Burkholderia susongensis sp. nov.,
a mineral-weathering bacterium isolated from
weathered rock surface

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A novel type of mineral-weathering bacterium was isolated from the weathered surface of rock
(mica schist) collected from Susong (Anhui, China). Cells of strain L226^T were Gram-stain
negative. The strain grew optimally at 30 °C, with 1 % (w/v) NaCl and at pH 7.0 in trypticase soy
broth. On the basis of 16S rRNA gene phylogeny, strain L226^T was shown to belong to the genus
Burkholderia and the closest phylogenetic relatives were Burkholderia sprentiae WSM5005^T
(98.3%), Burkholderia acidipaludis NBRC 101816^T (98.2%), Burkholderia tuberum STM678^T
(97.2%) and Burkholderia diazotrophica JPY461^T (97.1%). The DNA G+C content was
63.5 mol% and the respiratory quinone was Q-8. The major fatty acids were C_{16:0}, C_{17:0}\text{cyclo}
and C_{19:0}\text{cyclo\omega8c}. The polar lipid profile of strain L226^T consisted of a mixture of
phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, unknown lipids and
unidentified aminophospholipids. Based on the low level of DNA–DNA relatedness (ranging from
25.8 % to 34.4 %) to the tested type strains of species of the genus Burkholderia and unique
phenotypic characteristics, it is suggested that strain L226^T represents a novel species of the
genus Burkholderia, for which the name Burkholderia susongensis sp. nov., is proposed. The type
strain is L226^T (= CCTCC AB2014142^T = JCM 30231^T).

The genus Burkholderia, belonging to the family Burkholderiaceae of the class Betaproteobacteria was
proposed by Yabuuchi et al. (1992) and emended by Gillis et al. (1995). At the time of writing, 88 species with validly
published names were recognized as members of the genus Burkholderia (http://www.bacterio.net/burkholderia.html)
(De Meyer et al., 2013) including six recently described species, Burkholderia jiangsuensis (Liu et al., 2014),
Burkholderia cordobensis (Draghi et al., 2014), Burkholderia aspalathi (Mavengere et al., 2014), Burkholderia dlworthii
(De Meyer et al., 2014), Burkholderia eburnea (Kang et al., 2014) and Burkholderia pseudomultivorans (Peeters et al.,
2013). It has been reported that species of the genus Burkholderia have been isolated from humans (cystic
fibrosis), rhizosphere soil, root nodules, animals, plants, water and hospital equipment (Vandamme et al., 2007;
Suárez-Moreno et al., 2012). By a polyphasic approach, including 16S rRNA gene and recA and gyrB gene sequence
analysis, polar lipid profile and phenotypic and biochemical characterization, strain L226^T was shown to be affiliated
with the genus Burkholderia and may represent a novel species of this genus.

Strain L226^T was isolated from the weathered surface of rock (mica schist) collected from Susong, Anhui (China).
The medium used for isolation contained (l

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- 3
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K_2HPO_4, 0.5 g MgSO_4, 2.0 g K,HPO_4, 0.5 g MgSO_4, 0.1 g NaCl, 0.5 g CaCO_3 and 15.0 g agar. Weathered rock samples were added to flasks
containing physiological salt solution (0.85 % NaCl) and shaken at 200 r.p.m. for 30 min to allow bacteria to detach
from the rock particles. The suspensions were then allowed to stand for about 10 min. Serial 10-fold dilutions of sample suspensions (10^{-3}–10^{-5}) were plated onto agar plates to determine total culturable bacteria. The plates
were incubated for 3 days at 28 °C. Strain L226^T was picked. The mineral weathering experiment of strain L226^T
was performed according to the method of Huang et al. (2014). The concentrations of Al, Si and K released by
strain L226^T from biotite in the culture solutions were determined by inductively coupled plasma-optical emission
spectrometry. The mineral weathering experiment showed that the Al, Si and K released by strain L226^T from
biotite were increased by 2.7-fold, 180-fold and 18.8-fold,

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, recA and gyrB gene sequences of strain L226^T are KJ746438, KM246785
and KM246786, respectively.

