Microbacterium sediminicola sp. nov. and *Microbacterium marinilacus* sp. nov., isolated from marine environments

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Two novel *Microbacterium* species are described on the basis of phenotypic, chemotaxonomic and genotypic studies. The two strains, designated YM10-847^T and YM11-607^T, were isolated from river sediment and unidentified hydroid, respectively, of a marine lake. The strains were Gram-positive, catalase-positive bacteria with L-ornithine as the diagnostic diamino acid of the peptidoglycan. The acyl type of the peptidoglycan was *N*-glycolyl. The major menaquinones were MK-10 and MK-11 for YM10-847^T, and MK-11 and MK-12 for YM11-607^T. Mycolic acids were not detected. The DNA G + C content of strains YM10-847^T and YM11-607^T was 67.8 and 71.6 mol%, respectively. Comparative 16S rRNA gene sequence analysis revealed that the two strains belong to the genus *Microbacterium*. DNA–DNA relatedness data showed that YM10-847^T and YM11-607^T are two novel species of this genus. On the basis of these results, strains YM10-847^T and YM11-607^T represent two novel species of the genus *Microbacterium*, for which the names *Microbacterium sediminicola* sp. nov. and *Microbacterium marinilacus* sp. nov. are proposed. The type strains are YM10-847^T (=MBIC08264^T=DSM 18905^T) and YM11-607^T (=MBIC07778^T=DSM 18904^T), respectively.

The genus *Microbacterium* was first proposed by Orla-Jensen (1919) with the type species *Microbacterium lacticum*, and was emended by Takeuchi & Hatano (1998). Members of the genus *Microbacterium* are Gram-positive, obligately aerobic and catalase-positive. The peptide subunit of peptidoglycan consists of alanine, D-glutamic acid (plus hydroxyglutamic acid), and either L-lysine, L-ornithine or L-homoserine. Menaquinones are MK-10, MK-11, MK-12, MK-13 and MK-14. Muramic acid occurs in the *N*-glycolyl form. The G + C content of the genomic DNA ranges from 66 to 72 mol%. Members of the genus *Microbacterium* are widespread and can be isolated from various environmental habitats (Collins & Bradbury, 1992). The genus *Microbacterium* is a member of the family *Microbacteriaceae* of the order *Actinomycetales*.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains YM10-847^T and YM11-607^T are AB286021 and AB286020, respectively.

A phylogenetic tree based on 16S rRNA gene sequences of all species of the genus *Microbacterium* and the composition of the medium 'H0.3' are available as supplementary material with the online version of this paper.

Strain YM10-847^T was isolated from a sediment sample collected from the mouth of the Samambula River, Fiji (depth: 1 m; GPS location: 18° 7' 14" S, 178° 28' 18" E), in December 2003. Strain YM11-607^T was isolated from an unidentified hydroid collected from the Sano Marine Lake, Republic of Palau (depth: 1 m; GPS location: 07° 17' 74" N, 134° 26' 92" E), in March 2004. The samples (0.5–1.0 cm³) were homogenized with a glass rod in 5 ml sterile seawater. Bacteria were isolated from the homogenate (50 µl) by culturing at 25 °C for 30 days on P (YM10-847^T) or H0.3 (YM11-607^T) media. Compositions of medium P were described in Yoon *et al.* (2007) and compositions of medium H0.3 are listed in Supplementary material available in IJSEM Online.

Biomass for biochemical and chemotaxonomic characteristics was prepared by culturing in TSB broth at 27 °C.

Morphological observation under a scanning electron microscope (model JSM-5600; JEOL) was performed using cultures grown on glucose–peptone meat extract agar medium (1% D-glucose, 0.5% peptone, 0.5% meat extract, 0.3% NaCl and 1.2% agar; pH 7.0) at 27 °C for 3 days. The carbon-assimilation properties of the two strains and three related species were determined by using a

yeast nitrogen-based agar medium without amino acids (Nihon Pharmaceutical) (Pridham & Gottlieb, 1948). NaCl tolerance, pH and temperature ranges for growth were determined on 1/5 nutrient agar or marine agar (Difco). The two isolated strains and type strains of the three related species were characterized biochemically using API ZYM (bioMérieux) in accordance with the manufacturer's instructions.

The *N*-acyl types of muramic acid were determined by using the method of Uchida & Aida (1977). Cell walls were purified by using 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. The amino acid composition was determined by HPLC using the Pico Tag method (Waters). Samples were derivatized with phenylisothiocyanate and UV (254 nm) detection was used. The presence of mycolic acid was determined by using the TLC method described in Tomiyasu (1982). Menaquinones were extracted and purified by using the method described in Collins *et al.* (1977), and were then analysed by HPLC (model 802-SC; Jasco) using a chromatograph equipped with a Capcell Pak C18 column (Shiseido) (Tamaoka *et al.*, 1983). Methyl esters of cellular fatty acids were prepared and analysed by GLC (model HP6890; Hewlett Packard).

