**Microbacterium immunditiarum** sp. nov., an actinobacterium isolated from landfill surface soil, and emended description of the genus *Microbacterium*

Srinivasan Krishnamurthi,1,2 A. Bhattacharya,1† P. Schumann,3 Syed G. Dastager,2 Shu-Kun Tang,4 Wen-Jun Li4 and T. Chakrabarti1†

1Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Sector 39A, Chandigarh 160 036, India
2National Institute of Oceanography, Dona Paula, Goa 403004, India
3Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7b, D-38124 Braunschweig, Germany
4Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, PR China

Correspondence
T. Chakrabarti
tapan@nccs.re.in or tapanchakra@gmail.com

A Gram-positive, non-endospore-forming bacterium, designated strain SK 18T, was isolated from surface soil of a landfill site by dilution plating on trypticase soy broth agar. Preliminary characterization of strain SK 18T via biochemical tests, analysis of fatty acid methyl esters and partial 16S rRNA gene sequencing placed it within the genus *Microbacterium*. Analysis of the cell wall indicated that the peptidoglycan was of cross-linkage type B, containing the amino acids lysine and ornithine and with muramic acid in the N-glycolyl form. The polar lipids were phosphatidylglycerol, diphosphatidylglycerol, an unidentified phospholipid and an unidentified glycolipid. The major fatty acids of the cell membrane were anteiso-C17 : 0, anteiso-C15 : 0 and iso-C16 : 0. These data further strengthened placement of the strain within the genus *Microbacterium*. Strain SK 18T shared highest 16S rRNA gene sequence similarity (97.2 %) with *Microbacterium ulmi* DSM 16931T. Levels of similarity with the type strains of all other recognized *Microbacterium* species were less than 97.0 %. DNA–DNA hybridization experiments with strain SK 18T and its closest relative, *M. ulmi* DSM 16931T, revealed a low reassociation value of 39.0 % (σ=3.8 %). Moreover, strain SK 18T showed a number of differences in phenotypic characteristics (colony colour, catalase activity, hydrolysis of polymers, acid production from sugars and oxidation of various substrates), and its DNA G+C content was also higher than that of *M. ulmi* DSM 16931T. These data indicated that strain SK 18T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium immunditiarum* sp. nov. is proposed. The type strain is SK 18T (=MTCC 7185T=JCM 14034T). An emended description of the genus *Microbacterium* is also provided.

Members of the genus *Microbacterium* are widespread in nature and have been isolated from diverse habitats such as soil, water, plants, milk products, insects and humans (Collins & Bradbury, 1992; Richert et al., 2007). The genus was first described by Orla-Jensen (1919), and the description was emended by Collins et al. (1983) and again more recently by Takeuchi & Hatano (1998a). In the latter emendment, the genus *Aureobacterium* was united with the genus *Microbacterium*. The authors concluded that, although the diamino acid in the cell wall of members of these two genera differed (lysine in *Microbacterium* and ornithine in former members of the genus *Aureobacterium*), species of these two genera are intermixed phylogenetically and share similar physiological and chemotaxonomic properties (Takeuchi & Hatano, 1998a). Two species of other genera (*Flavobacterium marinotypicum* and *Brevibacterium oxydans*) have also been transferred to the genus *Microbacterium* as *Microbacterium maritypicum* (Takeuchi &
Hatano, 1998b) and Microbacterium oxydans (Schumann et al., 1999), respectively. To determine the phylogenetic significance of the peptidoglycan type in members of the genus Microbacterium, Richert et al. (2007) performed phylogenetic analysis of 27 species using four housekeeping genes in addition to 16S rRNA genes. The authors concluded that those species with lysine form a coherent cluster in the tree based on the ppp gene and in the concatenated tree and that the genus Aureobacterium ‘seems to be embedded within the genus Microbacterium’.

At the time of writing, the genus Microbacterium comprised 73 recognized species with validly published names (http://www.bacterio.cict.fr/). A review of the literature on Microbacterium revealed that DNA–DNA hybridization has played a crucial role in the identification of novel species in this genus, as levels of 16S rRNA gene sequence similarity between the type strains of the closest relatives are more than 97 % in some cases, 98–99 % in many cases and as high as 99.9 %, as reported for Microbacterium oxydans and Microbacterium luteolum (Schumann et al., 1999). Here, we describe a novel species of the genus on the basis of data from a polyphasic study, including 16S rRNA gene sequence analysis.