Four supplementary figures are available with the online Supplementary Material.
respectively, compared with the uninoculated controls. Strain L226<sup>T</sup> was cultured routinely on trypticase soy agar (TSA) (Pancreatic digest of casein 15 g, Papain digest of soybean meal 5 g, Nacl 5 g, Agar 15 g) for additional taxonomic experiments. The reference strains Burkholderia tuberum LMG 21444<sup>T</sup>, Burkholderia sprentiae LMG 27175<sup>T</sup> and Burkholderia diazotrophica LMG 26031<sup>T</sup> were obtained from the Belgian Coordinated Collections of Microorganisms (BCCM) and Burkholderia acidipaludis NBRC 101816<sup>T</sup> was obtained from the NITE Biological Research Center (NBRC). The four type strains were used as reference strains in biochemical, chemotaxonomic and DNA–DNA hybridization experiments.

Cellular morphology and motility were examined by light microscopy (CX21; Olympus) and transmission electron microscopy (H-7650; Hitachi) using cells from the exponential phase grown on TSA agar at 30 °C for 24 h. Motility was tested by the hanging drop method (Murray et al., 1994). A Gram reaction was determined as described by Murray et al. (1994). The determination of the optimal growth temperature was performed using TSA liquid medium at 4, 10, 15, 28, 37, 40 and 45 °C, and assayed after 5 days of incubation at 30 °C. The pH range for growth was determined in TSA liquid medium adjusted to pH 4.0–11.0 (at intervals of 1 pH unit) with NaOH and HCl solution before autoclaving. The buffers used to keep the pH of the medium stable (each at a final concentration of 100 mM) were acetate (for pH 4.0–5.0), phosphate (for pH 6.0–8.0) and Tris (for pH 9.0–11.0). Growth was also evaluated at 30 °C after 5 days of incubation. To investigate tolerance to NaCl, trypticase soy broth was used and the NaCl concentration was altered (0–10 %, w/v, at intervals of 1.0 %) after 5 days of incubation at 30 °C.

Standard physiological tests were performed according to the methods described by Gordon et al. (1974) and Lányi (1988). Susceptibility to antibiotics was tested on TSA plates using discs containing the following concentrations of antibiotics (per disc): ampicillin, 10 μg; gentamycin, 10 μg; kanamycin, 30 μg; neomycin, 30 μg; rifampicin, 5 μg; streptomycin, 10 μg; tetracycline, 30 μg; chloramphenicol, 30 μg. The plates were incubated at 30 °C and read after 5 days of incubation. The zone (≥ 2 mm) of inhibition was used to separate susceptibility from resistance. Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined based on the oxidation of 1 % (w/v) tetramethyl-p-phenylenediamine. Enzyme activities and acid production from different carbohydrates were determined using API ZYM systems (bioMérieux) according to the manufacturer’s instructions. Reaction to various substrates was determined by using API 20NE galleries (bioMérieux) and the Biolog GN2 micro plate assays as recommended by the manufacturers.

Growth of strain L226<sup>T</sup> was aerobic and cells were Gram-stain-negative. Rod-shaped cells were observed, but neither flagella nor spores were found (see Fig. S1, available in the online Supplementary Material). Colonies on TSA plates were pale yellow with entire margins, round, convex and smooth with a diameter of 1.0–1.5 mm after incubation for 48 h at 30 °C. Strain L226<sup>T</sup> could grow at 15–40 °C (optimum, 30 °C), at pH 4.0–8.0 (optimum, pH 7.0) and with 0–4 % NaCl (w/v; optimum, 1 %). Strain L226<sup>T</sup> was resistant to ampicillin, neomycin and rifampicin and sensitive to chloramphenicol, tetracycline, gentamicin and kanamycin. More detailed physiological and biochemical characteristics of strain L226<sup>T</sup> are given in the species description and a comparison of disparate characteristics between strain L226<sup>T</sup> and the four reference strains is given in Table 1.

