Microbacterium sorbitolivorans sp. nov., a novel member of Microbacteriaceae isolated from fermentation bed in pigpen

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Two bacterial strains SZDIS-1-1T and GZDIS-1-1 were isolated from fermentation bed of a pigpen in Fujian Province, China. Cells were Gram-stain-positive, facultatively anaerobic, short rods without flagellum. Their nearest phylogenetic neighbours were Microbacterium amylyticum NS5 (16S rRNA gene sequence similarity 98.2 %), Microbacterium indicum BBH6 (97.9 %) and Microbacterium gubbeenense DPC 5286 (97.8 %) with the DNA–DNA hybridization values to strain SZDIS-1-1T as 20.0±1.2, 14.3±5.8 and 19.1±1.6 %, respectively. The DNA G+C content of the new isolates was 67.6–71.1 mol% and anteiso-C15:0, anteiso-C17:0 and iso-C16:0 were their predominant cellular fatty acids. These results were consistent with classification into the genus Microbacterium. However, cell-wall sugars and characteristic amino acid were rhamnose, glucose, galactose and ornithine. Major menaquinones were MK-11 and MK-10. The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, glycolipids, unknown phospholipid and unknown lipids. These characteristics constructed a distinct profile of the two isolates. Therefore, based on polyphasic taxonomic characteristics, strains SZDIS-1-1T and GZDIS-1-1 represented a novel species of genus Microbacterium, for which the name Microbacterium sorbitolivorans sp. nov. is proposed, with strain SZDIS-1-1T (=CGMCC 1.15228=DSM 103422) as the type strain.

The genus Microbacterium, belonging to the family Microbacteriaceae (order Actinomycetales, class Actinobacteria) was established by Orla-Jensen (1919) and subsequently reclassified by Collins et al. (1983), who defined the genus for species containing peptidoglycan with lysine. Takeuchi & Hatano (1998) proposed to combine with genus Aureobacterium that its species containing peptidoglycan with ornithine based on 16S rRNA gene sequences. Thus, the variation of the amino acids is regarded as a species and not a genus characteristic. At the time of writing, Microbacterium comprised 97 species (http://www.bacterio.net/microbacterium.html), which were isolated from soil, sea sediments, insects, human clinical specimens, plants and oil-contaminated water (Anand et al., 2012; Wang et al., 2014). Their cells are generally strictly aerobic except for some species; the cell-wall peptidoglycan and sugars principally contain lysine or ornithine and galactose and rhamnose, respectively; major menaquinones are unsaturated MK11, MK12, MK13 and/or MK14; polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol and one or more glycolipids; cellular fatty acids are predominantly anteiso-C15:0, anteiso-C17:0 and iso-C16:0 (Suzuki et al., 2012); DNA G+C content range is 61–75 mol% (Alves et al., 2014).

During research into the microbial resources of a pigpen in Xiamen, Fujian Province of China, a novel species with candidate strains SZDIS-1-1T and GZDIS-1-1 was isolated from the fermentation bed by 10-fold dilution plating on dextran-inorganic salt agar plates (10–1; 10 g dextran, 2 g KNO₃, 0.05 g MgSO₄·7H₂O, 2 g KH₂PO₄·3H₂O, 0.02 g CaCO₃, 0.01 g FeSO₄·7H₂O, 10 g NaCl, 2 g KCl, 3 g MgSO₄·6H₂O) at 30 °C for 7 days. The isolates were then purified and incubated using R medium (JCM medium No. 26) (http://www.jcm.riken.jp).