DNA was isolated as described by Saito & Miura (1963). DNA base composition was estimated by HPLC (Tamaoka & Komagata, 1984). Levels of DNA–DNA relatedness were determined by the method of Ezaki *et al.* (1989) using photobiotin and a microplate format.

DNA was prepared using InstaGene matrix (Bio-Rad). The 16S rRNA gene was amplified by PCR using a forward primer corresponding to positions 8–27 and a reverse primer corresponding to positions 1492–1510 (*Escherichia coli* numbering system; Weisburg *et al.*, 1991) and sequenced with an automated sequence analyser (3730 DNA Analyser; Applied Biosystems) using BigDye Terminator v3.1 Cycle Sequencing kit (Applied

Biosystems). Species related to the novel strains were identified by performing sequence database searches using the BLAST program (Altschul *et al.*, 1990). Sequence data for related species were retrieved from GenBank. The CLUSTAL_X software (Thompson *et al.*, 1997) was used for a multiple alignment of nucleotide sequences. Neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004). Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura & Ohta, 1972) for the neighbour-joining method. Min-mini algorithm (Nei & Kumar, 2000) was used for the maximum-parsimony method. The maximum-likelihood tree was inferred by using PHYML (Guindon & Gascuel, 2003; Guindon *et al.*, 2005). The distance-based tree was used as a starting tree to be refined by the maximum-likelihood algorithm based on the HKY model (Hasegawa *et al.*, 1985), with a transition/transversion ratio and a gamma shape parameter, which were estimated by maximizing the likelihood of the phylogeny.

The 1465 and 1472 bp of 16S rRNA gene sequences were determined for YM11-607^T and YM10-847^T, respectively. Subsequent 16S rRNA-based phylogenetic analysis demonstrated that the strains affiliated with the genus *Microbacterium* as shown in Supplementary Fig. S1 (available in IJSEM Online). YM11-607^T was closely related to *Microbacterium paludicola* in three kinds of trees (Figs 1, 2 and 3). The level of sequence similarity between YM11-607^T and *M. paludicola* was 98.3 %. YM10-847^T was closely related to *Microbacterium arborescens*, *Microbacterium imperiale* and *Microbacterium ulmi* (Figs 1, 2 and 3). The level of sequence similarity between YM10-847^T and related *Microbacterium* species: *M. arborescens*, *M. imperiale* and *M. ulmi* was 98.3, 98.1 and 97.2 %, respectively.

The DNA G + C content of strains YM10-847^T and YM11-607^T was 67.8 and 71.6 mol%, respectively. The cell-wall peptidoglycans of both strains contained L-ornithine as the diagnostic diamino acid. The major menaquinones were MK-10 and MK-11 for YM10-847^T, and MK-11 and MK-12 for YM11-607^T. The acyl type of the peptidoglycans was

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences and constructed using the neighbour-joining method and K_{nuc} values. Numbers at branching points are bootstrap values (1000 resamplings). *Rarobacter faecitabidus* DSM 4813^T was used as an outgroup. Bar, 1 substitution per 100 nt.

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences and constructed using the maximum-likelihood method. *Rarobacter faecitabidus* DSM 4813^T was used as an outgroup.

N-glycolyl. The major cellular fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} (Table 1). These characteristics were well matched to the genus description of *Microbacterium*.

DNA–DNA relatedness was determined twice. Species with high 16S rRNA sequence similarity values to the isolated strains were used for this experiment. Representative values for YM10-847^T and *M. arborescens* was 13 %, YM10-847^T and *M. imperiale* was 14 %, and YM11-607^T and *M. paludicola* was 36 %. These values are well below the 70 % cut-off point for species classification, as recommended by Wayne *et al.* (1987), thus confirming that the isolated

strains are two independent novel species of the genus *Microbacterium*.

The chemotaxonomic and morphological characteristics (Tables 1 and 2) of these two isolated strains are consistent with their assignment to the genus *Microbacterium* (Takeuchi & Hatano, 1998). The phenotypes and characteristics that distinguish the isolated strains from one another and from their phylogenetic neighbours are listed in Table 2.

On the basis of the present results, we propose two novel *Microbacterium* species: *Microbacterium sediminicola* sp. nov. and *Microbacterium marinilacus* sp. nov.

Description of *Microbacterium sediminicola* sp. nov.

Microbacterium sediminicola (sedi.mi.ni.co'la. L. n. *sedimen*-*inis*, sediment; L. suff. *-cola*, inhabitant dweller; N.L. n. *sediminicola*, sediment-dweller).