During our study of the archaean and bacterial diversity of a landfill environment in Chandigarh, India, a Gram-positive coryneform bacterium was isolated from the surface soil by the dilution plate technique with trypticase soy broth (Hi-Media) solidified with 1.5 % agar (TSBA). The isolate was purified by subculturing on TSBA for 4 days at 30 °C and was subsequently designated strain SK 18T. The pure culture was maintained as a glycerol stock at 218T. The pure culture was maintained as a glycerol stock at 4 days at 30 °C. The isolate was purified by subculturing on TSBA for 4 days at 30 °C and was subsequently designated strain SK 18T. The pure culture was maintained as a glycerol suspension (10 %, v/v) at −70 °C. Preliminary characterization of the isolate indicated that it belonged to the genus Microbacterium, but it could not be assigned to any recognized species.

The Gram reaction and endospore staining were performed according to Smibert & Krieg (1994). Cell morphology and motility (determined by the hanging drop method) were observed under a phase-contrast microscope (Zeiss) at 1000 × magnification, with cells grown for 5 days at 30 °C on TSBA. Oxidase and catalase activities were tested by using oxidase discs (Hi-Media) and 3 % (v/v) H2O2 (Merck), respectively, according to methods described by Smibert & Krieg (1994). Growth at different temperatures, salt concentrations and pH (biological buffers used to adjust pH: acetate buffer for preparing media of pH 4 and 5, phosphate buffer for preparing media of pH 6 and 7, and Tris/HCl buffer for preparing media of pH 8 and 9) was investigated by using trypticase soy broth (Hi-Media) according to the methods described by Smibert & Krieg (1994) (cultures were incubated for up to 24 h in all three experiments at 30 °C for determination of optimum NaCl concentration and pH). Hydrolysis of casein and starch was tested on skimmed milk agar and starch agar, respectively (Hi-Media). Acid production from carbohydrates was studied in a medium containing (w/v): 1 % peptone, 0.5 % NaCl, 0.003 % bromothymol blue and 0.5 % carbohydrate (pH 7.2). Oxidation of substrates by strain SK 18T and by Microbacterium ulmi was tested on cells grown on TSBA (for 4 days at 30 °C) by using the GP2 plates of the Biolog system according to the manufacturer’s instructions. Biochemical tests, such as those for methyl red, the Voges–Proskauer reaction, indole production and nitrate reduction, were performed according to procedures described by Lányi (1987). For tests of hydrolysis of various polymers, xanthine, hypoxanthine, L-tyrosine, xylan, DNA, aesculin (all at 0.4 %, w/v) and Tweens 20, 40, 60 and 80 (0.4 %, w/v) were used as supplements in basal medium, trypticase soy agar (TSA; Hi-Media), L-Arginine, L-lysine and L-ornithine decarboxylase reactions were performed in their respective media according to the manufacturer’s instructions (Hi-Media).

For analysis of fatty acids, strain SK 18T was cultivated on TSBA at 30 °C for 5 days. M. ulmi DSM 16931T was used as a reference strain and was processed under the same conditions. Fatty acid methyl esters were prepared and analysed by using the standard method of the Microbial Identification System (MIDI-Sherlock version 4.02, library TSBAS50) as described by Pandey et al. (2002). Strain SK 18T was cultivated in TSB for 4 days at 30 °C on a rotary shaker (200 r.p.m.) and the dried cell mass was used for cell-wall and menaquinone analysis. Isoprenoid quinones were extracted and purified as described by Komagata & Suzuki (1987). The peptidoglycan was isolated after disruption of the cells by shaking with glass beads and subsequent trypsin digestion, according to the method of Schleifer (1985). Amino acids and peptides in the cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates by using the solvent systems described by Schleifer (1985). The molar ratios of the amino acids were determined by GC (GC 14A; Shimadzu) and GC-MS (320-MS Quadrupole GC/MS; Varian) of N-heptafluorobutyryl amino acid isobutyl esters (MacKenzie, 1987; Groth et al., 1996). The presence of glycolyl residues in the peptidoglycan was examined by using the colorimetric method of Uchida et al. (1999). Cell-wall sugars were analysed according to the procedure of Stanek & Roberts (1974). Polar lipids were extracted, separated by two-dimensional TLC and identified according to Minnikin et al. (1977).

