Microbacterium deminutum sp. nov., *Microbacterium pumilum* sp. nov. and *Microbacterium aoyamense* sp. nov.

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Three novel bacterial strains were isolated from a soil sample collected in Japan by culture on a GPM agar plate supplemented with superoxide dismutase and catalase. The strains were Gram-positive, catalase-positive, non-motile bacteria with L-ornithine as a diagnostic diamino acid of the peptidoglycan. The acyl type of the peptidoglycan was *N*-glycolyl. The major menaquinones were MK-12, 13 and 14. Mycolic acids were not detected. G + C contents of the DNA were in the range 69–71 mol%. Comparative 16S rRNA gene sequence analysis revealed that the isolates belonged to the genus *Microbacterium* and were closely related to *Microbacterium terregens*, *Microbacterium aurum*, *Microbacterium koreense*, *Microbacterium schleiferi* and *Microbacterium lacticum*. However, *M. aurum*, *M. koreense* and *M. lacticum* clearly differed from the isolated strains based on the presence of L-lysine as the cell-wall diamino acid and various other chemotaxonomic characteristics. Levels of DNA–DNA relatedness showed that the isolated strains represented three separate genomic species. Based on both phenotypic and genotypic data, the following novel species of the genus *Microbacterium* are proposed: *Microbacterium deminutum* sp. nov. (type strain KV-483^T = NRRL B-24453^T = NBRC 101278^T), *Microbacterium pumilum* sp. nov. (type strain KV-488^T = NRRL B-24452^T = NBRC 101279^T) and *Microbacterium aoyamense* sp. nov. (type strain KV-492^T = NRRL B-24451^T = NBRC 101280^T).

The genus *Microbacterium* was proposed by Orla-Jensen (1919) with the type species *Microbacterium lacticum*, and the description was emended by Takeuchi & Hatano (1998). Members of the genus are widespread and have been isolated from various environmental habitats (Collins & Bradbury, 1992). At the time of writing, the genus *Microbacterium* comprises 40 recognized species.

Strains KV-483^T, KV-488^T and KV-492^T were isolated from a soil sample collected from a cemetery in Aoyama, Tokyo, Japan. Two grams of soil was suspended in 18 ml sterile water and mixed. Soil particles were allowed to sediment, the liquid phase was diluted 10⁵-fold and 100 µl samples were spread onto the surface of each cultivation plate. GPM agar plates with superoxide dismutase (300 U per plate) and catalase (2100 U per plate) (Takahashi *et al.*, 2003) were used, and were cultured at 27 °C. Biomass for biochemical and chemotaxonomic characterization was prepared by

culturing in trypticase soy broth at 27 °C followed by cell harvesting by centrifugation.

Morphological observation under a scanning electron microscope (model JSM-5600; JEOL) was performed on cultures grown on GPM medium at 27 °C for 6 or 7 days. Assimilation of carbon sources was determined using agar medium of yeast nitrogen base without amino acids (Nihon Pharmaceutical Co., Ltd) (Pridham & Gottlieb, 1948). NaCl tolerance and pH and temperature ranges for growth were determined on one-fifth-strength nutrient agar. The three new isolates and reference strains *Microbacterium aurum* JCM 9179^T, *Microbacterium schleiferi* JCM 9175^T and *Microbacterium terregens* JCM 1342^T were characterized biochemically by using the API ZYM system according to the manufacturer's instructions (bioMérieux).

N-Acyl types of muramic acid were determined using the method of Uchida & Aida (1977). Purified cell wall was obtained by the method of Kawamoto *et al.* (1981). One milligram of purified cell wall was hydrolysed at 100 °C with 1 ml 6 M HCl for 16 h. The residue was dissolved in 100 µl water and was used for amino acid analysis. Composition of amino acids was determined by HPLC using the Pico Tag method (Waters). This method involves the use of

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KV-483^T, KV-488^T and KV-492^T are AB234026–AB234028.

