Humihabitans oryzae gen. nov., sp. nov.

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Strain KV-657^T was isolated from a paddy field soil sample collected in Japan using GPM agar plates supplemented with catalase. The strain was a Gram-positive, aerobic organism that formed branching hyphae with LL-diaminopimelic acid as the diagnostic peptidoglycan diamino acid. The major menaquinone was MK-8(H₄). Mycolic acids were not detected. The G + C content of the DNA was 70 mol%. 16S rRNA gene sequence analysis revealed that this strain is closely related to *Intrasporangium calvum* DSM 43043^T, with a similarity of 97.6 %. Based on the morphological, biochemical and chemotaxonomic properties of this strain and phylogenetic analysis, it was concluded that this isolate represents a new genus and species in the family *Intrasporangiaceae*, for which the name *Humihabitans oryzae* gen. nov., sp. nov. is proposed. The type strain of *Humihabitans oryzae* is KV-657^T (=NRRL B-24470^T =NBRC 101802^T).

In order to facilitate the discovery of novel micro-organisms, various isolation techniques have been developed (Magarvey *et al.*, 2004; Yamamura *et al.*, 2003; Hayakawa *et al.*, 2004). In a previous report, we described a new isolation method using agar medium containing oxidant scavengers, for example superoxide dismutase with or without catalase (Takahashi *et al.*, 2003). Using this method, we have succeeded in isolating representatives of two new genera, *Oryzihumus leptocrescens* (Kageyama *et al.*, 2005) and *Patulibacter minatonensis* (Takahashi *et al.*, 2006). Among 45 strains isolated from paddy soil in Saitama Prefecture, Japan, using this new isolation method (Takahashi *et al.*, 2003), 20 strains belonged to the class *Actinobacteria*, including strain KV-657^T. Strain KV-657^T forms branching hyphae, has LL-diaminopimelic acid (A₂pm) in its peptidoglycan and, on the basis of a phylogenetic tree constructed using 16S rRNA gene sequences, it was thought to be closely associated with *Intrasporangium calvum*.

In this paper, we report on the morphological, physiological and biochemical characteristics of strain KV-657^T and DNA–DNA hybridization and 16S rRNA gene sequence analysis in comparison with *I. calvum* NBRC 12989^T. Based on these results, KV-657^T represents a novel genus and species.

Abbreviation: A₂pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KV-657^T is AB282887.

Scanning electron micrographs of cells of strain KV-657^T and HPLC traces from menaquinone analysis of the novel strain and *I. calvum* NBRC 12989^T are available as supplementary material with the online version of this paper.

Strain KV-657^T was isolated from soil samples collected from a paddy field in Saitama Prefecture, Japan. Biomass for biochemical and chemotaxonomic studies was prepared by culture in TSB (Difco) at 27 °C for 3 or 4 days, followed by harvesting by centrifugation.

Morphological observations were carried out using a scanning electron microscope (model JSM-5600; JEOL) and cultures grown on GPM 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 2 days. The ability of the strain to grow on a range of sole carbon sources at 1 % (w/v) was determined using carbon utilization media (Pridham & Gottlieb, 1948) (Nihon Pharmaceutical Co., Ltd). NaCl tolerance (0–7 %) and pH (3–11) and temperature (5–45 °C) ranges for growth were determined on 1/5-strength nutrient agar. The isolate was characterized biochemically using API ZYM in accordance with the manufacturer's instructions (bioMérieux).

Whole-cell hydrolysates were analysed for A₂pm isomers using TLC (Becker *et al.*, 1965; Hasegawa *et al.*, 1983). Purified cell walls were obtained 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 this solution was used for amino acid analysis. Amino acid composition was determined by HPLC using the Pico Tag method (Waters). Samples were derivatized with phenylisothiocyanate and UV detection (at 254 nm) was used. The presence of mycolic acids was assessed by using the TLC method of Tomiyasu (1982). Menaquinones were extracted and purified by using the method of Collins *et al.* (1977) and then analysed by HPLC (model 802-SC; Jasco) using a chromatograph equipped with a Capcell Pak C18 column

Fig. 1. Phylogenetic tree constructed on the basis of 16S rRNA gene sequences, using the neighbour-joining method and K_{nuc} values. *Janibacter limosus* DSM 11140^T was used as an outgroup. GenBank accession numbers are given in parentheses. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 0.005 substitutions per nucleotide position.

(Shiseido) (Tamaoka *et al.*, 1983). Methyl esters of cellular fatty acids were prepared and analysed by GLC (model HP6890; Hewlett Packard).

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

DNA for PCR was prepared by sonication (Yu *et al.*, 2002). The 16S rRNA gene was amplified by PCR and sequenced using an automatic sequence analyser (ABI Prism 3130; 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 BLAST. Sequence data for related species were retrieved from GenBank. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura & Ohta, 1972) and phylogenetic trees were constructed by using the neighbour-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.

A nearly complete 16S rRNA gene sequence was determined for strain KV-657^T. A database search demonstrated that the strain belonged to the family *Intrasporangiaceae*. The phylogenetic tree was calculated using 16S rRNA gene sequence data as follows: *I. calvum* DSM 43043^T (1469 bp), *Terracoccus luteus* DSM 44263^T (1479 bp), *Terrabacter terrae* PPLB^T (1475 bp) and *Terrabacter tumescens* DSM 20306^T (1461 bp). In the 16S rRNA gene phylogenetic tree (Fig. 1), the strain was closely associated with *I. calvum* DSM 43043^T. The sequence similarity between KV-657^T and *I. calvum* DSM 43043^T was 97.6 %.

