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 HI08-69^T, 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 α type, with lysine as the diagnostic diamino acid. The predominant menaquinone was demethylmenaquinone DMK-9(H₄) and the major fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0} and C_{16:0}. 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 HI08-69^T 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 HI08-69^T 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 HI08-69^T 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 HI08-69^T (=NBRC 105856^T=DSM 24868^T).

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 β 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 sequence analysis indicated that the isolate, which was designated strain HI08-69^T, is a member of the sub-order *Micrococcineae*. In the present study, the taxonomic position of this strain was determined by using a polyphasic approach.

Strain HI08-69^T 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 × NBRC medium 802 [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 HI08-69^T was obtained. As strain HI08-69^T 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.

Colony 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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HI08-69^T is AB639012.

A supplementary figure is available with the online version of this paper.

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 HI08-69^T 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 ($0.4\text{--}0.6 \times 1.0\text{--}3.0\text{ }\mu\text{m}$), whereas stationary phase cells were oval to coccoid ($0.5\text{--}0.6 \times 0.5\text{--}1.0\text{ }\mu\text{m}$) (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 HI08-69^T 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 HI08-69^T 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 HI08-69^T 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,

Fig. 1. Scanning electron micrographs of cells of strain HI08-69^T grown on NBRC medium 802 for 1 (a) and 7 (b) days at 28 °C. Bars, 2 μm .

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 HI08-69^T 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 HI08-69^T is of the A4 α type (Schleifer & Kandler, 1972) with an interpeptide bridge comprising D-Glu, and an L-Ser residue

Fig. 2. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between strain HI08-69^T and its closest taxonomic neighbours. *Brevibacterium linens* DSM 20425^T was used as the outgroup. Bootstrap values (>70 %) based on 1000 replicates are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

at position 1 of the peptide subunit. 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-C_{15:0} (47.4 %), anteiso-C_{17:0} (16.8 %) and C_{16:0} (11.8 %) but iso-C_{16:0} (7.9 %), C_{14:0} (6.6 %), anteiso-C_{15:1} A (5.0 %), C_{15:0} (2.7 %) and iso-C_{15:0} (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 HI08-69^T 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 HI08-69^T was of the A4α 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 A4β type, with L-ornithine as the diagnostic diamino acid (Yi *et al.*, 2007). In terms of its peptidoglycan, therefore, strain HI08-69^T appears to be distinct from the current members of the genus *Demequina*. The chemotaxonomic characteristics of strain HI08-69^T that allow it to be distinguished from its closest phylogenetic neighbours are summarized in Table 1. Based on the chemotaxonomic and phylogenetic data, strain HI08-69^T represent a novel

Table 1. Chemotaxonomic characteristics that enable strain HI08-69^T to be differentiated from members of the closest related genera

Taxa: 1, strain HI08-69^T (data from this study); 2, *Demequina* (Yi *et al.*, 2007; Finster *et al.*, 2009; Matsumoto *et al.*, 2010; Ue *et al.*, 2011); 3, *Sanguibacter* (Fernández-Garayzabal *et al.*, 1995); 4, *Actinotalea* (Yi *et al.*, 2007); 5, *Oerskovia* (Stackebrandt *et al.*, 2002); 6, *Cellulomonas* (Schumann *et al.*, 2009). ND, No data available.

Characteristic	1	2	3	4	5	6
Peptidoglycan type	A4 α *	A4 β	A4 α	A4 β	A4 α	A4 β
Diamino acid	L-Lys	L-Orn	L-Lys	L-Orn	L-Lys	L-Orn
Interpeptide bridge	D-Glu	D-Glu or Ser-D-Asp or Ser-D-Glu	Ser-D-Glu	D-Asp	L-Thr-D-Asp or L-Thr-D-Glu	D-Asp or D-Glu
Major menaquinone(s)	DMK-9(H ₄)	DMK-9(H ₄)	MK-9(H ₄)	MK-10(H ₄)	MK-9(H ₄)	MK-9(H ₄), MK-8(H ₄)
Major fatty acids†	ai-C _{15:0} ; ai-C _{17:0} ; C _{16:0}	ai-C _{15:0} ; ai-C _{17:0} ; C _{16:0}	ai-C _{15:0} ; C _{16:0} ; C _{14:0}	ai-C _{15:0} ; C _{16:0}	ai-C _{15:0} ; C _{16:0}	ai-C _{15:0} ; C _{16:0} ; i-C _{15:0} ; ai-C _{17:0}
Polar lipids‡	DPG, PG, PI, PL	DPG, PG, PI	ND	ND	DPG, PG, PI	DPG, PG, PGL, PI
DNA G + C content (mol%)	71.7	65–72	69–73	76	70–75	69–76

