Microbacterium awajiense sp. nov., Microbacterium fluvii sp. nov. and Microbacterium pygmaeum sp. nov.

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The taxonomic positions of three novel strains isolated from soil, driftwood and sediment samples collected in Japan were investigated based on the results of chemotaxonomic, phenotypic and genotypic characteristics. The strains that we examined were Gram-positive, catalase-positive bacteria with L-ornithine as a diagnostic diamino acid of peptidoglycan. The acyl type of peptidoglycan was N-glycolyl. The major menaquinones were MK-11, -12, -13 and/or -14. Mycolic acids were not detected. The G+C content of the DNA was 68 to 70 mol%. These morphological and chemotaxonomical characters and comparative 16S rDNA analysis of the three isolated strains revealed that they belong to the genus Microbacterium. DNA-DNA relatedness data revealed that the three isolates are three new species of the genus Microbacterium. On the basis of the polyphasic evidence, the isolates should be classified as novel species of the genus Microbacterium: Microbacterium awajiense sp. nov., Microbacterium fluvii sp. nov. and Microbacterium pygmaeum sp. nov. with the type strains YM13-414ᵀ (=MBIC08276ᵀ, DSM 18907ᵀ), YSL3-15ᵀ (=MBIC08277ᵀ, DSM 18908ᵀ) and KV-490ᵀ (=NRRL B-24469ᵀ, NBRC 101800ᵀ), respectively.

INTRODUCTION

The genus Microbacterium was first proposed by Orla-Jensen (1919) with the type species Microbacterium lacticum, and was emended by Takeuchi & Hatano (1998). The genus Microbacterium is a member of the family Microbacteriaceae in the order Actinomycetales. In the present study, strain YM13-414ᵀ was isolated from a sediment sample, strain YSL3-15ᵀ was isolated from driftwood, and KV-490ᵀ was isolated from a soil sample. On the present phenotypic and chemotaxonomic data strongly indicate that these strains belong to the genus Microbacterium. Their phenotypic and phylogenetic characteristics, coupled with data for genomic DNA-DNA relatedness levels, suggest that these strains should be classified as the novel species Microbacterium awajiense sp. nov., Microbacterium fluvii sp. nov. and Microbacterium pygmaeum sp. nov.

MATERIALS AND METHODS

Bacterial strains and isolation

Strain YM13-414ᵀ was isolated from a sediment sample collected from the shore of Yura, Awaji Island, Japan (depth 20 cm, GPS location: N 34°16′25.7″, E 134°57′13.8″), in September 2004. The samples (0.5–1 cm³) were homogenized with a glass rod in 5 mL of sterile seawater. The homogenate (50 μL) was cultured on EGG medium at 25°C for 30 days. The components of EGG medium are shown in Table 1. The bacterium was then cultured on Marine Broth 2216 (Difco) containing 1.5% agar after being cultivated for 7–10 days. Strain YSL3-15ᵀ was isolated from driftwood collected in October 2005 at the estuary of Maera River on Iriomote Island, Japan. The driftwood was crushed in autoclaved artificial seawater. Next, 1/10 diluents of the sample was applied to seawater medium containing 0.1% lignan. Colonies were isolated after incubation for 1 week at 25°C. Strain KV-490ᵀ was isolated from a soil sample collected in the Aoyama Cemetery in Tokyo, Japan. Two grams of soil was suspended in 18 mL of sterile water and mixed. Soil particles were allowed to sediment, the liquid phase was diluted to 10⁵ and 100 μL samples were spread onto the surface of each plate. GPM agar plates (1.0% glucose, 0.5% peptone, 0.5% meat extract, 0.3% NaCl and 1.2% agar, pH 7.0) with SOD (300 unit/plate) and catalase (2100 unit/plate) (Takahashi et al., 2003) were used, and these were cultured at 27°C. KV-490ᵀ was isolated from GPM agar plates with SOD and catalase. Biomass for biochemical and chemotaxonomic characteristics was prepared by culturing in TSB broth at 27°C.

