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Burkholderia humptydooensis sp. nov., a New Species Related to *Burkholderia thailandensis* and the Fifth Member of the *Burkholderia pseudomallei* Complex

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ABSTRACT During routine screening for *Burkholderia pseudomallei* from water wells in northern Australia in areas where it is endemic, Gram-negative bacteria (strains MSMB43^T, MSMB121, and MSMB122) with a similar morphology and biochemical pattern to *B. pseudomallei* and *B. thailandensis* were coisolated with *B. pseudomallei* on Ashdown's selective agar. To determine the exact taxonomic position of these strains and to distinguish them from *B. pseudomallei* and *B. thailandensis*, they were subjected to a series of phenotypic and molecular analyses. Biochemical and fatty acid methyl ester analysis was unable to distinguish *B. humptydooensis* sp. nov. from closely related species. With matrix-assisted laser desorption ionization–time of flight analysis, all isolates grouped together in a cluster separate from other *Burkholderia* spp. 16S rRNA and *recA* sequence analyses demonstrated phylogenetic placement for *B. humptydooensis* sp. nov. in a novel clade within the *B. pseudomallei* group. Multilocus sequence typing (MLST) analysis of the three isolates in comparison with MLST data from 3,340 *B. pseudomallei* strains and related taxa revealed a new sequence type (ST318). Genome-to-genome distance calculations and the average nucleotide identity of all isolates to both *B. thailandensis* and *B. pseudomallei*, based on whole-genome sequences, also confirmed *B. humptydooensis* sp. nov. as a novel *Burkholderia* species within the *B. pseudomallei* complex. Molecular analyses clearly demonstrated that strains MSMB43^T, MSMB121, and MSMB122 belong to a novel *Burkholderia* species for which the name *Burkholderia humptydooensis* sp. nov. is proposed, with the type strain MSMB43^T (American Type Culture Collection BAA-2767; Belgian Co-ordinated Collections of Microorganisms LMG 29471; DDBJ accession numbers CP013380 to CP013382).

IMPORTANCE *Burkholderia pseudomallei* is a soil-dwelling bacterium and the causative agent of melioidosis. The genus *Burkholderia* consists of a diverse group of species, with the closest relatives of *B. pseudomallei* referred to as the *B. pseudomallei* complex. A proposed novel species, *B. humptydooensis* sp. nov., was isolated from a bore water sample from the Northern Territory in Australia. *B. humptydooensis* sp. nov. is phylogenetically distinct from *B. pseudomallei* and

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other members of the *B. pseudomallei* complex, making it the fifth member of this important group of bacteria.

KEYWORDS *Burkholderia humptydooensis* sp. nov., *Burkholderia pseudomallei* complex, MSMB43^T

Burkholderia species are abundant and occupy diverse ecological niches, including soil, plants, animals, and humans. Probably the most diverse and environmentally adaptable plant-associated bacteria also belong to the genus *Burkholderia* (1). Many species of *Burkholderia* have been described since the discovery of *B. cepacia* by W. H. Burkholder in 1949 as the cause of onion rot (2); this species was later recognized as a human pathogen. Currently, there are more than 90 identified species in this genus (3, 4). There has been a proposal to divide the species into two genera, one of which would retain the *Burkholderia* name and the other which would be *Paraburkholderia* gen. nov. (5). At least 20 closely related species belong to the *Burkholderia cepacia* complex, with many of these soil-dwelling species considered opportunistic pathogens for immunocompromised individuals and other species considered to have both mutualistic and pathogenic roles in plants (6).

