Microbacterium kribbense sp. nov., isolated from soil

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A Gram-positive, short, rod-shaped, cream-coloured actinobacterium, designated MSL-04\textsuperscript{T}, was isolated from a soil sample collected from an agricultural field on Bigeum Island (Republic of Korea) and was subjected to polyphasic characterization to unravel its taxonomic position. The optimum growth temperature and pH were 28–30 °C and pH 7.2–7.4. Chemotaxonomic characteristics such as B2\textsubscript{b}-type peptidoglycan with glycolyl residues, MK-11 and MK-12 as the major menaquinones and a fatty acid profile containing iso- and anteiso-branched fatty acids placed this organism within the genus Microbacterium. Phylogenetic analysis based on 16S rRNA gene sequences confirmed the position of strain MSL-04\textsuperscript{T} in the genus Microbacterium and showed that it belonged to the same clade as Microbacterium ulmi but was distinct from its nearest neighbours. The 16S rRNA gene sequence of strain MSL-04\textsuperscript{T} showed the highest levels of similarity with respect to Microbacterium ulmi LMG 20991\textsuperscript{T} (97.5 % similarity) and Microbacterium arborescens IFO 3750\textsuperscript{T} (97 %). The G+C content of the DNA was 71.0 mol%. The DNA–DNA relatedness between MSL-04\textsuperscript{T} and its closest neighbours (≤70 %) showed that the strain represents a distinct genomic species. On the basis of the phenotypic and genotypic results, strain MSL-04\textsuperscript{T} represents a novel species in the genus Microbacterium, for which the name Microbacterium kribbense sp. nov. is proposed. The type strain is MSL-04\textsuperscript{T} (=KCTC 19269\textsuperscript{T} = DSM 19265\textsuperscript{T}).

The genus Microbacterium, the type genus of the family Microbacteriaceae, was proposed by Orla-Jensen (1919) for Gram-positive, non-spore-forming, rod-shaped bacteria isolated during studies on lactic acid-producing bacteria. More recently, the description of the genus Microbacterium was emended to unite the genera Microbacterium and Aureobacterium (Takeuchi & Hatano, 1998). Members of the genus are widespread and have been isolated from a broad range of environmental habitats, e.g. plants, soil, water, air, steep liquor, milk products and humans. At the time of writing, the genus Microbacterium comprises more than 50 recognized species. Here, we describe the phenotypic and genotypic properties of a novel Microbacterium species.

Strain MSL-04\textsuperscript{T} was isolated from a soil sample from Bigeum Island, Republic of Korea, using R2A agar medium (Difco). Routine cultivation was performed using R2A broth at 28 °C. Bacterial growth was monitored for up to 7 days after inoculation by measuring the turbidity at 600 nm. Cell morphology was observed by using light microscopy after 24 h cell growth. Sporulation was observed by means of phase-contrast microscopy after the cells had reached stationary phase. Gram staining was carried out on exponentially growing cells, using Hucker’s modified method (Cowan & Steel, 1965). The presence of flagella was examined using Leifson’s method (Cowan & Steel, 1965). Growth at different temperatures, salt concentrations (w/v) and pH values was investigated as described by Tang et al. (2003). The pH was adjusted by using autoclaved Na\textsubscript{2}CO\textsubscript{3}. The following buffers were used to control the pH in R2A medium: 0.1 M KH\textsubscript{2}PO\textsubscript{4}/0.1 M NaOH (pH 6.0, 7.0 and 8.0); 0.1 M NaHCO\textsubscript{3}/0.1 M Na\textsubscript{2}CO\textsubscript{3} (pH 9.0 and 10.0); 0.05 M Na\textsubscript{2}HPO\textsubscript{4}/0.1 M NaOH (pH 11.0); and 0.2 M KCl/0.2 M NaOH (pH 12.0). The temperature range for growth of the organism was examined at 10–50 °C (in increments of 5 °C). Salt tolerance was tested from 0 to 7 % NaCl (w/v). Anaerobic growth was tested using incubation at 28 °C in 10 ml rubber-stoppered, screw-capped tubes containing R2A medium (9 ml) covered with liquid paraffin.

Biochemical and physiological tests were performed using the methods described by Shirling & Gottlieb (1966). Oxidase activity was investigated by testing for oxidation of 1 % benzidine (Deibel & Ewans, 1960) and tetramethyl-p-phenylenediamine (Tarrand & Groschel, 1982). Catalase

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MSL-04\textsuperscript{T} is EF466125.
activity was determined by checking bubble formation in a 3\% (v/v) hydrogen peroxide solution. Acid production from carbohydrates was determined as described by Leifson (1963). Substrate utilization and enzyme activities in strain MSL-04\(^T\) were determined using API ZYM test kits (bioMérieux) according to the manufacturer's instructions.

