Burkholderia oklahomensis sp. nov., a *Burkholderia pseudomallei*-like species formerly known as the Oklahoma strain of *Pseudomonas pseudomallei*

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C6786, the clinical isolate of the 'Oklahoma' strain of *Pseudomonas* (now *Burkholderia*) *pseudomallei*, was originally isolated in 1973 from a wound infection resulting from a farming accident in Oklahoma, USA. Environmental isolates C7532 and C7533 from the Oklahoma accident site were found to match C6786. These three isolates and a clinical isolate originally identified as *B. pseudomallei* that was recovered from a person in Georgia, USA, involved in an automobile accident were characterized by biochemical, 16S rRNA gene sequencing, multilocus sequence typing and DNA–DNA hybridization analyses. Results indicated that these strains comprise a novel species. The name *Burkholderia oklahomensis* sp. nov. is proposed, with strain C6786^T (=LMG 23618^T=NCTC 13387^T=CCUG 51349^T) as the type strain.

More than 30 *Burkholderia* species, comprising a variety of plant, animal and human pathogens, are currently described (Coenye & Vandamme, 2003; Yabuuchi *et al.*, 1992b). *Burkholderia pseudomallei*, a saprophytic soil bacterium that is the aetiological agent of melioidosis in animals and humans (White, 2003), has been designated a category B select agent by the CDC, due to its potential use for biological terrorism (Rotz *et al.*, 2002).

B. pseudomallei is typically seen in South-East Asia and northern Australia, but is occasionally reported in tropical areas around the world. Most melioidosis cases reported in other regions arise from travel to areas endemic for *B. pseudomallei*. In the USA, case reports of locally acquired melioidosis in the states of Oklahoma and Georgia have been documented (McCormick *et al.*, 1977; Nussbaum *et al.*, 1980). It is important to establish whether *B. pseudomallei* is native to the USA and to characterize further the *B. pseudomallei*-like organisms that were recovered from these cases.

The Oklahoma strain of *Pseudomonas* (now *Burkholderia*) *pseudomallei* was first isolated in 1973 from a 27-year-old farmer with a deep leg wound heavily contaminated with

soil as a result of a farming accident in Oklahoma, USA (McCormick *et al.*, 1977). The clinical isolate C6786^T was identified by using standard microbiological procedures. Environmental isolates C7532 and C7533 were recovered from the soil near the accident site and were similar to C6786^T. These three isolates comprise the original Oklahoma strain. The initial study indicated that the Oklahoma strain may represent a variant of *B. pseudomallei* or potentially a novel species based on the results of biochemical assays, guinea pig inoculations, fatty acid analysis and a fluorescent antibody test (McCormick *et al.*, 1977). Subsequent studies have produced conflicting interpretations regarding the identification of the Oklahoma strain as a strain of *B. pseudomallei* (Godoy *et al.*, 2003; Tomaso *et al.*, 2004, 2005; Yabuuchi *et al.*, 1992a). Yabuuchi *et al.* (1992a) performed a variety of standard microbiological tests as well as DNA–DNA hybridization and 16S rRNA gene sequencing with the clinical isolate of the Oklahoma strain and concluded that it was a strain of *B. pseudomallei*. Recently, Godoy *et al.* (2003) completed a study using multilocus sequence typing (MLST) that indicated that the Oklahoma isolates were divergent from all other *B. pseudomallei* isolates in their study panel and suggested that the Oklahoma strain may be a novel species. Interestingly, they observed that *B. mallei*, also a category B select agent and the pathogen responsible for the disease glanders, appeared to be a clone of *B. pseudomallei* (Godoy *et al.*, 2003). PCR assays recently developed by Tomaso *et al.* (2004, 2005) for detecting *B. pseudomallei* and *B. mallei* do not detect the Oklahoma strain.

Abbreviations: MLST, multilocus sequence typing; RBR, relative binding ratio; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains C6786^T, C7532, C7533, E0147 and ATCC 23343^T are DQ108388, DQ108389, DQ108390, DQ108391 and DQ108392, respectively.

An additional isolate recovered from an automobile accident victim was also evaluated. This isolate was recovered in 1977 from a 21-year-old male from Georgia, USA, who was involved in an automobile accident that propelled him into a clay embankment. His left eye was packed with soil and developed ulcers in the days after the accident. An organism assigned as *B. pseudomallei* (E0147, the Georgia strain) was isolated 8 weeks after the accident and identified by using biochemical assays (Nussbaum *et al.*, 1980).

Because molecular studies of the Oklahoma strain suggested that it may be a separate species, we decided to re-examine and characterize the Oklahoma strain, as well as the Georgia strain, by using standard microbiological procedures, 16S rRNA gene sequencing, MLST and DNA–DNA hybridization to determine whether they were strains of *B. pseudomallei*. We determined that these isolates comprise a novel *Burkholderia* species and therefore propose the name *Burkholderia oklahomensis* sp. nov.

