Expanded Range of *Burkholderia* Species in Australia

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**Abstract.** This study describes the isolation and characterization of several *Burkholderia* species from soil in northern Australia. Phenotypic and molecular tests indicate that these isolates belong to the species *Burkholderia thailandensis* and *Burkholderia ubonensis*. These observations significantly extend our knowledge of the geographic distribution of these 2 species. Evidence of these species in Australia has implications for bacterial identification in clinical laboratories, diagnostic serology tests, and environmental biodiversity studies.

**INTRODUCTION**

Three bacterial species have been identified in recent years with close taxonomic proximity to the human pathogen *Burkholderia pseudomallei*: *Burkholderia thailandensis*, *Burkholderia oklahomensis*, and the somewhat more distant relative *Burkholderia ubonensis*.1–3 All 4 species are found in surface soils, and careful polyphasic identification is required to distinguish the more pathogenic *B. pseudomallei* from the other 3 species.4 The first of these close relatives proposed as a separate species was *B. thailandensis*, whose distinctive feature is the ability to assimilate L-arabinose.1 *B. thailandensis* is widely regarded as nonpathogenic; however, there have been reports of human infection that resemble aspects of *B. pseudomallei* infection.5,6 The recently named *B. oklahomensis* is another soil saprophyte reported to have caused human infection.5 *B. ubonensis* is a species also originally reported from an environmental sample collected in Thailand that has yet to be identified in human infections.5 Of these 4 *Burkholderia* species, only *B. pseudomallei* has been previously reported in Australia. The assumption that species closely related to *B. pseudomallei* are absent from Australian habitats has simplified bacterial identification procedures in environmental microbiology studies.7 Furthermore, this assumption has resulted in all positive serological screening tests being attributed to *B. pseudomallei* exposure. However, it is possible that these tests may not distinguish between low-level exposure to *B. pseudomallei* and high-level exposure to *B. thailandensis*.8,9 In this report, we present evidence to support a wider biodiversity of *Burkholderia* species in northern Australia and examine their phylogenetic relationship to *B. pseudomallei* recovered from Australian soils.

**METHODS**

**Soil and water sampling.** Water and soil samples were collected from a range of environmental sources in northern Australia. Sterile 250-mL collection bottles containing no preservative were submerged several centimeters into the water body and allowed to fill. A small volume of air was retained in water samples. Soil samples were collected by pushing inverted sterile, 30-mL collection tubes into surface soil. Samples were not refrigerated and were transported in insulated containers. Processing was carried out within 48 hrs of sample collection.

**Isolation and selective enrichment of *Burkholderia* species.** Water samples were vacuum-filtered through 0.2-μm filter papers. The filter papers were then placed into 20 mL of tryptone soya broth (TSB) amended with 10 μg/mL gentamicin. For soil samples, 10-g volumes of soil were added to 15-mL sterile, demineralized water and shaken at room temperature for 4 hrs. The soils were allowed to sediment for another hour without shaking, after which time 1 mL of water was aspirated and inoculated into 20 mL of TSB amended with 10 μg/mL gentamicin. Control broths and plates containing *B. pseudomallei* or distilled water were inoculated concurrently with test samples.

After incubation, 1-mL volumes of TSB enrichment broths were transferred to 30 mL of Ashdown’s broth containing 50 mg/mL colistin. In addition, 10-μL amounts were streaked onto both Ashdown’s agar and *B. pseudomallei*-selective agar (BPSA). After growth in Ashdown’s broths, 10-μL volumes were streaked onto both types of selective agar. All broths and plates were allowed to grow at 37°C for 48 hrs.

**Phenotypic characterization.** Ashdown’s and TSB broths were monitored for pellicle formation, while selective agars were scrutinized for colonies resembling *B. pseudomallei* after both the initial incubation period of 48 hrs and following a further 5-days’ growth at room temperature. Suspect colonies were assessed for resistance to gentamicin and colistin sulfate (10-μg discs on blood agar), and Gram stains and oxidase tests were performed. For putative *Burkholderia* isolates, API20NE assays (bioMérieux, Marcy l’Etoile, France) were also performed. Growth on single-carbon sources were assessed using minimal-salts medium, 5× (pH 6.8; Difco, Detroit, MI), prepared according to the manufacturer’s instructions. Single-carbon sources (Sigma, St. Louis, MO) and agar were added.10 Nitrate utilization was determined according to a standard method11 by inoculating isolates into nitrate broth with Durham tubes (Excel Laboratory Products, Belmont, Western Australia). Isolates were also subjected to fatty acid methyl ester (FAME) analysis by fine-capillary column gas chromatography (MIDI Systems Inc., Newark, DE), according to the manufacturer’s instructions.12

