Burkholderia bannensis sp. nov., an acid-neutralizing bacterium isolated from torpedo grass (*Panicum repens*) growing in highly acidic swamps

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Two strains of acid-neutralizing bacteria, E25^T and E21, were isolated from torpedo grass (*Panicum repens*) growing in highly acidic swamps (pH 2–4) in actual acid sulfate soil areas of Thailand. Cells of the strains were Gram-negative, aerobic, non-spore-forming rods, 0.6–0.8 µm wide and 1.6–2.1 µm long. The strains showed good growth at pH 4.0–8.0 and 17–37 °C. The organisms contained ubiquinone Q-8 as the predominant isoprenoid quinone and C_{16:0}, C_{17:0} cyclo and C_{18:1ω7c} as the major fatty acids. Their fatty acid profiles were similar to those reported for other *Burkholderia* species. The DNA G + C content of the strains was 65 mol%. On the basis of 16S rRNA gene sequence similarity, the strains were shown to belong to the genus *Burkholderia*. Although the calculated 16S rRNA gene sequence similarity of E25^T to strain E21 and the type strains of *Burkholderia unamae*, *B. tropica*, *B. sacchari*, *B. nodosa* and *B. mimosarum* was 100, 98.7, 98.6, 97.6, 97.4 and 97.3%, respectively, strains E25^T and E21 formed a group that was distinct in the phylogenetic tree; the DNA–DNA relatedness of E25^T to E21 and *B. unamae* CIP 107921^T, *B. tropica* LMG 22274^T, *B. sacchari* LMG 19450^T, *B. nodosa* LMG 23741^T and *B. mimosarum* LMG 23256^T was 90, 42, 42, 42, 45 and 35%, respectively. The results of physiological and biochemical tests including whole-cell protein pattern analysis allowed phenotypic differentiation of these strains from previously described *Burkholderia* species. Therefore, strains E25^T and E21 represent a novel species, for which the name *Burkholderia bannensis* sp. nov. is proposed. The type strain is E25^T (=NBRC 103871^T =BCC 36998^T).

Yabuuchi *et al.* (1992) created the genus *Burkholderia* by the transfer of seven species from *Pseudomonas*, with *Burkholderia cepacia* as the type species. At the time of writing, the genus *Burkholderia* comprises more than 60 species, occupying a wide range of ecological niches and showing a variety of metabolic activities (Coenye & Vandamme, 2003). During the course of a study to characterize waterweeds adapted to highly acidic aquatic environments (pH 2–4) in actual acid sulfate soils (AASS) in South-East Asia (Aizawa *et al.*, 2008; Sasaki *et al.*, 2008), we have isolated a number of bacteria associated with the waterweeds and reported several novel bacterial species

(Aizawa *et al.*, 2007, 2010b, c; Kimoto *et al.*, 2010). In the present study, we characterized two acid-neutralizing bacteria, E25^T and E21, isolated from torpedo grass (*Panicum repens*; see Supplementary Fig. S1, available in IJSEM Online) growing in a highly acidic swamp (pH 2.9) at Banna Experimental Station in Nakhon Nayok Province, Thailand. By a polyphasic approach, including 16S rRNA gene sequence analysis, DNA–DNA hybridization, whole-cell protein analysis, fatty acid methyl ester analysis and phenotypic and biochemical characterization, the strains were shown to be affiliated with the genus *Burkholderia*. The data obtained suggest that the strains represent a novel species of the genus *Burkholderia*.

Each *P. repens* plant was divided into leaves, aerial stems, underwater stems and roots. After gentle washing in excess saline for 1 min to remove loosely attached soil, each part was transferred to fresh saline and shaken vigorously for 15 min; this procedure was repeated three times. The

Abbreviation: AASS, actual acid sulfate soil.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains E25^T and E21 are AB561874 and AB561875.

Three supplementary figures and three supplementary tables are available with the online version of this paper.

extracts were combined (to give sample LBM). The LBM sample was transferred to fresh saline and shaken vigorously for 18 h (to give sample TBM). A number of bacteria were isolated from samples LBM and TBM by using one-tenth-strength tryptic soy (1/10 TS) agar plates [3.0 g tryptic soy broth l⁻¹ solidified with 15.0 g agar l⁻¹ (both from Difco)] at pH 4.0. For preparation of these acidic plates, double-strength medium and 3% agar solution were autoclaved separately to prevent hydrolysis of the agar and then mixed. Among the isolates, acid-neutralizing bacteria were selected on the basis of neutralization of 1/10 TS liquid medium (pH 4.0), determined by measuring the pH of the culture supernatants after 3 days of cultivation.

