Morphological Alteration and Survival of *Burkholderia pseudomallei* in Soil Microcosms

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Abstract. The resilience of *Burkholderia pseudomallei*, the causative agent of melioidosis, was evaluated in control soil microcosms and in soil microcosms containing NaCl or FeSO₄ at 30°C. Iron (Fe(II)) promoted the growth of *B. pseudomallei* during the 30-day observation, contrary to the presence of 1.5% and 3% NaCl. Scanning electron micrographs of *B. pseudomallei* in soil revealed their morphological alteration from rod to coccoid and the formation of microcolonies. The smallest *B. pseudomallei* cells were found in soil with 100 µM FeSO₄ compared with in the control soil or soil with 0.6% NaCl ($P < 0.05$). The colony count on Ashdown's agar and bacterial viability assay using the LIVE/DEAD® BacLight™ stain combined with flow cytometry showed that *B. pseudomallei* remained culturable and viable in the control soil microcosms for at least 120 days. In contrast, soil with 1.5% NaCl affected their culturability at day 90 and their viability at day 120. Our results suggested that a low salinity and iron may influence the survival of *B. pseudomallei* and its ability to change from a rod-like to coccoid form. The morphological changes of *B. pseudomallei* cells may be advantageous for their persistence in the environment and may increase the risk of their transmission to humans.

INTRODUCTION

Human infection by *Burkholderia pseudomallei* through inhalation, percutaneous inoculation, or the ingestion of contaminated soil or water can potentially cause fatal melioidosis.^{1–3} The increasing number of melioidosis cases is causing concern not only in northeastern Thailand and northern Australia, which are melioidosis-endemic hot spots, but also throughout most of Asia, in regions of South America, in some countries in Africa, and on various Pacific and Indian Ocean islands, as well as in travelers returning from these disease-endemic regions.^{3–7} The increased incidence of melioidosis generally arises after heavy monsoonal rains, cyclones, or typhoons have occurred, leading to a shift toward the inhalation of *B. pseudomallei* that is significantly correlated with pneumonia, bacteremia, septic shock, and a high mortality rate.^{8–10}

The extraordinary survival period of *B. pseudomallei*, which ranges from months to years in the endemic regions during the dry season and in a laboratory setting, highlights its persistence, adaptability, and ability to survive.^{11–13} An examination of *B. pseudomallei* maintained in distilled water at 25°C for 16 years provided evidence of its ability to survive under extreme conditions.¹⁴ *Burkholderia pseudomallei* can persist for 2 years in soil with a water content of greater than 40%, whereas it dies within 70 days in soil with a water content of less than 10%.¹⁵ Moreover, this soil-borne pathogen can survive for 7 days in soils with pH values ranging from 4 to 7 or those containing 0.25–1.0% sodium chloride (NaCl).¹⁶ A study of soil microcosms demonstrated that dry soil in tropical endemic regions may act as a reservoir of *B. pseudomallei* during the dry season, with an increase in their number and the potential for their mobilization from the soil to water occurring during the wet season.¹⁷ The association of the presence of *B. pseudomallei* with that of iron and salt in water and soil has been demonstrated in endemic areas.^{16,18–22} These results may facilitate understanding of the ability of *B. pseudomallei* to adapt to soil in northeastern Thailand that has different environmental features, including high

salinity and the presence of iron. The resilience exhibited by *B. pseudomallei* in endemic areas has resulted in a high risk of pathogen exposure.

Whereas the morphological alterations undergone by many bacterial pathogens are correlated with their viability, their entrance into a viable but nonculturable (VBNC) state also has been demonstrated. *Vibrio parahaemolyticus* cells induced to enter the VBNC state under certain stress conditions exhibited a change in shape from rod-like to coccoid.²³ In addition, the morphological transition of *Helicobacter pylori* from rods to cocci resulted in decreased culturability, although their viability was demonstrated. The rapid entrance of *H. pylori* into the VBNC state in the environment results in a public health hazard.²⁴ Cell-shape changes from the bacillus form to coccoid and spiral forms and the entrance into the VBNC state under conditions of pH, temperature, or salt stress were observed in *B. pseudomallei* in liquid media; these alterations may play a role in their ability to persist in melioidosis-endemic environments, which can lead to a public health risk.^{12,25}