The novel strain was also examined for a set of phenotypic and chemotaxonomic characteristics. The strain formed branching hyphae and also fragmentary hyphae. Cell length varied from 1.0 to 2.2 μ m, with a mean diameter of around 0.5 μ m (see Supplementary Fig. S1 in IJSEM Online). The cells were Gram-positive and catalase-positive. The DNA base composition of KV-657^T was 70 mol% G+C according to the HPLC nucleoside method. The cell-wall peptidoglycan of KV-657^T contained LL-A₂pm, Ala, Gly and Glu and was of type A3 γ (Schleifer & Kandler, 1972). The predominant menaquinone was MK-8(H₄). Mycolic acids were not detected. The cellular fatty acid components were iso-C_{14:0} (7.3 %), iso-C_{15:0} (39.6 %), anteiso-C_{15:0} (5.8 %), C_{15:0} (4.8 %), iso-C_{16:0} (13.3 %), iso-C_{17:0} (2.0 %), anteiso-C_{17:0} (2.0 %), C_{17:0} ω 8c (6.4 %) and C_{17:0} (5.8 %).

In order to confirm that the strain belonged to a novel species, DNA–DNA hybridization relatedness was determined. The level of DNA–DNA relatedness between the isolated strain and *I. calvum* NBRC 12989^T was determined to be 33–34 %, which was well below the 70 % cut-off point for species classification recommended by Wayne *et al.* (1987).

The differential characteristics of strain KV-657^T and other LL-A₂pm-containing members of the family *Intrasporangiaceae* are listed in Table 1. The major cellular fatty acids of these isolates are a complex mixture of straight-chain saturated, monounsaturated, iso- and anteiso-methyl-branched acids. Members of the genus *Arsenicicoccus* have a similar complex of major cellular fatty acids, but differ in cell morphology. On the basis of the 16S rRNA gene sequence-based phylogenetic tree, KV-657^T is thought to

Table 1. Differential characteristics of strain KV-657^T and other LL-A₂pm-containing members of the family *Intrasporangiaceae*

Data for reference genera were taken from Collins *et al.* (2004) (*Arsenicicoccus*), Schumann *et al.* (1997) (*Intrasporangium*), Martin *et al.* (1997) (*Terrabacter*) and Groth *et al.* (2002) (*Terracoccus*).

Characteristic	Strain KV-657 ^T	<i>Arsenicicoccus</i>	<i>Intrasporangium</i>	<i>Terrabacter</i>	<i>Terracoccus</i>
Cell morphology	Hyphae	Cocci	Hyphae	Cocci to rods	Cocci
DNA G + C content (mol%)	70	72	68	70–73	73
Major menaquinone	MK-8(H ₄)	MK-8(H ₄)	MK-8	MK-8(H ₄)	MK-8(H ₄)
Fatty acid types*	SAIU	SAIU	SAI	SAI	SAI

*A, Anteiso-methyl branched; I, iso-methyl branched; S, straight-chain saturated; U, monounsaturated.

be closely related to the genus *Intrasporangium*, but can be differentiated by cell morphology and the major menaquinones (Supplementary Fig. S2). Our findings show that KV-657^T belongs to new genus in the family *Intrasporangiaceae*.

Based on its distinct phylogenetic position within the family *Intrasporangiaceae* and the differential cell morphology, cell-wall murein type and fatty acid composition, the name *Humihabitans oryzae* gen. nov., sp. nov. is proposed.

Description of *Humihabitans* gen. nov.

Humihabitans (Hu.mi.ha'bi.tans. L. masc. n. *humus* soil; L. part. adj. *habitans* inhabiting; N.L. part. adj. used as a masc. n. *Humihabitans* inhabitant of soil).

Gram-positive, catalase-positive, aerobic organisms that form branching hyphae. Fragmentary vegetative mycelium is produced. Peptidoglycan is of the A type of direct cross-linkage and contains LL-A₂pm, glycine, alanine and glutamic acid. Mycolic acids are absent. The major menaquinone is MK-8(H₄). Phylogenetically, the genus is a member of the family *Intrasporangiaceae*. The type species is *Humihabitans oryzae*.

Description of *Humihabitans oryzae* sp. nov.

Humihabitans oryzae (o.ry'zae. L. gen. n. *oryzae* of rice, pertaining to the isolation of the type strain from rice paddy soil).

In addition to the characteristics that define the genus, it has the characteristics described below. Cell length is 1.0–2.2 µm with a mean diameter of around 0.5 µm. Colonies are pale yellow. Growth occurs at pH 5–11 and at 8 and 40 °C. In 1/5-strength nutrient agar medium, NaCl is tolerated up to 3%. D-Fructose, D-glucose, maltose, D-mannitol, D-mannose, sucrose, trehalose and D-xylose are assimilated, but L-arabinose, D-galactose, raffinose and L-rhamnose are not. Esterase (C4), esterase lipase (C8), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase and α-glucosidase are detected by the API ZYM enzyme assay; alkaline phosphatase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. Weak reactions for lipase (C14), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and β-glucosidase are detected in API ZYM tests. The DNA G+C content of the type strain is 70 mol%.

The type strain, KV-657^T (=NRRL B-24470^T =NBRC 101802^T), was isolated from rice paddy soil in Japan.

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