The genomic DNA of strain SK 18T was isolated according to the method described by Pitcher et al. (1989). The 16S rRNA gene was amplified by PCR by using the universal primers 8-27F (5′-AGAGTTTGTATCCTGGCCTAG-3′) and 1492R (5′-TACGGYTACCTTGTAGGCTAC-3′). The amplification reaction and purification of the product were performed according to Pandey et al. (2002). The amplified 16S rRNA gene was sequenced by the dyeoxy chain terminator method by using the Big Dye Terminator kit followed by capillary electrophoresis on an ABI 310 genetic analyser (Applied Biosystems). The primers used for sequencing were 342R (5′-CTGCTGCSYCCCGTAC-3′), 685R (5′-TCTACGCATTTCACCCTGCTAC-3′), 1100R
DNA–DNA hybridization experiments between strain SK 18<sup>T</sup> and its method (Mandel & Marmur, 1968).

Phylogenetic analysis based on 16S rRNA gene sequences of the type strains of all recognized Microbacterium species, using the MAXIMUM-PAKYNYTHY method in the PHYLIP software package version 3.5c (Felsenstein, 1993).

DNA for G+C content analysis and DNA–DNA hybridization was prepared according to the protocol of Marmur (1961) except that GES reagent [5 M guanidium thiocyanate, 100 mM EDTA, 0.5 % (w/v) Sarkosyl] was used as the lysis solution. The G+C content of the genomic DNA was determined spectrophotometrically (Lambda 35 spectrophotometer; Perkin Elmer) with the thermal denaturation system (Table 1).

DNA–DNA hybridization between strain SK 18<sup>T</sup> and its closest phylogenetic relative, M. ulmi DSM 16931<sup>T</sup>, was carried out by using the membrane filter method (Tourova & Antonov, 1988) as described by Reddy et al. (2000).

Strain SK 18<sup>T</sup> showed visible colonies (1–2 mm in diameter) on TSBA after 4 days of incubation at 30 °C. Colonies were creamish yellow, circular and opaque with entire edges. Cells were Gram-positive, non-motile, non-spore-forming rods. Strain SK 18<sup>T</sup> differed from M. ulmi DSM 16931<sup>T</sup> in terms of colony colour, acid production from carbohydrates and a few other biochemical characteristics (Table 1). Differences were also observed between strain SK 18<sup>T</sup> and M. ulmi in the oxidation of some carbon substrates (D-psicose, pyruvic acid methyl ester, galactose, D-sorbitol and sucrose) in the Biolog system (Table 1).

The cell-wall sugars of strain SK 18<sup>T</sup> were galactose, xylose and rhamnose. The hydrolysate (4 M HCl, 100 °C, 16 h) of the peptidoglycan contained the amino acids lysine, ornithine, alanine, glycine, serine and glutamic acid at a molar ratio of 1.0 : 0.2 : 2.0 : 2.7 : 0.8 : 1.0. Homoserine was present in trace amounts. 3-Hydroxyglutamic acid and amino acids indicative of contaminating proteins (such as leucine or isoleucine; Schleifer & Kandler, 1972) were absent. The partial hydrolysate (4 M HCl, 100 °C, 0.75 h) of the peptidoglycan contained the peptide Gly–Gly. Strain SK 18<sup>T</sup> thus appears to represent a hitherto unknown peptidoglycan of the B-type which differs from the B1β, B1β, B2α and B2β types reported for members of the genus Microbacterium (Evtushenko & Takeuchi, 2006), based on the combined occurrence of lysine and ornithine and the presence of serine. Strain SK 18<sup>T</sup> matches the description of the genus Microbacterium in containing glycolyl residues in the peptidoglycan (Takeuchi & Hatano, 1998a). The major fatty acids in strain SK 18<sup>T</sup> were anteiso-C<sub>17 : 0</sub> (40.1 % of the total), anteiso-C<sub>15 : 0</sub> (22.7 %) and iso-C<sub>16 : 0</sub> (20.2 %). Other fatty acids detected were iso-C<sub>15 : 0</sub> (8 %), iso-C<sub>17 : 0</sub> (6.1 %) and trace amounts (<5 %) of C<sub>16 : 0</sub>, C<sub>18 : 0</sub> and iso-C<sub>14 : 0</sub>. The fatty acid profile of strain SK 18<sup>T</sup> was quantitatively different from that of M. ulmi DSM 16931<sup>T</sup>, especially in the composition of the major fatty acids anteiso-C<sub>15 : 0</sub> and anteiso-C<sub>17 : 0</sub> (Table 1). These two fatty acids were present in almost equal amounts (31.7 and 32.7 %, respectively) in M. ulmi. In strain SK 18<sup>T</sup>, by contrast, the amount of anteiso-C<sub>17 : 0</sub> (40.1 %) was nearly double that of anteiso-C<sub>15 : 0</sub> (22.7 %). The major menaquinones of strain SK 18<sup>T</sup> were MK-13 (39.7 %) and MK-12 (38.6 %). Minor amounts of MK-10 (8.9 %), MK-11 (6.4 %) and MK-14 (6.4 %) were also detected. The major polar lipids were phosphatidyglycerol, diphasphatidyglycerol and an unknown glycolipid; minor quantities of an unknown phospholipid and an unknown lipid were also detected (Fig. S1 in IJSEM Online). Detailed comparative chemotaxonomic characteristics are listed in Table 1.