We amplified the 16S rRNA gene for strains C6786^T, C7532, C7533, E0147 and ATCC 23343^T (the type strain of *B. pseudomallei*). Bacterial culture and DNA template preparation were performed as described previously (Gee *et al.*, 2003). Amplification of the 16S rRNA gene was performed by using a protocol and primers F229 (5'-CGCAAGC-GAAAGTATCAAGA-3') and R1908 (5'-TTTACAGCCG-ATAAGCGTGAG-3'), described previously (Gee *et al.*, 2003). The 16S rRNA gene sequences were obtained by using a previously described protocol and set of primers (Gee *et al.*, 2003). We were able to determine the nearly complete sequence (corresponding to positions 26–1514 in the *Escherichia coli* numbering system) of the 16S rRNA gene for these five isolates.

We found that C6786^T, C7532 and C7533 had identical 16S rRNA gene sequences, which we designated the OK 16S rRNA gene consensus sequence. A BLAST query of GenBank on 1 September 2005, using the OK 16S rRNA gene consensus sequence, did not yield an exact match to any 16S rRNA gene sequence in the database (Altschul *et al.*, 1990). Although Yabuuchi *et al.* (1992a) stated that the 16S rRNA gene sequence for the Oklahoma strain was identical to the 16S rRNA gene sequence for *B. pseudomallei*, the sequence was not included in their paper, nor was an entry available in GenBank.

We performed an analysis with the Wisconsin package, ver. 10.3 (Accelrys), using BESTFIT. The 16S rRNA gene sequence for strain E0147, the Georgia strain, differed from the OK 16S rRNA gene consensus sequence at only two positions, yielding a match of 99.9%. The OK 16S rRNA gene consensus sequence, as represented by the sequence derived from strain C6786^T, was aligned with 16S rRNA gene sequences from related bacteria available in GenBank by using PILEUP. Gaps were removed for neighbour-joining analysis (Kimura two-parameter, 1000-step bootstrap) in MEGA 3 (Kumar *et al.*, 2004) (Fig. 1).

By using BESTFIT, we compared the OK 16S rRNA gene consensus sequence with those of reference strains representing *B. pseudomallei* and *Burkholderia thailandensis*. We found that there was a 98.5% match to the sequence of ATCC 23343^T (the *B. pseudomallei* type strain). Comparison of the OK 16S rRNA gene consensus sequence to that from the *B. thailandensis* type strain, ATCC 700388^T (GenBank accession no. U91838), which is a close relative of *B. pseudomallei* (Brett *et al.*, 1998), also indicated a match of 98.5%.

Fig. 1. Neighbour-joining phylogenetic tree of *Burkholderia oklahomensis* based on comparisons with 16S rRNA gene sequences of related bacteria. *Pseudomonas aeruginosa* DSM 50071^T (GenBank accession no. X06684) was used as an outgroup for this analysis. Bar, 5% sequence dissimilarity.

MLST of *B. pseudomallei* and closely related species is based on sequencing discrete segments of seven housekeeping genes and assigning sequence types (STs) based on sequence variations of the loci. Godoy *et al.* (2003) found that the three Oklahoma isolates were identical by MLST, and they assigned them to ST81. The alleles of the seven loci of the Oklahoma strain were not shared with either *B. pseudomallei* or *B. thailandensis* (Godoy *et al.*, 2003).

We performed MLST on E0147 (the Georgia strain) and ATCC 23343^T (the *B. pseudomallei* type strain). Bacterial culture and DNA template preparation were performed as described previously (Gee *et al.*, 2003). We used the panel of primers described on <http://www.mlst.net> and by Godoy *et al.* (2003) for both amplification and sequencing. For amplification, we used the Expand Hi Fidelity PCR system (Roche). The amplification mix consisted of 1 × buffer 2, 200 µM dNTP mix, 0.4 µM forward and reverse primers, 0.9 units Expand polymerase and 1 µl cell extract. PCR conditions consisted of an initial 4 min hold at 95 °C. This was followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min with a final hold at 72 °C for 10 min. PCR products were purified with a QIAquick PCR purification kit (Qiagen). Sequencing was performed by using an Applied Biosystems (ABI) BigDye Terminator cycle sequencing ver. 3.0 kit as per the manufacturer's instructions, except that 3 µl BigDye was used instead of 8 µl (Applied BioSystems). Sequencing products were purified by using Centri-Sep spin columns (Princeton Separations) and were

resolved by using an ABI model 3100 automated DNA sequencing system.