**Nucleic acid extraction, polymerase chain reaction, sequencing, and analysis.** Crude deoxyribonucleic acid (DNA) preparations were generated by suspending 1 large (> 1 mm diameter) bacterial colony in 50 μL of a solution of 0.05 M sodium hydroxide and 0.025% sodium dodecyl sulfate (v/v).13 The suspension was heated at 100°C for 15 min before the addition of 950 μL of sterile DNase/RNase-free water. This

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was thoroughly vortexed and briefly centrifuged to pellet the cell debris. The extracts were diluted 1/10 to produce the inoculum used for all subsequent molecular assays.

Two separate polymerase chain reaction (PCR) assays were performed to exclude *B. pseudomallei* and *B. mallei*. Semi-nested 16s–23s spacer and real-time *fpX* PCRs were performed as previously described. Amplification, sequencing, and analysis of the BUR3–BUR4 amplicon of the *recA* gene was carried out as previously described. The consensus sequence generated for each isolate was compared with the available *recA* sequences to confirm the gene identity and determine the highest percentage match using the basic local alignment search tool (BLAST; www.ncbi.nlm.nih.gov). The consensus sequences and any *Burkholderia recA* sequences publicly available were aligned and trimmed to the length of the BUR3–BUR4 amplicon using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). From alignment, the phylogenetic tree was produced using MEGA v2.1 (http://www.megasoftware.net/) with published computational settings. *Pseudomonas aeruginosa* PA01 *recA* sequence (NC002156) was used as the root for all trees.

**DNA macrorestriction analysis.** Pulsed-field gel electrophoresis (PFGE) was performed using the PulseNet (Centers for Disease Control, Atlanta, GA) *Escherichia coli* method, with the following amendments. Isolates were cultured on brain–heart infusion agar in a humidified environment at 37°C for 18 hrs. Chromosomal DNA was digested overnight with restriction endonuclease *XbaI* and run on a 1% SeaKem Gold (Cambrex Bio Science, Rockland, ME) agarose gel, after pre-electrophoresis, with a pulse time and ramp of 5.5–52 s for 20.2 hrs at 6 V/cm on a CHEF DRIII apparatus (Bio-Rad, Hercules, CA). The standard H49812 was used. After ethidium bromide staining, a gel image was obtained using the Gel Doc imaging system (Bio-Rad) and relatedness of PFGE patterns was analyzed with the Bionumerics software package Version 3.2 (Applied Maths, Kortrijk, Belgium). The dendrogram was calculated by using the un-weighted pair group method (UPGM) using average linkages and the Dice coefficient, with a position tolerance of 1% and an optimization of 0.5%. In addition to several newly isolated strains, a panel of *B. thailandensis* strains was also subjected to DNA macrorestriction analysis. The Western Australian *B. pseudomallei* outbreak strain, National Collection of Type Cultures (NCTC) 13177, was included for comparison purposes.

**Whole-genome sequencing and in-silico multilocus sequence typing analysis.** *Burkholderia* isolates 49639 and A1301 were submitted for whole-genome shotgun sequencing to Tim Reid at The Naval Medical Research Center Annex (Rockville, MD). Following public release of the genomes via DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank, multilocus sequence typing (MLST) analysis was performed by extracting the target genes sequences from the sorted contigs using BioEdit software. Isolate 49639 was examined using the *B. pseudomallei* MLST website (http://bpsuomallei.mlst.net/misc/info.asp) developed by Daniel Godoy and Gaynor Randle (Imperial College). Isolate A1301 was examined using the *Burkholderia cepacia* complex MLST website (http://pubmlst.org/bcc/).

**RESULTS**

During the course of environmental isolation, 4 *Burkholderia* isolates were identified that presented with atypical characteristics. Isolates A1301 and A10A02 were recovered from environmental samples collected in the east Kimberley region of Western Australia. Isolates 17540 and 49639 were found in soil and sediment samples from the Northern Territory. The sample sites in the Northern Territory were geographically separated from those in Western Australia by approximately 500 km. All 4 *Burkholderia* isolates were subjected to a range of biochemical and molecular tests to evaluate their taxonomic status.

