Burkholderia acidipaludis sp. nov., aluminium-tolerant bacteria isolated from Chinese water chestnut (*Eleocharis dulcis*) growing in highly acidic swamps in South-East Asia

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Two strains of aluminium-tolerant bacteria, SA33^T and 7A078, were isolated from Chinese water chestnut (*Eleocharis dulcis*) growing in highly acidic swamps (pH 2–4) in actual acid sulfate soil areas of Vietnam (SA33^T) and Thailand (7A078). The strains were Gram-negative, aerobic, non-spore-forming rods, 0.6–0.7 µm wide and 1.3–1.7 µm long. These strains showed good growth at pH 3.0–8.0 and 17–37 °C. The organisms contained ubiquinone Q-8 as the predominant isoprenoid quinone and C_{16:0}, C_{18:1ω7c} and C_{17:0} cyclo as the major fatty acids. Their fatty acid profiles were similar to those reported for other *Burkholderia* species. The DNA G + C content of these strains was 64 mol%. On the basis of 16S rRNA gene sequence similarity, the strains were shown to belong to the genus *Burkholderia*. Although the 16S rRNA gene sequence similarity values calculated for strain SA33^T to 7A078 and the type strains of *Burkholderia kururiensis*, *B. sacchari* and *B. tuberum* were 100, 97.3, 97.1 and 97.0 %, respectively, strains SA33^T and 7A078 formed a group that was distinct in the phylogenetic trees; the DNA–DNA relatedness of strain SA33^T to 7A078 and these three type strains were respectively 90, 47, 46 and 45 %. The results of physiological and biochemical tests, including whole-cell protein pattern analysis, allowed phenotypic differentiation of these strains from described *Burkholderia* species. Therefore, strains SA33^T and 7A078 represent a novel species, for which the name *Burkholderia acidipaludis* sp. nov. is proposed. The type strain is SA33^T (=NBRC 101816^T =VTCC-D6-6^T). Strain 7A078 (=NBRC 103872 =BCC 36999) is a reference strain.

Yabuuchi *et al.* (1992) created the genus *Burkholderia* by the transfer of seven species from *Pseudomonas*, with *Burkholderia cepacia* as the type species. During the course of a study to develop bioremediation measures for actual acid sulfate soils (AASS; Aizawa *et al.*, 2008; Sasaki *et al.*,

2008), we isolated a number of bacteria associated with plants growing in highly acidic aquatic environments (pH 2–4) of AASS in South-east Asia (Aizawa *et al.*, 2007, 2010; Kimoto *et al.*, 2010). In this present study, we characterized two pH-neutralizing bacteria, strain SA33^T, isolated from Chinese water chestnut (*Eleocharis dulcis*) growing in highly acidic swamps (pH 2–4) in AASS areas in Vietnam, and 7A078, isolated from *E. dulcis* growing in similar areas in 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*.

Abbreviation: AASS, actual acid sulfate soil.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SA33^T and 7A078 are AB513180 and AB513181.

A scanning electron micrograph of cells of strain SA33^T, SDS-PAGE profiles of whole-cell extracts and physiological characteristics and fatty acid compositions of the novel strains and type strains of related species are available as supplementary material with the online version of this paper.

A number of bacteria were isolated from plants of *E. dulcis* by using one-tenth-strength tryptic soy (1/10 TS) agar plates [3.0 g tryptic soy broth l⁻¹ (Difco) solidified with 15.0 g agar l⁻¹ (Difco)] (pH 4.0). For preparation of acidic plates, double-strength medium and 3 % agar solution were autoclaved separately and mixed after autoclaving to prevent hydrolysis of the agar. Among these isolates, pH-neutralizing bacteria were selected on the basis of neutralization of 1/10 TS liquid medium (pH 4.0), determined by measuring the pH of culture supernatants after a 3-day cultivation period. Strains SA33^T and 7A078 were isolated from an aquatic stem sample collected in Vietnam and internal tissues of a surface-sterilized root sample collected in Thailand, respectively. Strains SA33^T and 7A078 formed round, smooth, convex, pale-yellow colonies with entire margins on 1/10 TS (pH 4.0) agar plates. Both strains 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. The strains showed good growth at pH 3–8, with optimum growth at pH 4–7, but no or little growth below pH 2 or above pH 9 when cultured at 30 °C for 5 days. Growth was as good on TS (pH 4.0) agar plates as on 1/10 TS (pH 4.0) agar plates. Growth under anaerobic conditions was determined after 5 days of incubation at 30 °C in an AnaeroPack (Mitsubishi Gas Chemical Co., Ltd). The strains showed good growth on 1/10 TS (pH 4.0) agar plates under aerobic conditions but not under anaerobic conditions. Cells grown on 1/10 TS (pH 4.0) agar plates 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.*, 2010). The cells were irregular rods, 0.6–0.7 µm wide and 1.3–1.7 µm long, when the organisms were cultured on 1/10 TS (pH 4.0) agar plates at 28 °C for 3 days (Supplementary Fig. S1, available in IJSEM Online).