Biomass for biochemical and chemotaxonomic characterization was prepared by culturing strain MSL-04\(^T\) in trypticase soy broth at 28\(^\circ\)C for 5 days and then harvesting cells by centrifugation. The peptidoglycan structure was determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Qualitative analyses of amino acids and peptides in peptidoglycan hydrolysates were carried out as described by Schleifer (1985) and Schleifer & Kandler (1972) with the modification that TLC on cellulose plates was used instead of paper chromatography. Quantitative analysis of amino acids in the total cell hydrolysate was performed using GC and GC-MS as described by MacKenzie (1987) and Groth et al. (1996). The methods used for sugar analysis of whole-cell hydrolysates, determination of the menaquinone pattern and analysis of fatty acids were those of Li et al. (2004a, b) and Chen et al. (2004). Polar lipid profiles were determined by means of two-dimensional TLC (Minnikin et al., 1977). The genomic DNA of the bacterium was extracted and purified by using the method of Marmur (1961). The DNA G+C content of strain MSL-04\(^T\) was measured using the thermal denaturation method of Marmur & Doty (1962).

The 16S rRNA gene sequence of the isolate was amplified by using a PCR as described by Cui et al. (2001). A phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein, 1993) and MEGA, version 2.1 (Kumar et al., 2001), following multiple alignment of data using CLUSTAL_X (Thompson et al., 1997). Evolutionary distances (with distance options according to Kimura’s two-parameter model; Kimura, 1980, 1983) and clustering were calculated with the neighbour-joining (Saitou & Nei, 1987) (Fig. 1) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap analysis (based on 1000 resamplings)

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Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain MSL-04\(^T\) among species of the genus Microbacterium. Asterisks indicate branches that were also recovered using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and maximum-parisimony (Kluge & Farris, 1969) algorithms. Numbers at branching points refer to bootstrap values (based on 1000 resamplings). Curtobacterium luteum DSM 20542\(^T\) was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.
was used to evaluate the tree topology of the neighbour-joining data (Felsenstein, 1985).

Strain MSL-04<sup>T</sup> was found to comprise Gram-positive, aerobic, non-motile, non-spore-forming, short rods. Colonies were lemon-yellowish, circular, convex with entire margins, shiny, moist and approximately 0.4–0.6 × 0.5–0.8 mm in size after 48 h growth at 28 °C in R2A medium. Growth occurred over a wide range of pH (7.0–11.0). The physiological and biochemical characteristics, metabolic properties and substrate-utilization results obtained for strain MSL-04<sup>T</sup> are presented in Table 1 and in the species description below.

Strain MSL-04<sup>T</sup> contained ornithine, alanine, glycine, homoserine and hydroxyglutamic acid in the total hydrolysate of the peptidoglycan. From these data, we concluded that the peptidoglycan type was B2 (Schleifer & Kandler, 1972). Its cell-wall hydrolysate contained ribose, galactose, glucose and trace amounts of xylose. The predominant menaquinones were MK-11 and MK-12. The main polar lipids were diphasphatidylglycerol and phosphatidylglycerol. The major cellular fatty acids of strain MSL-04<sup>T</sup> are C<sub>18:1</sub>v<sub>7c</sub> (35.7 %), anteiso-C<sub>15:0</sub> (23.5 %), iso-C<sub>16:0</sub> (23.1 %), anteiso-C<sub>17:0</sub> (9.7 %), iso-C<sub>15:0</sub> (3.3 %) and iso-C<sub>17:0</sub> (2.3 %). To ascertain the phylogenetic position of strain MSL-04<sup>T</sup>, the almost-complete 16S rRNA gene sequence (1428 nt) was determined. A phylogenetic tree demonstrated that the isolate is a member of the genus Microbacterium, and that it forms an independent phyletic line within a monophyletic subclade. The 16S rRNA gene sequence similarity between the isolate and Microbacterium type strains ranged from 93.7 to 97.5 %. Microbacterium ulmi LMG 20991<sup>T</sup> showed the highest sequence similarity (97.5 %) with respect to strain MSL-04<sup>T</sup>. DNA–DNA hybridization was carried out using the optical renaturation method (De Ley et al., 1970; Huß et al., 1983; Jahnke,

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### Table 1. Physiological and biochemical characteristics that serve to differentiate strain MSL-04<sup>T</sup> from its closest phylogenetic neighbours