*L-Ser occupies position 1 of the peptide subunit.

†ai, anteiso-branched; i, iso-branched.

‡DPG, Diposphatidylglycerol; PG, phosphatidylglycerol; PGL, unknown phosphoglycerolipid; PI, phosphatidylinositol; PL, unknown polar lipid.

species of a new genus for which the name *Lysinimicrobium mangrovi* gen. nov., sp. nov. is proposed.

Description of *Lysinimicrobium* gen. nov.

Lysinimicrobium (Ly.si.ni.mi.cro'bi.um. N.L. n. *lysinum* lysine; N.L. neut. n. *microbium* microbe; N.L. neut. n. *Lysinimicrobium* a microbe with lysine in the cell wall).

Cells are Gram-staining-positive, facultatively anaerobic and non-spore-forming and exhibit a rod-to-coccus cycle. The cell-wall peptidoglycan is of the A4 α type and contains L-lysine as the diagnostic diamino acid, with an L-serine residue at position 1 of the peptide subunit. The major cell-wall sugars are arabinose, galactose and mannose. The predominant menaquinone is DMK-9(H₄) and the principal polar lipids are diposphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unknown polar lipid. The major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and C_{16:0}. Phylogenetically, the genus *Lysinimicrobium* belongs to the family *Demequinaceae* within the suborder *Micrococcineae*. The type species is *Lysinimicrobium mangrovi*.

Description of *Lysinimicrobium mangrovi* sp. nov.

Lysinimicrobium mangrovi (man.gro'vi. N.L. gen. neut. n. *mangrovi* of a mangrove, referring to the isolation of the type strain from the rhizosphere of a mangrove).

Displays the following properties in addition to those given for the genus. Cells of exponentially growing cultures are rod-shaped (0.4–0.6 $\times$ 1.0–3.0 μ m) while stationary-phase cells are oval to coccoid (0.5–0.6 $\times$ 0.5–1.0 μ m). Cells are non-motile. Catalase-positive and oxidase-negative. Colonies are pale yellow, circular, transparent and smooth. Grows at 15–28 °C (optimum 28 °C) and pH 6.0–9.0 (optimum between pH 7.0 and pH 8.0). Growth occurs with NaCl at 0–7 % (w/v) (optimum 0 %) but not at 10 % (w/v). Acid is produced from amygdalin, L-arabinose, arbutin, D-cellobiose, D-fructose, D-galactose, gentiobiose, D-glucose, glycogen, D-lactose, D-maltose, D-mannose, L-rhamnose, D-ribose, salicin, starch, D-sucrose, D-trehalose and D-xylose. Positive result in tests for leucine arylamidase, β -galactosidase and α - and β -glucosidase activities but negative result for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cysteine arylamidase, trypsin, chymotrypsin, acid phosphatase, phosphohydrolase, α -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, pyrazinamidase, pyrrolidonyl arylamidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease activities. H₂S and indole are not produced. Aesculin is hydrolysed but gelatin is not. Nitrate is not reduced. The interpeptide bridge of the cell-wall peptidoglycan is D-glutamic acid. The major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and C_{16:0}, while iso-C_{16:0}, C_{14:0}, anteiso-C_{15:1}, A, C_{15:0} and iso-C_{15:0} are found in minor amounts.

The type strain, HI08-69^T (=NBRC 105856^T=DSM 24868^T) was isolated from the rhizosphere of a mangrove growing on

Iriomote Island, Japan. The genomic DNA G + C content of the type strain is 71.7 mol%.

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