**Phenotypic characterization.** All 4 isolates were Gram-negative rods with examples of bipolar staining. Colonies grew well on the selective agars, developing distinctive appearances as colonies matured (Figure 1). API20NE identified all 4 isolates as *B. cepacia*. All 4 isolates were oxidase-positive, gentamicin- and colistin-resistant, and capable of *L*-arginine and *L*-arabinose assimilation. Isolates A1301 and 17540 grew well on maltose or *L*-arabinose as single-carbon sources, whereas isolates A10A02 and 49639 grew on *L*-arabinose but not on maltose when supplied as single-carbon sources. Isolates A10A02 and 49639 were able to convert nitrate to gas, whereas isolates A1301 and 17540 were only able to convert nitrate to nitrite.

FAME analysis indicated none of the 4 strains contained 2-hydroxymyristic acid (14:0 2OH) residues. This phenotypic marker is useful in differentiating *B. thailandensis* from *B. pseudomallei*. Results of FAME analysis are summarized in Table 1.

**PCR and sequencing.** Both *B. pseudomallei*-specific PCR assays used yielded negative results for all 4 isolates. Isolates A10A02 and 49639 were both close matches to *B. thailandensis* E264 (CP000086), with 99.2% and 98.2% matches across the 386-bp–sized amplicons, respectively. Isolates A1301 and 17540 were close matches to *B. ubonensis* LMG 20358 (AY780511), with 97.7% and 98.4% matches, respectively, for the same amplicons. Using the sequenced *recA* PCR products, a phylogenetic tree was generated. This tree included DNA sequences from any other *Burkholderia* species spanning the same region (Figure 2). The close relationship between isolates A10A02, 49639, and *B. thailandensis* E264 is illustrated. This tree also illustrates the close relationship of *B. ubonensis* LMG 20358, isolate A1301, and isolate 17540. These sequences were uploaded to the GenBank database in November 2006 and are available with the following accession numbers: EF043037 (*Burkholderia* species A1301), EF043038 (*Burkholderia* species A10A02), EF043039 (*Burkholderia* species 49639), and EF050105 (*Burkholderia* species 17540).

**DNA macrorestriction analysis.** DNA macrorestriction analysis by PFGE of *B. thailandensis*-like isolates A10A02 and 49639 indicated a greater similarity to the *B. thailandensis* isolates than to *B. pseudomallei* NCTC 13177 (Figure 3). According to the UPGM dendrogram, the 2 *B. thailandensis* isolates were linked to each other at the 86% level and to the panel of known *B. thailandensis* isolates at 74–83.5%. The Western Australian outbreak strain of *B. pseudomallei* was linked at 62% relatedness to A10A02 and 49639.

**Whole-genome sequencing and in-silico MLST analysis.** *Burkholderia* isolates’ 49639 and A1301 Whole-Genome Shotgun projects were deposited at DDBJ/EMBL/GenBank
in June 2007 under the project accession numbers ABBH00000000 and ABBE00000000, respectively. The versions described in this paper are the first versions, ABBH01000000 and ABBE01000000, respectively.

*Burkholderia* isolate 49639 returned MLST type sequences of 6 (*ace*), 10 (*gltB*), 15 (*gmhD*), 29 (*lepA*), 9 (*lipA*), 14 (*narK*), and 9 (*ndh*). This was most similar to 3 profiles in the database. In all 3 cases, the MLST profile of isolate 49639 differed at only one locus: *lipA* for strains 132/02 and E0177 (Sequence Type 352) and *lepA* for E0433 (Sequence Type 361). All 3 of these profiles were generated from isolates described as *B. thailandensis*. Further widening of the terms of the profile comparison to “5 or more loci matching” returned a greater number of results, all of which were identified as *B. thailandensis*. In the case of isolate *Burkholderia* A1301, none of the MLST sequences corresponded to known alleles, and a sequence type (ST) could not be determined. These sequences were submitted to the curator of the *B. cepacia* complex MLST website (Adam Baldwin) to create new ST and allele profiles.