Notably, there are two *Burkholderia* species that can cause severe human and animal diseases: *B. pseudomallei* and *B. mallei*, the causative agents of melioidosis and glanders, respectively. *B. pseudomallei* is a major cause of community-acquired sepsis in northeast Thailand and northern Australia (7). Due to the concerns of their potential use as weapons of mass destruction, federal health agencies in the United States have recently classified these species as Tier 1 (top tier) disease agents (8). It has been well established that *B. mallei* is a clone of *B. pseudomallei* that became a host-adapted pathogen in equines, resulting in a massive genome reduction (9). Genetically, both *B. pseudomallei* and *B. mallei* are members of the *B. pseudomallei* phylogenetic group or complex (10). Three additional closely related species have been identified so far in this group: *B. thailandensis* (11), *B. oklahomensis* (12), and a newly identified *B. thailandensis*-like species (13, 14). These closely related species are soil saprophytes and are considered nonpathogenic, although a few strains of *B. thailandensis* and *B. oklahomensis* have been described as causing clinical infection in humans (12, 15).

RESULTS AND DISCUSSION

Bacterial growth and characteristics. As described previously (14), *B. humptydooensis* sp. nov. MSMB43^T did not grow when incubated at temperatures higher than 42°C and also produced little or no gas from nitrate. On Columbia blood agar, smooth and creamy white colonies were observed after 24 h, whereas red, convex, and small (1- to 2-mm) colonies were observed on MacConkey medium after 48 h. Dry and wrinkled colonies were observed on Ashdown's agar after 72 h of growth (Fig. 1), similar to the appearance of *B. pseudomallei*, while slimy, confluent, honey-like growth appeared on Standard I medium after 48 h (Fig. 1). Variations in colony morphology may exist within MSMB43^T, as the morphology of MSMB43^T on Ashdown's agar was previously reported as smooth and round colonies (16). Bacterial growth was visible on all media after incubation at 25 to 42°C for at least 24 h, with the best growth observed on Columbia blood agar. No growth was observed at 8°C and 45°C. The optimal temperatures for growth were between 28 and 37°C aerobically. All strains showed Gram-negative bipolar staining, appearing as rods of 2 to 3 μm in length and 0.4 to 0.8 μm in diameter. All strains were motile in semisolid media. Biochemical differentiation of *B. humptydooensis* sp. nov. from *B. pseudomallei* and *B. thailandensis* was possible by screening for the presence of tryptophan, esculin, or the assimilation of arabinose (to distinguish from *B. pseudomallei*) and the assimilation of maltose (to differentiate from *B. thailandensis*) (Table 1). All strains were positive for nitrate, gelatin, glucose, mannose, mannitol, *N*-acetylglucosamine, gluconate, caprate, adipate, malate, citrate, and phenylacetate. All strains were negative for glucose (acidification) and urea (Table 1).