For fatty acid methyl esters analysis, stationary phase cells of strains L226<sup>T</sup>, B. tuberum LMG 21444<sup>T</sup>, B. sprentiae LMG 27175<sup>T</sup>, B. diazotrophica LMG 26031<sup>T</sup>, and B. acidipaludis NBRC 101816<sup>T</sup> were harvested from TSA plates after incubation for 2 days at 30 °C. The fatty acid methyl esters were extracted and prepared according to the protocol of the Sherlock Microbial Identification System (Sherlock version 6.1; MIDI). Fatty acids were analysed by GC (model 6890;...
Hewlett Packard) and identified by using the Microbial Identification software package (Sasser, 1990). The cellular fatty acid profiles of strain L226T and the type strains of related species of the genus *Burkholderia* are shown in Table 2. The major cellular fatty acids (>10 %) of strain L226T and the type strains of related species of the genus *Burkholderia* were C17:0 cyclo, C16:0 and C19:0 cyclo o8c. The presence of C16:0 3-OH supports the placement of these strains in the genus *Burkholderia* (Gillis et al., 1995; Garrity et al., 2005). Although the fatty acid profile of strain L226T was similar to those of other closely related species of the genus *Burkholderia*, trace amounts (<1 %) of C18:1ω5c were detected in *B. sprentiae* LMG 27175T, *B. acidipaludis* NBRC 101816T, *B. tuberum* LMG 21444T and *B. diazotrophica* LMG 26031T but not detected in strain L226T. Furthermore, a trace amount (<1 %) of summed feature 3 (C16:1ω7c and/or C16:1ω6c) was detected in strain L226T. Summed feature 3 (C16:1ω7c and/or C16:1ω6c) comprising more than 1 % was detected in *B. sprentiae* LMG 27175T, *B. acidipaludis* NBRC 101816T, *B. tuberum* LMG 21444T and *B. diazotrophica* LMG 26031T. Trace amounts (<1 %) of C8:0 3-OH were detected only in strain L226T and *B. sprentiae* LMG 27175T.

For polar lipid analysis, cells of strain L226T were harvested from TSA after incubation for 24 h at 30°C. Polar lipids were extracted from 100 mg freeze-dried cells by the modified method of Minnikin *et al.* (1984) and separated by TLC on Merck Kieselgel 60-HPTLC. Total polar lipids were detected by spraying with phosphomolybdic acid solution (Sigma-Aldrich) followed by heating at 150°C for 10 min. Aminolipids were detected by spraying the plate with a 0.2% (w/v) solution of ninhydrin in butanol saturated with water followed by heating at 105°C for 10 min (Ross *et al.*, 1985). Phospholipids were detected by spraying the plate with the Zinzadze reagent of Dittmer & Lester (1964). Glycolipids were detected with 1-naphthol spray reagent by heating at 100°C for 3–5 min (Jacin & Mishkin, 1965). The DNA G+C content was determined using the thermal denaturation method as described by Mesbah *et al.* (1989) using reversed-phase HPLC. Menaquinones were extracted and purified by the methods of Collins *et al.* (1977) and Tamaoka *et al.* (1983) and then analysed by HPLC (model 1100; Agilent) with a Zorbax EclipseXB-C18.5 column (250 × 64.6 mm).

Strain L226T exhibited a complex polar lipid profile consisting of phosphatidylethanolamine (PE), phosphatidyglycerol (PG), diposphatidylglycerol (DPG), two unknown lipids and four unidentified aminophospholipids (APLs) (see Fig. S2). Strain L226T exhibited a very similar polar lipid profile to its closest relatives, *B. acidipaludis* NBRC 101816T, *B. tuberum* STM678T and *B. diazotrophica* JPY461T, with the major polar lipids PE, PG, DPG and APL (Sheu *et al.*, 2013). However, unknown lipids were only detected in strain L226T. Furthermore, the phospholipids and unidentified aminolipid of these strains could not be found in strain L226T. The DNA G+C content of strain L226T was 63.5 mol%, which is within the range reported for members of the genus *Burkholderia* (Table 1). The respiratory quinone detected in strain L226T was Q-8, which is in agreement with that of other species of the genus *Burkholderia* (Valverde *et al.*, 2006; Sheu *et al.*, 2012, 2013). Thus, the fatty acid profile, polar lipid profile, DNA G+C content and major isoprenoid quinone of strain L226T were all typical of species within the genus *Burkholderia*.