After cells were grown in R medium at 30 °C for 2 days, genomic DNA extraction, 16S rRNA gene PCR
amplification and sequencing were performed as described by Cai et al. (2011). The similarities of 16S rRNA gene sequences between two isolates and other related species were analysed by EzTaxon server (http://www.ezbiocloud.net) (Kim et al., 2012) and their multiple sequence alignments were performed using CLUSTAL_W (Thompson et al., 1994). Phylogenetic analyses were performed by neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods in MEGA version 5.0 software (Tamura et al., 2011). Distances for neighbour-joining method were calculated using distance options according to Kimura’s two-parameter model (Kimura, 1980). The topologies of the phylogenetic trees were assessed by bootstrap analysis (Felsenstein, 1985) of 1000 resampled datasets. DNA–DNA hybridization between strain SZDIS-1-1T and strains GZDIS-1-1, Microbacterium amylyticum N5T, M. indicum BBH6T and M. gubbeenense DPC 5286T were conducted using thermal denaturation and renaturation method (De Ley et al., 1971). The G+C content of genomic DNA was determined using HPLC as described by Mesbah et al. (1989) with reference DNA of Lambda phage (Sigma) (49.8 mol%).

For analysis of chemotaxonomic characteristics, cells of two isolates and the above mentioned three reference strains were incubated in R medium at 30 °C and harvested at the same physiological age as the initial stationary phase. To standardize the physiological age, growth curve for each strain was tested respectively. For fatty acid analysis, all strains were harvested in the optical density reached 70% of the values to stationary phase in each growth curve. Cell walls were prepared by the method of Lechevalier & Lechevalier (1980), cell-wall sugars were determined following procedures described by Staneck & Roberts (1974) and cell-wall characteristic diamino acids were examined using high-speed amino acid analyzer L-8900 (Hitachi). Respiratory quinones were analysed as described by Komagata & Suzuki (1987). Polar lipids were extracted and examined by one- and two-dimensional TLC on Merck silica gel 60 F254 aluminum-backed thin-layer plates, according to the procedures of Kates (1986) and Collins et al. (1980). TLC plates were sprayed with various specific reagents for the detection of sugars (α-naphthol/sulfuric acid), phosphates (Zinadze reagent, Schiff reagent), amino groups (ninhydrin/n-butyl alcohol, Dragendorff’s reagent) (Guo et al., 2011) and total lipids (molybdatephosphoric acid), respectively. Cellular fatty acids were obtained according to the protocol of the Sherlock Microbial Identification System (MIDI), analysed by GC (6890; Hewlett Packard) and identified using the Microbial Identification software package version 6.0 with method TSBA6 and database TSBA6 6.00.

Morphological characteristics of the isolates were examined after cells were grown on R medium at 30 °C for the initial stationary phase. Cell morphology and flagellum were observed using transmission electron microscopy (JEM14000, JEOL). Gram staining, motility, relationship of oxygen, catalase activity and antibiotic resistance tests were carried out according to Dong & Cai (2001). Growth at different temperatures (4, 10, 15, 20, 25, 30, 37, 40, 42, 55 and 65 °C), NaCl concentrations [0–15 % (w/v) at 1 % intervals] and pH values (4.0–10.0 at 1.0 pH unit intervals) were assessed after incubation in R medium for 7 days. For the pH endurance test, biological buffers such as citric acid/ sodium citrate (for pH 4–5), NaOH/KH₂PO₄ (for pH 6–8) and Na₂CO₃/NaHCO₃ (for pH 9–10) were used to adjust the pH (Tang et al., 2003). Biochemical characteristics, sugar fermentation tests, enzyme activities, oxidase activity and utilization of carbon and nitrogen sources were performed using the API 20E, API 20NE, API 50 CH, API ZYM kits (bioMérieux) and Biolog GEN III Microstation system following manufacturers’ instructions.