Cells are rod-shaped, vary in cell size from 0.4 to 0.7 by 0.8 to 1.5 µm. Gram-positive, catalase-positive, aerobic. Colonies are pale yellow. Growth occurs between pH 6 and 11, and 19 and 38 °C. In 1/5 nutrient agar medium, NaCl is tolerated up to 7 %. L-Arabinose, D-galactose, D-glucose, maltose, D-mannitol, D-mannose, L-rhamnose, trehalose and D-xylose are assimilated, but D-fructose, raffinose and sucrose are not. Esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase are detected by the API ZYM enzyme assay; alkaline phosphatase, chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, α-mannosidase and α-fucosidase are negative. Weak reaction for lipase (C14) and trypsin is detected. The acyl type of the peptidoglycan was *N*-glycolyl. The major menaquinones are MK-10 and MK-11. The major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The DNA G+C content is 67.8 mol%.

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences and constructed using the maximum-parsimony method. Numbers at branching points are bootstrap values (100 resamplings). *Rarobacter faecitabidus* DSM 4813^T was used as an outgroup. Bar, 5 substitution per 100 nt.

Table 1. Fatty acid composition (%) of isolated strains

Fatty acid	YM10-847 ^T	YM11-607 ^T
iso-C _{14:0}	1.42	2.03
iso-C _{15:0}	—	2.08
anteiso-C _{15:0}	40.63	59.11
iso-C _{16:0}	24.85	14.37
anteiso-C _{17:0}	31.93	20.91

The type strain, YM10-847^T (=MBIC08264^T=DSM 18905^T), was isolated from sediment collected from the mouth of the Samambula River, Fiji.

Description of *Microbacterium marinilacus* sp. nov.

Microbacterium marinilacus (mari.ni.la'cus. L. adj. *marinus*, marine; L. n. *lacus* -us, lake; N.L. gen. n. *marinilacus*, of a marine lake).

Cells are rod-shaped, vary in cell size from 0.4 to 0.6 by 0.9 to 1.4 µm. Gram-positive, catalase-positive, aerobic.

Colonies are lemon yellow. Growth occurs between pH 6 and 11, and 12 and 38 °C. In 1/5 nutrient agar medium, NaCl is tolerated up to 8 %. L-Arabinose, D-fructose, D-glucose, maltose, D-mannitol, D-mannose, L-rhamnose, trehalose and D-xylose are assimilated, but D-galactose, raffinose and sucrose are not. Esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase and α-fucosidase are detected by the API ZYM enzyme assay; alkaline phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase and α-mannosidase are negative. Weak reaction for lipase (C14), chymotrypsin, acid phosphatase and trypsin is detected. The acyl type of the peptidoglycan was N-glycolyl. The major menaquinones are MK-11 and MK-12. The major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The DNA G + C content is 71.6 mol%.

The type strain, YM11-607^T (=MBIC07778^T=DSM 18904^T), was isolated from an unidentified hydroid collected from the Sano Marine Lake, Republic of Palau.

Table 2. Differential characteristics between the isolated strains and related *Microbacterium* species

Strain/species: 1, YM10-847^T; 2, YM11-607^T; 3, *M. paludicola*; 4, *M. arborescens*; 5, *M. imperiale*; 6, *M. ulmi*. Data for the type strains of *M. paludicola*, *M. arborescens*, *M. imperiale* and *M. ulmi* regarding API ZYM tests and assimilation of carbon sources are from the present study. Other data on *M. imperiale* and *M. arborescens* are from Behrendt *et al.* (2001) and Takeuchi & Hatano (1998), respectively, other data on *M. paludicola* are from Park *et al.* (2006), and other data on *M. ulmi* are from Rivas *et al.* (2004). +, Positive; —, negative; w, weakly positive; ND, no data; Orn, L-ornithine; Lys, L-lysine.

Characteristic	1	2	3	4	5	6
Growth at 37 °C	+	+	+	—	+	+
Colony colour	Pale yellow	Lemon yellow	Lemon yellow	Dirty orange	Red–orange	White
Assimilation of:						
L-Arabinose	+	+	+	+	+	—
D-Fructose	—	+	+	+	+	+
D-Galactose	+	—	+	+	+	+
D-Mannitol	+	+	+	+	+	—
Raffinose	—	—	+	+	+	—
L-Rhamnose	+	+	+	+	+	—
Sucrose	—	—	+	+	+	—
D-Xylose	+	+	+	+	+	—
Enzyme assay (API ZYM):						
Alkaline phosphatase	—	—	—	+	+	+
Trypsin	w	+	+	+	+	—
Chymotrypsin	—	w	w	+	+	—
Naphthol-AS-BI-phosphohydrolase	—	+	+	w	w	+
α-Galactosidase	+	—	—	+	+	—
β-Galactosidase	+	—	—	+	+	+
N-Acetyl-β-glucosaminidase	+	+	—	+	+	+
α-Mannosidase	—	—	+	+	+	—
α-Fucosidase	—	+	—	—	—	—
pH range	6–11	6–11	6–8	ND	ND	5–8
DNA G + C content (mol%)	67.8	71.6	66.5	71.0	71.2	69.0
Cell wall diamino acid	Orn	Orn	Orn	Lys	Lys	Orn
Major menaquinones (MK)	10, 11	11, 12	11, 12	11, 12	11, 12	11, 12, 13

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