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Survival, Sublethal Injury, and Recovery of Environmental *Burkholderia pseudomallei* in Soil Subjected to Desiccation

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Environmental *Burkholderia pseudomallei* isolated from sandy soil at Castle Hill, Townsville, in the dry tropic region of Queensland, Australia, was inoculated into sterile-soil laboratory microcosms subjected to variable soil moisture. Survival and sublethal injury of the *B. pseudomallei* strain were monitored by recovery using culture-based methods. Soil extraction buffer yielded higher recoveries as an extraction agent than sterile distilled water. *B. pseudomallei* was not recoverable when inoculated into desiccated soil but remained recoverable from moist soil subjected to 91 days' desiccation and showed a growth response to increased soil moisture over at least 113 days. Results indicate that endemic dry tropic soil may act as a reservoir during the dry season, with an increase in cell number and potential for mobilization from soil into water in the wet season.

Recent studies of *Burkholderia pseudomallei*, the etiological agent of the disease melioidosis, suggest a complex relationship between the organism and factors in its host environment, particularly soil, water, climate, landscape position, and human interaction with that environment (1, 2). Fatal pneumonia and sepsis can result from exposure to soil and water in areas where the agent is endemic (Southeast Asia and northern Australia), particularly during the wet season (3, 4). Whereas high rainfall, surface water, and soil moisture appear to be common factors associated with disease distribution in the wet tropics (1), the high incidence of disease in dry tropical settings suggests that *B. pseudomallei* has the capacity to adapt to extremes of soil moisture in a normal annual cycle (5, 6).

Townsville, a coastal, dry tropic Australian city, records high incidences of melioidosis during wet months (January to May; 60% mean annual rainfall, ~1,076 mm) (7–10). June-to-September dry season monthly rainfall averages are <10 mm. Consequently, surface soil varies from very boggy to extremely hard setting (7). Although environmental sampling has demonstrated this bacterium exists in dry soils, there is little quantitative information on its longevity and physiological responses in such soil (8, 9). This includes sublethal injury: the inability to recover otherwise-viable stressed or environmentally adapted cells using growth-based selective culture techniques, the most common method for enumeration of *B. pseudomallei* in soil. We examined the response of a *Burkholderia pseudomallei* strain isolated from Townsville soil to wetting and drying cycles in laboratory microcosms to determine how soil moisture affects the quantitative recovery, sublethal injury, and survival of *B. pseudomallei* in an endemic-area soil. The efficacy of buffer versus distilled water as recovery agents was also tested.

MATERIALS AND METHODS

Soil cores extracted to a depth of 30 cm were collected from sites associated with high disease incidence (10) on the lower western slopes of Castle Hill, Townsville, adjacent to an ephemeral creek (Table 1). For experiments 1 [survival and injury effect(s) in soil] and 2 [soil extraction buffer (SEB) versus water extraction method], a composite sample (depths, 0 to 30 cm) from soil sample 2010-WE3 was used. The moist-versus-dry-soil survival experiment, 3, used soil sample 2011-1. Both soil samples comprise medium-coarse sand with minor clay (pH 6 to 6.5) (Table 1).

Isolation of *B. pseudomallei*. *B. pseudomallei* was isolated from 2010-WE3 (10 to 30 cm depth). Soil screening consisted of 3 5-g subsamples shaken in 5 ml soil extraction buffer (SEB) (200 rpm for 1 h at room temperature). SEB comprised 0.20% Tween 20, 0.85% NaCl, 0.01% Antifoam A, and 0.2% tetrasodium pyrophosphate in sterile distilled water. Soil suspension aliquots were spread plated onto Ashdown's agar (including colistin at 50 mg liter^{-1}) (ASH) and incubated at 37°C for 2 to 14 days. Presumptive *B. pseudomallei* criteria and isolate confirmation by quantitative real-time PCR (qRT-PCR) were as previously described (11).

Survival, sublethal injury, and extraction of *B. pseudomallei* in soil. Soil was sterilized by autoclaving (121°C for 30 min) 3 times over 3 days. Soil was dried at 110°C for 24 h and soil moisture adjusted to 9.5% (wt/wt) with sterile distilled water, typical of *in situ* water content when sampled.