Therefore, the aims of this study were to examine the culturability of *B. pseudomallei* R3E, the most common ribotype of environmental isolates obtained in northeastern Thailand,²⁶ growing in a soil microcosm in the presence of 0–3% NaCl and 0–1,000 µM ferrous sulfate (FeSO₄) at 30°C for 30 days. These conditions reflect the endemic soil and climate conditions of Khon Kaen, Thailand (Land development Department of Thailand), which is a melioidosis-prevalent hot spot. The morphological alteration of *B. pseudomallei* R3E in the soil microcosms was examined using scanning electron microscopy. In addition, the viability and culturability of the *B. pseudomallei* R3E strain, the biofilm-forming wild-type H777 strain and the mutant of this strain, the M10 strain, were monitored in endemic soil microcosms containing 1.5% NaCl for 120 days using a LIVE/DEAD® BacLight™ (Invitrogen, Eugene, OR) assay and plate counts to determine the effects of soil salinity and biofilm formation ability on the survival of this soil-borne pathogen.

MATERIALS AND METHODS

Bacterial strains. Three strains of *B. pseudomallei*: R3E, the most common ribotype of environmental isolates obtained

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from northeastern Thailand²⁶; H777, a moderate biofilm-formation isolate; and its mutant M10²⁷ were used in this study. A single colony of *B. pseudomallei* on an Ashdown's agar plate from a glycerol stock solution was inoculated in 3 mL Luria-Bertani (LB) broth and was incubated at 37°C for 18 hours with shaking at 200 rpm. The inocula for the soil microcosms were prepared by subculturing the 1% inocula in 100 mL LB at 37°C until an optical density (OD₅₅₀) of 0.8 (equal to 10⁷ colony-forming units [CFU]/mL) was achieved. For the morphological observations on a glass slide, 1% inocula were grown in modified Vogel-Bonner medium (MVBM) until an OD₅₅₀ of 0.9 was reached.²⁷

Soil microcosms. Sandy loam soil obtained from a *B. pseudomallei*-positive site (site 39, Ban Kai Na) in the Nam Phong District, Khon Kaen (pH 5.60, electrical conductivity [EC] of 0.02 dS/m [nonsaline soil], and total iron content of 50 mg/kg soil)^{16,22} was used throughout this study. The soil sample was sieved through a 2-mm pore size sieve and was air-dried for 1 day. One hundred grams of soil placed in a 250-mL flask was autoclaved at 121°C at 15 psi for 15 minutes. The sterility of the soil was tested by suspending it in 200 mL PEG-DOC solution (2.5% w/v polyethylene glycol 600 [PEG 600; AR grade; Merck KGaA, Darmstadt, Germany] and 0.1% w/v sodium deoxycholate [DOC; AR grade; Sigma-Aldrich, St. Louis, MO]). After shaking at 150 rpm for 2 hours, the soil particles were allowed to settle for 1 hour before the supernatants were serially diluted and were plated on LB agar.²⁸ Of sterile distilled water, 60 mL was added to each 100 g of autoclaved soil to provide the moisture content existing in rice paddy soils during the wet season.

The effect of soil salinity and iron content on the survival of *B. pseudomallei*. NaCl and FeSO₄ solutions were added to the soil microcosms to final concentrations of 0.6% and 1.5% NaCl and 100, 500, and 1,000 µM FeSO₄ of soil dry weight. The soil microcosms were inoculated with 1 mL of 10⁷ CFU/mL *B. pseudomallei* by dropping 10 100-µL aliquots of the suspension evenly on the soil surface, and the preparations were incubated at 30°C for 30 days. Two independent experiments were performed in duplicate.

Culturability and viability of *B. pseudomallei* obtained from the soil microcosms. To assess the culturability of *B. pseudomallei* cells grown in each 100-g soil microcosm throughout the 120-day observation period, the cells were recovered using 200 mL of the PEG-DOC solution. The culturability of these cells was examined by spreading aliquots of serial dilutions of cell suspensions on Ashdown's agar plates in duplicate and incubating the plates at 37°C for 48 hours; the results were stated as CFU/g of soil. The assays were performed on at least two independent occasions.

