Lysinimicrobium mangrovi gen. nov., sp. nov., an actinobacterium isolated from the rhizosphere of a mangrove

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A novel strain of Gram-staining-positive bacterium, designated HI08-69T, was isolated from the rhizosphere of a mangrove on Iriomote Island, Japan, and its taxonomic position was investigated by a polyphasic approach. The strain had peptidoglycan of the A4\a type, with lysine as the diagnostic diamino acid. The predominant menaquinone was demethylmenaquinone DMK-9(H4) and the major fatty acids were anteiso-C15:0, anteiso-C17:0 and C16:0. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unidentified polar lipid but minor amounts of other polar lipids were also detected. The genomic DNA G+C content of strain HI08-69T was 71.7 mol%. In phylogenetic analyses based on 16S rRNA gene sequences, the novel strain and members of the genus Demequina formed a monophyletic cluster, with pairwise sequence similarities of 95.6–96.8%. However, strain HI08-69T was clearly distinguishable from all established members of the genus Demequina in terms of several chemotaxonomic characteristics. On the basis of the phenotypic and genotypic characteristics, strain HI08-69T represents a novel species in a new genus for which the name Lysinimicrobium mangrovi gen. nov., sp. nov. is proposed. The type strain of the type species is HI08-69T (=NBRC 105856T =DSM 24868T).

The family Demequinaceae, which was proposed by Ue et al. (2011), currently contains just one genus: Demequina (Yi et al., 2007). At the time of writing, the genus Demequina consists of six recognized species, all of which have demethylmenaquinone DMK-9(H4) as their diagnostic isoprenoid quinone and a cell-wall peptidoglycan of the A4\b type (Schleifer & Kandler, 1972). During the course of a study of bacterial diversity in cool-temperate and subtropical areas, a novel actinobacterium was isolated from a soil sample collected from the rhizosphere of a tree on a subtropical island in Japan. Comparative 16S rRNA gene sequences indicated that the isolate, which was designated strain HI08-69T, is a member of the suborder Micrococlaceae. In the present study, the taxonomic position of this strain was determined by using a polyphasic approach.

Strain HI08-69T was isolated from a soil sample that had been collected from the rhizosphere of a mangrove (Bruguiera gymnorhiza) growing on Iriomote Island, Okinawa, Japan. Approximately 1 g of the sample was diluted 10-, 100- and 1000-fold with saline before 0.2 ml of each dilution was spread on plates of 0.2 % (w/v) polypeptone, 0.04 % (w/v) yeast extract, 0.02 % (w/v) MgSO4.7H2O and 1.5 % (w/v) agar; at pH 7.0 supplemented with 5.0 % (w/v) NaCl, 0.005 % (w/v) cycloheximide and 0.002 % (w/v) nalidixic acid. After cultivation at 30 °C for 1 week and repeated isolation, strain HI08-69T was obtained. As strain HI08-69T did not require NaCl for growth, full-strength NBRC medium 802 [1.0 % (w/v) polypeptone, 0.2 % (w/v) yeast extract, 0.1 % (w/v) MgSO4.7H2O and, if required, 1.5 % (w/v) agar; at pH 7.0] was used as the basal medium for this study. Biomass for all of the chemotaxonomic and molecular systematic studies except the fatty acid analysis was obtained by incubating the novel strain in shake flasks at 28 °C and 100 r.p.m. for 48 h.

Colonies appearance and pigment production were investigated after incubation at 28 °C for 3 days. The morphology of cells cultured for up to 7 days was determined under a light microscope (BX-51; Olympus) and in a scanning electron microscope (JSM-6060; JEOL). Cell motility, pH
range for growth, NaCl tolerance, anaerobic growth, Gram staining and oxidase activity were determined using the methods outlined by Hamada et al. (2010). The temperature range and optimum temperature for growth were determined by incubating the novel strain at 5, 10, 15, 20, 25, 28, 37, 45 and 60 °C on plates of NBRC medium 802 agar for 4 days. Growth at 5 and 10 °C was also evaluated after 14 days of incubation. Other physiological and biochemical tests were performed using API ZYM, API Coryne, API 20E and API 50CH strips (bioMérieux) according to the manufacturer’s instructions.