DNA–DNA relatedness values among strains KV-483^T, KV-488^T and KV-492^T and related *Microbacterium* type strains are available as supplementary material in IJSEM Online.

phenylisothiocyanate for creation of phenylthiocarbamyl derivatives. Cell-wall sugars were obtained according to the method of Kawamoto *et al.* (1981), and samples were analysed by using the method of Becker *et al.* (1965); the presence of mycolic acid was examined by the TLC method of Tomiyasu (1982). Menaquinones were extracted and purified according to the method of Collins *et al.* (1977), and were then analysed by HPLC (model 802-SC; Jasco) on a chromatograph equipped with a CAPCELL PAK C18 column (Shiseido) (Tamaoka *et al.*, 1983). Methyl esters of cellular fatty acids were prepared by direct transmethylation with methanolic hydrochloric acid. They were then analysed by GLC (model GC-17A; Shimadzu) with a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific) (Suzuki & Komagata, 1983). Cells grown for 3 or 4 days were collected and used for these experiments.

DNA was isolated as described by Saito & Miura (1983). DNA base composition was estimated by HPLC (Tamaoka & Komagata, 1984). Levels of DNA–DNA relatedness were determined according to the method of Ezaki *et al.* (1989) using photobiotin and a microplate format.

For 16S rRNA gene sequence analysis, DNA was prepared and amplified as reported by Yu *et al.* (2002) and Takahashi *et al.* (2003), respectively, and the gene was sequenced with an automatic analyser (ABI PRISM 377A; PE Applied Biosystems) using a PRISM Ready Reaction dye primer cycle sequencing kit (PE Applied Biosystems). Species related closely to those of the new isolates were determined by performing sequence database searches using the BLAST program. Sequence data for related species were retrieved from GenBank. Phylogenetic analysis was performed using CLUSTAL W software (Thompson *et al.*, 1994). Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura & Ohta, 1972) and phylogenetic trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987). Sequence similarity values were determined by visual comparison and manual calculation.

Cells of strains KV-483^T, KV-488^T and KV-492^T were irregular rods, with cell size in the range 0.2–0.7 × 0.4–1.2 μm. Cells of all three strains were Gram-positive, catalase-positive and non-motile. The DNA G+C content of the three strains was in the range 69–71 mol%. Cell-wall peptidoglycans of KV-483^T, KV-488^T and KV-492^T contained glycine, homoserine, glutamic acid, 3-hydroxyglutamic acid, ornithine and alanine. The predominant menaquinones were MK-12, MK-13 and MK-14, with ratios of 14:50:9 for KV-483^T, 6:10:3 for KV-488^T and 7:60:9 for KV-492^T. The acyl type of the peptidoglycan was *N*-glycolyl. Mycolic acids were not detected. The predominant cellular fatty acid components were anteiso-*C*_{15:0}, anteiso-*C*_{17:0} and iso-*C*_{16:0} (Table 1).

Nearly complete 16S rRNA gene sequences were determined for the three isolated strains. Phylogenetic analysis based on these sequences demonstrated that the three strains belonged to the genus *Microbacterium*. Fig. 1 shows

Table 1. Fatty acid compositions of the novel strains

Data are percentages of the total fatty acids. —, Not detected or <1%.

Fatty acid	KV-483 ^T	KV-488 ^T	KV-492 ^T
iso- <i>C</i> _{14:0}	—	1.07	1.05
iso- <i>C</i> _{15:0}	—	3.37	8.91
anteiso- <i>C</i> _{15:0}	28.3	46.61	48.2
iso- <i>C</i> _{16:0}	7.04	25.92	17.2
<i>C</i> _{16:0}	2.88	2.33	1.15
iso- <i>C</i> _{17:0}	1.57	4.98	2.28
anteiso- <i>C</i> _{17:0}	58.9	10.20	21.3
iso- <i>C</i> _{18:0}	—	1.90	—
<i>C</i> _{18:0}	—	2.88	—

the relationship between the three novel strains and their nearest phylogenetic relatives. 16S rRNA gene sequencing based on the 1433 bp of strain KV-483^T revealed peak similarity levels of 98.8% with the sequence of *M. terregens* IFO 12961^T (Takeuchi & Hatano, 1998) and 98.5% with that of *M. schleiferi* DSM 20489^T (Yokota *et al.*, 1993b). Strain KV-488^T (1359 bp) showed peak similarities of 98.9% with the 16S rRNA gene sequence of *M. terregens* IFO 12961^T and 98.7% with that of *M. aurum* DSM 8600^T (Yokota *et al.*, 1993a). Strain KV-492^T (1443 bp) had peak similarity levels of 99.1% with *M. terregens* IFO 12961^T and 98.9% with *M. aurum* DSM 8600^T. 16S rRNA gene sequence similarity values among the three novel strains were 98.8–99.8%.