Genomic DNA was prepared according to the method of Marmur (1961). The 16S rRNA gene sequence of strain L226T was determined following PCR amplification using primers 27f and 1492r (Lane, 1991). Amplification of the partial recA and gyrB genes was performed with primers as described previously by Spilker *et al.* (2009). Amplification products were purified and cloned into pEASY-T3 vector (TransGen Biotech). Sequencing was performed directly on a sequencer (ABI 3730; Invitrogen). Phylogenetic analysis was performed using MEGA software, version 5 (Tamura *et al.*, 2011), after multiple alignments of the data by CLUSTAL X (Thompson *et al.*, 1997). Distances (corrected according to Kimura’s two-parameter model; Kimura, 1983) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony trees were generated by using the treeing algorithms in the PHYLIB software package (Felsenstein, 1993). Bootstrap analysis with 1000 replicates was performed to assess the support of the clusters.

### Table 2. Fatty acid composition of the members of the genus *Burkholderia*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Strain L226T</th>
<th>Strain STM678</th>
<th>Strain JPY461</th>
<th>Strain NBRC 101816</th>
<th>Strain LM26031</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8:1ω7c</td>
<td>4.7</td>
<td>2.9</td>
<td>4.2</td>
<td>4.9</td>
<td>4.7</td>
</tr>
<tr>
<td>C14:0</td>
<td>29.3</td>
<td>23.7</td>
<td>18.1</td>
<td>22.1</td>
<td>22.4</td>
</tr>
<tr>
<td>C16:0</td>
<td>1.1</td>
<td>1.6</td>
<td>3.6</td>
<td>4.4</td>
<td>4.6</td>
</tr>
<tr>
<td>C16:0 2-OH</td>
<td>4.5</td>
<td>4.2</td>
<td>5.1</td>
<td>4.9</td>
<td>4.7</td>
</tr>
<tr>
<td>C16:0 3-OH</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>C17:0 cyclo</td>
<td>29.4</td>
<td>28.0</td>
<td>22.4</td>
<td>22.6</td>
<td>16.7</td>
</tr>
<tr>
<td>C18:0</td>
<td>TR</td>
<td>1.0</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C18:1ω6c</td>
<td>TR</td>
<td>ND</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C19:0 cyclo</td>
<td>18.8</td>
<td>20.1</td>
<td>18.9</td>
<td>15.9</td>
<td>23.8</td>
</tr>
<tr>
<td>Summed feature 2*</td>
<td>5.7</td>
<td>5.6</td>
<td>6.6</td>
<td>6.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Summed feature 3*</td>
<td>1.4</td>
<td>1.0</td>
<td>2.0</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Summed feature 8*</td>
<td>2.1</td>
<td>7.9</td>
<td>12.7</td>
<td>13.5</td>
<td>10.9</td>
</tr>
</tbody>
</table>

*Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 2 comprises C14:0 3-OH and iso-C15:0 1. Summed feature 3 comprises C16:1ω7c and/or C16:1ω6c. Summed feature 8 comprises C18:1ω7c and/or C18:1ω6c.
An almost-complete 16S rRNA gene sequence (1453 bp) was obtained for strain L226T. The sequence was subjected to similarity searches by using the sequence matching tool of the NCBI BLAST program (http://www.ncbi.nlm.nih.gov), Ribosomal Database Project II (http://rdp.cme.msu.edu/) and EzTaxon (http://www.eztaxon.org/; Kim et al., 2012). Comparative 16S rRNA gene sequence analysis showed that strain L226T was affiliated phylogenetically with species of the genus *Burkholderia*, the highest similarities being found with the sequences of *B. sprentiae* WSM5005T (98.3 % similarity), *B. acidipaludis* NBRC 101816T (98.2 % similarity), *B. tuberum* STM678T (97.2 % similarity) and *B. diazotrophica* JPY461T (97.1 % similarity). Phylogenetic trees based on the neighbour-joining (NJ) and maximum-likelihood (ML) methods are shown in Fig. 1. Strain L226T and *B. acidipaludis* NBRC 101816T formed an independent cluster (Fig. 1). The overall topologies of the phylogenetic trees obtained with the ML and NJ methods were rather similar. The 739 bp segment of the *gyrB* gene and 708 bp segment of the *recA* gene were amplified and sequenced. They exhibited 91.0 % and 96.0 % similarity to those from *B. sprentiae* WSM5005T, respectively. The phylogenetic tree based on the *gyrB* gene sequences is shown in Fig. S3. Strain L226T formed an independent cluster and the phylogenetic tree based on the *recA* gene sequences (Fig. S4) also revealed an independent cluster.

