Ubiquity of Putative Type III Secretion Genes among Clinical and Environmental *Burkholderia pseudomallei* Isolates in Northern Australia

H. C. Smith-Vaughan,1* D. Gal, 1 P. M. Lawrie, 2 C. Winstanley, 3 K. S. Sriprakash, 4 and B. J. Currie 1

University of Queensland 2 and Queensland Institute of Medical Research, 4 Brisbane, and Menzies School of Health Research, Royal Darwin Hospital, Darwin, Australia, and Department of Medical Microbiology and Genitourinary Medicine, University of Liverpool, Liverpool, United Kingdom 3

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Horseradish peroxidase-like type III secretion (TTS1) genes were present in all 116 Northern Australian *Burkholderia pseudomallei* isolates tested but were not detected in other common environmental *Burkholderia* species. PCR of TTS1 genes may prove valuable as a diagnostic test.

Meliodosis is potentially a life-threatening disease and is endemic in Northern Australia and Southeast Asia (5). The etiological agent of meliodosis, *Burkholderia pseudomallei*, is a soil- and water-dwelling saprophyte capable of causing catastrophic systemic infections in humans and animals (7). Meliodosis is now recognized as the most common cause of fatal community-acquired bacteremic pneumonia in two hyperendemic regions, northeastern Thailand and the Top End of the Northern Territory of Australia (5).

An intriguing difference in meliodosis epidemiology in these two regions where melioidosis is hyperendemic is the lack of correlation between disease incidence and seropositivity in the respective populations. The average annual incidence of melioidosis in the Top End is 16.5 out of 100,000 (34.5 out of 100,000 in the heavy 1998 monsoonal season) with seropositivity ranging between 5 and 13% (5). In comparison, the annual incidence in Ubon Ratchathani in northeastern Thailand is 4.4 out of 100,000, where the majority of the rural population is seropositive (15). Several explanations may account for these observations. In Thailand, underreporting of cases and protective immunity resulting from recurrent exposure may be factors, whereas in the Top End, a higher prevalence of risk factors for disease, such as diabetes and alcohol excess, and/or the presence of more virulent strains may be important (5). Indeed, different isolates appear to have various levels of pathogenicity, as demonstrated in animal models and epidemiological studies. For example, the 50% lethal dose of 42 Australian and Papua New Guinean isolates ranged from 10 CFU to the least virulent of >10^6 CFU in *B. pseudomallei*-susceptible BALB/c mice (17). Furthermore, our studies with isolates from a goat farm demonstrated that the predominant *B. pseudomallei* clone causing disease in the goats was underrepresented in the soil, while the common soil clones were rarely cultured from goats with melioidosis (unpublished data).

Many different approaches are presently under way to elucidate the mechanisms of *B. pseudomallei* pathogenesis, and several factors have been identified to date (10, 12, 18, 21). Recently, the presence of type III secretion (TTS)-associated genes in *B. pseudomallei* was described (1, 19, 20). A variety of gram-negative pathogens of animals and plants can use TTS systems to deliver virulence factors to host cells. The range of diseases caused by these pathogens in different hosts is reflected in the myriad of type III-secreted proteins (9). Winstanley et al. (19) identified a cluster of genes in *B. pseudomallei* with high similarity to genes of a horseradish peroxidase (hypersensitivity response and pathogenicity) locus in *Ralstonia solanacearum* and developed probes targeting a corresponding region in *B. pseudomallei*. Furthermore, mining of the *B. pseudomallei* genome has been used to identify a second related TTS gene cluster (11) and a region resembling the SPI-1 locus of *Salmonella enterica* serovar Typhimurium, including putative homologues of the effectors SipB and SopE which may be involved in apoptosis, cell fusion, and actin-associated membrane protrusion (1). PCR and genome sequence analyses indicate that, unlike the 50% lethal dose-like TTS cluster (TTS1), the other two TTS gene clusters are also present in *Burkholderia thailandensis* and *Burkholderia mallei* (11).

In this study, 116 clinical and environmental *B. pseudomallei* isolates collected in the Top End were screened for TTS1 genes. Some of these isolates exhibited relatively low virulence in mouse models and/or colonized patients without apparent disease. Environmental isolates were cultured from soil and water samples collected from various outback communities and rural and urban properties in the Top End by using Ashdown's medium (2). Environmental *B. pseudomallei* organisms were identified by using an API 20E (bioMerieux, Marcy l'Etoile, France), polyclonal antibody-based latex agglutination, and diagnostic PCR (8). Clinical isolates were identified by MicroScan Walkaway (Dade Diagnostics, Calif.), and veterinary isolates were identified by GN MicroPlate (Biolog, Calif.). Genomic DNA was extracted from *Burkholderia* strains (3), and TTS1 genes were amplified by using conditions previously described (20). For the detection of TTS1 genes by Southern blotting and hybridization, samples of 1 μg of DNA were hybridized to a DIG-labeled TTS1 probe. The probe was generated by using a DIG DNA Labeling Kit (Roche Diagnostics) according to the manufacturer's instructions. E. coli DH5α cells were transformed with a pBluescript II SK+ (Stratagene) vector containing the TTS1 gene cluster (19) and grown on a nitrocellulose membrane (Zeta-Probe; Bio-Rad, Richmond, Calif.). After washing with a DIG blocking reagent, the membrane was hybridized at 45°C with a DIG-labeled TTS1 probe. After washing the membrane, signals were detected by using an enhanced chemiluminescence system (ECL; Amersham Biosciences, Piscataway, N.J.) according to the manufacturer's instructions. DIG-labeled TTS1 genes were amplified by using PCR with primers corresponding to the conserved region of the TTS1 gene cluster (12). The amplified TTS1 genes were cloned into a plasmid and sequenced to confirm the identity of the amplified TTS1 genes.