MLST analysis of E0147 revealed that four of the seven MLST loci (*ace*, *gltB*, *lepA* and *lipA*) of E0147 had alleles identical to the Oklahoma strain. Three loci were unique (*gmhD*, *narK* and *ndh*) and the resulting alleles did not match any of the alleles of the Oklahoma strain, *B. pseudomallei* or *B. thailandensis*. A comparison of these three alleles with the corresponding alleles of the Oklahoma strain using BESTFIT indicates 2/468 bp difference (99.6 % match) for *gmhD*, 2/561 bp difference (99.6 % match) for *narK* and 2/443 bp difference (99.6 % match) for *ndh*. The allelic profile of E0147 was submitted to <http://www.mlst.net> and was assigned as ST234. We also determined that ATCC 23343^T is a match to ST51.

To compare STs directly, the sequences for the seven loci were combined or concatenated to form a sequence of 3999 bp as described by Godoy *et al.* (2003). Neighbour-joining analysis (Kimura two-parameter, 500-step bootstrap) of the concatenated allele sequences by using MEGA 3 indicated that E0147 clusters with the Oklahoma isolates (Fig. 2). A BESTFIT analysis indicated a match of 99.9 % between the concatenated sequences for the Oklahoma strain (ST81) and E0147 (ST234). A BESTFIT analysis comparing the concatenated sequences of ST81 with ST51, the ST of the type strain of *B. pseudomallei*, indicated a match of 95 %.

Fig. 2. Neighbour-joining phylogenetic tree derived from concatenating all seven allele sequences from a representative set of MLST sequence types. All STs are available at <http://www.mlst.net>. Bar, 0.5 % sequence dissimilarity.

Table 1. Relative binding ratio and divergence of DNA

ND, Not determined.

Source of unlabelled DNA	Results with labelled DNA from:								
	<i>B. oklahomensis</i> C6786 ^T			<i>B. pseudomallei</i> ATCC 23343 ^T			<i>B. thailandensis</i> E254		
	RBR* at 65 °C	D†	RBR at 80 °C	RBR* at 65 °C	D†	RBR at 80 °C	RBR* at 65 °C	D†	RBR at 80 °C
<i>B. oklahomensis</i> C6786 ^T	100	0·0	100	50	5·0	25	55	5·0	19
<i>B. pseudomallei</i> ATCC 23343 ^T	64	5·5	40	100	0·0	100	63	4·5	30
<i>B. thailandensis</i> E254	60	5·5	35	53	4·5	26	100	0·0	100
<i>B. oklahomensis</i> C7532	100	0·0	100	ND	ND	ND	ND	ND	ND
<i>B. oklahomensis</i> C7533	100	1·0	100	ND	ND	ND	ND	ND	ND
<i>B. oklahomensis</i> E0147	92	1·0	92	ND	ND	ND	ND	ND	ND
<i>B. cepacia</i> ATCC 25416 ^T	36	10·5	14	21	11·0	5	18	10·5	8

*Relative binding ratio: the amount of double-stranded DNA formed between labelled and unlabelled DNAs from different strains divided by the amount of double-stranded DNA formed between labelled and unlabelled DNA from the same strain. RBR is expressed as a percentage.

†Divergence within related sequences, calculated on the assumption that each 1 °C decrease in the thermal stability of a DNA duplex is caused by 1 % unpaired bases within that duplex. *D* was calculated to the nearest 0·5 %.

When Yabuuchi *et al.* (1992a) performed DNA–DNA hybridization in their study, they concluded that the Oklahoma clinical isolate did not represent a novel species. However, they made this determination by using a lower threshold of species delineation (i.e. a 60 % threshold) (Yabuuchi *et al.*, 1992a). We decided to perform DNA–DNA hybridization on the Oklahoma and Georgia strains. Cells were harvested and lysed and the chromosomal DNA was isolated and purified as described previously (Brenner *et al.*, 1982). DNA from strain C6786^T was labelled with [³²P]dCTP by using a commercial nick-translation kit (Invitrogen Life Technologies) and tested for reassociation to unlabelled DNA from the same strain (homologous reaction), as well as to C7532, C7533, E0147 and the type or reference strains of *Burkholderia cepacia* (ATCC 25416^T), *B. thailandensis* (strain E254) and *B. pseudomallei* (ATCC 23343^T) (heterologous reactions). Relative binding ratios (RBR) and percentage divergence were calculated as described previously (Brenner *et al.*, 1982). The G+C content of the

Burkholderia-like strains was determined by the thermal-denaturation method of Mandel *et al.* (1970).