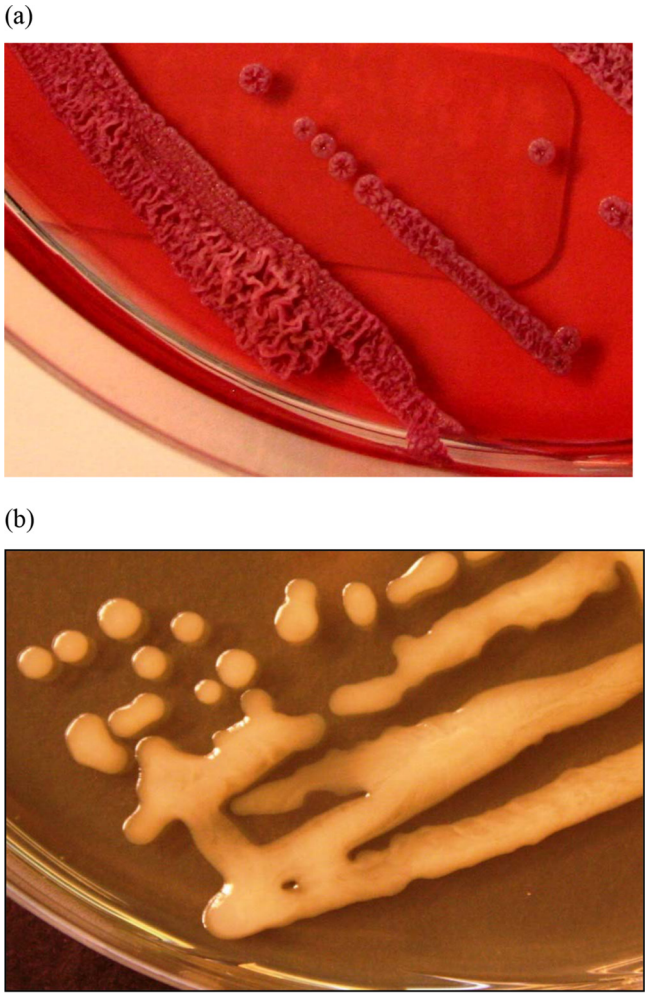

FIG 1 Colony morphology of *B. humptydooensis* sp. nov. MSMB43^T. Cultures were grown on Ashdown's agar (a) or on Standard I nutrient agar (b).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) of the three isolates showed a cluster with other members of the *B. pseudomallei* complex (see Fig. S1 in the supplemental material). Fatty acid methyl ester analysis was unable to distinguish among the fatty acid profiles from the three *B. humptydooensis* sp. nov. strains and the closely related species (five *B. thailandensis*, two *B. oklahomensis*, and three *B. ubonensis* strains) (Fig. S2).

Antimicrobial susceptibility and virulence screening. Based on the CLSI breakpoints for *B. pseudomallei*, all strains were determined to be susceptible *in vitro* to ceftazidime, imipenem, trimethoprim-sulfamethoxazole, and doxycycline, whereas re-

TABLE 1 Phenotypic characteristics of *B. humptydooensis* sp. nov. and closely related species within the *B. pseudomallei* group

Biochemical reaction	Characteristic (compound present in medium or assimilated by strain)				
	B. pseudomallei K96243[a]	*B. thailandensis* E264^T	*B. humptydooensis* sp. nov. MSMB43^T	*B. humptydooensis* sp. nov. MSMB121	*B. humptydooensis* sp. nov. MSMB122
Tryptophan	+	−	−	−	−
Arginine	+	−	+	−	−
Esculin	−	+	+	+	+
PNPG	−	−	−	−	+
Arabinose assimilation	−	+	+	+	+
Maltose assimilation	−	+	−	−	−

[a]Data for *B. pseudomallei* K96243 were obtained from Wuthiekanun et al. (29).

TABLE 2 Summary of MICs determined in triplicate by the broth microdilution method

Antimicrobial substance	MIC (mg/liter)		
	MSMB43^T	MSMB121	MSMB122
Amoxicillin-clavulanic acid[a]	32/16	32/16	32/16
Ceftazidime	4	2	4
Imipenem	0.5	0.5	0.5
Rifampin	>8	>8	8
Chloramphenicol	8	4	4
Trimethoprim-sulfamethoxazole	0.5/9.5	≤0.25/4.75	≤0.25/4.75
Streptomycin	>32	>32	>32
Gentamicin	32	32	>32
Doxycycline	1	1	1
Tigecycline	2	4	4
Ciprofloxacin	0.5	1	1
Levofloxacin	0.5	1	1

[a]Resistance was observed, based upon the CLSI breakpoints of *B. pseudomallei*.

sistance to amoxicillin-clavulanic acid was observed (Table 2). The antimicrobial susceptibility pattern of *B. humptydooensis* sp. nov. generally resembled that of *B. pseudomallei* (17–20). No significant differences were observed either among the three strains or between the two different susceptibility testing methods. As the maximum concentration of aminoglycosides in the microtiter plates was 32 mg/liter, high-level streptomycin resistance but low-level gentamicin resistance were confirmed using the Etest method (data not shown).

Neither *B. humptydooensis* sp. nov. nor *B. thailandensis* caused mortality in any mice when delivered via the subcutaneous (s.c.) route, nor did any mice show outward signs of illness. In comparison, s.c. infections of fully virulent *B. pseudomallei* results in 50% mortality within 10 days at a dose of 10^3 CFU (21). It remains unknown if the inhalation route increases the pathogenicity of species tested in the same way as *B. thailandensis* E264^T, which can cause high mortality in mice at doses of 10^4 to 10^6 CFU when delivered as an aerosol (22–24).