The prevalence of TTS1 genes among 116 Northern Australian clinical and environmental *B. pseudomallei* isolates tested and a comparison of the prevalence of TTS1 genes among *B. pseudomallei* isolates from Thailand and Australia are shown in Table 1. The prevalence of TTS1 genes was similar between Australian and Papua New Guinean isolates and the isolates from Thailand. PCR was used to type the TTS1 genes into one of two types, A or B. Type A was detected in all isolates from Australia and Papua New Guinea, whereas type B was detected in all isolates from Thailand. The prevalence of TTS1 genes was higher in the Top End of Australia than in Papua New Guinea. The prevalence of TTS1 genes was also higher in isolates from the Top End than in isolates from Thailand. The prevalence of TTS1 genes was similar between Australian and Papua New Guinean isolates and isolates from Thailand.
genomic DNA cut with EcoRI were electrophoresed on a 0.8% agarose gel (13). The probe was prepared by PCR amplification by using the primers described previously (20) and a cosm id clone containing the TTS1 genes as a template. The amplicon was labeled through five additional PCR cycles with [α-32P]dATP. All methods used were as described previously (13). L-arabinose assimilation was determined by using GN MicroPlate, MicroScan Walkaway, or API 20E. Early isolates for which biochemical profiles were not available were tested on minimal agar medium supplemented with 0.2% (wt/vol) L-arabinose as described previously (16).

None of the Top End human, veterinary, and environmental B. pseudomallei isolates in this study assimilated L-arabinose (Ara−); in fact, no Ara− strains (B. thailandensis) have been detected in Australia to date. PCR amplification of TTS1 genes was performed on 116 Top End B. pseudomallei isolates, 12 B. thailandensis isolates from Thailand, and a further 7 isolates of other Burkholderia species (Table 1 and Fig. 1). All human, environmental, and veterinary B. pseudomallei isolates from the Top End were positive for TTS1 genes by PCR. Other Burkholderia species (B. cepacia, B. spinosa, and B. vietnamiensis) commonly encountered by our laboratory in environmental specimens and the B. thailandensis isolates were negative by this test. These results were confirmed by Southern hybridization with the TTS1-specific probe (data not shown).

The ability of a strain of B. pseudomallei to cause clinical illness and the severity of the infection is undoubtedly related to host factors. In the Top End, 80% of melioidosis cases were identified as having one or more associated risk factors, including excessive alcohol consumption (39%), diabetes (37%), chronic lung disease (27%), chronic renal disease (10%), and other risk factors (20%) (4). Furthermore, all but 1 of the 49 fatal cases had at least one identified risk factor (4).

Nevertheless, results from several studies suggest that host factors are not alone in determining the onset and severity of melioidosis and that virulence levels vary between strains of B. pseudomallei. Further variables, including inoculating dose, time between infection and presentation, and the adequacy of treatment can influence clinical severity of melioidosis (17). Our finding that all isolates contained TTS1 genes could not explain the reported variations in virulence among Top End isolates in BALB/c mice (17).

An important marker of B. pseudomallei virulence is its inability to utilize L-arabinose as a sole energy source (Ara−). In contrast, the antigenically cross-reacting Ara+/Ara− ratio in environmental isolates and incidence of melioidosis; a ratio of 1.7 in the northeastern hyperendemic region compared to 0.4 in central Thailand, where melioidosis cases are uncommon (16). To date, all Top End environmental B. pseudomallei isolates have proven to be Ara− (5), which is consistent with the higher incidence of melioidosis in this region. Quantitative counts of environmental B. pseudomallei have also been correlated with disease incidence (14) and may play a role in this region.

In conclusion, TTS1 genes appear to be ubiquitous among Top End clinical and environmental strains of B. pseudomallei. This is consistent with its environmental existence and its relationship with plant hosts. The TTS1 amplicon was not present in other environmental Burkholderia species commonly encountered by our laboratory. Thus, PCR for TTS1 may prove highly useful for primary identification of B. pseudomallei in environmental samples. We are presently investigating its general applicability for diagnostic PCR.

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REFERENCES

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*University of Queensland and Queensland Institute of Medical Research, Brisbane, and Menzies School of Health Research, Royal Darwin Hospital, Darwin, Australia, and Department of Medical Microbiology and Genitourinary Medicine, University of Liverpool, Liverpool, United Kingdom*


Page 883, column 2, lines 10 and 11: “a horseradish peroxidase (hypersensitivity response and pathogenicity) locus” should read “an HRP (hypersensitivity response and pathogenicity) locus.”