Both soil survival and sublethal injury as well as soil extraction method (SEB versus water) experiments used sterile soil (2010-WE3; 8 × 100 g) inoculated with environmental *B. pseudomallei*. *B. pseudomallei* was cultured on nutrient agar (NA) (37°C for 48 h), resuspended in sterile saline, and inoculated at ~440 CFU per g soil. Inoculum cell concentration was estimated using a predetermined relationship between A_{600} and CFU and then confirmed by serial dilution and spread plating on NA (37°C for 48 h). Over 14 days, 5 g of soil from each sample was shaken in 5 ml SEB at 200 rpm for 1 h. The silty suspension was serially diluted in duplicate in sterile saline before spread plating onto NA and ASH and incubation at 37°C for 3 days. Sublethal injury was assessed by the difference between recoveries on ASH versus NA.

Extraction method efficiency was tested as follows: 3 5-g soil subsamples from the aforementioned inoculated soil (after 70 days' incubation) plus 5 ml sterile SEB or distilled sterile water were shaken (200 rpm at room temperature for either 15 min, 1 h, 2 h, or overnight). Duplicate subsamples were taken from the silty supernatant layer following suspension settling and serially diluted in sterile saline; 100 μl was spread plated onto ASH (37°C for 3 days), and the resultant colonies were enumerated.

Soil drying effects on *B. pseudomallei* survival were monitored as follows: 2 100-g inoculated soil samples (2011-1) were dried at 30°C in a sealed container with desiccant. Another soil sample (100 g) was intermit-

Received 18 October 2012 Accepted 29 January 2013
Published ahead of print 1 February 2013
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doi:10.1128/AEM.03168-12

TABLE 1 Soil characteristics for experimental soil substrate

Parameter	Result[a] for soil sample	
	2010-WE3	2011-1, Castle Hill
Date collected	02 Feb 2010	08 Feb 2011
Location (latitude, longitude)	19°15′27″S, 146°47′34″E	19°15′30″S, 146°47′32″E
Site description	Stream terrace at base of Castle Hill; very low relief, alluvial depositional processes, grass vegetation cover	Sloped creek (ephemeral) bank, alluvial depositional processes, medium to low relief, grassed and treed land reserve adjacent to walking path
Soil description	Overbank sediment deposit; very dark brown; gravelly coarse sandy clay loam, poorly drained, seasonally saturated	Overbank deposit, brown, very coarse sand, clayey, medium to poorly drained, saturated at depth seasonally
Physicochemical features	pH 6 to 6.5; weak pedality; quartz, feldspar, clay (minor kaolinite, illite/mica and smectite); medium- to coarse-grained sand	pH 6; massive texture; quartz, feldspar, clay (major kaolinite, tr to absent smectite, minor to tr illite/mica); medium- to coarse-grained sand

[a] tr, trace.

tently wetted with sterile distilled water and incubated at ca. 84% relative humidity (EasyLog USB datalogger; data not shown). To determine soil moisture loss, soil samples were weighed before and after aseptically removing 5 g subsamples. Bacterial enumeration was as per the aforementioned SEB 1-h extraction procedure (adjusted for soil dry weight). From day 70 to day 105, microcosms were maintained at soil moistures of <0.1% for the desiccated soil and 9.9% (wt/wt) for the moist soil. At day 105, the water content of both microcosms was adjusted to ca. 15% (wt/wt), and *B. pseudomallei* enumeration was performed within 24 h and again after 7 days.

Statistical analyses. Results are presented as arithmetic means. One-way analysis of variance (ANOVA) was performed on SEB versus water soil extraction results and two-way ANOVA was performed for SEB and water versus time, respectively, to determine significance of differences. One-way ANOVA was performed on results for desiccated versus moist soil over time, with $P < 0.05$ (95% level of confidence) considered significant for all analyses.

RESULTS

Survival, sublethal injury, and recovery of *B. pseudomallei* after culture were monitored through recoverable cell extraction over 16 weeks. In contrast to sterile distilled water (dH_2O) ($P = 0.03$), SEB extraction efficiency progressively increased with increased homogenization time, although within one log unit of variation ($P = 5.03E{-}6$; Fig. 1). Sigmoidal *B. pseudomallei* population growth characterizes days 1 to 14 (soil sample 2010-WE3), with no significant difference in recovery on ASH and NA (Fig. 2).