After incubation at 28 °C for 3 days, strain HI08-69T formed pale yellow, circular, transparent, smooth colonies with diameters of 0.5–1.0 mm. Pigment production was not observed. The cells were Gram-staining-positive, non-motile and non-spore-forming and exhibited a rod-to-coccus growth cycle. Cells of exponentially growing cultures were rod-shaped (0.4–0.6 × 1.0–3.0 μm), whereas stationary phase cells were oval to coccoid (0.5–0.6 × 0.5–1.0 μm) (Fig. 1). The cells were seen to fragment by the formation of septa. Growth occurred at 15–28 °C (optimum 28 °C) and pH 6.0–9.0 (optimum between pH 7.0 and pH 8.0). The strain exhibited good growth with NaCl concentrations of 0–5 % (w/v) and moderate growth with 7 % (w/v); no growth was observed with NaCl at 10 or 15 % (w/v). Optimal growth occurred in the absence of NaCl. Weak growth was observed under anaerobic conditions. The results of other physiological and biochemical analyses have been summarized in the species description.

The PCR-based amplification and sequencing of the 16S rRNA gene of strain HI08-69T were performed as described previously (Hamada et al., 2010). The novel strain’s phylogenetic neighbours were identified and pairwise 16S rRNA gene sequence similarities were calculated, using the EzTaxon server (Chun et al., 2007). The CLUSTAL_X program (Thompson et al., 1997) was used to align the almost-complete 16S rRNA gene sequence of strain HI08-69T with the corresponding sequences of members of the genus Demequina and some related taxa. Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms and version 5.01 of the MEGA program (Tamura et al., 2011). The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates. In the trees, strain HI08-69T formed a clade with members of the genus Demequina (Fig. 2) although 16S rRNA gene sequence similarities of only 95.6–96.8 % were recorded in the pairwise comparisons between the novel strain and the type strain of each Demequina species.

A cell-wall sample was prepared, from approximately 1 g wet cells of the novel strain, by mechanical disruption with an ultrasonic oscillator and glass beads and then differential centrifugation in distilled water. The sample of cell walls was purified by boiling in 4 % (w/v) SDS for 40 min, washed several times with distilled water and then hydrolysed in 4 M HCl for 16 h. The molar ratios of the amino acids in the resultant hydrolysate were determined according to the method described by Hamada et al. (2010). The amino acid isomers in the same hydrolysate were determined by LC-MS as described by Nozawa et al. (2007), in an LCMS-2020 spectrometer (Shimadzu). Biomass for the investigation of cellular fatty acids was grown on tryptic soy agar (Difco) for 24 h at 28 °C. Cellular fatty acid methyl esters were prepared from the biomass and analysed by following the standard protocol of version 4.0 of the Sherlock Microbial Identification System (MIDI) and using version 4.0 of the TSBA database (Sasser, 1990) and a 6890N GC (Agilent). Polar lipids were analysed by TLC, as described by Hamada et al. (2010), using chloroform-methanol-water (65 : 25 : 4, v/v/v) in the first direction and chloroform-acetic acid-methanol-water (80 : 18 : 12 : 5, v/v/v/v) in the second. Isoprenoid quinones, cell-wall sugars and genomic DNA G+C content were investigated by the methods outlined by Hamada et al. (2010).

The peptidoglycan of strain HI08-69T contained alanine (Ala), glutamic acid (Glu), serine (Ser) and lysine (Lys) in a molar ratio of 1.0 : 2.0 : 1.0 : 0.8. Enantiomeric analysis of the peptidoglycan amino acids revealed the presence of D-Ala, D-Glu, L-Ser and L-Lys but no L-Ala. These results indicate that the cell-wall peptidoglycan of strain HI08-69T is of the A4z type (Schleifer & Kandler, 1972) with an interpeptide bridge comprising D-Glu, and an L-Ser residue.
Arabinose, galactose and mannose were detected as the cell-wall sugars. The only isoprenoid quinone detected was demethylmenaquinone DMK-9(H4). The major cellular fatty acids were anteiso-C15 : 0 (47.4 %), anteiso-C17 : 0 (16.8 %) and C16 : 0 (11.8 %) but iso-C16 : 0 (7.9 %), C14 : 0 (6.6 %), anteiso-C15 : 1 A (5.0 %), C15 : 0 (2.7 %) and iso-C15 : 0 (1.8 %) were also detected. The predominant polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unidentified (phosphorus-free) polar lipid. Minor or trace amounts of phosphatidylinositol mannoside, an unidentified phosphoglycolipid, two unidentified glycolipids and three unidentified (phosphorus-free) polar lipids (Fig. S1, available in IJSEM Online) were also detected. The genomic DNA G+C content of the novel strain was 71.7 mol%.

