several environmentally relevant factors (salinity, iron, manganese and temperature) on biofilm formation of four clinical ribotypes of *B. pseudomallei* commonly found in Northeast Thailand. The results showed that biofilm formation increased when *B. pseudomallei* were grown in modified Vogel and Bonner's medium containing 0.85-1.7 M NaCl or 100-500 μM iron ($FeSO_4$). Low temperature (20ºC) also induced more biofilm formation than 30ºC or 37ºC. On the other hand, protease production and bacterial motility were adversely affected but not in the case of low temperature. Results from this study should be useful in the development of prevention measures or controlling *B. pseudomallei* biofilm formation in the environment.

Keywords: *Burkholderia pseudomallei*, biofilm, environmental factors

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis, a life-threatening bacterial infection of humans and animals mainly in Southeast Asia and northern Australia (White, 2003; Cheng and Currie, 2005). *B. pseudomallei* can be transmitted through the population in the endemic areas by percutaneous inoculation, inhalation or ingestion of contaminated soil and water. In Thailand, the mortality rate of treated melioidosis patients is as high as 40% (White, 2003) because *B. pseudomallei* is inherently resistant to a variety of antibiotics including β-lactams, aminoglycosides, macrolides, and polymyxins (Howe *et al*, 1971; Leelarasamee and Bovornkitti, 1989). In addition, *B. pseudomallei* is known to form biofilm and microcolonies (Vorachit *et al*, 1995) and *B. pseudomallei* growing in biofilm become resistant to several conventional antibiotics including doxycycline, cef-

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tazidime, imipenem, and trimethoprim/sulfamethoxazole (Sawasdidoln *et al*, 2010) offering an explanation of relapse cases in melioidosis patients.

The incidence of melioidosis in Northeast Thailand largely restricted to Khon Kaen and Ubon Ratchathani Provinces (Leelarasamee, 1998) is 10-fold higher than that in Central Thailand (Palasatien *et al*, 2008). Also, the incidence of *B. pseudomallei* in soil in Northeast Thailand is 20-fold higher than that in Central Thailand and this high incidence is associated with certain physicochemical characteristics of the soil in Northeast Thailand including a pH of 5.0-6.0, a moisture content >10%, and higher oxygen demand and total nitrogen (Palasatien *et al*, 2008). About 75-85% of melioidosis cases are during the rainy season (White, 2003; Cheng and Currie, 2005). Association of the high incidence of melioidosis with the monsoon season, is observed not only in Thailand (June to November) (Srisurat *et al*, 2008) but also in Australia (October to March) (Cheng *et al*, 2008) after a long period of drought.

Several environmental factors such as soil (Chen *et al*, 2003; Palasatien *et al*, 2008) and water (Draper *et al*, 2010) characteristics are assumed to affect the distribution of *B. pseudomallei*. In terms of adaptation of bacteria to environment, biofilm formation is considered as an important survival strategy (Davey and O'Toole, 2000; Hall-Stoodley *et al*, 2004). *Pseudomonas* sp has been demonstrated to have an ability to produce extracellular polysaccharide, which alters the microenvironment in order to facilitate bacteria survival in desiccated condition (Roberson and Firestone, 1992). *B. pseudomallei* is resistant to a number of environmental factors, including salinity, although the precise mechanism(s) for its adaptive response to environmental changes remain largely unknown (Inglis and Sagripanti, 2006). The northeast region of Thailand has a severe problem of soil salinization with 8.5% of the region being affected (Dissataporn *et al*, 2002). In addition, iron and manganese oxide nodules have been found at a depth of 5-30 cm in soils of Nam Phong catena (Thanachit *et al*, 2010). High salt, iron and manganese concentrations in soils of Northeast Thailand together with the soil temperature may affect the survival of *B. pseudomallei* as 27°C could enhance microcolony formation of *B. pseudomallei* compared to 37°C (Boddey *et al*, 2006). However, no experimental evidence has been presented to support this notion.