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. These sequences showed high similarity to species within the genus *Burkholderia*. The 16S rRNA gene sequence similarities of strain SA33^T to 7A078, *Burkholderia kururiensis* KP23^T, *B. sacchari* LMG 19450^T and *B. tuberum* STM678^T were 100, 97.3, 97.1 and 97.0 %, respectively. The phylogenetic relationship with closely related species was determined by using MEGA version 4 (Tamura *et al.*, 2007) and the PHYLIP 3.65 package (Felsenstein, 2005) after multiple sequence alignments performed with CLUSTAL_X (Thompson *et al.*, 1997). Evolutionary distances were computed as described by Jukes & Cantor (1969). Phylogenetic trees were reconstructed by using the maximum-parsimony (Kluge & Farris, 1969), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) methods. The reliability of these tree topologies was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985). The phylogenetic trees reconstructed by these three methods showed that strains

SA33^T and 7A078 belonged to the genus *Burkholderia*, in which these strains formed a cluster with 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 mimosa, *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 heleia* (Aizawa *et al.*, 2010) from Chinese water chestnut, *Burkholderia sacchari* (Brämer *et al.*, 2001) isolated from soil of a sugar cane plantation and *B. 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 constructed 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 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) with the modification that the hybridization temperature was 51 °C. The DNA–DNA relatedness value of strain SA33^T to 7A078 was 90 %, which is higher than the recommended threshold value (70 %) for the delineation of genomic species (Wayne *et al.*, 1987). Strain SA33^T exhibited relatively low levels of DNA–DNA relatedness with respect to *B. kururiensis* JCM 10599^T (47 %), *B. sacchari* LMG 19450^T (46 %) and *B. tuberum* LMG 21444^T (45 %), indicating that strain SA33^T was not related to them at the species level. These data suggest that strains SA33^T and 7A078 represent a novel species of the genus *Burkholderia*.

Additionally, strains SA33^T and 7A078 could be distinguished from closely related species of *Burkholderia* in the phylogenetic trees (Fig. 1) on the basis of physiological characteristics (Supplementary Table S1). Strains SA33^T and 7A078 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 the closely related type strains in the phylogenetic trees (Fig. 1), except *B. sacchari* LMG 19450^T and *B. ferrariae* LMG 23612^T, were positive. Interestingly, strains SA33^T and 7A078, as well as *B. ferrariae* LMG 23612^T, gave positive results in the PCR assay for *nifH* (Caballero-Mellado *et al.*, 2007), 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

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (positions 97–1417 of the *Escherichia coli* 16S rRNA gene), showing the positions of strains SA33^T and 7A078 among species of the genus *Burkholderia*. Numbers at branch nodes are percentages based on 1000 resamplings; only values $\geq 50\%$ are given. The sequence of *Pandora* norimbergensis LMG 13019^T was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.

the diazotrophy of these *nifH*-positive strains. Strains SA33^T and 7A078 could grow in the presence of 5 mM AlCl₃ in 0.1% BactoTryptone (Difco) adjusted to pH 3.5 with sulfuric acid, whereas the closely related type strains except those of *B. tropica* and *B. unamae* did not. Standard physiological tests were carried out according to the 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, the API ZYM and API 20E systems and the ATB VET system (bioMérieux), respectively, according to the manufacturer's instructions. Utilization of various substrates as sole carbon sources was tested by using Biolog GN2 microplates in accordance with the manufacturer's instructions. Analyses of DNA G+C content and cellular fatty acids were performed as described by Tamura *et al.* (1994). The major isoprenoid quinone was determined as described previously (Aizawa *et al.*, 2010). The DNA G+C content of strains SA33^T and 7A078 was 64 mol%. 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_{18:1ω7c} and C_{17:0} cyclo (Supplementary Table S2); similar patterns were obtained

from cells of the type strains of closely related species of the genus. The major isoprenoid quinone of the strains was determined to be Q-8, as is the case for 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.*, 2010).

Whole-cell protein extracts were prepared from strains SA33^T and 7A078 and the type strains of several related species and analysed by SDS-PAGE, as described previously (Pot *et al.*, 1994), and the results are shown in Supplementary Fig. S2. The newly isolated strains showed protein profiles that were clearly different from those of type strains of related *Burkholderia* species. 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 support the notion that strains SA33^T and 7A078 represent a novel aluminium-tolerant *Burkholderia* species.