The viability of *B. pseudomallei* cells was determined using a LIVE/DEAD[®] BacLight[™] Kit according to the manufacturer's directions. The bacterial cells were harvested from 5 mL of the soil/PEG-DOC supernatant described above and were washed three times using sterile distilled water by centrifugation at 1,000 × g for 20 minutes before resuspending them in 5 mL sterile distilled water. A 2-mL aliquot of the bacterial suspension was mixed with 1.5 µL of Syto-9 solution (3.34 mM in dimethyl sulfoxide [DMSO]) and 1 µL propidium iodide (PI) solution (20 mM in DMSO), and then the mixture was incubated in the dark at room temperature for 15 minutes. Syto-9 would penetrate all of the cells to stain the nucleic acids

and thus was used to determine the total cell counts, whereas the red-fluorescing dye PI would enter only the cells with damaged cytoplasmic membranes and thus was used to determine the number of dead bacteria. PI quenches the Syto-9 fluorescence in dead cells.²⁹ Thus, live bacteria with intact membranes emitted green fluorescence at 530 nm, whereas dead bacteria with damaged membranes emitted red fluorescence at 630 nm after excitement at 488 nm.^{30,31} The green and red fluorescence levels of LIVE/DEAD[®] BacLight[™]-stained cells in 100,000 events were measured using flow cytometry (BD FACSCanto II system; BD Biosciences, Oxford, United Kingdom). Unstained and singly stained cells from each sample were also analyzed to determine the level of auto-fluorescence and to correct the fluorescence compensation effect, respectively. The data were analyzed using BD FACSDiva v.6.1.3 software (San Jose, CA). The results were presented as the ratio of live bacterial cells to total bacterial cells.

To validate the staining and flow cytometric methods, LIVE/DEAD[®] BacLight[™]-stained cells of three groups of *B. pseudomallei*, including mid-log *B. pseudomallei* R3E cells (representing live cells), heat-killed (70°C for 3 hours) *B. pseudomallei* R3E cells (representing dead cells), and *B. pseudomallei* R3E cells recovered from the control soil on day 0 were used. Flow cytometric and microscopic assessments of these cells, the latter conducted using an epifluorescence microscope (Ni-U; Nikon, Melville, NY) at 1000× magnification with excitation at 488 nm, were performed to validate the staining procedure.

Morphological observation. On day 0, three sterile glass slides were placed in each flask containing a soil microcosm to allow bacterial attachment. Uninoculated soil microcosms were used as the negative control. The slides were taken on day 30 and were immediately fixed using 2% v/v glutaraldehyde (EM grade; Electron Microscopy Sciences, Hatfield, PA) for 1 hour at room temperature, followed by post-fixing using 1% v/v osmium tetroxide (EM grade; Electron Microscopy Sciences) in 0.2 M cacodylate buffer for 1 hour. The slides were washed twice for 15 minutes using 0.2 M cacodylate buffer solution, subjected to an ethanol dehydration series, and then coated with gold and examined under a scanning electron microscope (SEM) (LEO, SEM-EDSX) using the procedures outlined in the *Electron Microscopy Procedures Manual* of the Fred Hutchinson Cancer Research Center Electron Microscopy Resource (Available at: <http://sharedresources.fhcrc.org/training/electron-microscopy-procedures-manual>, accessed December 1, 2012). The length and width at least 100 bacterial cells in the SEM images were measured using ImageJ software (National Institutes of Health, Bethesda, MD).

To examine the cells in a biofilm, a sterile glass slide was immersed in 20 mL of *B. pseudomallei* cultured in MVBM in a 50-mL tube (Corning[®], Corning, NY) and was incubated for 2 days under static conditions at 37°C to allow biofilm formation at the water-air interface.

Statistical analysis. The statistical analyses were performed using the SPSS software, version 17 (SPSS Inc., Chicago, IL). The Shapiro-Wilk test was used to assess the normality of the bacterial culturability (log₁₀CFU/g soil) and viability (live/total) data. If the data distributions were not normal, the nonparametric Kruskal-Wallis test was used to evaluate the differences between the values determined on the sampling day and those obtained on day 0. The differences between the widths and lengths of bacteria grown in the control soil and in

MVBM were analyzed using an independent Student's *t* test, whereas differences between the widths and lengths of bacteria grown in the control soil microcosm and those grown in soil supplemented with salt or iron were analyzed using one-way analysis of variance followed by Tukey's honestly significant difference post hoc test. Differences with $P < 0.05$ were considered significant.