Morphological and biochemical tests

Morphological observation under a scanning electron microscope (model JSM-5600; JEOL) was performed using

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cultures grown on GPM agar medium at 27°C for 3 or 6 days. The carbon-assimilation properties of the two strains YM13-414T and KV-490T were determined using Pridham-Gottlieb agar medium (Nihon Pharmaceutical Co., Ltd.) (Pridham & Gottlieb, 1948), and the carbon-assimilation properties of strain YSL3-15T were determined using ten-times diluted Pridham-Gottlieb agar medium supplemented with ten-times diluted nutrient agar medium (Difco). NaCl tolerance and the pH and temperature ranges for growth were determined using 1/5 nutrient agar. The three isolates were characterized biochemically using API ZYM (bio-Mérieux) according to the manufacturer’s instructions.

Chemotaxonomic tests

The N-acyl type of muramic acid was determined using the method of Uchida & Aida (1977). Purified cell walls were obtained using the method of Kawamoto et al. (1981). One milligram of purified cell walls was hydrolyzed at 100°C with 1 mL of 6 N HCl for 16 h. The residue was dissolved in 100μL of water, and was used for amino acid analysis by thin layer chromatography (TLC). Mycolic acid was assayed using the TLC method of Tomiyasu (1982). Menaquinones were extracted and purified using the method of Collins et al. (1977), and were then analyzed by HPLC (model 802-SC; Jasco) equipped with a CAPCELL PAK C18 column (Shiseido Co., Ltd.) (Tamaoka et al., 1983). Methyl esters of cellular fatty acids were prepared, and were analyzed by GLC (model HP6890; Hewlett-Packard).

G+C content of DNA and DNA-DNA hybridization

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

16S rDNA sequencing and phylogenetic analysis

DNA was prepared by sonication (Yu et al., 2002) or using InstaGene matrix (Bio-Rad). 16S rDNA was amplified by PCR and sequenced with an automatic sequence analyzer (ABI Prism™ 3130 or 3730; PE Applied Biosystems) using a dye terminator cycle sequencing kit (PE Applied Biosystems).

Species related to the new isolate were identified by performing sequence database searches using the BLAST program (Altschul et al., 1990). Sequence data of related species were retrieved from GenBank. Nucleotide substitution rates (Knuv values) were calculated (Kimura & Ohta, 1972) and phylogenetic trees were constructed using the
The neighbor-joining method (Saitou & Nei, 1987). The statistical significance of the tree topology was evaluated by bootstrap analysis of sequence data using CLUSTAL W software (Thompson et al., 1994). Sequence similarity values were determined by visual comparison and manual calculation.

RESULTS AND DISCUSSION

The three strains were Gram-positive, aerobic, irregular rods (Fig. 1). The DNA base composition of the three strains was 68 to 70 mol% G+C. The cell wall peptidoglycans of the three isolates contained L-ornithine as a diagnostic diamino acid. The major menaquinones were MK-12, -13 and -14 for YM13-414T, MK-11 and -12 for YSL3-15T and MK-11, -12 and -13 for KV-490T. The acyl type of peptidoglycan was N-glycolyl. The major cellular fatty acids were anteiso-C15:0 and anteiso-C17:0 for YM13-414T, anteiso-C15:0 for YSL3-15T and anteiso-C15:0 and anteiso-C17:0 for KV-490T (Table 2).

We obtained nearly complete 16S rDNA gene sequences of the three isolated strains. Subsequent 16S rDNA-based phylogenetic analysis demonstrated that the strains belonged to the genus Microbacterium. Figure 2 shows the relationship between the three isolated strains and their near phylogenetic relatives. YM13-414T and YSL3-15T were related to M. deminutum and M. pumilum, and the similarity values of 16S rDNA sequences among these four strains ranged from 98.2 to 98.9%. On the other hand, KV-490T was too similar to deduce the interspecies relationship between type strains of Microbacterium, bootstrap values in the neighbor-joining tree were not so high. The similarity values of the 16S rDNA sequence between KV-490T and other Microbacterium species are as follows: M. terregens (98.8%), M. lacus (98.8%), M. aurum (98.4%) and M. aoyamense (99.1%).