Almost complete 16S rRNA gene sequences of the isolates SZDIS-1-1T (1445 bp) and GZDIS-1-1 (1445 bp) were determined. They shared 99.5 % 16S rRNA gene sequence similarity and 82.9±6.0 % DNA–DNA hybridization value to each other, therefore, they belong to the same species. However, they were distinct strains in the same species because of different physiological characteristics, e.g. gelatin hydrolysis, trypsin activity and acid production from 1-xylose, as shown in Table S1. Furthermore, strain SZDIS-1-1T shared the highest 16S rRNA gene sequence similarities with M. amylyticum N5T (98.2 %), M. indicum BBH6T (97.9 %) and M. gubbeenense DPC 5286T (97.8 %). Moreover, DNA–DNA hybridization values between strain SZDIS-1-1T and the three reference strains were 20.0±1.2, 14.3±5.8 and 19.1±1.6 %, respectively. These values were significantly lower than the threshold of 70 % for the delineation of genomic species (Wayne et al., 1987). In phylogenetic trees calculated with different algorithms, the two isolates formed a stable clade with the above three closely related species (Figs 1 and S1, available in the online Supplementary Material). Based on phylogenetic analysis and DNA–DNA hybridization, strains SZDIS-1-1T and GZDIS-1-1T represent a novel species of the genus Microbacterium.

The DNA G+C contents of strains SZDIS-1-1T and GZDIS-1-1 were 67.6 and 71.1 mol%, respectively. The major cellular fatty acids were anteiso-C₁₅:₀ (47.8–49.8 %), anteiso-C₁₇:₀ (22.5–23.5 %) and iso-C₁₆:₀ (22.6–22.9 %), which were consistent with the related species in genus Microbacterium, but the presence of anteiso-C₁₃:₀ (0.1 %) in the two isolates distinguished them from others (Table S1). The peptidoglycan of the two isolates was found to be of B2 type with ornithine as the cell-wall characteristic diamino acid (Yokota et al., 1993). The cell-wall sugars were rhamnose, glucose and galactose. The predominant respiratory quinones were MK-10 and MK-11, which were also identified in M. amylyticum N5T but were different from M. indicum BBH6T, M. gubbeenense DPC 5286T and type strain M. lactica KCTC 9230T. Additionally, the polar lipid profiles were distinct for each related strain as shown in Fig. S2. On the other hand, strains SZDIS-1-1T and GZDIS-1-1 were able to grow at 40 °C, utilize sorbitol as the sole carbon source, produce acid from xylitol and gentiobiose, and output N-acetyl-β-glucosaminidase,
Table 1. Differential characteristics of two isolates and three closely related species, as well as the type strain of the type species in the genus Microbacterium

Strains: 1, strain SZDIS-1-1; 2, strain GZDIS-1-1; 3, *M. amylolyticum* N57; 4, *M. gubbeenense* DPC 5286T; 5, *M. indicum* BBH6T; 6, *M. lacticum* KCTC 9230T. The six strains are Gram-stain positive. All are positive for catalase, VP reaction, aesculin degradation. Negative for oxidase, urease, H₂S and indole production and arginine dihydrolase. Acid is produced from D-glucose, D-mannose, aesculin ferric citrate, salicin, cellobiose, maltose, but not from dulcitol, inositol, D-sorbitol, D-tagatose, D-fucose, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. All are able to utilize dextrin, maltose, cellobiose, lactose, D-salicin, D-glucose, D-mannose, D-galactose, inosine, D-mannitol, D-gluconic acid, Tween 40, acetoacetic acid and acetic acid, but not N-acetylneuraminic acid, 3-methyl glucose, D-aspartic acid, L-alanine, L-histidine, mucic acid, quinic acid, a-ketoglutaric acid, D-malic acid, formic acid as sole carbon or nitrogen source. +, Positive; −, negative; Orn, ornithine; Lys, lysine; Rha, rhamnose; Glc, glucose; Gal, galactose; Man, mannose; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PGM, phosphatidylglycerol mannosides; DMG, dimannosyldiacylglycerol; GL, glycolipid; PL, unidentified phospholipid; Ls, unidentified lipids.

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<td>Yellow</td>
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<td>10–40, 30</td>
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<td>8–30, 28†</td>
<td>8–30, 22†</td>
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<td>Rha, Glc, Gal</td>
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<td>Rha, Glc, Gal</td>
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<td>Major menaquinones</td>
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<td>MK-11, MK-10</td>
<td>MK-11, MK-10*</td>
<td>MK-11, MK-12†</td>
<td>MK-11, MK-12†</td>
<td>MK-11, MK-12§</td>
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Table 1. cont.