**DISCUSSION**

This is the first confirmation that either *B. thailandensis* or *B. ubonensis* are present in Australia. Recognition that these

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<th>Fatty acid methyl ester (FAME) peak*</th>
<th>Percentage of total FAME content† for</th>
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<tr>
<td></td>
<td><em>Burkholderia thailandensis</em> A10A02</td>
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<tr>
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<td>18:0 2OH</td>
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* FAME (retention time) peak corresponding to the respective fatty acid methyl ester.
† Values are percentages of the total bacterial FAME content made up by the derivative of the named fatty acid. Additional fatty acid derivatives quantified but present in only trace amounts (< 0.5%) are not shown but contribute to the total cellular fatty acids on which the percentage calculations were based. Cellular fatty acids that were not detected at all for a given strain are shown as “ND.”
§ Data from Ref. 3. Cellular fatty acid contents not available denoted as “NA.”
¶ Summed feature 2 comprises 14:0 3OH and/or 16:1-iod.
†† Summed feature 3 comprises 16:1 ω7c and/or 16:1 ω6c for A10A02 only. All other isolates are 16:1 ω7c and/or 15-iso 2OH.

**TABLE 1**

FAME analysis of *Burkholderia* species

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**FIGURE 1.** Colonies of *Burkholderia thailandensis* A10A02 (A), *B. thailandensis* 49639 (B), *Burkholderia ubonensis* A1301 (C), and *B. ubonensis* 17540 (D) on *Burkholderia* pseudomallei-selective agar after 5-days’ growth.
Burkholderia species are present in Australia has important practical implications for bacterial identification in clinical laboratories, diagnostic serology tests, and environmental bacterial biodiversity studies.

Maltose utilization was found to distinguish B. thailandensis from B. ubonensis, as described previously. Similarly, assimilation of L-arabinose effectively differentiated the B. thailandensis isolates from B. pseudomallei in our collection. These tests were performed by incorporating these sugars as sole carbon sources into agar plates. Oxidation–fermentation type reactions involving complex media, with acid production leading to a color change, were not found to be suitable for isolate discrimination based on maltose, L-arabinose, or L-arginine utilization. In these tests, a false-positive result could be recorded for an isolate that did not possess the ability to metabolize the single-carbon source in question. Nitrate utilization profiles of isolates A10A02 and 49639 were consistent with the established nitrate to gas phenotype of B. thailandensis.

FIGURE 2. Phylogenetic tree based on recA sequences showing proximity of Australian isolates A10A02, 49639, A1301, and 17540 to the Burkholderia thailandensis type strain E264 and the Burkholderia ubonensis type strain LMG 20358 with bootstrap values at the nodes. Bar represents 0.1 substitution per site.

FIGURE 3. Results of pulsed-field gel electrophoresis of Burkholderia thailandensis isolates from Western Australia with comparison B. thailandensis strains from Thailand. A10A02 and 49639 are the two newly described Australian isolates. The E strains are all B. thailandensis from Thailand. National Collection of Type Cultures (NCTC) 13177 is an Australian Burkholderia pseudomallei, which was included for comparative purposes.
densis. Similarly, nitrate metabolism of isolates A1301 and 17540 (nitrate to nitrite only) was in agreement with the description of *B. ubonensis*.

The FAME peak 14:0 20H, corresponding to the 2-hydroxymyristic acid commonly detected in *B. pseudomallei* was not present in any of these 4 isolates. Some fatty acids corresponded with type strains, whereas others differed markedly from published fatty acid profiles. These discrepancies could be due to small variations in fatty acid content between assays performed at different laboratories under varying growth conditions or may reflect genuine differences between Australian and southeast Asian *Burkholderia* species.