B. pseudomallei recovery from intermittently irrigated soil was significantly different from that from desiccated soil on days 7 to 63 ($P < 0.05$). *B. pseudomallei* remained viable and recoverable in completely desiccated soil to day 70, whereupon recoveries from both soils converged (Fig. 3). From days 14 to 70, *B. pseudomallei* cell counts from the desiccated soil remained within a range of about one order of magnitude (Fig. 3), averaging ca. 2.7×10^5 cells per g soil, and *B. pseudomallei* remained recoverable for up to 105 days in the desiccated soil. No water was added to the intermittently irrigated soil between days 50 and 105. At day 105, after the water content of both microcosms was adjusted to ca. 15% (wt/wt), water-recoverable *B. pseudomallei* showed an average increase of ca. 2.2 orders of magnitude within 24 h (results not shown) and increased to 1.1×10^8 cells per g desiccated soil and 7.1×10^7 cells per g intermittently irrigated soil after 7 days (results not shown). The environmental *B. pseudomallei* survived in microcosm soils for a total of 113 days, after which the experiment was terminated.

DISCUSSION

Although researchers have extracted and isolated *B. pseudomallei* from environmental matrices and in clinical settings for decades, the focus has been largely on the selective growth medium rather than extraction procedure (12–14). However, quantitative extraction and enumeration of microorganisms from soil generally in-

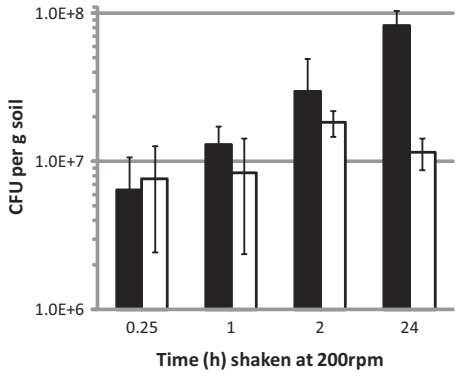

FIG 1 Comparison of extraction efficiency (CFU per g soil) over time (h) using soil extraction buffer (SEB) (■) versus sterile distilled water (□).

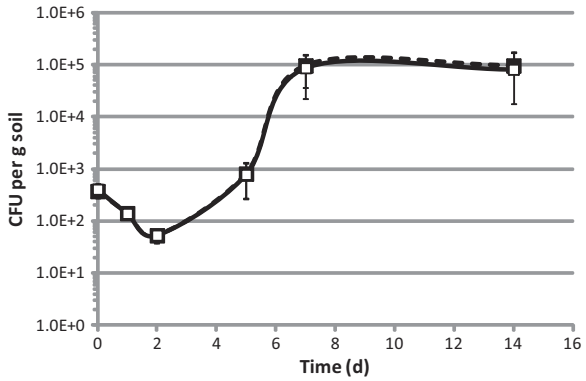

FIG 2 Survival of *B. pseudomallei* strain in soil over 14 days; recovery on selective (ASH) (solid line) and nonselective (NA) (dashed line) media. The difference between recovery on selective and nonselective media was used to assess sublethal injury.

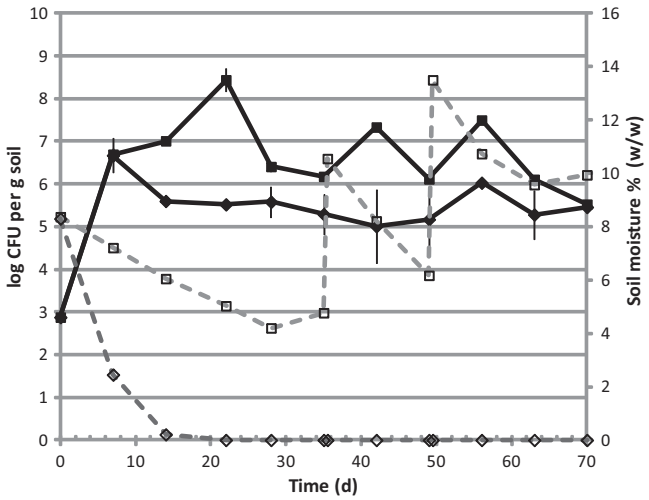

FIG 3 Survival of *B. pseudomallei* strain in desiccated soil (◆, log CFU per g soil; ◇, percentage of soil moisture content) and intermittently irrigated soil (■, log CFU per g soil; □, percentage of soil moisture content).