The G+C content of strain C6786^T was determined to be 66 mol%, so the hybridization reactions were done at 65 °C (optimum) and 80 °C (stringent). The results of DNA relatedness studies are given in Table 1. Isolates C7532, C7533 and E0147 exhibited >90 % relatedness (RBR) under both the optimum and stringent reassociation criteria and had divergence (*D*) of <5 % from the labelled reference (C6786^T) strain. The reference strain was <65 % related to strains of *B. cepacia* (ATCC 25416^T), *B. thailandensis* (E254) and *B. pseudomallei* (ATCC 23343^T), with *D* values >5·5 % and the stringent RBR <40 %. Thus, we propose that the Oklahoma and the Georgia strains represent a single species, which we designate *Burkholderia oklahomensis* sp. nov., that is separate from the previously described *Burkholderia* species according to the molecular criteria for species-level relatedness established by Wayne *et al.* (1987). This

Table 2. Phenotypic characteristics of the four strains of *Burkholderia oklahomensis* and comparison with similar *Burkholderia* species

v, Results are variable from strain to strain; (v), results vary within strain.

Test	C6786 ^T	C7532	C7533	E0147	<i>B. mallei</i> *	<i>B. pseudomallei</i> *	<i>B. thailandensis</i>	<i>B. cepacia</i> *	<i>B. gladioli</i> *
Salmonella–Shigella agar	–	+	+	–	–	v	–	v	–
Cetrimide	+	–	–	–	–	–	–	v	–
Urea	–	–	–	–	v	v	–	+	v
Simmons' citrate	+	+	+	–	–	v	+	v	+
Nutrient broth 6 % NaCl	–	+	–	+	–	v	–	–	–
Gas from nitrate	(v)	(v)	(v)	–	–	+	+	–	–

*Data taken from Weyant *et al.* (1995).

assignment is supported strongly by the near identity of the concatenated sequences of the seven housekeeping (MLST) loci of the Oklahoma and Georgia isolates and their ~5 % divergence from those of *B. pseudomallei*.

The standard biochemical identification scheme for *B. pseudomallei* and other closely related species is unable to provide a reliable and reproducible phenotypic method to differentiate between *B. oklahomensis* and *B. pseudomallei* (Weyant *et al.*, 1995). Therefore, frontline diagnostic laboratories will probably continue to identify an isolate of *B. oklahomensis* presumptively as *B. pseudomallei*. Molecular-based methods described in this work performed in reference laboratories may then be used to aid in identifying the isolate. As these methods are not commonly used in frontline diagnostic laboratories, it is encouraging that PCR tests have recently been developed that exclude the Oklahoma strain, but detect *B. pseudomallei* and the closely related select agent *B. mallei* (Tomaso *et al.*, 2004, 2005).

Although a previous study indicated that *B. oklahomensis* had a lower level of virulence than *B. pseudomallei* in an animal model, little is known about the virulence of *B. oklahomensis* in humans (McCormick *et al.*, 1977; Nussbaum *et al.*, 1980). More work is needed to assess the geographical prevalence and clinical significance of *B. oklahomensis*.

Description of *Burkholderia oklahomensis* sp. nov.

Burkholderia oklahomensis (ok.la.hom.en'sis. N.L. fem. adj. *oklahomensis* pertaining to Oklahoma, of Oklahoma).

The following description is based on the results of studies of four isolates. Cells are Gram-negative, bipolar-staining rods. The cells are aerobic and motile and grow well at 25 and 37 °C, but slowly at 42 °C. Colonies on 5 % sheep blood agar are irregular, white, smooth and show a diffuse lysis of red cells extending out from heavy growth with no action by individual colonies. They may become wrinkled or mucoid after 72 h incubation. Cells grow well on MacConkey agar. Cells are oxidase-, catalase-, arginine decarboxylase (dihydrolase)- and nitrate reduction-positive. Cells grow in nutrient broth with 0 % NaCl. The three Oklahoma strains (C6786^T, C7532 and C7533) produce variable results for gas from nitrate. Both production of gas and of little to no gas have been observed (McCormick *et al.*, 1977; Yabuuchi *et al.*, 1992a). The Georgia strain (E0147) is negative for production of gas from nitrate. Tests for indole, urease, lysine decarboxylase, ornithine decarboxylase and hydrogen sulfide are negative. Cells show oxidative metabolism and, on triple-sugar iron agar, produce an acid reaction on the slant and no change in the butt of the medium. All strains are positive for assimilation of glucose, D-xylose, mannitol, lactose, sucrose and maltose. All are negative on minimal salts agar with 10 % arabinose (Wuthiekanun *et al.*, 1995). Purple, wrinkled colonies are seen on Ashdown's agar after 1 week incubation at 37 °C (Ashdown, 1979). Differential tests are listed in Table 2.

First isolated from human wound infection in Oklahoma, USA. Type strain is C6786^T (=LMG 23618^T=NCTC 13387^T=CCUG 51349^T).

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