Of the 4 organisms described in this report, none was found to be positive by either of the *B. pseudomallei*-specific PCR assays used. Both of these assays have previously been shown to discriminate between *B. pseudomallei* and *B. thailandensis*. However, *B. ubonensis* was not available during validation of these assays, so potential cross-reactivity could not be excluded. Sequencing of a portion of the *recA* gene enables differentiation of closely related *Burkholderia* species. This approach was shown to distinguish *B. thailandensis* and *B. ubonensis* both from each other and from all other known *Burkholderia* species. Both pairwise and multiple sequence comparisons demonstrated that isolates A10A02 and 49639 were most closely related to *B. thailandensis*, and isolates A1301 and 17540 were equally well matched to *B. ubonensis*. DNA macrorestriction analysis was performed on the 2 *B. thailandensis*-like organisms to further evaluate their relationship to other *B. thailandensis* strains and to *B. pseudomallei*. These 2 isolates were more closely grouped with the *B. thailandensis* strains from Thailand than with an Australian *B. pseudomallei* isolate. Whole-genome sequencing followed by *in-silico* MLST indicated that isolate 49639 is closely related to *B. thailandensis*. MLST of isolate A1301 did not produce a match to any isolates in the *B. cepacia* MLST database. However, the database did not include any *B. ubonensis* profiles.

On the basis of these results, we propose the following provisional designation for the isolated organisms: *Burkholderia* thailandensis A10A02, *Burkholderia* thailandensis 49639, *Burkholderia* ubonensis A1301, and *Burkholderia* ubonensis 17540.

The recognition that both *B. thailandensis* and *B. ubonensis* are present means that systematic surveys in northern Australia are needed to map the extent of their distribution in the tropical rhizosphere and other habitats. In addition, culture collections of Australian *Burkholderia* should be re-evaluated for the presence of these species. The 2 *B. thailandensis* strains described here were not isolated from the same location in Northern Australia but were separated by more than 500 km. Likewise, the 2 *B. ubonensis* strains were isolated from the same distinct areas. Discrimination between *B. pseudomallei* and *B. thailandensis* requires at least an additional series of substrate utilization tests, as previously documented. The well-recognized limitation of routine laboratory identification tests for *B. pseudomallei* argues in favor of a more formal polyphasic identification process, including molecular methods. In view of reports claiming melioidosis-like infection caused by strains identified as *B. thailandensis*, this species may not retain its status as a strict non-pathogen.

The argument that *B. thailandensis* and *B. ubonensis* did not exist in Australia made identification of *Burkholderia* species from clinical samples a little easier. Recognition of the presence of these species on this continent places a burden on the clinical laboratory to use more-discriminating bacterial identification methods. L-Arabinose utilization is one such simple preliminary test which can discriminate between *B. pseudomallei* and closely related species. Without such tests we cannot now be certain whether *B. thailandensis* isolates are missed either as a cause of pulmonary disease or as contaminants of surface lesions. The additional significance of these species to the diagnostic laboratory is in the interpretation of serological tests, particularly the indirect hemagglutination assay (IHA), which relies on a crude antigen preparation. The IHA is widely used because of its low cost and speed, despite its low specificit and sensitivity. In Australia, the specificity of the IHA has not been questioned because *B. thailandensis* was not previously known to be present. The assumption that *B. thailandensis* was absent has possibly contributed to an over-reliance on serodiagnostic methods for melioidosis. In view of our observations, this specificity of melioidosis serodiagnosis needs to be re-evaluated in Australia. It is possible that some borderline IHA results may reflect recent or high-level exposure to *B. thailandensis* rather than to *B. pseudomallei*. In a recent study in Australia, 88% of serum samples from patients with culture-confirmed melioidosis contained antibodies reactive with *B. thailandensis* antigen. However, there is a lack of data on the proportion of individuals with low-to-medium *B. pseudomallei* IHA titers but with high *B. thailandensis* IHA titers. If borderline melioidosis IHA results can reflect exposure to *B. thailandensis*, then serological surveys in some remote Australian communities may overestimate subclinical melioidosis disease load.

The increased diversity of *Burkholderia* species we have observed in northern Australia raises questions as to the evolutionary origins of these isolates and, indeed, their process of speciation. Our observations do not indicate an obvious evolutionary source of *B. thailandensis* or *B. ubonensis* either in southeast Asia or Australia. If the geographic distribution of *B. thailandensis* includes North America and Australia, and the distribution of *B. ubonensis* also includes Australia, then have these species been transported between continents or has there been a series of parallel speciation events? Whole-genome sequencing of additional isolates may help to clarify their phylogenetic origin.

In conclusion, our observations of *B. thailandensis* and *B. ubonensis* in northern Australia indicate that the geographic distribution of these species is much wider than previously thought. This recognition of an expanded biodiversity of *Burkholderia* species in the topical north of Australia highlights the value of further studies into the distribution of *Burkholderia* species in our region.

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