Levels of DNA–DNA hybridization were determined from duplicate experiments with the three novel strains and their

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences created using the neighbour-joining method and K_{nuc} values, showing the phylogenetic positions of strains KV-483^T, KV-488^T and KV-492^T. Numbers at branch points are bootstrap percentages (based on 1000 resamplings). The tree was unrooted and *Microbacterium liquefaciens* DSM 20638^T was used as an outgroup.

three closest phylogenetic relatives. Representative values among strains KV-483^T, KV-488^T and KV-492^T and the type strains of *M. aurum*, *M. schleiferi* and *M. terregens* were less than 29 % (see Supplementary Table S1 in IJSEM Online). These values were well below the 70 % cut-off point for species classification recommended by Wayne *et al.* (1987).

The morphological and chemotaxonomic characteristics (Tables 1 and 2) of the isolated strains are consistent with their assignment to the genus *Microbacterium* (Takeuchi & Hatano, 1998). A number of phenotypic characteristics that can be used to distinguish the isolated strains from each other and from their nearest phylogenetic neighbours are presented in Table 2.

From the phenotypic and genotypic data presented, it is apparent that these isolated strains represent three distinct species within the genus *Microbacterium*. We propose the names *Microbacterium deminutum* sp. nov., *Microbacterium pumilum* sp. nov. and *Microbacterium aoyamense* sp. nov.

Description of *Microbacterium deminutum* sp. nov.

Microbacterium deminutum (de.mi.nu'tum. L. part. adj. *deminutum* diminutive).

Cells are irregular rods, 0.3–0.7 × 0.5–0.9 µm in size. Gram-positive, non-motile, catalase-positive and aerobic. Colonies are pale yellow. Growth occurs at pH 6–9 and 17–31 °C. In one-fifth-strength nutrient agar medium, NaCl is tolerated up to 4 %. Glucose, galactose, maltose, mannose, rhamnose and sucrose are assimilated, but arabinose, fructose, mannitol, raffinose, trehalose and xylose are not. Esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosamidase are detected with the API ZYM enzyme assay; α-fucosidase is not detected. Weak reactions for alkaline phosphatase, lipase (C14) and α-mannosidase are detected. The diagnostic diamino acid of the peptidoglycan is L-ornithine. The acyl type of the peptidoglycan is *N*-glycolyl. Major menaquinones are MK-12, 13 and 14. Major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. Cell-wall sugars contain rhamnose, fucose, galactose, glucose and xylose. The DNA G + C content is 69 mol%.

The type strain, KV-483^T (=NRRL B-24453^T=NBRC 101278^T), was isolated from soil from Aoyamareien, Tokyo, Japan.

Description of *Microbacterium pumilum* sp. nov.

Microbacterium pumilum (pu'mi.lum. L. neut. adj. *pumilum* dwarfish, diminutive, little).

Cells are irregular rods, 0.2–0.6 × 0.4–1.2 µm in size. Gram-positive, non-motile, catalase-positive and aerobic. Colonies are pale yellow. Growth occurs at pH 7–10 and 17–32 °C. In one-fifth-strength nutrient agar medium, NaCl is tolerated up to 2 %. Glucose, arabinose, galactose, maltose, mannose and sucrose are assimilated, but fructose, mannitol, raffinose, rhamnose, trehalose and xylose are not. Alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosamidase and α-fucosidase are detected with the API ZYM enzyme assay; α-mannosidase is not detected. Weak reactions for esterase (C4) and lipase (C14) are detected. The diagnostic diamino acid of the peptidoglycan is L-ornithine. The acyl type of the peptidoglycan is *N*-glycolyl. Major menaquinones are MK-12, 13 and 14. Major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. Cell-wall sugars contain rhamnose and galactose. The DNA G + C content is 71 mol%.

The type strain, KV-488^T (=NRRL B-24452^T=NBRC 101279^T), was isolated from soil from Aoyamareien, Tokyo, Japan.

Description of *Microbacterium aoyamense* sp. nov.