Genetic and genomic comparative analyses. Four rRNA operons are present on the MSMB43^T chromosomes, of which two unique versions were found (AQ610_12930/AQ610_01425 and AQ610_21350/AQ610_02540). The genomes of strains MSMB43^T, MSMB121, and MSMB122 each consisted of two chromosomes (Table 3). These two copies of the 16S rRNA genes were different, which led to ambiguities in conventional sequencing (Fig. S3). The 16S rRNA gene sequence similarities of *B. humptydooensis* sp. nov. to other members of the *B. pseudomallei* complex (*B. thailandensis*, *B. mallei*, and *B. oklahomensis*) were 99%. Phylogenetic reconstruction of 16S rRNA and *recA* sequences confirmed genetic proximity to the *B. pseudomallei* complex but also determined that all *B. humptydooensis* sp. nov. strains formed their own group within this complex (Fig. S3 and S4).

All three *B. humptydooensis* sp. nov. strains, MSMB43^T, MSMB121, and MSMB122, are sequence type 318 (ST318), and there are no other representatives of this ST. Overall, phylogenetic analysis using multilocus sequence typing (MLST) data supports the

TABLE 3 Whole-genome data[a] for *B. pseudomallei* group organisms

Species and strain	GC content (%)	Genome size (Mb)	No. of CDS[c]
B. humptydooensis sp. nov. MSMB43^T	67.1	7.3[b]	6,324
B. humptydooensis sp. nov. MSMB121	67.5	6.7	5,795
B. humptydooensis sp. nov. MSMB122	67.5	6.8	5,845
B. thailandensis E264^T	67.6	6.7	5,652
B. oklahomensis C6786^T	66.9	7.1	6,097
B. pseudomallei K96243	68.1	7.2	5,948
B. mallei ATCC 23344^T	68.5	5.8	5,006

[a]Two chromosomes are present in all genomes shown.
[b]One plasmid present.
[c]CDS, coding DNA sequences.

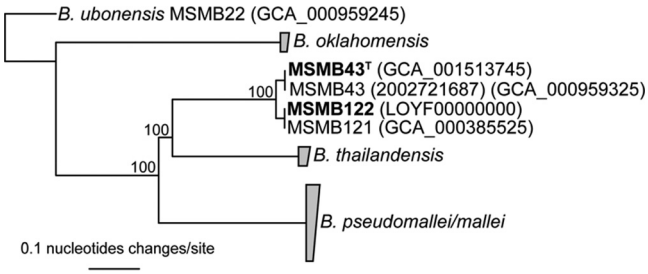

FIG 2 Core genome phylogeny of *B. humptydooensis* sp. nov. SNPs from the comparison of four *B. humptydooensis* sp. nov. genomes and representatives of the other closely related species were used to reconstruct the phylogenetic relationships. Genomes from this study are shown in bold and assembly numbers are provided inside parentheses. Numbers at nodes indicate bootstrap support values. Collapsed nodes are shown in gray.

separation of *B. humptydooensis* sp. nov. from the other *B. pseudomallei* complex members, as described previously (14).

The PacBio sequencing resulted in one finished assembly (for MSMB43^T) and one mostly finished assembly with 4 contigs (MSMB122) (Table 3). The MSMB43^T genome had one circular contig ~305 kb long that appears to be a plasmid; this same sequence is also present in the previously completed genome of *Burkholderia* sp. MSMB43^T (alternately known as 2002721687 [BioProject no. PRJNA239255]). A comparative genomics approach using large-scale BLAST score ratios (LS-BSR) (25) demonstrated that a large (~35-kb) stretch of the *B. pseudomallei* K96243 genome (UniProt gene names BPSS1165 [accession number Q63L43] to BPSS1184 [accession number Q63L24]) on chromosome 2 is highly conserved (>98% identity) in the plasmid sequence, suggesting a shared origin for these regions. The core genome phylogeny demonstrated the position of *B. humptydooensis* sp. nov. in relation to other clades in the *B. pseudomallei* complex and confirms the results from other methods (Fig. 2).