Strains E25^T and E21 were isolated from TBM samples prepared from a root and an aerial stem, respectively, from the same plant collected in Thailand. Both strains formed round, smooth, convex, pale-yellow colonies with entire margins on 1/10 TS agar plates (pH 4.0). Each showed good growth on these plates at 17–37 °C, with optimum growth at 28–32 °C, but did not grow at 5 or 42 °C. Strains E25^T and E21 showed good growth at pH 3–8, with optimum growth at pH 4–7 and no or little growth below pH 2 or above pH 9 when cultured at 30 °C for 5 days. The strains grew as well on TS plates (pH 4.0) as on 1/10 TS agar plates (pH 4.0). Growth under anaerobic conditions was determined after 5 days of incubation at 30 °C in an AnaeroPack (Mitsubishi Gas Chemical Co., Ltd). The strains grew well on 1/10 TS plates (pH 4.0) under aerobic conditions but not under anaerobic conditions, indicating that they are aerobes. Cells grown on 1/10 TS agar plates (pH 4.0) were Gram-negative (Ryu, 1938), non-endospore-forming and non-motile. Their morphology was observed by scanning electron microscopy, as described previously (Aizawa *et al.*, 2010b). The cells were irregular rods, 0.6–0.8 µm wide and 1.6–2.1 µm long, after growth on 1/10 TS agar plates (pH 4.0) at 28 °C for 3 days.

The 16S rRNA genes of the strains were amplified by PCR using universal primers (Tamura & Hatano, 2001), and nearly complete 16S rRNA gene nucleotide sequences were determined. The NCBI BLAST software tool (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) and the EzTaxon software (Chun *et al.*, 2007) were applied to compare the determined 16S rRNA gene sequences with those of type strains. These sequences showed high similarity to species within the genus *Burkholderia*. The 16S rRNA gene sequence similarities of strain E25^T to strain E21, *Burkholderia unamae* MTL-641^T, *B. tropica* Ppe8^T, *B. sacchari* LMG 19450^T, *B. nodosa* Br3437^T and *B. mimosarum* PAS44^T were 100, 98.7, 98.6, 97.6, 97.4 and 97.3 %, respectively. Phylogenetic relationships of E25^T and E21 with closely related species were determined by using MEGA version 4 (Tamura *et al.*, 2007) and the PHYLIP 3.65 package (Felsenstein, 2005) after performing multiple alignments of the data with CLUSTAL_X (Thompson *et al.*, 1997). Evolutionary distances were computed as described previously (Jukes & Cantor, 1969). Phylogenetic trees were

reconstructed by using the maximum-parsimony (Kluge & Farris, 1969), maximum-likelihood (Felsenstein, 1981), minimum-evolution (Rzhetsky & Nei, 1992) and neighbour-joining (Saitou & Nei, 1987) methods. The reliabilities of these tree topologies were evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985). Phylogenetic trees reconstructed by these four methods were topologically similar and showed that strains E25^T and E21 belonged to the genus *Burkholderia*, within which the strains formed a cluster with strains of some *Burkholderia* species isolated from plants and soil: *Burkholderia tuberum* (Vandamme *et al.*, 2002), *B. mimosarum* (Chen *et al.*, 2006) and *B. nodosa* (Chen *et al.*, 2007), isolated from leguminous *Mimosa* species, *Burkholderia silvatlantica* (Perin *et al.*, 2006), *B. unamae* (Caballero-Mellado *et al.*, 2004) and *B. tropica* (Reis *et al.*, 2004), isolated from maize and sugar cane, *Burkholderia heleaia* (Aizawa *et al.*, 2010b) and *B. acidipaludis* (Aizawa *et al.*, 2010c), from Chinese water chestnut, *Burkholderia sacchari* (Brämer *et al.*, 2001), isolated from soil of a sugar cane plantation, and *Burkholderia kururiensis* M130 (Caballero-Mellado *et al.*, 2007), isolated from the root of a rice plant growing in Brazil (Baldani *et al.*, 1997; Weber *et al.*, 1999), as well as *Burkholderia ferrariae* (Valverde *et al.*, 2006), isolated from iron ore. The phylogenetic tree reconstructed by using the neighbour-joining method is shown in Fig. 1. Although the strains showed a high degree of 16S rRNA gene sequence similarity to established species of the genus, they formed a separate line of descent in the phylogenetic cluster of the genus (Fig. 1). Stackebrandt & Goebel (1994) pointed out that a high degree of 16S rRNA gene sequence similarity (97% or higher) is of limited value for differentiating species, and that DNA–DNA hybridization studies must be performed to determine species affiliation under these

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (positions 150–1418 of the *Escherichia coli* 16S rRNA gene), showing the positions of strains E25^T and E21 among their phylogenetic neighbours. Numbers at branch nodes are percentages based on 1000 bootstrap resamplings; only values over 70% are given. The sequences of *Burkholderia cepacia* ATCC 25416^T and *Burkholderia ginsengisoli* KMY03^T were used as the outgroup. Bar, 0.005 substitutions per nucleotide position.

