Marihabitans asiaticum gen. nov., sp. nov., a meso-diaminopimelic acid-containing member of the family *Intrasporangiaceae*

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Strain HG667^T, isolated from surface seawater collected at the Kesennuma ferry port in Miyagi Prefecture, Japan, was found to be a Gram-positive, catalase-positive bacterium comprising irregular short rods and cocci. The diagnostic diamino acid in the cell-wall peptidoglycan was meso-diaminopimelic acid. The major menaquinone was MK-8(H₄). Mycolic acids were not detected. The G + C content of the DNA was 70 mol%. Analysis of the 16S rRNA gene sequence revealed that the strain represents a novel lineage within the family *Intrasporangiaceae*, order *Actinomycetales*, being associated with the genus *Kribbia*. On the basis of morphological, biochemical and chemotaxonomic properties of the strain, together with phylogenetic data relating to the 16S rRNA gene sequence, HG667^T represents a novel genus and species in the family *Intrasporangiaceae*, for which the name *Marihabitans asiaticum* gen. nov., sp. nov. is proposed. The type strain of *Marihabitans asiaticum* is HG667^T (=MBIC07497^T =DSM 18935^T).

The family *Intrasporangiaceae* was proposed by Rainey *et al.* (in Stackebrandt *et al.*, 1997) and its description was emended by Stackebrandt & Schumann (2000). The genera belonging to this family can be divided into three groups on the basis of the diagnostic diamino acid types in the cell-wall peptidoglycan: the LL-diaminopimelic acid (LL-A₂pm) type, the meso-A₂pm type and the L-ornithine type. The genera *Janibacter* (Martin *et al.*, 1997), *Knoellia* (Groth *et al.*, 2002), *Kribbia* (Jung *et al.*, 2006), *Oryzihumus* (Kageyama *et al.*, 2005), *Phycoccus* (Lee, 2006) and *Tetrasphaera* (Maszenan *et al.*, 2000) belong to the meso-A₂pm group. The strain described in the present study, HG667^T, also contained meso-A₂pm as the diagnostic diamino acid in the cell-wall peptidoglycan.

Strain HG667^T was isolated from surface seawater collected in November 2003 at the Kesennuma ferry port in Miyagi Prefecture, Japan (GPS location: 38° 53' 52" N 141° 35' 35" E). A colony was picked from 1/10-strength marine agar 2216 (Difco) after incubation for 1 month at 25 °C and was subcultured on marine agar 2216 (MA; Difco).

Biomass for the study of biochemical and chemotaxonomic characteristics was prepared by culturing cells in tryptic soy

broth (Difco) at 27 °C for 3 or 4 days and then harvesting them by centrifugation.

The cell morphology of the strain was studied with a light microscope during growth (at 21 h and at 2, 3, 4 and 5 days) in marine broth at 27 °C. Bacterial cells (3-day-old culture on MA) were also studied with a scanning electron microscope (model JSM-5600; JEOL). Motility was investigated using microscopic analysis. Colonies were suspended in artificial seawater (Lyman & Fleming, 1940) after incubation for 3 days, 1 week and 1 month at 27 °C on MA medium. NaCl tolerance was determined on 1/5-strength nutrient agar and pH and temperature ranges for growth were determined on MA. The ability of the strain to grow on various substrates serving as sole carbon sources (at 1 %, w/v) was determined using a carbon-utilization medium (Pridham & Gottlieb, 1948) (Nihon Pharmaceutical). The isolate was characterized biochemically using API ZYM (bioMérieux) in accordance with the manufacturer's instructions.

Whole-cell hydrolysates were analysed for A₂pm isomers by using TLC (Becker *et al.*, 1965; Hasegawa *et al.*, 1983). Purified cell walls were obtained using the method of Kawamoto *et al.* (1981). Purified cell wall (1 mg) was hydrolysed at 100 °C with 1 ml 6 M HCl for 16 h. The residue was dissolved in 100 µl water and then used for amino acid analysis by TLC. Sugars in the purified cell wall

Abbreviation: A₂pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HG667^T is AB286025.

were determined according to the procedures described by Stanek & Roberts (1974). The presence of mycolic acid was checked using the TLC method of Tomiyasu (1982). Menaquinones were extracted and purified using the method of Collins *et al.* (1977) and then analysed by HPLC (model 802-SC; Jasco) on a chromatograph equipped with a Capcell Pak C18 column (Shiseido) (Tamaoka *et al.*, 1983). Polar lipids were extracted according to the method described by Minnikin *et al.* (1984). The two-dimensional TLC method (Komagata & Suzuki, 1987) was used to determine the phospholipid profile. To determine the cellular fatty acid methyl esters, cell mass of strain HG667^T was harvested from TSA plates after cultivation for 3 days at 30 °C and subjected to analysis using GLC (model HP6890; Hewlett Packard). The method described in the manual (version 6) of the Sherlock Microbial Identification System (version 5.0) (MIDI) was used for sample preparation and analysis.