Microbacterium aoyamense (ao.ya.men'se. N.L. neut. adj. *aoyamense* referring to Aoyama, Tokyo, Japan, where the type strain was isolated).

Cells are irregular rods, 0.3–0.5 × 0.4–0.8 µm in size. Gram-positive, non-motile, catalase-positive and aerobic. Colonies are pale yellow. Growth occurs at pH 5–11 and 14–34 °C. In one-fifth-strength nutrient agar medium, NaCl is tolerated up to 5 %. Glucose, galactose, maltose, mannitol, mannose, raffinose, rhamnose, sucrose and trehalose are assimilated, but arabinose, fructose and xylose are not. Esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosamidase are detected with the API ZYM enzyme assay; alkaline phosphatase, cystine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase are negative. Weak reaction for valine arylamidase. The diagnostic diamino acid of the peptidoglycan is L-ornithine. The acyl type of the peptidoglycan is *N*-glycolyl. Major menaquinones are MK-12, 13 and 14. Major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. Cell-wall sugars contain rhamnose, galactose and xylose. The DNA G + C content is 69 mol%.

The type strain, KV-492^T (=NRRL B-24451^T=NBRC 101280^T), was isolated from soil from Aoyamareien, Tokyo, Japan.

Table 2. Differential characteristics of strains KV-483^T, KV-488^T and KV-492^T and related *Microbacterium* species

Taxa: 1, KV-483^T; 2, KV-488^T; 3, KV-492^T; 4, *M. aurum* JCM 9179^T; 5, *M. terregens* JCM 1342^T; 6, *M. schleiferi* JCM 9175^T; 7, *M. koreense*. In addition to the displayed differential characteristics, strain KV-488^T differs from KV-483^T and KV-492^T in assimilation of L-arabinose and inability to assimilate L-rhamnose as a sole carbon source. Data for the type strains of *M. aurum*, *M. terregens* and *M. schleiferi* are from this study (API ZYM tests), Lee *et al.* (2006) (assimilation of carbon sources) and Takeuchi & Hatano (1998) and Behrendt *et al.* (2001) (chemotaxonomic characteristics and growth at 37 °C). Data for *M. koreense* are from Lee *et al.* (2006). +, Positive; w, weakly positive; –, negative; ND, no data.

Characteristic	1	2	3	4	5	6	7
Growth at 37 °C	–	–	–	+	–	+	+
Assimilation of:							
Glucose	+	+	+	+	–	–	+
Mannitol	–	–	+	+	–	–	+
Maltose	+	+	+	+	–	–	+
Mannose	+	+	+	+	–	–	+
Enzyme assay (API ZYM) results:							
Alkaline phosphatase	w	+	–	w	w	–	ND
Valine arylamidase	+	+	w	+	+	+	ND
Cystine arylamidase	+	+	–	+	+	w	ND
Trypsin	+	+	–	+	w	+	ND
α-Galactosidase	+	+	–	+	–	–	ND
β-Galactosidase	+	+	+	+	–	+	ND
β-Glucuronidase	+	+	–	–	–	w	ND
N-Acetyl-β-glucosamidase	+	+	+	–	+	+	ND
α-Mannosidase	w	–	–	+	–	–	ND
DNA G+C content (mol%)	69	71	69	69	69	67	68
Cell-wall diamino acid*	Orn	Orn	Orn	Lys	Orn	Orn	Lys
Cell-wall sugars†	Rha, Fuc, Gal, Glc, Xyl	Rha, Gal	Rha, Gal, Xyl	Fuc, Gal, Glc	Rha, Gal, 6dTal	6dTal, Man, Gal	Gal, Xyl
Major menaquinones (ratio)	12, 13, 14 (14:50:9)	12, 13, 14 (6:10:3)	12, 13, 14 (7:60:9)	11, 12	12, 13	10, 11, 12	11, 12
Major fatty acids‡	ai17, ai15, i16	ai15, i16, ai17	ai15, ai17, i16	ai15, ai17, i16	ai15, ai17, i16	ai15, ai17, i16	ai15, ai17, i16

*Lys, Lysine; Orn, ornithine.

†6dTal, 6-Deoxytalose; Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Xyl, xylose.

‡ai, Anteiso; i, iso. All fatty acids listed are saturated.

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