DNA–DNA hybridization experiments were carried out as described by De Ley et al. (1970) using a UV/VIS spectrophotometer (UV1201; Rayleigh) to evaluate the DNA–DNA relatedness between strain L226T and its closest neighbours, *B. tuberum* LMG 21444T, *B. sprentiae* LMG 27175T, *B. diazotrophica* LMG 26031T and *B. acidipaludis* NBRC 101816T. The DNA–DNA relatedness between strain L226T and *B. tuberum* LMG 21444T, *B. sprentiae* LMG 27175T, *B. diazotrophica* LMG 26031T and *B. acidipaludis* NBRC 101816T was 26.4 %, 27.1 %, 29.8 % and 30.5 % (mean ± SD, n = 3) respectively. All of the values were significantly lower than 70 %, the threshold value recommended for the assignment of genomic species (Wayne et al., 1987).

In view of the combined morphological, physiological and chemotaxonomic data and the phylogenetic tree presented here, it is evident that strain L226T belongs to the genus *Burkholderia*. However, diverse phenotypic characteristics as well as phylogenetic and genomic discrepancy clearly distinguish strain L226T from closely related species of the genus *Burkholderia*. Thus, on the basis of the data presented, we suggest that strain L226T represents a novel species of the genus *Burkholderia*, for which the name *Burkholderia susongensis* sp. nov. is proposed.

**Description of Burkholderia susongensis** sp. nov.

*Burkholderia susongensis* (su.song.en’sis. N.L. fem. adj. *susongensis* referring to Susong county, Anhui Province, PR China, where the organism was isolated).

Cells are Gram-stain-negative, motile, aerobic, non-spore-forming rods. Colonies on TSA agar are pale yellow, circular, smooth and convex with entire edges. The colony size is approximately 1.0–1.5 mm in diameter on TSA agar after 48 h incubation at 30 °C. Growth occurs at 15–40 °C (optimum, 30 °C), at pH 4.0–8.0 (optimum, pH 7.0) and with 0–4 % (w/v) NaCl (optimum, 1 %) in trypticase soy broth. Catalase- and oxidase-positive. Gives a positive result in tests for hydrolysis of Tween 20, 40 and 80 and starch, but a negative result in tests for methyl red and Voges–Proskauer reactions. In API 20NE tests, positive reactions for urease and β-galactosidase activities, arginine dihydrolase activity, aesculin hydrolysis and assimilation of glucose, mannose, mannitol, N-acetylgalactosamine, glucurionate, citrate, adipate, malate and phenylacetate, and negative reactions for nitrate reduction, indole production, glucose fermentation, gelatin hydrolysis and assimilation of malose and caprate. In the API ZYM kit, alkaline phosphatase, C4 esterase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present and C8 esterase lipase, C14 lipase, valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent.

According to the Biolog GN2 system, the following carbon sources are utilized: α-cyclodextrin, dextrin, glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, D-arabitol, cellobiose, D-fructose, L-fructose D-galactose, gentiobiose, myo-inositol, α-lactose, lactulose, melibiose, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, methyl pyruvate, monomethyl succinate, acetic acid, cis-aconitic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninate, D-alanine, L-alanine, L-α-lanin glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyI-L-glutamic acid, histidine, hydroxy-L-proline,
L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, L-serine, DL-carnitine, γ-aminobutyric acid, urocanic acid, isonoic, uridine, 2-aminoethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate; L-arabinose, i-erythritol, methyl β-D-glucoside, α-ketovaleric acid, D-serine, DL-carnitine, thymidine, phenylethylamine and putrescine are not utilized. Resistant to ampicillin, neomycin and rifampicin and sensitive to chloramphenicol, tetracycline, gentamicin and kanamycin. The major fatty acids (>5%) are C17:0 cyclo, C16:0 cyclo ω8c and summed feature 2 (comprising C14:0 3-OH and iso-C16:1 I). The major respiratory quinone is Q-8. The polar lipid profile consists of a mixture of phosphatidylethanolamine, phosphatidylglycerol, diphasphatidylglycerol, two unknown lipids and four unidentified aminophospholipids.

The type strain, L226T (=CCTCC AB2014142T=JCM 30231T), was isolated from the weathered surfaces of rock (mica schist) in Susong, Anhui province, PR China. The DNA G + C content of the type strain is 63.5 mol%.

Acknowledgements

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References