In the present study the effects of physical and chemical conditions on biofilm formation were examined for 4 clinical *B. pseudomallei* isolates with ribotypes 3, 8, 19 and 23. In Thailand, three ribotypes (3, 8 and 23) account for 48.2% of both clinical and environmental isolates (Sermswan *et al*, 2001). Types 3 and 8 have been found to be prevalent worldwide, with type 3 being the most prevalent in Asian countries (Pitt *et al*, 2000; Sermswan *et al*, 2001). Type 23 is associated with high mortality (Pitt *et al*, 2000). Knowledge of the factors that affect *B. pseudomallei* biofilm formation is important for understanding the environmental cues that can lead to biofilm formation and bacterial survival.

MATERIALS AND METHODS

Bacterial strains

Four clinical isolates of *B. pseudomallei* (R3C, R8C, R19C and R23C) representing 4 different ribotypes (ribotypes 3, 8, 19 and 23) commonly found in Thailand (Sermswan *et al*, 2001) were used in this study. In addition, the biofilm-producing clinical

isolate (H777) and its non-biofilm producing mutant (M10) (Taweechaisupapong *et al*, 2005) were used as a positive and a negative control, respectively. *B. thailandensis* UE5 was also included as a reference strain for calculation of biofilm-forming capacity in all experiments (Taweechaisupapong *et al*, 2005). Each bacterial strain was grown on Ashdown's agar plate at 37°C for 48 hours. A single colony of *B. pseudomallei* then was streaked on a Luria-Bertani (LB) agar plate before being grown further in modified Vogel and Bonner's medium (MVBM) (Lam *et al*, 1980) under various growth conditions.

Biofilm formation

Biofilm formation of bacteria under various culture conditions was determined in a 96-well plate according to the method of Taweechaisupapong *et al* (2005) in the presence of various concentrations of NaCl (0, 0.85, 1.19, 1.36, 1.53 and 1.7 M), $Fe(SO_4)$. $7H_2O$ (0, 1, 50, 100, 500 and 1,000 mM) or $MnSO_4$ (0, 1, 50, 500 and 1,000 µM). The effect of temperature on biofilm formation was investigated at 20, 30 and 37°C. A 2% (v/v) inoculum of culture (OD_{550} = 0.9) was pretreated in MVBM with each environmental stress as described above for 18 hours. Then 200 µl aliquots of the pretreated culture of each bacterial suspension were added into 8 wells of a 96-well flat-bottomed plastic tissue culture plate (Nunclon^{Tm}, Roskilde, Denmark). Non-treated bacteria in MVBM were used as the negative control. Plates were incubated at 37°C or each test temperature for 3 hours to allow adhesion. Non-adhering bacterial cells of each well were removed and replaced with 200 µl aliquot of fresh MVBM containing test compounds. After incubation for an additional 21 hours, the planktonic cells were removed and the wells were washed with 200 µl of sterile distilled water and replaced with 200 µl of fresh medium containing test compounds. The plates were further incubated for another 24 hours. Then, the wells were washed three times with 200 µl of sterile distilled water, and the attached bacteria (representing 2-day biofilm culture) were fixed with 200 µl of methanol per well for 15 minutes before being air-dried. Bacteria then were stained with 200 µl of 2% (w/v) crystal violet per well for 5 minutes and excess stain was removed with running tap water. Dye bound to adherent bacterial cells was solubilized by adding 200 µl of 33% (v/v) glacial acetic acid per well and the absorbance (A) of each well was measured at 630 nm using a microplate reader (TECAN Safire, Port Melbourne, Australia). The ability of biofilm formation of all tested strains was determined as ratio between test strains and *B. thailandensis* UE5 (reference strain) (Taweechaisupapong *et al*, 2005).

In order to monitor biofilm formation of bacteria under different stress conditions that may affect the bacterial cell mass, the average A_{630} (crystal violet) was normalized to average A_{540} (cell mass) of each experiment. The relative biofilm formation was calculated using the equation:

$$\text{Relative biofilm} = \frac{A_{630} \text{ of tested strain}/A_{540} \text{ of tested strain}}{A_{630} \text{ of reference strain}/A_{540} \text{ of reference strain}}$$

Three independent experiments were performed in duplicate determinations each time.