![Fig. 1. Scanning electron micrographs of cells from 3- or 6-day-old cultures of strains YM13-414T (A), YSL3-15T (B) and KV-490T (C) grown on GPM agar medium at 27°C. Bar, 2 μm.](image)

![Fig. 2. Phylogenetic tree derived from 16S rDNA sequences and created using the neighbor-joining method. Numbers at branching points refer to bootstrap values (1000 resamplings). Only values with >40% are indicated. The tree was unrooted, and Microbacterium liquefaciens was used as an outgroup.](image)

Table 2. Fatty acid composition (%) of isolated strains.

<table>
<thead>
<tr>
<th></th>
<th>YM13-414T</th>
<th>YSL3-15T</th>
<th>KV-490T</th>
</tr>
</thead>
<tbody>
<tr>
<td>iso-C15:0</td>
<td>14.04</td>
<td>3.48</td>
<td>1.18</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>31.69</td>
<td>48.43</td>
<td>30.45</td>
</tr>
<tr>
<td>iso-C16:0</td>
<td>16.65</td>
<td>18.06</td>
<td>7.17</td>
</tr>
<tr>
<td>C16:0</td>
<td>1.62</td>
<td>5.91</td>
<td>1.77</td>
</tr>
<tr>
<td>iso-C17:0</td>
<td>5.74</td>
<td>1.52</td>
<td>1.82</td>
</tr>
<tr>
<td>anteiso-C17:0</td>
<td>28.81</td>
<td>19.58</td>
<td>56.81</td>
</tr>
<tr>
<td>C18:0</td>
<td>—</td>
<td>1.48</td>
<td>—</td>
</tr>
</tbody>
</table>

![Diagram](image)
DNA-DNA hybridization relatedness was determined. The relatedness value between YM13-414T and YSL3-15T, and *M. deminutum* and *M. puilum* was <23%. The results showed that these two isolates are independent new species. Species with ornithine in the cell wall and high 16S rDNA sequence similarity values were used to characterize KV-490T. Representative values of DNA-DNA relatedness values between KV-490T and *M. lacus, M. aoyamense*, and *M. terregens* were <21%. These values were well below the 70% cut-off point for species classification, as recommended by Wayne et al. (1987). These results confirm that the three isolated strains belonged to three independent new species of the genus *Microbacterium*.

The chemotaxonomic and morphological characteristics of these three isolated strains are consistent with their assignment to the genus *Microbacterium* (Takeuchi & Hatano, 1998). The phenotypic characters showed that the isolated strains were distinguished from their nearest phylogenetic neighbors is presented in Table 3.

Based on the present results, we propose three novel *Microbacterium* species: *Microbacterium awajiense* sp. nov., *Microbacterium fluvii* sp. nov. and *Microbacterium pygmaeum* sp. nov.

**Description of Microbacterium awajiense sp. nov.**

*Microbacterium awajiense* (awa.ji. en.se. N.L. neut. adj. *awajiense*, referring to Awaji Island, Hyogo, Japan, where the strain was isolated).

Cells are irregular rods, varying in their cell size from 0.5 to 0.8 by 0.7 to 1.2 μm. Gram-positive, non-motile, catalase positive, aerobic. Colonies are light yellow. Growth occurs between pH 6 and pH 11, and 13°C and 38°C. In 1/5 Nutrient agar medium, NaCl is tolerated up to 5%. Glucose, galactose, maltose, mannitol, mannose, raffinose, rhamnose, trehalose and xylose are assimilated. Sucrose is not assimilated. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase and α-fucosidase are detected by the API ZYM enzyme assay; trypsin, α-galactosidase, β-glucuronidase and α-mannosidase are negative. Weak reaction for β-galactosidase is detected. The acyl type of the peptidoglycan was N-glycolyl. The cell wall peptidoglycans contained L-ornithine as a diagnostic diamino acid. The major menaquinone =MK-12, -13 and -14. The major cellular fatty acids are anteiso-C15:0 and anteiso-C15:0. The DNA G+C content is 70 mol%. The type strain is YM13-414T (=MBIC08276T, DSM 18907T), which was isolated from the sediment sample collected from the shore of Yura, Awaji Island, Japan.