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
<th>Strain 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony colour</td>
<td>Lemon yellow</td>
<td>White</td>
<td>Dirty orange</td>
<td>Red–orange</td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>–</td>
<td>w</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydrogen sulfide</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>v</td>
</tr>
<tr>
<td>Urease</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth on sole carbon sources</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td>l-Arabinose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>–</td>
<td>–</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Malate</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Phenyl acetate</td>
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<td>–</td>
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<td>Propionate</td>
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<td>–</td>
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<td>Hydrolysis of:</td>
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<tr>
<td>Gelatin</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Starch</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>Acid production from:</td>
<td></td>
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<tr>
<td>Glycerol</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Glucose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Melezitose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Chemotaxonomy</td>
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<tr>
<td>Menaquinones</td>
<td>11, 12</td>
<td>12, 13, 11, 14, 10</td>
<td>11, 12</td>
<td>11, 12</td>
</tr>
<tr>
<td>Cell-wall diamino acid</td>
<td>Lys</td>
<td>Orn</td>
<td>Lys</td>
<td>Lys</td>
</tr>
<tr>
<td>Cell-wall sugars*</td>
<td>Rib, Gal, Glc, Xyl</td>
<td>Gal, Fuc, Xyl, Rha</td>
<td>6dTal, Man, Gal</td>
<td>Rha, Man, Gal</td>
</tr>
<tr>
<td>Polar lipids†</td>
<td>PG, DPG</td>
<td>PG, DPG, L</td>
<td>ND</td>
<td>DPG, PG, DMG</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>71.0</td>
<td>69.0</td>
<td>71.0</td>
<td>71.0–75.4‡</td>
</tr>
</tbody>
</table>

*6dTal, 6-Deoxytalose; Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.
†DMG, Dimannosyldiaclylglycerol; DPG, diphasphatidylglycerol; PG, phosphatidylglycerol; L, unknown polar lipid.
‡Range of values for a number of strains (Collins et al., 1983).
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1992), under optimal hybridization conditions, between strain MSL-04<sup>T</sup>, M. ulmi LMG 20991<sup>T</sup> and Microbacterium arborescens NBRC 3750<sup>T</sup>. The DNA–DNA relatedness between strain MSL-04<sup>T</sup> and M. ulmi LMG 20991<sup>T</sup> and M. arborescens NBRC 3750<sup>T</sup> was 45 and 51.2 %, respectively. These values are significantly lower than the 70 % threshold recommended for the delineation of genomic species (Wayne et al., 1987). Therefore, the phylogenetic and DNA–DNA hybridization results from this work demonstrate that the isolate is phylogenetically closely related to members of the genus Microbacterium and is not related to any previously described Microbacterium species.

Strain MSL-04<sup>T</sup> shares some chemotaxonomic features with members of the genus Microbacterium, i.e. the major menaquinones are MK-11 and MK-12, iso- and anteiso-branched cellular fatty acids predominate and the DNA G+C content is 71.0 mol%. However, it shows considerable phenotypic and genomic differences from other recognized species of the genus Microbacterium.

On the basis of the results obtained using this polyphasic approach, strain MSL-04<sup>T</sup> represents a novel species of the genus Microbacterium, for which the name Microbacterium kribbense sp. nov. is proposed.

Description of Microbacterium kribbense sp. nov.

Microbacterium kribbense (krib.ben’se. N.L. neut. adj. kribbense arbitrary adjective formed from the acronym of the Korea Research Institute of Bioscience and Biotechnology, KRIBB, where the taxonomic studies on the type strain were performed).

Gram-positive, non-motile, catalase-negative and aerobic. Colonies are pale to lemon yellow in colour. Cells are irregular rods, 0.4–0.6 × 0.5–0.8 μm in size. Growth occurs at pH 7–11 and 20–37 °C; optimal growth occurs at pH 7.2–7.4 and 28 °C. NaCl is tolerated at concentrations up to 3 %. Arabinose, galactose, glucose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, sucrose and trehalose are assimilated, but ribose, fructose and xylose are not. Nitrate is reduced to nitrite. Aesculin, starch and Tween 80 are hydrolysed, but cellulose, gelatin and urea are not. Esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase are detected with the API ZYM enzyme assay; alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-galactosidase, α-mannosidase and α-fucosidase are negative. The diagnostic diaminod acid isomer of the peptidoglycan is L-lysine. The acyl type of the peptidoglycan is N-glycolyl. The predominant menaquinones are MK-11 and MK-12. The major polar lipids are diphosphatidylglycerol and phosphatidylethanolamine. The major cellular fatty acids are C<sub>16:0</sub>, anteiso-C<sub>15:0</sub>, iso-C<sub>16:0</sub>, anteiso-C<sub>17:0</sub>, iso-C<sub>15:0</sub> and iso-C<sub>17:0</sub>. Whole-cell hydrolysate contains ribose, galactose and glucose and xylose. The DNA G+C content of the type strain is 71.0 mol%.

The type strain, MSL-04<sup>T</sup> (=KCTC 19269<sup>T</sup> =DSM 19265<sup>T</sup>), was isolated from a soil sample collected from Bigeum Island, Korea.

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