RESULTS

Survival of *B. pseudomallei* in soil microcosms. The 30-day survival rate of the *B. pseudomallei* R3E strain grown in the soil microcosms in the presence of 0%, 0.6%, 1.5%, and 3% NaCl or 100, 500, and 1,000 μM FeSO_4 at 30°C were calculated as CFU/g of soil (Figure 1). Culturable *B. pseudomallei* R3E cells were recovered from the soil microcosms throughout the experimental period. A soil salinity level of 0.6–3% NaCl affected the culturability of the *B. pseudomallei* R3E strain, with a significantly decreased CFU value obtained on days 21 and 30 ($P < 0.05$). At the end of the study period, the high-salt conditions (1.5% and 3% NaCl) had drastically diminished the culturability rate of *B. pseudomallei* by 4 log CFUs. *Burkholderia pseudomallei* cells exposed to 3% NaCl in the soil microcosms had lost their culturability by day 30. In contrast, bacterial growth was observed in the control soil and the soil supplemented with 100 and 500 μM FeSO_4 throughout the 30-day observation period. However, the highest concentration of iron (1,000 μM FeSO_4) resulted in reduced bacterial counts on day 30 (Figure 1). These results indicated that the culturability of *B. pseudomallei* was diminished by salinity stress, whereas the survival of *B. pseudomallei* was promoted by the presence of an appropriate concentration of iron in the soil microcosm.

Morphological alteration. The morphology of *B. pseudomallei* cells after cultivation for a period of 30 days in the control soil microcosms and the 2-day biofilm formation in MVBM were examined using SEM (Figures 2 and 3). We found that the *B. pseudomallei* cells had transformed to coccoid shape

FIGURE 1. Number ($\log_{10}$ CFU/g soil) of *Burkholderia pseudomallei* R3E in 100 g of the control soil microcosm and in soil microcosms containing 0.6–3% NaCl or 100–1,000 μM FeSO_4 after incubation at 30°C for 30 days. The bacteria recovered in the PEG-DIC solution (2.5% w/v polyethylene glycol 600 and 0.1% w/v sodium deoxycholate) were 10-fold serially diluted and then each dilution was spread on Ashdown's agar using the spread plate technique, after which the plates were incubated at 37°C for 48 hours. The error bars correspond to the standard deviation of the values determined in duplicate experiments. * $P < 0.05$ for $\log_{10}$ CFU/g soil on the test day compared with the value determined on day 0 of each experiment.

in the control soil microcosm and the soil supplemented with 0.6% NaCl or 100 μM FeSO_4 (Figure 2A–C). A representative image of the uninoculated control soil is shown in Figure 2D. The median length and width of *B. pseudomallei* cells grown for 30 days in the control soil were significantly shorter and wider, respectively, than those of the bacteria cells grown in MVBM ($P < 0.001$) (Table 1). The median length of *B. pseudomallei* cells grown in soil containing 0.6% NaCl was significantly shorter than that of cells grown in the control soil ($P < 0.001$) (Table 2). In addition, *B. pseudomallei* cells grown in soil containing 100 μM FeSO_4 were significantly smaller than were those grown in the control soil or in soil containing 0.6% NaCl ($P < 0.01$) (Table 2). We observed clusters of coccoid *B. pseudomallei* cells embedded in a pellicle-like substance that glued them to the surface and represented the formation of a microcolony in the control soil microcosm (Figure 3A). This occurrence indicated the formation of a biofilm because of microbial growth. Moreover, rod-shaped bacilli blanketed with an extracellular polymer matrix were observed in the 2-day cultures of *B. pseudomallei* grown in MVBM under biofilm formation conditions (Figure 3B). These findings suggested that the morphological transformation of *B. pseudomallei* cells from rod-like forms to coccoid forms occurred in the soil microcosm during the experimental period.

The culturability and viability of *B. pseudomallei* grown in the soil microcosms. The decrease in the number of culturable *B. pseudomallei* cells that were grown in the soil microcosms supplemented with 1.5% and 3% NaCl compared with their stability in the control soil (Figure 1) emphasized the need to address whether *B. pseudomallei* entered a VBNC state while dwelling under salinity stress conditions. Therefore, the culturabilities of the *B. pseudomallei* R3E, H777, and M10 strains were determined using the spread plate technique and their viabilities were determined using the LIVE/DEAD[®] BacLight[™] assay during a 120-day period of cultivation in the control soil microcosm and the soil microcosm supplemented with 1.5% NaCl.