To determine the G+C content, chromosomal DNA was isolated as described by Saito & Miura (1963) and the content estimated using HPLC (Tamaoka & Komagata, 1984).

DNA for 16S rRNA gene sequence analysis was prepared using an InstaGene matrix (Bio-Rad). The 16S rRNA gene was amplified by using the PCR with 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 using an automatic sequence analyser (3730 DNA analyser; Applied Biosystems) with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Species related to the novel strain were identified by performing sequence database searches with BLAST (Altschul *et al.*, 1990). Sequence data for related species were retrieved from GenBank and used for a detailed phylogenetic analysis. The 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 using the neighbour-joining method as described by Saitou & Nei (1987). The DNAML program in the PHYLIP 3.5c package (Felsenstein, 1985) was used for maximum-likelihood analysis, with a default transition/transversion ratio of 2.000000. Sequence similarity values were determined by means of visual comparison and manual calculation.

The cells of strain HG667^T were aerobic, Gram-positive, non-spore-forming, irregular short rods or cocci (Fig. 1). Observation using light microscopy showed that the cells occur singly and also in pairs, short chains or clumps. No distinct life cycle and no changes in cell morphology were observed under the light microscope during growth (for 21 h to 5 days) in marine broth. Motility was not observed under the conditions used. The DNA G+C content of the strain was 70 mol%. The cell wall contained *meso*-A₂pm as the diagnostic diamino acid. The cell-wall sugars were galactose, glucose and ribose. The major menaquinone (constituting about 90 %) was MK-8(H₄). The phospho-

Fig. 1. Scanning electron micrograph of cells from a 3-day-old culture of strain HG667^T grown on MA medium at 27 °C. Bar, 2 µm.

lipids were phosphatidylglycerol, an unknown phospholipid, diphosphatidylglycerol and phosphatidylinositol mannosides. The cellular fatty acids were iso-C_{17:0} (38.5 %), C_{17:1}ω8c (19.3 %), C_{18:1}ω9c (10.5 %), iso-C_{15:0} (7.4 %), iso-C_{17:1}ω9c (5.2 %), C_{16:0} (3.0 %), C_{17:0} (2.7 %), C_{18:1}ω7c (1.7 %), iso-C_{16:0} (1.1 %) and anteiso-C_{17:0} (1.1 %).

To determine the phylogenetic position of strain HG667^T, an almost-complete 16S rRNA gene sequence was determined. A database search demonstrated that the strain belonged to the family *Intrasporangiaceae* in the suborder *Micrococcineae*. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain HG667^T (1435 bp) was closely related to *Kribbia dieselivorans* N113^T (1476 bp), with 96.0 % similarity (Fig. 2). The 16S rRNA gene sequence identities of HG667^T with respect to type strains of type species of other genera with *meso*-A₂pm in the cell wall were as follows: 94.9 % with *Janibacter limosus* DSM 11140^T (1473 bp), 94.8 % with *Knoellia sinensis* DSM 12331^T (1479 bp), 92.8 % with *Oryzihumus leptocrescens* KV-628^T (1429 bp), 93.4 % with *Phycococcus jejuensis* KSW2-15^T (1413 bp) and 93.0 % with *Tetrasphaera japonica* ACM 5116^T (1445 bp).

Strain HG667^T can be differentiated from other genera of the family *Intrasporangiaceae* on the basis of its cell-wall diamino acid, cell morphology and major fatty acid composition. Table 1 shows the phenotypic characteristics of the isolate and of *meso*-A₂pm-containing members of the family *Intrasporangiaceae*. The phylogenetically closest genus is *Kribbia*, members of which also contain *meso*-A₂pm. Strain HG667^T can be differentiated from the genus *Kribbia* on the basis of major fatty acids and growth temperature. While strains of the genus *Kribbia* can grow over a wide range of temperatures (8–42 °C), strain HG667^T grows at 18–34 °C. Strain HG667^T can be differentiated from the genera *Janibacter*, *Knoellia*, *Tetrasphaera* and *Phycococcus* on the basis of cell morphology and the pattern of phospholipids. The differential

Fig. 2. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, for strain HG667^T and members of the family *Intrasporangiaceae*. Only values with greater than 40% significance are shown. Filled circles indicate that the corresponding nodes were also recovered in the maximum-likelihood tree. The tree was unrooted and *Arthrobacter globiformis* DSM 20124^T was used as an outgroup. Bar, 0.01 K_{nuc}.

characteristic between strain HG667^T and the genus *Oryzihumus* is cell morphology. Other characteristics of strain HG667^T are given in the genus and species descriptions.

On the basis of the data presented here, we consider that strain HG667^T merits description as a novel genus and species within the family *Intrasporangiaceae*, for which the name *Marihabitans asiaticum* gen. nov., sp. nov. is proposed.

Description of *Marihabitans* gen. nov.

Marihabitans (Ma.ri.ha'bi.tans. L. neut. n. *mare* sea; L. part. adj. *habitans* inhabiting; N.L. adj. used as a neut. subst. *Marihabitans* inhabitant of the sea).