Among the three tested *B. humptydooensis* sp. nov. genomes, the calculated genome-to-genome distance calculation (GGDC) and average nucleotide identity (ANI) values were in the range of 93 to 99% and 98 to 99%, respectively (Table 4). The high GGDC and ANI values indicate that all of these tested strains belong to a single species, including the proposed *B. humptydooensis* sp. nov. type strain MSMB43^T. As expected from whole-genome sequencing (WGS) analyses, strain MSMB43^T had a slightly lower GGDC similarity (93%) than the other two *B. humptydooensis* sp. nov. strains, which were approximately 97% similar. Further GGDC analysis (Table 4) determined that the similarities of all *B. humptydooensis* sp. nov. strains to all other tested *Burkholderia* species in the *B. pseudomallei* complex were less than 70%, with the highest detected similarity being between *B. humptydooensis* sp. nov. and *B. thailandensis* (51.1% [± 3.2%]) (mean ± confidence interval [CI]). This confirmed that the three tested strains are not *B. thailandensis* but rather a distinct species. The GGDC similarity between *B. mallei* and *B. pseudomallei* was 92.5%, which confirmed previous conventional DNA-DNA hybridization (DDH) results and demonstrated that, from a strict taxonomic point of view, they belong to a single species (9).

In conclusion, we have utilized comprehensive genotyping techniques, including 16S rRNA, *recA*, MLST, and whole-genome-based GGDC, to further support the existence of a new species that is distinct but genetically related to the four members of the *B. pseudomallei* complex (*B. pseudomallei*, *B. mallei*, *B. thailandensis*, and *B. oklahomensis*). These analyses confirm the speciation of *B. humptydooensis* sp. nov., a soil bacterial saprophyte found in the Northern Territory of Australia, where melioidosis is highly endemic. The addition of *B. humptydooensis* sp. nov. as a new member of the *B. pseudomallei* complex will benefit evolutionary studies of *B. pseudomallei*, the serious bacterial pathogen that shares a similar ecological niche with this new species.

TABLE 4 GGDC and ANI values for whole-genome sequence similarities

Species and strain	GGDC or ANI value for comparison with genome of[a]:						
	B. humptydooensis sp. nov. MSMB43^T	*B. humptydooensis* sp. nov. MSMB121	*B. humptydooensis* sp. nov. MSMB122	*B. thailandensis* E264^T	*B. oklahomensis* C6786^T	*B. pseudomallei* K96243	*B. mallei* ATCC 23344^T
B. humptydooensis sp. nov. MSMB43^T		98.81	98.98	93.56	91.80	91.17	93.22
B. humptydooensis sp. nov. MSMB121	93.1 ± 2.3		99.84	93.59	91.80	93.39	93.26
B. humptydooensis sp. nov. MSMB122	93.1 ± 2.3	97.3 ± 0.02		93.60	91.86	93.23	93.25
B. thailandensis E264^T	51.1 ± 3.2	51.4 ± 3.2	51.3 ± 3.2		91.48	93.01	93.05
B. oklahomensis C6786^T	43 ± 3.1	43.1 ± 3.1	43.1 ± 3.1	40.7 ± 3.1		91.17	91.33
B. pseudomallei K96243	48.6 ± 3.1	48.6 ± 3.2	48.6 ± 3.2	45.6 ± 3.3	39.6 ± 3.1		99.04
B. mallei ATCC 23344^T	48.7 ± 3.1	49.1 ± 3.1	49.1 ± 3.1	45.7 ± 3.2	40.3 ± 3.1	92.5 ± 2.8	

[a]Genome-to-genome distance calculations (with confidence intervals) are shown in the bottom left half of the matrix (below the line of identity, i.e., the line formed by blank cells for comparisons of strains with themselves); average nucleotide identities are shown in the top right half of the matrix. Values in shaded boxes represent values above the similarity threshold that defines members of the same species.

TAXONOMY

Burkholderia humptydooensis sp. nov. (hump.ty.doo.en'sis. L. gen. adj. *humptydooensis*, pertaining to Humpty Doo, a small town in Northern Territory of Australia, where the first member of this species was isolated).