circumstances. Therefore, we performed DNA–DNA hybridization on these strains and their neighbours on the phylogenetic tree by using the microplate hybridization method (Ezaki *et al.*, 1988, 1989) as described by Tamura *et al.* (1999). Hybridization was performed at 51 °C for 3 h using a hybridization solution containing 50 % deionized formamide, 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02 % BSA, 0.02 % polyvinylpyrrolidone and 0.02 % Ficoll 400), 0.1 mg heat-denatured, sonicated salmon testes DNA ml⁻¹ and 2.5 % dextran sulfate. The DNA–DNA relatedness of strain E25^T to E21 was 90 %, which is higher than the recommended threshold value (70 %) for the delineation of genomic species (Wayne *et al.*, 1987). Strain E25^T exhibited relatively low levels of DNA–DNA relatedness with respect to *B. nodosa* LMG 23741^T (45 %), *B. unamae* CIP 107921^T (42 %), *B. tropica* LMG 22274^T (42 %), *B. sacchari* LMG 19450^T (42 %) and *B. mimosarum* LMG 23256^T (35 %), indicating that strain E25^T was not related to them at the species level. These data suggest that strains E25^T and E21 represent a novel species of the genus *Burkholderia*.

Strains E25^T and E21 could also be distinguished from closely related species of *Burkholderia* in the phylogenetic trees (Supplementary Fig. S2) on the basis of physiological characteristics (Supplementary Table S1). All physiological and biochemical experiments were done at least in triplicate using not only strains E25^T and E21 but also the type strains of phylogenetically closely related *Burkholderia* species, *B. unamae* CIP 107921^T, *B. tropica* LMG 22274^T, *B. sacchari* LMG 19450^T, *B. nodosa* LMG 23741^T and *B. mimosarum* LMG 23256^T. Strains E25^T and E21 did not show positive results in acetylene reduction experiments (Burris, 1972) using Winogradsky's nitrogen-free mineral soft gel medium containing 1 % mannitol (Hashidoko *et al.*, 2002), whereas closely related type strains were positive, with the exception of *B. acidipaludis* SA33^T, *B. sacchari* LMG 19450^T, *B. kururiensis* JCM 10599^T, *B. tuberum* LMG 21444^T, *B. silvatlantica* LMG 23149^T, *B. heleaia* SA41^T and *B. ferrariae* LMG 23612^T. These data showed good agreement with results obtained by ¹⁵N₂ incorporation assays (Martinez-Aguilar *et al.*, 2008). The *nifH* PCR assay (Caballero-Mellado *et al.*, 2007) was performed in this study for strains E25^T and E21 and also the type strains of phylogenetically closely related *Burkholderia* species. Interestingly, strains E25^T and E21, as well as *B. acidipaludis* SA33^T and *B. ferrariae* LMG 23612^T, gave positive results, whereas *B. sacchari* LMG 19450^T gave a negative result; the results for *B. sacchari* LMG 19450^T and *B. ferrariae* LMG 23612^T were in accordance with a previous report (Caballero-Mellado *et al.*, 2007). Further study might be necessary to determine the diazotrophy of these *nifH*-positive strains. Strains E25^T and E21 could grow in the presence of 1 mM AlCl₃ in 0.1 % BactoTryptone (Difco) adjusted to pH 3.5 with sulfuric acid.

Standard physiological tests were carried out according to methods described previously (Smibert & Krieg, 1994).

Acid production from carbon sources, enzyme activities and resistance to antibiotics were assessed by using the API 50 CH system, API ZYM and API 20 E systems and ATB VET system (bioMérieux), respectively, according to the manufacturers' instructions. The utilization of various substrates as sole carbon sources was tested by using Biolog GN2 Microplates in accordance with the manufacturer's instructions. Physiological characteristics that differ between the novel strains and closely related species are summarized in Supplementary Table S1, and physiological diversity within the species is shown in Supplementary Table S2. The DNA G + C content was determined by the method described by Tamaoka & Komagata (1984). The DNA G + C content of strains E25^T and E21 was 65 mol%. Cellular fatty acids of the two strains and reference strains were saponified, methylated and extracted as described by Tamura *et al.* (1994). Fatty acid methyl esters were then analysed by using a Shimadzu model GCMS-QP5050 GC/MS equipped with an SPB-1 column (Supelco). The major fatty acids of both strains grown on 1/10 TS agar plates containing 1 % glucose (pH 5.0) at 28 °C for 3 days were C_{16:0}, C_{17:0} cyclo and C_{18:1}ω7c (Supplementary Table S2); similar patterns were obtained from cells of the type strains of closely related species. Isoprenoid quinones were prepared as described previously (Tamura *et al.*, 1994) and analysed by using a Quattro premier MS coupled to an Acuity UPLC (UPLC/MS/MS; Waters) as described by Aizawa *et al.* (2010a). The major isoprenoid quinone of the strains was Q-8, as in the case of other species of the genus *Burkholderia* (Yamada *et al.*, 1982; Zhang *et al.*, 2000; Yang *et al.*, 2006; Valverde *et al.*, 2006; Aizawa *et al.*, 2010b, c).