corporates a homogenization step where soil is shaken and/or incubated with diluent. Our study directly compared the efficiency of water and surfactant-containing extraction buffer as extraction agents for recovery of *B. pseudomallei* from soil (Fig. 1). Although Trung et al. demonstrated increased yield with an extraction buffer (PEG-DOC) versus water, the extraction procedures differed in both the method and length of time for shaking soil in the emulsion and the subsequent soil/supernatant separation step (15). We avoided centrifugation after shaking soils in extraction buffer due to the potential loss of *B. pseudomallei* attached to, or associated with, soil silt and clay fractions. Cells associated with the more mobile clay fraction may be relevant to understanding potential transport of the bacterium in soil and suspension of soil particles in runoff after a rain event (16).

Whereas PCR-based methods of detection of *B. pseudomallei* in soil are potentially more sensitive than culture-based methods for environmental surveys of organism distribution, molecular biological methods yield little information about viability or physiology (17). Insignificant differences in recovery of this environmental *B. pseudomallei* from soil extracts cultured on selective (ASH) and nonselective (NA) media indicate a lack of sublethal injury on long-term soil exposure using this assessment method (Fig. 2). This suggests that the selective agents in modified Ashdown's medium, i.e., crystal violet, gentamicin, and colistin, had no significant effect on the quantitative recovery of the *B. pseudomallei* strain from microcosm soil extracts. In addition, beyond 14-day laboratory incubation, fungal contaminants interfered with bacterial colony counts on NA but not on ASH (results not shown). This result, combined with the increase in extraction efficiency using SEB over 24 h, demonstrates the usefulness of this extraction-enumeration method and a lack of stress-induced sublethal injury associated either with incubation in soil or with shaking with water or surfactant-containing extraction buffer. It is noted that Tween may be used as a carbon source by *Burkholderia* spp. (see, e.g., reference 18), and it is possible that that SEB containing this surfactant stimulated growth of the *B. pseudomallei* environmental isolate.

Our methodology differed from previous studies in that we aimed to elucidate a physiological response of environmental *B. pseudomallei* within a host substrate to varying soil moisture only, a parameter linked to climate-related disease incidence (6). The environmental *B. pseudomallei* exposed in this study remained viable in desiccated soil for at least 91 days and in intermittently irrigated soil for 113 days. This is in contrast to results for *B. pseudomallei* strains isolated from soil and water in China, which survived in desiccated soil only up to 30 days, and clinical strains, which did not survive greater than ca. 25 days at a soil moisture content of 5% (19, 20). Chen et al. (20) concluded that *B. pseudomallei* could survive extended periods in soil only at a minimum 15% water content (20). Similarly, Tong et al. (19) could not recover *B. pseudomallei* within 70 days of exposure to a soil of $<$10% moisture, although the soil type was not described (19). In addition, Palasatien et al. found that sandy soils in northeast Thailand which were positive for *B. pseudomallei* had an average moisture content of 14.92%, while the average soil moisture of negative sample sites was 7.77% (1). Our *B. pseudomallei* environmental isolate incubated in soil at $\leq$10% water content reached a maximal CFU per g soil (ca. 10^8) that was similar to those for clinical isolates incubated in soil with a higher water content (up to 20% [wt/wt]) and 4 orders of magnitude greater inoculum than used in our study (20). In contrast to findings of the study by Tong et al. (19), our environmental *B. pseudomallei* isolate was not recoverable after direct inoculation of bacterial culture into desiccated soil, even after successive, postinoculation soil irrigation (results not shown). While it is possible that our environmental *B. pseudomallei* isolate entered a viable but nonculturable (VBNC) state, our finding suggests that survival of this bacterium is mediated by a life cycle stage which is responsive to environmental conditions (21). Our observations from a dry tropic setting also suggest that *B. pseudomallei* strains may exhibit regional physiologic variability. This hypothesis is supported by the discovery of many varied genes in the *B. pseudomallei* genome which putatively encode secondary metabolic functions, e.g., nutrient acquisition and chemical defense, which could enhance environmental adaptation and survival, such as in soils (22). A VBNC response has been demonstrated in *B. pseudomallei* in response to several stressors, including chlorine, pH, and increased temperature (2). A whole-population VBNC response in soils would preclude culture-based *B. pseudomallei* enumeration and potentially explain discrepancies between nucleic-acid and culture-based assessments of the organism's presence (17). This finding, along with the lack of sublethal injury observed in our study via application of the commonly used Ashdown's medium, suggests that VBNC responses may influence recoverability from soil more than sublethal injury. However, it should be noted that a single soil type was used for exposures, and the soil was sterilized in order to assess injury using a nonselective medium, negating potential soil microbiota-mediated impacts (e.g., grazing, bacteriophages, competition; see reference 2 for a review of potential impacts). Considering the temporal variability in recovery of *B. pseudomallei* from soils in areas where the disease is endemic and soil heterogeneity generally, the relationship between soil characteristics, *B. pseudomallei* presence, and physiological status warrants further investigation.