Bacilli, 0.4 to 0.8 μm in diameter and 2 to 3 μm in length, arranged individually or in irregular clusters. The organism is Gram negative with bipolar staining, motile, and non-spore forming. Growth is observed in a temperature range of 25 to 42°C within 24 to 48 h on various standard solid media. Within 24 h, small colonies (0.5 to 1 mm) are formed on nonselective media (Columbia blood and Standard I) and after 48 h also on selective media (Ashdown's, MacConkey). Best growth occurs at 28 to 37°C after $\geq$24 h. Colonies become confluent and honey-like in appearance on glycerol-containing medium (Standard I) after 48 h. On Ashdown's selective agar, highly wrinkled purple colonies are observed at $\geq$48 h, thus resembling the growth of *B. pseudomallei*.

Assimilation (API 20NE) was found for D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid, while results were negative for D-maltose. Esculin and gelatin are hydrolyzed. Variable reactions with L-arginine and 4-nitrophenyl-β D-galactopyranoside (PNPG).

Positive (API ZYM) for alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, acidic phosphatase, and naphthol-AS-BI-phosphohydrolase. Enzymes absent on API ZYM are valine arylamidase, cystin arylamidase, trypsin, α-chymotrypsin, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-frucosidase. This species is aerobic, catalase and oxidase positive, and urease and indole negative. Nitrate and nitrite are reduced (with no gas formation from nitrite) and no production of H_2S.

B. humptydooensis sp. nov. strains are resistant to aminoglycosides and amoxicillin-clavulanic acid but susceptible to trimethoprim-sulfmethoxazole, doxycycline, imipenem, and ceftazidime. All *B. humptydooensis* sp. nov. strains are seroreactive with sera from melioidosis patients who were infected with *B. pseudomallei* serotype B strains. All strains produced O-antigen ladder type B2, except that strain MSMB43^T produced a novel O-antigen ladder type (26). The type strain, MSMB43^T, has been previously referred to as *B. thailandensis*-like species in multiple studies (13, 14). MSMB43^T was isolated in 1995 from an automated water well (bore) in Humpty Doo, Australia. *B. humptydooensis* sp. nov., like *B. thailandensis*, is nonvirulent in mice. In addition, MSMB43^T is known to produce thailanstatins, which possess antiproliferative activities in representative human cancer cell lines (27). The type strain MSMB43^T has been deposited in the American Type Culture Collection as BAA-2767 and the Belgian Co-ordinated Collections of Microorganisms as LMG 29471.

MATERIALS AND METHODS

Strain isolation. Strain MSMB43^T was isolated from a water sample from an automated water bore (well) collected in 1995 and examined for *B. pseudomallei* in the Northern Territory (NT) of Australia. This strain was initially thought to be *B. thailandensis* due to its ability to assimilate arabinose as a sole carbon source, which is a trait used to discriminate *B. thailandensis* from *B. pseudomallei* (arabinose negative) (14). The bore from which MSMB43^T was discovered is located in Humpty Doo, a region of rural properties outside the capital of the NT, Darwin. The Top End of the NT has a high incidence rate of melioidosis (28). In fact, the water sample from which MSMB43^T was recovered also yielded *B. pseudomallei*. An additional two strains (MSMB121 and MSMB122) were both isolated in 2007 from a single separate bore water sample within the NT collected approximately 950 km south of the territory capital, Darwin, resulting in a 910-km separation between the two sample sites of MSMB121/MSMB122 and MSMB43^T. To date, the proposed *B. humptydooensis* sp. nov. has not been identified outside the NT, and it has not been isolated from any clinical specimens from patients within the NT.

The specific epithet "humptydooensis" given to this new species was adopted from the location name Humpty Doo, where this new species was first discovered.