Phenotype assay

A single colony of bacteria from LB agar was inoculated into M9 medium with each environmental stress and incubated for 18 hours. The secreted protease production in each culture supernatant of

B. pseudomallei was assayed by centrifuging bacterial cultures at 10,000*g* for 10 minutes and incubating 20 µl of 18-hour bacterial culture supernatant on skim milk agar [3% (w/v) skim milk in 0.75% (w/v) Bacto agar] at 37°C for 24 hours (DeShazer *et al*, 1999). Protease production of bacteria was expressed as the diameter (mm) of clear zone. Swimming and swarming motilities were assessed using agar plate assay as previously described (Deziel *et al*, 2001). In brief, 2 µl of each 18-hour bacterial culture in M9 medium were inoculated onto Tryptone swim agar (1% tryptone, 0.5% NaCl, 0.3% Bacto agar) or swarm agar (0.8% nutrient broth, 5% dextrose and 0.5% Bacto agar) at 37°C for 24 hours. Then the resulting diameter of bacterial growth was measured.

Statistical analysis

Comparisons of the relative biofilm formation, protease production, swarm and swim diameter under various conditions of the same ribotype and of the same condition among different ribotypes were evaluated using one way ANOVA and statistical significance is when $p < 0.05$.

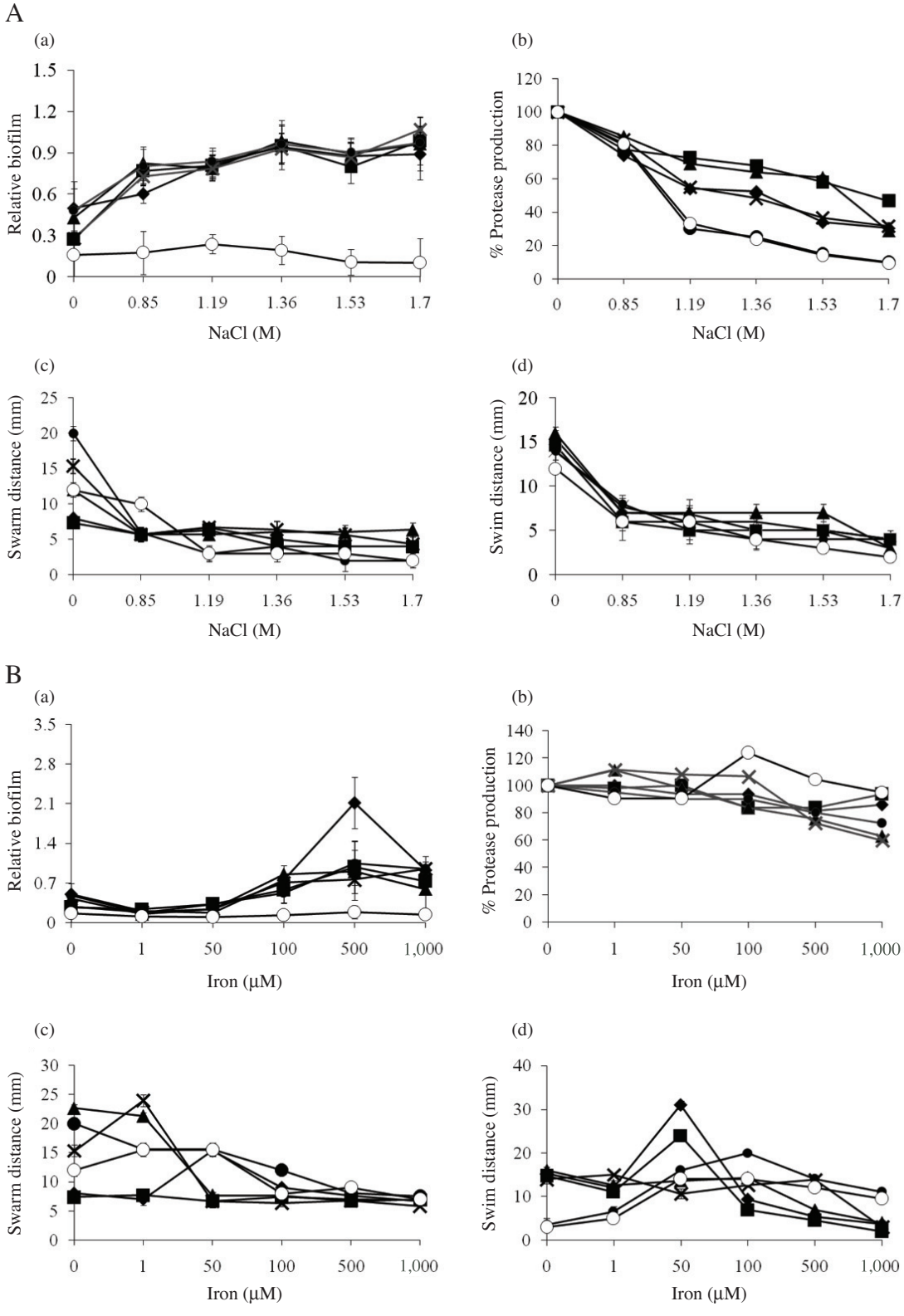
RESULTS

The 4 clinical isolates of *B. pseudomallei* (R3C, R8C, R19C and R23C), biofilm-producing isolate (H777) (positive control) and M10 (negative control) were grown in MVBM in the presence of 0.85-1.7 M NaCl. Biofilm formations in all NaCl concentrations of the 4 isolates and positive control are statistically higher than that without NaCl ($p < 0.001$) and the amount of biofilm of each ribotype increased in a concentration dependent manner [Fig 1A(a)]. Biofilm formations of these 5 isolates of *B. pseudomallei* were stimulated also by iron, with R3C biofilm formation being the highest at 500 µM iron ($p < 0.001$) [Fig 1B(a)]. On the other hand, biofilm production was suppressed in the presence of manganese, with the exception of R8C isolate, where biofilm formation was enhanced at 1 µM manganese ($p < 0.001$) [Fig 1C(a)]. Biofilm formation by the 4 *B. pseudomallei* clinical isolates was very limited at 30°C and 37°C but is significantly increased ($p < 0.001$) at 20°C, with isolate R23C demonstrating the highest biofilm formation ($p < 0.001$) [Fig 1D(a)]. Under all tested stress conditions, biofilm of mutant *B. pseudomallei* M10 was minimal.

Protease production of *B. pseudomallei* was suppressed in the presence of chemical agents ($p < 0.05$) but not by low temperature [Fig 1A(b) -1D(b)]. Higher concentrations of NaCl, iron and manganese significantly diminish the motility of *B. pseudomallei* clinical isolates as measured by swarm and swim distances ($p < 0.05$) [Fig 1A-C(c) and 1A-C(d)]. However, higher bacterial motility was evident with increase in temperature ($p < 0.05$) [Fig 1D(c) -1D(d)].

DISCUSSION

Biofilm formation of bacteria serves as a protection against environmental insults and adverse ecological stress (Davey and O'Toole, 2000). Osmolarity, inorganic molecules and temperature were reported to regulate biofilm formation of a number of bacterial species (O'Toole and Kolter, 1998; Romling *et al*, 1998; Singh *et al*, 2002; White-Ziegler *et al*, 2008). However, little is known regarding the adaptations of *B. pseudomallei* under stress conditions. In this study we found that high osmolarity (NaCl), high amount of iron and low temperature promoted *B. pseudomallei* biofilm production. Under these conditions, a decrease in protease production, swimming


A
(a)
Relative biofilm
1.5
1.2
0.9
0.6
0.3
0
0 0.85 1.19 1.36 1.53 1.7
NaCl (M)
(b)
% Protease production
120
100
80
60
40
20
0
0 0.85 1.19 1.36 1.53 1.7
NaCl (M)
(c)
Swarm distance (mm)
25
20
15
10
5
0
0 0.85 1.19 1.36 1.53 1.7
NaCl (M)
(d)
Swim distance (mm)
20
15
10
5
0
0 0.85 1.19 1.36 1.53 1.7
NaCl (M)
B
(a)
Relative biofilm
3.5
2.8
2.1
1.4
0.7
0
0 1 50 100 500 1,000
Iron (μM)
(b)
% Protease production
140
120
100
80
60
40
20
0
0 1 50 100 500 1,000
Iron (μM)
(c)
Swarm distance (mm)
30
25
20
15
10
5
0
0 1 50 100 500 1,000
Iron (μM)
(d)
Swim distance (mm)
40
30
20
10
0
0 1 50 100 500 1,000
Iron (μM)