Therefore, based on the physiological, biochemical, chemotaxonomic and molecular-genetic results described above, strains SA33^T and 7A078 were concluded to represent a novel species of the genus *Burkholderia*, for which the name *Burkholderia acidipaludis* sp. nov. is proposed.

Description of *Burkholderia acidipaludis* sp. nov.

Burkholderia acidipaludis [a.ci'di.pa.lu'dis. N.L. adj. *acidus* -a -um (from L. n. *acidum*) acid; L. gen. n. *paludis* of a swamp; N.L. gen. n. *acidipaludis* of an acidic swamp].

Cells are Gram-negative, strictly aerobic, non-spore-forming, non-motile rods ($0.6\text{--}0.7 \times 1.3\text{--}1.7\ \mu\text{m}$). Colonies are smooth, round, convex and pale-yellow with entire margins after 3 days of cultivation at $32\ ^\circ\text{C}$ on 1/10 TS (pH 4.0) agar plates. Growth occurs at $17\text{--}37\ ^\circ\text{C}$ with optimum growth at $28\text{--}32\ ^\circ\text{C}$. The pH range for growth is 3–8, with optimum growth at pH 4–7. Positive for hydrolysis of Tweens 20, 40 and 60. Positive for activities of oxidase, catalase, acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and urease and reduction of nitrate to nitrite, but negative for hydrolysis of DNA and gelatin, activities of lipase (C4), trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase and proteinase, indole production and reduction of nitrate to nitrogen gas (API ZYM, API 20E and API 20NE systems). 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, inositol, D-mannitol, D-sorbitol, cellobiose, maltose, lactose, D-lyxose, D-tagatose, D- and L-fucose and D- and L-arabitol, but negative for acid production from methyl β -D-xylopyranoside, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, amygdalin, arbutin, salicin, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, xylitol, turanose, potassium gluconate and potassium 5-ketogluconate (API 50CH). Positive for utilization of Tweens 40 and 80, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, *i*-erythritol, D-fructose, L-fucose, D-galactose, α -D-glucose, *myo*-inositol, D-mannitol, D-mannose, D-psicose, L-rhamnose, D-sorbitol, pyruvic acid methyl ester, succinic acid monomethyl ester, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, β -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, DL-lactic acid, sebacic acid, succinic acid, bromosuccinic acid, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, hydroxy-L-proline, L-phenylalanine, L-proline, L-pyrroglutamic acid, L-threonine, DL-carnitine, urocanic acid, phenylethylamine and glycerol as sole carbon sources, but negative for utilization of α -cyclodextrin, *N*-acetyl-D-galactosamine, gentiobiose, lactose, lactulose, maltose, melibiose, methyl β -D-glucoside, raffinose, sucrose, turanose, xylitol, *cis*-aconitic acid, citric acid, D-glucuronic acid, itaconic acid, α -ketoglutaric acid, α -ketovaleric acid, malonic acid, D-saccharic acid, glucuronamide, glycyl L-aspartic acid, glycyl L-glutamic acid, L-ornithine, inosine, thymidine, putrescine, 2-aminoethanol and 2,3-butanediol (Biolog GN2 Microplate). Resistant to ($\mu\text{g ml}^{-1}$) penicillin (0.25), amoxicillin (4), amoxicillin/clavulanic acid (4/2), oxacillin (2), cephalothin (8), cefoperazone (4), chloramphenicol (8), erythromycin (1), lincomycin (2), pristnamycin (2), tylosin (2), sulfamethizole (100), nitrofurantoin (25), fusidic acid (2), rifampicin (4) and metronidazole (4), but sensitive to

streptomycin (8), spectinomycin (64), kanamycin (8), apramycin (16), tetracycline (4), doxycycline (4), gentamicin (4), colistin (4), co-trimoxazole (2/38) and oxolinic acid (2) (ATB VET). The major isoprenoid quinone is Q-8. The predominant cellular fatty acids are $\text{C}_{16:0}$, $\text{C}_{18:1\omega7c}$ and $\text{C}_{17:0}$ cyclo. The DNA G+C content of the two known strains is 64 mol%.

The type strain, SA33^T (=NBRC 101816^T =VTCC-D6-6^T), was isolated from an aquatic plant, *Eleocharis dulcis*, that grows luxuriantly in a highly acidic swamp (pH 3) in an AASS area in Vietnam. Strain 7A078 (=NBRC 103872 =BCC 36999), isolated from a similar habitat in Thailand, is a reference strain.

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