Bacterial growth and characteristics. All three strains were grown at temperatures of 8, 25, 37, 42, and 45°C for 24, 48, 72, and 144 h on Columbia blood agar, MacConkey agar, Ashdown's selective agar, and Standard I nutrient agar with and without supplementary CO_2. Cell morphology was examined using a Zeiss light microscope at 1,000$\times$ magnification with cells grown for 2 days at 37°C. Biochemical data were obtained for all three strains of *B. humptydooensis* sp. nov. (MSMB43^T, MSMB121, and MSMB122)

and compared to data for strains of *B. pseudomallei* (K96243) (29) and *B. thailandensis* (E264^T) by using the API ONE and API Zym systems (bioMérieux) according to the manufacturer's instructions.

MALDI-TOF MS and fatty acid methyl ester analysis was performed for all three *B. humptydooensis* sp. nov strains (see the text in the supplemental material for a detailed description of these methods).

Antimicrobial susceptibility screening. MICs were determined by the broth microdilution method using commercially available CE-certified Micronaut-S 96-well microtiter plates (Merlin, Bornheim-Hersel, Germany) containing 2-fold serial dilutions of the following antibiotics: amoxicillin-clavulanic acid (0.5 to 64/0.25 to 32 mg/liter), ceftazidime (0.5 to 64 mg/liter), imipenem (0.25 to 32 mg/liter), rifampin (0.0625 to 8 mg/liter), chloramphenicol (0.5 to 64 mg/liter), trimethoprim-sulfamethoxazole (0.25 to 32/4.75 to 608 mg/liter), streptomycin (0.25 to 32 mg/liter), gentamicin (0.25 to 32 mg/liter), doxycycline (0.25 to 32 mg/liter), tigecycline (0.03125 to 4 mg/liter), ciprofloxacin (0.03125 to 4 mg/liter), and levofloxacin (0.0625 to 4 mg/liter). One well without antibiotic was used as a growth control. All plates containing the lyophilized antimicrobial substances were stored at room temperature until use.

Testing conditions were in accordance with the current Clinical and Laboratory Standards Institute (CLSI) recommendations for *B. pseudomallei* (30). Single colonies of MSMB43^T, MSMB121, and MSMB122 were picked from agar plates and inoculated in physiological saline (0.85% NaCl) until the turbidity matched that of a 0.5 McFarland standard. The suspension was diluted 221-fold in cation-adjusted Mueller-Hinton II broth (catalog number 297701; Becton Dickinson). After incubation for 24 h at 37°C in a 5% CO_2 atmosphere, bacterial growth was verified photometrically at a wavelength of 620 nm using a commercial photometer (Merlin, Bornheim-Hersel, Germany), and each strain was tested in triplicate. Additionally, a gradient strip method (Etest; bioMérieux) was applied to investigate a broader range of antibiotic concentrations.

Virulence testing in mouse models. The pathogenic potential of *B. humptydooensis* sp. nov. MSMB43^T was investigated in a BALB/c mouse model and compared to the pathogenic potential of *B. thailandensis* (type strain E264^T). Live cultures were grown to logarithmic phase (optical density at 600 nm, ~1.0) in Luria-Bertani (LB) broth as previously described (22). Sterile 1× phosphate-buffered saline (PBS) was used to wash cells twice before making dilutions for injecting mice. Viability counts of the final inocula were made on LB agar plates. Six- to 8-week-old female BALB/c mice in treatment groups of 5 mice per cage were used. Food and water were provided *ad libitum*. All mice in a single cage received the same infectious dose (*B. humptydooensis* sp. nov.: 1.05×10^4, 10^5, or 10^6 CFU; *B. thailandensis*: 3.4×10^4, 10^5, or 10^6 CFU) via a single s.c. injection in the scruff of the neck. Mice were monitored daily for health status. All mice were euthanized on day 21 postinjection. This work was conducted under approved protocols from the NAU IACUC (protocol 14-011) and DOD ACURO (HDTRA1-12-C-0066_Wagner).

16S rRNA and *recA* gene analysis. 16S rRNA and *recA* gene sequencing analyses were performed on three *B. humptydooensis* sp. nov. strains: MSMB43^T, MSMB121, and MSMB122, as previously described (11, 31). From whole-genome analysis of strain MSMB43^T using the SSU-ALIGN program (32), we investigated the number of rRNA operons present. Phylogenetic reconstruction of 16S rRNA and *recA* sequences was conducted using MEGA version 6 (33).