Extraction of *B. pseudomallei* from soil undergoing successive wetting-drying cycles shows a trend of responsiveness to increased moisture. This indicates that growth of *B. pseudomallei* in dry soil can be stimulated by an increase in soil moisture due to rainfall. A qualitative survey demonstrated the presence and persistence (2 to

12 months) of *B. pseudomallei* in a variety of sampled soils of various levels of moisture content, with bacterial counts from dry soils orders of magnitude lower than those in very moist soil (9).

Soils of the Townsville district are exposed to extremes of rainfall variation both in the short term (daily to weekly) and over an annual cycle of wet and dry seasons. The dry season usually extends from May to October and the wet season from November to April (23). The rainfall pattern can be highly variable in intensity and duration. For example, it is not uncommon to receive half the annual rainfall in a single rain event of 3 to 5 days. Evaporation is high, and even during the wet season, evaporation may on most days exceed rainfall. Hence, formation of soil surface crusts results in high surface runoff. Soil moisture content varies between close to 0% in the dry season to up to 100% saturated in the wet season. The incidence of melioidosis is associated with onset of the wet season, particularly intense rainfall events (6), suggesting that *B. pseudomallei* can remain within the environment during the dry season in a less infectious form and/or at levels and/or distributions of lower overall infectiveness.

The peak risk of septicemic disease in Australia occurs within 2 weeks of the onset of summer rainfall in the tropical north (6). Tong et al. (19) and Baker et al. (24) have postulated that the low water content of dry-season soils reduces the persistence of *B. pseudomallei*, and the prevalence of the bacterium in soils in the wet season may be due to mobilization via the water cycle from other more favorable soil horizons (19, 24). While these observations demonstrate that *B. pseudomallei* can be mobilized in water, our results indicate that *B. pseudomallei* can remain residual and viable in dry soil, an often-conjectured but not previously shown response. This suggests that the relationship between soil and water is one of reservoir and transportation medium, respectively. The precise physiological mechanisms by which *B. pseudomallei* is able to survive and persist in dry soil are yet to be determined. To better understand the persistence of this bacterium in the environment, it is necessary to screen a broader variety of isolates for their responses to environmental exposure in the presence of native biota in heterogeneous soils. In addition, the effect of the relationship between soil moisture and soil physicochemical characteristics on the survival and mobilization of *B. pseudomallei* in the environment will provide insight into the interactions between its complex microbiology, the environment, and human epidemiology that result in disease risk.

ACKNOWLEDGMENTS

This research was supported by a grant from the Townsville Health Service District Private Practice Trust Fund.

Stefan Löher and James Clarke assisted with field and laboratory work. Michael Corkeron and two unnamed reviewers provided helpful editorial advice.

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