Whole-cell protein extracts were prepared from strains E25^T and E21 and the type strains of several related species and analysed by SDS-PAGE, as described previously (Pot *et al.*, 1994); the results are shown in Supplementary Fig. S3. The newly isolated strains showed a clearly different protein profile from related species of *Burkholderia*. Since it is well known that bacteria with identical or very similar protein patterns possess high genome similarity (Vandamme *et al.*, 1996), these SDS-PAGE results also support the notion that strains E25^T and E21 represent a novel acid-neutralizing *Burkholderia* species.

Therefore, based on the physiological, biochemical, chemotaxonomic and molecular genetic results described above, strains E25^T and E21 represent a novel species of the genus *Burkholderia*, for which the name *Burkholderia bannensis* sp. nov. is proposed.

Description of *Burkholderia bannensis* sp. nov.

Burkholderia bannensis (ban.nen'sis. N.L. fem. adj. *bannensis* pertaining to the Banna region of Nakhon Nayok Province, Thailand, where the first strains were isolated).

Cells are Gram-negative, strictly aerobic, non-spore-forming, non-motile rods (0.6–0.8 × 1.6–2.1 μm). Colonies are smooth, round, convex and pale yellow with entire margins after 3 days of cultivation at 32 °C on 1/10 TS agar plates

(pH 4.0). Growth occurs at 17–37 °C, with optimum growth at 28–32 °C. Grows at pH 3–8, with optimum growth at pH 4–6. Positive for hydrolysis of Tweens 20, 40, 60 and 80. Positive for oxidase, catalase, acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and reduction of nitrate to nitrite, but negative for hydrolysis of DNA and gelatin, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, proteinase, urease, arginine dihydrolase, indole production and reduction of nitrate to N₂ (API ZYM, API 20E and API 20NE). Positive for acid production from glycerol, D- and L-arabinose, D-ribose, D-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, D-sorbitol, arbutin, aesculin, cellobiose, maltose, lactose, melibiose, trehalose, gentiobiose, D-lyxose, D- and L-arabitol; negative for acid production from methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, sucrose, inulin, melezitose, raffinose, starch, glycogen and turanose (API 50CH). Positive for utilization of Tweens 40 and 80, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-fructose, L-fucose, D-galactose, gentiobiose, α -D-glucose, lactulose, D-mannitol, D-mannose, inositol, L-rhamnose, trehalose, pyruvic acid methyl ester, succinic acid mono-methyl ester, *cis*-aconitic acid, citric acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, β -hydroxybutyric acid, α -ketoglutaric acid, D-saccharic acid, succinic acid, succinamic acid, glucuronamide, D-alanine, L-alanyl glycine, L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-phenylalanine, L-serine, L-threonine, γ -aminobutyric acid, urocanic acid, thymidine, glycerol, DL- α -glycerol phosphate and D-glucose 6-phosphate as sole carbon sources; negative for utilization of α -cyclodextrin, glycogen, lactose, melibiose, methyl β -D-glucoside, D-psicose, raffinose, xylitol, α -hydroxybutyric acid, itaconic acid, α -ketobutyric acid, putrescine and 2,3-butanediol (Biolog GN2). Resistant to (μ g ml⁻¹) penicillin (0.25), amoxicillin (4), amoxicillin/clavulanic acid (4/2), oxacillin (2), cephalothin (8), cefoperazone (4), erythromycin (1), lincomycin (2), pristinamycin (2), tylosin (2), colistin (4), sulfamethizole (100), nitrofurantoin (25), fusidic acid (2) and metronidazole (4); sensitive to (μ g ml⁻¹) streptomycin (8), spectinomycin (64), kanamycin (8), gentamicin (4), apramycin (16), tetracycline (4), doxycycline (4), co-trimoxazole (2/38), flumequin (4), oxolinic acid (2), enrofloxacin (0.5) and rifampicin (4) (ATB VET). The major isoprenoid quinone is Q-8. The predominant cellular fatty acids are C_{16:0}, C_{18:1 ω 7c} and C_{17:0} cyclo. The DNA G+C content of the two known strains is 65 mol%.

The type strain, E25^T (=NBRC 103871^T =BCC 36998^T), and a second strain, E21, were isolated from a root and an aerial stem, respectively, of *Panicum repens*, an aquatic plant growing luxuriantly in a highly acidic swamp (pH 2.9) in an AASS area in Thailand.

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