MLST. MLST was performed on all three *B. humptydooensis* sp. nov. strains as previously described (9). As of 7 October 2016, a total of 1,439 sequence types had been identified in *B. pseudomallei* and closely related species by MLST (http://www.MLST.net). The seven genes that comprise this MLST are *ace*, *gltB*, *gmhD*, *lepA*, *lipA*, *narK*, and *ndh*.

Genome assembly and core genome phylogeny. Two genomes (MSMB43^T and MSMB122) were sequenced on the PacBio platform. Two other genomes that group with *B. humptydooensis* sp. nov. are present in GenBank and consist of strains MSMB121 and MSMB43^T (which is the same type strain used in this study but is listed with an alternative BioProject strain identifier, 2002721687 [BioProject no. PRJNA239255]) (34, 35) with GenBank assembly accession numbers GCA_000385525 and GCA_000959325, respectively. A comparative genomics approach using LS-BSR (25) was also performed with the *B. pseudomallei* genome strain K96243 (BPSS1165 to BPSS1184).

For the core genome phylogeny, genomes were aligned against *B. pseudomallei* K96243 by using NUCmer (36). The reference genome was also aligned against itself to identify duplicated regions, which were masked from subsequent analyses; the NASP pipeline was used to wrap these methods (http://tgennorth.github.io/NASP/). A phylogeny was inferred by using RAxML v8 (37) on a large set (n = 331,000) of concatenated single nucleotide polymorphisms (SNPs) using a time-reversible model incorporating the Lewis ascertainment bias correction.

Genome-to-genome distance calculations. DDH is the current gold standard for bacterial species delineation. DDH is necessary for the description of a new species within a taxon when strains share more than 98.65% 16S rRNA gene sequence similarity (38). If DNA-DNA relatedness between two strains is less than 70%, the two strains are considered different species. However, DDH is laborious and difficult to standardize, and interlaboratory reproducibility is relatively low. DDH was previously performed on MSMB43^T and showed a relative binding ratio of 91% with *B. thailandensis* (ATCC 700388) with a divergence of 4% (14). Because of the drawbacks of conventional DDH and the rapid progress in genome sequencing techniques, various *in silico* algorithms for calculating genome-to-genome similarities or distances have been developed. In addition to the commonly used ANI method (39), recently a highly reliable estimator for the relatedness of genomes was developed by Jan P. Meier-Kolthoff and colleagues (40). GGDC produces digital DDH values that correlate well with values obtained by conventional DDH, which is of utmost importance for compatibility with the current species concept and also provides confidence interval estimation. DDH values were calculated using formula 2 in GGDC; this formula summed the identities found in high-scoring segment pairs (HSP) and then divided them by the overall HSP length. The GGDC service is available from the German Collection of Microorganisms and Cell

Cultures home page (http://ggdc.dsmz.de/distcalc2.php). PacBio assemblies were used to determine the distances among *B. humptydooensis* sp. nov. and other closely related species. The genomes were subjected to GGDC analysis and compared to the available genome sequences of *B. pseudomallei*, *B. mallei*, *B. oklahomensis*, and *B. thailandensis* reference strains (K96243, ATCC 23344^T, C6786^T, and E264^T, respectively). For comparison, the ANI values were also calculated for all reference sequences by using JSpecies (41); the authors of JSpecies determined that ANI values of <95% indicate separate species.

Accession number(s). GenBank accession numbers for the 16S rRNA gene sequences of *B. humptydooensis* sp. nov. strains MSMB121 and MSMB122 are KF378608 and KF378609, respectively. The complete whole-genome sequence of the strain MSMB121 was published under GenBank accession numbers CP004095 and CP004096 (34, 35). The assembly for MSMB43^T was published under GenBank assembly number GCA_001513745 and that for MSMB122 under SRA numbers SRR1956040 and LNPD00000000.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02802-16.

TEXT S1, PDF file, 0.44 MB.

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We declare no conflicts of interest.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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