To determine the phylogenetic position of strain SK 18<sup>T</sup>, its almost-complete 16S rRNA gene sequence (1432 bp) was determined. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain SK 18<sup>T</sup> was related most closely to M. ulmi DSM 16931<sup>T</sup> (97.2 % similarity). Levels of similarity with the type strains of other species of the genus Microbacterium were less than 97.0 %. The phylogenetic relationships of strain SK 18<sup>T</sup> with other closely related species belonging to the genus Microbacterium are shown in Fig. 1. A similar topology was observed in the tree generated with the maximum-parsimony algorithm. DNA–DNA hybridization experiments between strain SK 18<sup>T</sup> and its closest neighbour, M. ulmi DSM 16931<sup>T</sup>, revealed a low level of DNA–DNA relatedness, 39.0 % (σ=3.8 %), which is lower than the threshold value prescribed for delineation of bacterial species (Wayne et al., 1987). This result indicated that strain SK 18<sup>T</sup> does not belong to M. ulmi and should be considered as a representative of a novel species. As it is generally accepted that species sharing less than 70 % DNA–DNA relatedness are to be considered as distinct species, strain SK 18<sup>T</sup> thus appears to represent a novel species of the genus Microbacterium, for which the name Microbacterium immunditiarum sp. nov. is proposed. An emended description of the genus Microbacterium is also given.

Microbacterium immunditiarum sp. nov. (5′-GGGGTGCGCTCGTTG-3′) and 1492R. The 16S rRNA gene sequence of strain SK 18<sup>T</sup> was used as a query to search for homologous sequences in GenBank. The sequence of the strain was aligned with 16S rRNA gene sequences of the type strains of all recognized Microbacterium species using the CLUSTAL X (Thompson et al., 1997) and the alignment was corrected manually. Levels of 16S rRNA gene sequence similarity were calculated from the alignment. Gaps at the 5′ and 3′ ends of the alignment were omitted from further analysis. Evolutionary distance matrices were calculated by using the algorithm of Jukes & Cantor (1969) with the DNADIST program within the TREECON software package (Van de Peer & De Wachter, 1997). A 16S rRNA gene-based phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) and bootstrap analysis was performed to assess the confidence limits of the branching. A 16S rRNA gene-based tree was also constructed by using the maximum-parsimony method in the PHYLIP software package version 3.5c (Felsenstein, 1993).

The genus description is as emended by Takeuchi & Hatano (1998a), but with the following changes: the peptidoglycan contains alanine, D-glutamic acid, and L-lysine and/or L-ornithine and/or L-homoserine. Serine may also be present in the peptidoglycan. The G+C content of the DNA is 64–77 mol%.

Description of *Microbacterium immunditiarum* sp. nov.

*Microbacterium immunditiarum* (im.mun.di.ti.a’rum. L. gen. pl. n. immunditiarum of dirt, filth, mires, refuse, reflecting isolation of the type strain from a municipal landfill site).