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<td>DPG, PG, PL, GLs, Ls</td>
<td>DPG, PG, PL, GLs, Ls</td>
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<td>PG, PI†</td>
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<td>DNA G+C content (mol%)</td>
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<td>71.1</td>
<td>68*</td>
<td>70*</td>
<td>65.5†</td>
<td>71§</td>
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All data were from this study, except *Anand et al. (2012), †Shivaji et al. (2007), §Brennan et al. (2001), ‡Lee et al. (2006), ||Suzuki et al. (2012).

to name a few characteristics. These characteristics were also specific for the two isolates and absent in each of the reference species (Table 1). Detailed morphological, physiological and biochemical characteristics of two isolates SZDIS-1-1T and GZDIS-1-1 are given in the species description and characteristics that differentiate the two isolates from reference strains are given in Table 1. It is therefore evident that strains SZDIS-1-1T and GZDIS-1-1 represent a novel species in the genus Microbacterium, for which the name Microbacterium sorbitolivorans sp. nov. is proposed.

Description of Microbacterium sorbitolivorans sp. nov.

Microbacterium sorbitolivorans (sor.bi.to.li.vo’rans. N. L. n. sorbitol sorbitol; L. v. vorare to eat; N. L. part. adj. sorbitolivorans sorbitol-devouring).

Cells are Gram-stain-positive, facultatively anaerobic short-rod (1.52–1.83×0.65–1.1 μm) without flagellum that undergo binary fission (Fig. S3). The colonies are smooth, circular, convex and pale yellow in colour. Growth occurs at 10–40°C, pH 6.0–9.0 and 0–11% (w/v) NaCl. Optimum growth occurs at 30°C, pH 7.0 and 0% (w/v) NaCl. Positive for catalase, VP reaction, ascorbic degradation, esterase (C8), leucine arylamidase, cystine arylamidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase. Negative for H2S and indole production, urea hydrolysis, nitrate reduction, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, deaminase, alkaline phosphatase, lipase, valine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase. Acid is produced from glycerol, erythritol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, N-
acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, cellulbiose, maltose, lactose, melibiose, sucrose, trehalose, melizitose, raftinose, starch, glycogen, xyitol, gentiobiose, turanose and D-Lyxose, but not from L-sorbose, dulcitol, inositol, D-sorbitol, D-tagatose, fucose, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. Utilizes dextrin, maltose, trehalose, cellulbiose, gentiobiose, sucrose, turanose, stachyose, raftinose, lactose, melibiose, methyl β-D-glucoside, D-salicin, N-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, glycerol, D-fructose 6-phosphate, L-ascartic acid, L-glutamic acid, pectin, L-galactonic acid lactone, D-gluconic acid, L-lactic acid, citric acid, Tween 40, α-hydroxybutyric acid, acetooacetic acid and acetic acid as sole carbon or nitrogen sources. Resistant to floxacin, lomefloxacin, chloramphenicol, azithromycin, clindamycin, tetracycline, sulfamer thoxazole, ceftazidime, oxacillin and nitrofurantoin. The cell wall sugars and characteristic amino acid are rhamnose, glucose, galactose and ornithine, respectively. Major menaquinones are MK-11 and MK-10. Anteiso-C<sub>15</sub>:0, anteiso-C<sub>17</sub>:0 and iso-C<sub>16</sub>:0 are the predominant cellular fatty acids. The polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, glycolipid, unknown phospholipids and unknown lipids. The DNA G+C content of the new isolates is 67.6–71.1 mol%. The type strain SZDIS-1-1<sup>T</sup> (=CGMCC 1.15228<sup>T</sup> = DSM 103422<sup>T</sup>) was isolated from fermentation bed of a pigpen in Xiamen, Fujian Province of China.

Acknowledgements

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