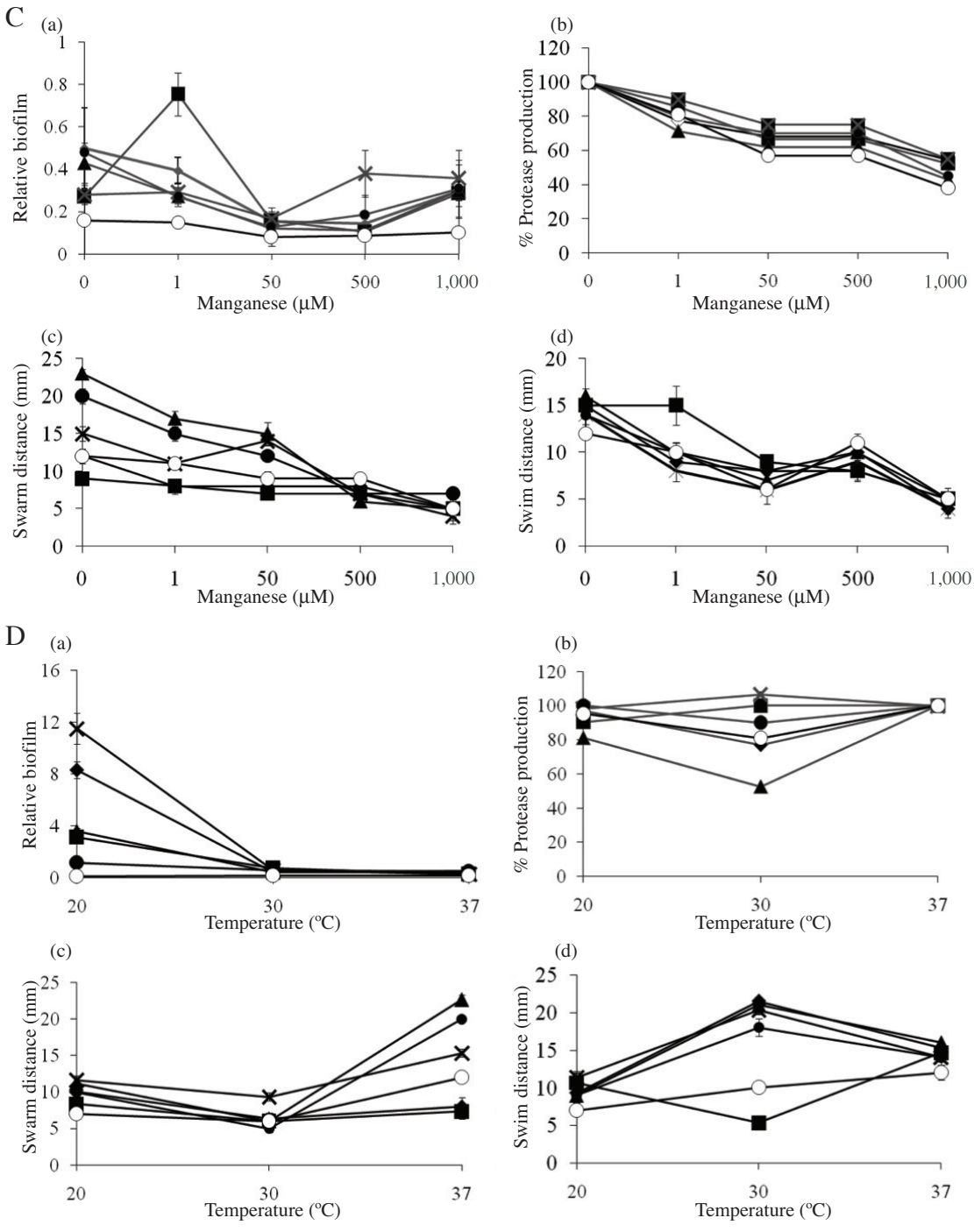


Fig 1–Effects of sodium chloride (A), iron (B), manganese (C) and temperature (D) on *B. pseudomallei* biofilm production, secreted protease, and swarm and swim distance. (a) Relative biofilm of 2-day incubation in MVBM, (b) protease production (%) on skim milk agar, (c) swarm distance on Bacto swarm agar plate, (d) swim distance on Tryptone swim agar. R3C (◆), R8C (■), R19C (▲), R23C (×), H777 (biofilm-producing) (●), M10 (non biofilm-producing) (○).

and swarming motility were observed.