In the phylogenetic analysis based on 16S rRNA gene sequences, strain HI08-69T formed a monophyletic cluster with members of the genus Demequina irrespective of the treeing algorithm that was employed. In the neighbour-joining tree, the branch corresponding to this cluster was supported by a bootstrap value of 100 % (Fig. 2). The predominant menaquinone of the novel strain was demethylmenaquinone DMK-9(H4) – the diagnostic isoprenoid quinone of the genus Demequina. It is this menaquinone that permits the current members of the genus Demequina to be distinguished from those of other genera within the suborder Micrococcineae. However, the peptidoglycan of strain HI08-69T was of the A4a type, with L-lysine as the diagnostic cell-wall diamino acid, whereas the peptidoglycan of each member of the genus Demequina is reported to be of the A4b type, with L-ornithine as the diagnostic diamino acid (Yi et al., 2007). In terms of its peptidoglycan, therefore, strain HI08-69T appears to be distinct from the current members of the genus Demequina. The chemotaxonomic characteristics of strain HI08-69T that allow it to be distinguished from its closest phylogenetic neighbours are summarized in Table 1. Based on the chemotaxonomic and phylogenetic data, strain HI08-69T represent a novel species of the genus Demequina.
Table 1. Chemotaxonomic characteristics that enable strain H108-69T to be differentiated from members of the closest related genera

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>Peptidoglycan type</td>
<td>A4s*</td>
<td>A4β</td>
<td>A4s</td>
<td>A4β</td>
<td>A4s</td>
<td>A4β</td>
</tr>
<tr>
<td>Diamino acid</td>
<td>L-Lys</td>
<td>L-Orn</td>
<td>L-Lys</td>
<td>L-Orn</td>
<td>L-Lys</td>
<td>L-Orn</td>
</tr>
<tr>
<td>Interpeptide bridge</td>
<td>D-Glu</td>
<td>D-Glu or Ser–D-Asp or Ser–D-Glu</td>
<td>D-Asp</td>
<td>D-Asp</td>
<td>D-Asp or D-Glu</td>
<td></td>
</tr>
<tr>
<td>Major menaquinone(s)</td>
<td>DMK-9(H₄)</td>
<td>DMK-9(H₄)</td>
<td>MK-9(H₄)</td>
<td>MK-10(H₄)</td>
<td>MK-9(H₄)</td>
<td>MK-9(H₄), MK-8(H₄)</td>
</tr>
<tr>
<td>Major fatty acids†</td>
<td>ai-C₁₅ : 0, ai-C₁₇ : 0, C₁₆ : 0</td>
<td>ai-C₁₅ : 0, ai-C₁₇ : 0, C₁₆ : 0</td>
<td>ai-C₁₅ : 0, C₁₆ : 0, C₁₄ : 0</td>
<td>C₁₄ : 0, ai-C₁₅ : 0, C₁₆ : 0</td>
<td>ai-C₁₅ : 0, C₁₆ : 0, i-C₁₅ : 0, D-ai-C₁₇ : 0</td>
<td></td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>DPG, PG, PI, PL</td>
<td>71.7</td>
<td>65–72</td>
<td>69–73</td>
<td>76</td>
<td>70–75</td>
</tr>
</tbody>
</table>

*1-L-Ser occupies position 1 of the peptide subunit.
†ai, anteiso-branched; i, iso-branched.
‡DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PGL, unknown phosphoglycolipid; PI, phosphatidylinositol; PL, unknown polar lipid.

Description of Lysinimicrobium mangrovi gen. nov., sp. nov.

Lysinimicrobium mangrovi (mangrovi, N.L. neut. n. mangrovi, referring to the isolation of the type strain from the rhizosphere of a mangrove). Cells are Gram-staining positive, facultatively anaerobic and non-motile. Cells grow at pH 7.0 and pH 8.0 (optimum between pH 7.5 and pH 8.0). Growth occurs with NaCl at 0–7 % (w/v). Growth is inhibited at 10 % (w/v) NaCl. Aesculin is hydrolysed but gelatin is not. Nitrate is not reduced. The interpeptide bridge of the cell-wall peptidoglycan is L-lysine as the diagnostic diamino acid, with an L-serine residue in minor amounts.

The type strain, H108-69T (=NBRC 105856 = DSM 24868T), was isolated from the rhizosphere of a mangrove growing on a sandy beach near Yokosuka, Japan.
Iriomote Island, Japan. The genomic DNA G+C content of the type strain is 71.7 mol%.

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References