The three tested *B. pseudomallei* strains retained similar levels of culturability and viability throughout the 120-day period of cultivation in the control soil ($P > 0.05$) (Figure 4A and B). These results indicated that *B. pseudomallei* could survive in endemic soil microcosms and suggested that these are environmental niches favoring bacterial persistence. In contrast, the culturability of *B. pseudomallei* grown in a saline soil microcosm containing 1.5% NaCl had declined by days 90 and 120 ($P < 0.05$) (Figure 5A). Notably, the viability of all three of the tested *B. pseudomallei* strains had diminished by day 120 of cultivation ($P < 0.05$) (Figure 5B). These findings reflect the consequences of prior osmotic stress on the ability of these bacteria to grow and survive on agar plates. In contrast, no difference in the culturability or the viability of the *B. pseudomallei* H777 (biofilm forming wild type) and M10 (biofilm forming mutant) strains was observed (Figure 5A and B).

Fluorescence images and cytograms of flow cytometric analysis of the LIVE/DEAD[®] BacLight[™] viability assay demonstrated a green fluorescence signal of Syto-9 of the mid-log *B. pseudomallei* R3E cells (Supplemental Figure 1A and B) and the *B. pseudomallei* R3E cells recovered from the control soil on day 0 (Supplemental Figure 1E and F), whereas the heat-killed *B. pseudomallei* R3E cells

FIGURE 2. Morphology of *Burkholderia pseudomallei* R3E grown in the soil microcosms. The bacteria were grown in the soil microcosms for 30 days and then examined using scanning electron microscopy. *Burkholderia pseudomallei* R3E cells grown in the control soil (A), in soil supplemented with 0.6% NaCl (B), in soil supplemented with 100 μ M of FeSO_4 (C); and in the control soil not inoculated with *B. pseudomallei* (D). Magnification = 5,000 $\times$.

emitted red fluorescence signal of PI (Supplemental Figure 1C and D).

DISCUSSION

The ability of *B. pseudomallei* to persist in environments has been demonstrated to be correlated with environmental factors of melioidosis-endemic zones. The adaptations that facilitate the survival of *B. pseudomallei* in various environments, including water and soil, have been previously linked to the incidence or outbreaks of the disease.^{19,32,33}

In this study, sterile control soil microcosm models with or without the addition of salt or iron were established to evaluate the performance of *B. pseudomallei* grown in soil for up to 30 days compared with that on the first day of inoculation. The results obtained demonstrated the ability of *B. pseudomallei* to survive for extended periods in the control soil, the low-salinity soil, and the iron-containing soil ($P < 0.05$) (Figure 1). Our results supported the results of a previous study that demonstrated that saline soil and water

were the preferred growth conditions for *B. pseudomallei* in northeastern Thailand.²¹ Moreover, the growth-promoting effect of iron on *B. pseudomallei* reinforced the significant association between *B. pseudomallei*-positive sites and the soil being reddish gray or reddish brown, indicating its iron content and the presence of animals.¹⁹ Red to reddish-yellow loamy sand containing iron and iron-impregnated nodules has been reported in the melioidosis-endemic area of Nam Phong catena in northeastern Thailand.³⁴ Our results provide additional support of the findings of a soil survey in the Nam Phong and Muang Districts, Khon Kaen, conducted in 2009 that revealed a correlation between culturable *B. pseudomallei* and the presence of higher iron levels in the soil during the dry season compared with that during the rainy season.²² Iron-oxidizing microbial populations in soil and sediments can use divalent ferrous (Fe(II)) as an electron source under both oxic and anoxic conditions and can use trivalent ferric (Fe(III)) as a terminal electron acceptor under anoxic conditions. In addition, the microbe-mediated oxidation of Fe(II) coupled to nitrate reduction

A **B**

FIGURE 3. Scanning electron micrographs of *Burkholderia pseudomallei* R3E. Morphological characteristics of an established microcolony formed by *B. pseudomallei* R3E grown in a control soil microcosm for 30 days at 30°C (**A**) and a biofilm colony formed by cells grown on a glass slide immersed in modified Vogel–Bonner medium (MVBm) for 2 days at 37°C (**B**). Magnification = 5,000 $\times$.

was also demonstrated in saline environmental systems, including paddy soil, under anoxic conditions in the dark.³⁵ A reasonable interpretation of these results is that the iron-containing soil in endemic areas may facilitate the survival of *B. pseudomallei*. However, excess iron could restrict bacterial growth and modify its metabolic patterns because of oxidative stress, thereby leading to DNA damage.^{36,37}

To the best of our knowledge, this is the first investigation to demonstrate morphological alterations of *B. pseudomallei* living in soil. Our results revealed clusters of short rod-like or coccoid bacterial cells blanketed with extracellular polymeric substances or microcolony formation (Figures 2 and 3).