Cells are Gram-positive, non-motile, non-spore-forming short rods occurring singly or in irregular clusters. Colonies are creamish yellow, opaque and circular with entire edges. Good growth occurs on TSBA at 15–37 °C (optimum 30 °C), but only weak growth is observed at 42 °C. Grows at pH 6.0–8.0, and optimally between pH 7.0 and 8.0. Growth occurs in the presence of up to 3 % (w/v) NaCl. Catalase-positive and oxidase-negative; indole and H₂S are not produced. Nitrate is reduced to nitrite. Hydrolyses aesculin, starch and gelatin, but not xylan, xanthine, hypoxanthine, L-tyrosine, DNA, casein or Tweens 20, 40, 60 or 80. Produces arginine dihydrolase.

### Table 1. Differential phenotypic properties of strain SK 18² and its closest phylogenetic relative, *Microbacterium ulmi* DSM 16931²

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
</tr>
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<tbody>
<tr>
<td>Colony colour</td>
<td>Creamish yellow</td>
<td>Creamish white</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Skimmed milk</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Production of acid from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Xylose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>L-Sorbosose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Oxidation of (Biolog):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Alaninamide</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>α-Cyclodextrin</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>β-Cyclodextrin</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>L-Lactic acid</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>D-Psicose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Pyruvic acid methyl ester</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Cell-wall sugars‡</td>
<td>Gal, Rha, Xyl</td>
<td>Gal, Fuc, Xyl, Rha*</td>
</tr>
<tr>
<td>Cell-wall diamino acid(s):‡</td>
<td>Lys (Orn)</td>
<td>Orn*</td>
</tr>
<tr>
<td>Major polar lipids§</td>
<td>PG, DPG, UKGL, UKPL, UL</td>
<td>PG, DPG, UKGL*</td>
</tr>
<tr>
<td>Major menaquinones</td>
<td>MK-13, MK-12</td>
<td>MK-12, MK-13*</td>
</tr>
<tr>
<td>Major fatty acids (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anteiso-C₁₅:₀</td>
<td>22.7</td>
<td>31.7</td>
</tr>
<tr>
<td>anteiso-C₁₇:₀</td>
<td>40.1</td>
<td>32.7</td>
</tr>
<tr>
<td>iso-C₁₆:₀</td>
<td>20.2</td>
<td>19.0</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>76.6</td>
<td>69.0*</td>
</tr>
</tbody>
</table>

*Data from Rivas et al. (2004).
‡Gal, Galactose; Xyl, xylose; Rha, rhamnose; Fuc, fucose.
§Lys, Lysine; Orn, ornithine.
§PG, Phosphatidylglycerol; DPG, diphasphatidylglycerol; UKGL, unknown glycolipid; UKPL, unknown phospholipid; UL, unknown lipid.
but not lysine decarboxylase or ornithine decarboxylase. The methyl red and Voges–Proskauer tests are negative. Acid is produced from D-glucose, L-rhamnose, sucrose, D-mannitol and weakly from D-galactose, D-mannose, maltose and D-fructose, but not from D-xyllose, lactose or L-sorbose. The major menaquinones are MK-13 and MK-12; minor amounts of MK-10, MK-11 and MK-14 are also present. The predominant fatty acids are anteiso-C17:0, anteiso-C15:0 and iso-C16:0. The peptidoglycan is of the B-type and contains the diamino acids lysine and ornithine, as well as amino acids serine, alanine, glycine, glutamic acid and traces of homoserine. The acyl type is glycolyl.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain SK18T and the type strains of all recognized Microbacterium species. Bootstrap values as a percentage of 1000 replications are shown at nodes; only values > 70% are shown. The sequence from Leifsonia aquatica DSM 20146 was used as an outgroup.

Filled circles indicate nodes also recovered by using the maximum-parsimony method. Bar, 0.02 substitutions per site.
The cell-wall sugars are galactose, xylose and rhamnose. The lipids comprise diphosphatidylglycerol, phosphatidylglycerol, an unknown glycolipid, an unknown phospholipid and an unknown lipid.

The type strain, SK 18T (=MTCC 7185T=JCM 14034T), was isolated from landfill surface soil in Chandigarh, India. The G+C content of the genomic DNA of the type strain is 76.6 mol%.

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