Our findings are in accordance with a previous study of *Pseudomonas aeruginosa* in which biofilm formation and swarming are negatively associated, while swarming is positively associated with secretion of proteases (Murray *et al*, 2010). Garrett *et al* (1999) reported that in *P. aeruginosa* down-regulation of flagellum synthesis is linked with up-regulation of alginate synthesis. These data point out two important players in the regulation of biofilm development. As cells adjust to an immobile life style on a surface, they lose their flagella and increase production of exopolymeric substances.

The production of extracellular proteases has been implicated in the biofilm disassembly mechanism, and several reports have demonstrated an inverse correlation between protease expression and biofilm formation. For example, in *Staphylococcus aureus,* deletion of genes encoding proteases resulted in a significant increase in biofilm formation, and a concomitant decrease in disassembly upon activation of accessory gene regulator (*agr*) of the quorum-sensing system (Boles and Horswill, 2008). In addition, protease inhibitors have been shown to promote *S. aureus* biofilm formation under environmental conditions that normally accelerate biofilm disassembly (Boles and Horswill, 2008; Lauderdale *et al*, 2009; Boles *et al*, 2010).

High osmolarity is known to inhibit biofilm formation of various bacteria. For example, *Pseudomonas fluorescens* biofilm formation is inhibited by high osmolarity environment produced by addition of NaCl (O'Toole and Kolter, 1998). In *Salmonella typhimurium*, growth in medium containing high concentrations of NaCl abolishes transcription of *csgD*, a central regulator gene for biofilm formation and curli production (Romling *et al*, 1998). On the other hand, *Vibrio cholerae* can form a biofilm under high salt conditions (Kapfhammer *et al*, 2005). Two hundred mM NaCl activates transcription of *Escherichia coli pga* operon, which encodes proteins required for synthesis of poly-*N*-acetylglucosamine, an essential polymer for biofilm formation (Goller *et al*, 2006). Species-specific differences in the regulation of genes involved in biofilm production may account for the observation.

Iron, an essential element for bacterial growth, is also involved in biofilm formation in several bacterial species. For example, biofilm formation is suppressed at low iron condition in cultures of *P. aeruginosa* (Singh *et al*, 2002), *V. cholerae* (Mey *et al*, 2005) and *E. coli* (Wu and Outten, 2009). Analysis of soil samples in Australia indicated a significant association between *B. pseudomallei* positive sites and reddish gray and reddish brown soil (Kaestli *et al*, 2007). The red soil color might reflect iron-containing soil. Analysis of soil from Nam Phong catena, Northeast Thailand revealed red to reddish yellow loamy sand and sandy clay loam containing iron and iron impregnated nodules (Thanachit *et al*, 2010). Higher biofilm formation of *B. pseudomallei* under high iron and salt concentrations found in this study may provide an explanation for *B. pseudomallei* survival in soil of Northeast Thailand, which may lead to the high incidence of melioidosis cases.

Manganese is also known to affect microbial biofilm formation (Wang *et al*, 2009). In a study of human dental caries, manganese was demonstrated to be involved in strain variability in biofilm formation of *Streptococcus mutans* (Arirachakaran *et al*, 2007).

In the present study, low temperature (20ºC) promoted *B. pseudomallei* biofilm formation. Low temperature triggers the general stress response of bacteria (Boddey *et al*, 2006; White-Ziegler *et al*, 2008). Low temperature may promote adherence of *B. pseudomallei* and contribute to biofilm formation. Biofilm formation of *Hafnia alvei*, an opportunistic pathogen of animal and humans, is accelerated dramatically when grown at 25 ºC but not at 37 ºC (Vivas *et al*, 2008). Expression of *E. coli* K12 genes involved in biofilm formation is upregulated at 23 ºC (White-Ziegler *et al*, 2008). Northeast Thailand has a tropical savanna climate, with an average temperature of 26 ºC (Thanachit *et al*, 2010). However, the temperature of soil in deeper layers is lower than surface temperature and thus biofilm formation of *B. pseudomallei* may be facilitated in deeper soil layer.

To the best of our knowledge, this is the first study that demonstrates the effects of various environmental-related stresses on *B. pseudomallei* biofilm formation. The present results may be useful in developing prevention measures through controlling *B. pseudomallei* biofilm formation in the environment.

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