TABLE 1

Results of an independent Student's *t* test analysis of the length and width of *Burkholderia pseudomallei* cells in MVBm and in control soil

	Length (μm)	Width (μm)
	Median (IQR)	Median (IQR)
MVBm	1.151 (1.030–1.300)	0.368 (0.307–0.422)
Control soil	0.606 (0.531–0.718)*	0.460 (0.380–0.523)*

IQR = interquartile range; MVBm = modified Vogel–Bonner medium.
*Statistically significant ($P < 0.001$).

TABLE 2

Results of a one-way ANOVA of the length and width of *Burkholderia pseudomallei* cells in control soil and in soil containing either 0.6% NaCl or 100 μM FeSO_4

	Length (μm)	Width (μm)
	Median (IQR)	Median (IQR)
Control soil	0.606 (0.531–0.718)	0.460 (0.380–0.523)
Soil + 0.6% NaCl	0.555 (0.478–0.633)*	0.442 (0.369–0.483)
Soil + 100 μM FeSO_4	0.473 (0.274–0.586)†§	0.395 (0.377–0.456)‡

ANOVA = analysis of variance; IQR = interquartile range.

*Length of *B. pseudomallei* in soil + 0.6% NaCl was significantly shorter than in control soil ($P < 0.001$).

†Length of *B. pseudomallei* in soil + 100 μM FeSO_4 was significantly shorter than in control soil ($P < 0.001$).

‡Width of *B. pseudomallei* in soil + 100 μM FeSO_4 was significantly wider than in control soil ($P < 0.01$).

§Length of *B. pseudomallei* in soil + 100 μM FeSO_4 was significantly shorter than in soil + 0.6% NaCl ($P < 0.001$).

||Width of *B. pseudomallei* in soil + 100 μM FeSO_4 was significantly shorter than in soil + 0.6% NaCl ($P < 0.01$).

Our findings demonstrated that, within the soil microcosm, *B. pseudomallei* transformed into cocci that were significantly shorter than the cocci within the biofilms that formed in MVBm broth ($P < 0.001$) (Table 1) and the cells previously

A **B**

FIGURE 4. Culturability and viability of *Burkholderia pseudomallei* grown in the control soil microcosm. The counts of culturable bacteria determined using an Ashdown's agar plate assay (**A**) and the live/total cells identified using the LIVE/DEAD[®] BacLight[™] viability kit in combination with flow cytometry (**B**) of the *B. pseudomallei* H777 wild-type strain (◆), its mutant M10 strain (◇), and the *B. pseudomallei* R3E environmental isolate strain (▲) grown in the control soil microcosm at 30°C for 120 days. The error bars represent the standard deviation of the values obtained in duplicate experiments.

FIGURE 5. Culturability and viability of *Burkholderia pseudomallei* grown in soil microcosms supplemented with 1.5% NaCl. The counts of culturable bacteria determined using an Ashdown's agar plate assay (A) and the live/total cells identified using the LIVE/DEAD[®] BacLight[™] viability kit in combination with flow cytometry of the *B. pseudomallei* H777 wild-type strain (◆), its mutant M10 strain (◇), and the *B. pseudomallei* R3E environmental isolate strain (▲) grown in soil supplemented with 1.5% NaCl at 30°C for 120 days. The error bars represent the standard deviation of the values obtained in duplicate experiments. * $P < 0.05$ for log₁₀ CFU/g soil and the live/total cells on the test day compared with the values determined on day 0 of each experiment.