Cells are Gram-positive, non-motile, non-spore-forming, catalase-positive, aerobic, irregular short rods and cocci. The peptidoglycan is of the A type and contains *meso*-A₂pm, alanine and glutamic acid. The cell-wall sugars are galactose, glucose and ribose. Mycolic acids are absent. The major menaquinone is MK-8(H₄). The phospholipids comprise phosphatidylglycerol, an unknown phospholipid, diphosphatidylglycerol and phosphatidylinositol mannosides. The cellular fatty acids are a complex mixture of straight-chain saturated, monounsaturated and iso- and anteiso-methyl-branched acids and consist mainly of iso-methyl-branched saturated and straight-chain monounsaturated acids, with iso-C_{17:0}, C_{17:1}ω8*c* and C_{18:1}ω9*c* predominating. Phylogenetically, the genus is a member of the family *Intrasporangiaceae*, suborder *Micrococcineae*. The type species is *Marihabitans asiaticum*.

Description of *Marihabitans asiaticum* sp. nov.

Marihabitans asiaticum (a.si.a'ti.cum. N.L. neut. adj. *asiaticum* of Asia, the source of the type strain).

Displays the properties of the genus with the following additions. Cells are 0.6–1.0 μm wide and 0.8–1.3 μm long. Colonies are light yellow. Growth occurs between 18 and 34 °C. The optimum pH for growth is 9 on MA. On 1/5-strength nutrient agar, NaCl is tolerated at concentrations up to 7%. Positive for nitrate reduction and hydrolysis of

Table 1. Differential characteristics of strain HG667^T and *meso*-A₂pm-containing members of the family *Intrasporangiaceae*

Data for reference genera were taken from Jung *et al.* (2006) (*Kribbia*), Martin *et al.* (1997), Lang *et al.* (2003) and Yoon *et al.* (2004) (*Janibacter*), Groth *et al.* (2002) (*Knoellia*), Kageyama *et al.* (2005) (*Oryzihumus*), Lee (2006) (*Phycococcus*) and Maszenan *et al.* (2000) and Hanada *et al.* (2002) (*Tetrasphaera*). ND, No data available.

Taxon	Cell morphology	Major fatty acids*	Polar lipids†	DNA G+C content (mol%)
Strain HG667 ^T	Irregular short rods and cocci	i-C _{17:0} , C _{17:1} ω8 <i>c</i> , C _{18:1} ω9 <i>c</i>	DPG, PG, PL, PIMs	70
<i>Kribbia</i>	Irregular short rods or cocci	10-Methyl C _{18:0} , i-C _{16:0} , C _{18:1} ω9 <i>c</i> , C _{16:0} , C _{18:0}	ND	69–70
<i>Janibacter</i>	Coccoid to rod-shaped	i-C _{16:0} , C _{17:1} ω8 <i>c</i> , C _{18:1} ω9 <i>c</i> , C _{17:0}	DPG, PG, PI	69–73
<i>Knoellia</i>	Irregular rods and cocci	i-C _{15:0} , i-C _{17:0} , i-C _{16:0} , ai-C _{17:0}	DPG, PE, PI, PG, PL	68–69
<i>Oryzihumus</i>	Irregular rods	i-C _{16:0} , i-C _{15:0} , i-C _{14:0}	ND	72–73
<i>Phycococcus</i>	Cocci	C _{17:1} ω8 <i>c</i> , i-C _{16:0} , i-C _{15:0}	DPG, PE, PI	74
<i>Tetrasphaera</i>	Cocci or short rods	ai-C _{15:0} and i-C _{15:0} ^a , i-C _{16:0} , ai-C _{17:0} and i-C _{15:0} ^b or i-C _{16:0} and ai-C _{17:0} ^{c‡}	DPG, PG, PI, PE§, PL§	68–71

*Major fatty acids are defined as constituting >10% of the total fatty acid content. ai, Anteiso; i, iso.

†DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, unknown phospholipid(s); PIMs, phosphatidylinositol mannosides.

‡Data for: a, *T. elongata*; b, *T. australiensis*; c, *T. japonica*.

§Detected in only some representatives of the genus.

casein and gelatin. Negative for urease activity, starch hydrolysis and oxidase activity. D-Galactose, D-glucose and trehalose are assimilated, but L-arabinose, D-fructose, maltose, D-mannitol, D-mannose, raffinose, L-rhamnose, sucrose and D-xylose are not assimilated. Esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, acid phosphatase, α -glucosidase and β -glucosidase are detected in the API ZYM enzyme assay; tests for alkaline phosphatase, lipase (C14), valine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are negative. Weakly positive for naphthol-AS-BI-phosphohydrolase. The DNA G + C content of the type strain is 70 mol%.

The type strain, HG667^T (=MBIC07497^T =DSM 18935^T), was isolated from surface seawater collected at the Kesennuma ferry port in Miyagi Prefecture, Japan.

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