**Description of Microbacterium fluvii sp. nov.**


Cells are irregular rods, varying in their cell size from 0.4 to 0.6 by 0.6 to 1.2 μm. Gram-positive, non-motile, catalase positive, aerobic. Colonies are pale yellow. Growth occurs between pH 6 and pH 11, and 16°C and 36°C. In 1/5 Nutrient agar medium, NaCl is tolerated up to 3%. Glucose, galactose, maltose, mannitol, mannose, and rhamnose are assimilated. Raffinose, sucrose, trehalose and xylose are not assimilated. Esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase and α-fucosidase are detected by the API ZYM enzyme assay; trypsin, α-galactosidase, β-glucuronidase and α-mannosidase are negative. Weak reaction for β-galactosidase is detected. The acyl type of the peptidoglycan was N-glycolyl. The cell wall peptidoglycans contained L-ornithine as a diagnostic diamino acid. The major menaquinone

| Characteristics of the isolated strains and related *Microbacterium* species. |
|------------------|---|---|---|---|---|---|---|---|
| Growth at 37°C   | + | - | - | - | - | - | - | - |
| Growth in NaCl at 5% | + | - | - | - | - | - | - | + |
| Enzyme assay (APIZYM) | | | | | | | | |
| Alkaline phosphatase | + | w | - | w | + | w | - | w |
| Lipase | + | + | - | w | w | + | + | + |
| Leucine arylamidase | + | + | + | + | + | + | + | + |
| α-Galactosidase | - | - | - | + | + | + | - | - |
| β-Galactosidase | w | w | - | + | + | + | - | - |
| β-Glucuronidase | - | - | - | + | - | - | - | - |
| α-Glucosidase | + | - | + | + | + | + | + | + |
| β-Glucosidase | + | + | + | + | + | - | - | w |
| α-Fucosidase | + | - | - | + | - | - | - | - |
| G+C content | 70 | 70 | 68 | 69 | 71 | 69 | 69 | 69 |
| Major menaquinones (MK) | 12,13,14 | 11,12 | 11,12,13 | 12,13,14 | 12,13,14 | 12,13 | 12,13,14 | 12,13 |

1, YM13-414T; 2, YSL3-15T; 3, KV-490T; 4, M. deminutum; 5, M. puilum; 6, M. lacus; 7, M. aoyamense; 8, M. terregens. Data are from present study, Kageyama et al. (2006 & 2007). Abbreviations: +, positive; w, weakly positive; -, negative; ND, no data.
is MK-11 and -12. The major cellular fatty acid is anteiso-C\textsubscript{15:0}. The DNA G+C content is 70 mol\%. The type strain is YSL3-15\textsuperscript{T} (=MBIC08277\textsuperscript{T}, DSM 18908\textsuperscript{T}), which was isolated from driftwood collected at the estuary of Maera River in Iriomote Island, Japan.

**Description of Microbacterium pygmaeum** sp. nov.


Cells are irregular rods, rods vary in cell size from 0.3 to 0.4 by 0.5 to 0.9 \( \mu \text{m} \). Gram-positive, non-motile, catalase positive, aerobic. Colonies are pale yellow. Growth occurs between pH 5 and pH 9, and 17°C and 31°C. In 1/5 Nutrient agar medium, NaCl is tolerated up to 5%. Glucose, galactose, maltose, mannitol, mannose, raffinose, sucrose, and trehalose are assimilated, but rhamnose, and xylose are not. Esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, \( \alpha \)-glucosidase, \( \beta \)-glucosidase, and \( N \)-acetyl-\( \beta \)-glucosaminidase are detected by the API ZYM enzyme assay; phosphatase alkaline, lipase (C4), cystine arylamidase, trypsin, chymotrypsin, \( \alpha \)-galactosidase, \( \beta \)-galactosidase, \( \beta \)-glucuronidase, \( \alpha \)-mannosidase and \( \alpha \)-fucosidase are negative. Weak reaction for valine arylamidase. The acyl peptidoglycans contained L-ornithine as a diagnostic diamino acid. The major menaquinone is MK-11, -12 and -13. The major cellular fatty acids are anteiso-C\textsubscript{15:0} and anteiso-C\textsubscript{17:0}. The DNA G+C content is 68 mol\%. The type strain is KV-490\textsuperscript{T}, DSM 18908\textsuperscript{T}, which was isolated from soil, Aoyamareien, Japan.

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