described by Sagripanti and others.³⁸ Furthermore, the smaller size of *B. pseudomallei* in soil microcosms in the presence of low salt or iron may reflect the bacterial survival strategies used in these habitats (Table 2). Our results have similarities with the results of previous studies showing the morphological alterations of *B. pseudomallei* cells under pH or salt stress in a nutrient-depleted liquid environment.^{12,25} Moreover, the ability of *B. pseudomallei* to accumulate granules of polyhydroxybutyrate as energy stores for their long-term survival was also observed in coccoid bacterial cells by Inglis and Sagripanti.¹² A decrease in size would result in a coccoid cell having a larger surface-to-volume ratio than that of a rod-shaped cell, which has the greatest surface area for nutrient uptake while maintaining the least amount of cell mass,³⁹ thereby reducing the need to expend energy for nutrient transport during starvation.⁴⁰ Our findings may have important biological implications because the nonspore-forming bacteria that develop in response to environmental

stress would also benefit from a fitness strategy that allows their long-term survival in natural environments. The morphological alteration of *B. pseudomallei* dwelling in soil appeared to be a long-term dormant-like survival mechanism used under nutrient-deprived conditions that may facilitate the persistence of this bacterium in nature. Our results support those of previous studies showing that a decrease in the size of *B. pseudomallei* can potentially create aerosolized particles. The median length (0.606 μ m) and median width (0.460 μ m) of *B. pseudomallei* cells in the endemic soil microcosm demonstrated in this study (Table 1) suggest the possibility that this pathogen could be retained in the lungs and therefore highlights the risk of transmission of melioidosis by inhalation, as was previously suggested by Sagripanti and others³⁸ and Thomas and others.⁴¹ The Balb/c murine model of inhalational melioidosis exhibited a shorter mean time to death when 1- μ m *B. pseudomallei* aerosol particles were inhaled compared with when 12- μ m particles were inhaled, as well as a lower median lethal dose of 4 and 12 CFU, respectively. Thus, greater lung pathology occurred after the inhalation of the 1- μ m aerosolized particles.⁴¹ These findings have important implications for the risk of inhalational melioidosis in endemic countries during the monsoon season, which facilitates the aerosol transmission of melioidosis infections and leads to pneumonic infections.

Numerous bacteria, including human pathogens, are known to respond to various environmental stresses by undergoing a morphological transition from rod to coccoid forms to increase their surface-to-volume ratio as they enter the long-term survival phase in a VBNC state.³⁸ In this study, the observed morphological alteration of *B. pseudomallei* cells from rod to coccoid forms might indicate that they had entered a VBNC state, as previously observed in many other pathogenic bacteria.^{42,43} However, further investigations are needed to clarify whether *B. pseudomallei* can exist in a VBNC state while dwelling in soil. The VBNC state is important because the unculturability of VBNC cells on routine agar leads to an underestimation of the total viable cells in the environment. Moreover, several studies have reported that VBNC cells had higher physical and chemical resistance than culturable cells.^{44–46}

Our investigation into the fate of *B. pseudomallei* during a 120-day period in various soil microcosms using the LIVE/DEAD[®] BacLight[™] assay demonstrated both the culturability and viability of a *B. pseudomallei* environmental isolate grown in a control soil microcosm (Figure 4A and B). Our results strengthen previous results demonstrating that endemic soils provided environmental niches for the survival of *B. pseudomallei*. The adaptation of *B. pseudomallei* through morphological alterations may increase its longevity in soil, particularly in endemic areas in which the soil has physicochemical properties suitable for soil-borne pathogens.^{16,17,32} Conversely, the loss of culturability and viability observed in soils with high-osmotic stress (i.e., 1.5–3% NaCl), Figure 5A and B) suggests a method of controlling the *B. pseudomallei* population because our results showed that *B. pseudomallei* lost its viability after 120 days of exposure to osmotic stress. This result confirmed our earlier findings that these conditions could be used to control bacterial numbers.¹⁶

Our results are consistent with the results of previous studies showing that *B. pseudomallei* undergoes a transition from a rod form to a coccoid form in response to environmental

adaptation to soil⁴⁷ under pH or salt stress.^{12,25} The presence of either iron or a low level of salt in the soil affected the culturability of *B. pseudomallei*; in contrast, high soil salinity could eradicate this bacterium. The ability of *B. pseudomallei* cells to alter their morphology and adopt a small particle shape in soil is beneficial for the survival of this species in harsh environments and allows its efficient aerosol transmission from contaminated endemic soil, thus spreading melioidosis.

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