Proposal of *Intrasporangium mesophilum* sp. nov., and reclassification of *Humihabitans oryzae* Kageyama *et al.* 2007 as *Intrasporangium oryzae* comb. nov.

Ling-Ling Yang,^{1†} Tao Ao,^{1†} Xing-Hong Wang,¹ Jie He,¹
Hans-Peter Klenk,² Shu-Kun Tang¹ and Wen-Jun Li¹

Correspondence

Xing-Hong Wang
wangxh@ynu.edu.cn
Wen-Jun Li
wjli@ynu.edu.cn

¹Key Laboratory of Microbial Diversity in Southwest China, Ministry of Education and Laboratory for Conservation and Utilization of Bio-resources, Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, PR China

²DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7b, D-38124 Braunschweig, Germany

A Gram-positive and aerobic bacterium, designated strain YIM 49065^T, was isolated from rhizospheric soil of *Jatropha curcas* in Yunnan, China. This isolate formed branched and fragmented mycelia containing LL-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The major cellular fatty acid profile was characterized by iso-C_{14:0}, iso-C_{15:0} and iso-C_{16:0}, and the predominant menaquinone was MK-8(H₄). The DNA G + C content was 69.6 mol%. A phylogenetic analysis based on 16S rRNA gene sequence demonstrated that strain YIM 49065^T was closely associated with *Intrasporangium calvum* DSM 43043^T and *Humihabitans oryzae* KV-657^T, exhibiting 98.8% and 98.6% 16S rRNA gene sequence similarities, respectively. Furthermore, DNA–DNA hybridizations among strains YIM 49065^T, *I. calvum* DSM 43043^T and *H. oryzae* DSM 22331^T indicated that this isolate represented a novel species in the genus *Intrasporangium*. On the basis of these data, we proposed one novel species, *Intrasporangium mesophilum* sp. nov., for strain YIM 49065^T (=DSM 23217^T = CCTCC AA 209077^T). Additionally, the comparison of biochemical and phylogenetic characters supported the reclassification of *Humihabitans oryzae* as a separate species within the genus *Intrasporangium*, *Intrasporangium oryzae* comb. nov. (type strain KV-657^T = JCM 15924^T = NBRC 101802^T = NRRL B-24470^T).

During a screening programme of bacteria with lipase activity used for biodiesel production, strain YIM 49065^T was isolated from the rhizospheric soil sample of an oil-seed plant, *Jatropha curcas*, collected from Chuxiong, Yunnan province, south-west China. The isolate was obtained after incubation at 28 °C for 14 days and was subsequently maintained on trypticase soy agar (TSA; Difco) at 4 °C, and as a glycerol suspension (20%, v/v) at –20 °C. To determine the taxonomic position of this isolate, a preliminary sequence analysis based on the 16S rRNA gene was performed. Genomic DNA extraction and PCR amplification of the 16S rRNA gene were carried out by using the method described by Li *et al.* (2007). Sequence comparison via BLAST searches (Altschul *et al.*, 1990) against

sequences from the GenBank, EMBL or DDBJ databases revealed that strain YIM 49065^T had a close relationship with members of the family *Intrasporangiaceae*, especially the genera *Intrasporangium* (Kalakoutskii *et al.*, 1967) and *Humihabitans* (Kageyama *et al.*, 2007), both being monospecific. At the time of writing, the family *Intrasporangiaceae* (Stackebrandt *et al.*, 1997; Stackebrandt & Schumann, 2006; Zhi *et al.*, 2009) contains 19 genera with validly published names, including *Intrasporangium* as the type genus. The genus *Humihabitans* was proposed mainly according to differences of menaquinone and fatty acid types from its closest phylogenetic neighbour *Intrasporangium* (Kageyama *et al.*, 2007).

In order to discriminate these micro-organisms and identify strain YIM 49065^T, a polyphasic approach was used. Cultural characteristics and colour of the aerial and substrate mycelia were determined following the method described by Shirling & Gottlieb (1966). Morphological characteristics were observed by using light microscopy (BH2; Olympus) and scanning electron microscopy (JSM5600LV; JEOL) after

[†]These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 49065^T is HQ204221.

Two supplementary tables and five figures are available with the online version of this paper.

14 days' growth on ISP (International *Streptomyces* Project) 2 agar medium (Shirling & Gottlieb, 1966). Growth at various temperatures (4, 10, 20, 28, 32, 37, 40 and 45 °C), pH (5, 6, 7, 8, 9 and 10) and NaCl concentrations (0–10%, w/v, at intervals of 0.5%) were performed using ISP 2 as basal medium. Cells of the strain were Gram-positive as determined according to Gregersen's method (Gregersen, 1978). Strain YIM 49065^T developed well on most test media, including ISP 2, ISP 3, ISP 5 (Shirling & Gottlieb, 1966), potato-glucose agar, nutrient agar and Czapek's agar (Dong & Cai, 2001), but weakly on ISP 4 (Shirling & Gottlieb, 1966). White to pale orange-yellow substrate mycelia and white aerial mycelia were produced. Strain YIM 49065^T formed extensively branched and fragmented mycelia (Fig. S1, available in IJSEM Online). The isolate grew well at 20–37 °C (optimum 28 °C), pH 6–8 (optimum pH 7–8) and in the presence of 3% NaCl (w/v). No diffusible pigments were produced on any media tested here.

Physiological and biochemical characteristics were determined following standard procedures. Catalase activity was determined by assessing bubble production in 3% (v/v) H₂O₂. Degradation of cellulose, gelatin, urea and Tweens 20, 40 and 80 was determined as described by Cowan & Steel (1965). Carbon utilization was tested using ISP 9 (Pridham & Gottlieb, 1948) containing each carbon source at a final concentration of 0.5% (w/v). Other physiological characteristics, such as H₂S and melanin production, and milk coagulation and peptonization, were examined according to the protocols described by Gordon *et al.* (1974). These phenotypic characteristics are listed in the species description and Table 1. Strain YIM 49065^T exhibited many

phenotypic features in common with the type strains of *Intrasporangium calvum* and *Humihabitans oryzae*. They all grew with 3% NaCl and were positive for catalase activity, nitrate reduction and utilization of fructose, sucrose and D-xylose as sole carbon sources, but negative for production of H₂S, hydrolysis of urea and utilization of arabinose and lactose. Some characteristics differentiating strain YIM 49065^T from the type strains of *I. calvum* and *H. oryzae* are given in Table 1.

Biomass for the study of chemotaxonomic characteristics, except cellular fatty acid analysis, was prepared by cultivation in shaken flasks of tryptic soy broth (TSB; Difco) at 170 r.p.m. at 28 °C and then harvested by centrifugation. Isomers of diaminopimelic acid were analysed according to the procedure developed by Hasegawa *et al.* (1983). Whole cell sugars were detected by precolumn derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) by HPLC (Tang *et al.*, 2009). Polar lipids were extracted and identified by two-dimensional TLC (Minnikin *et al.*, 1979; Collins & Jones, 1980). Menaquinones were extracted and purified as described by Minnikin *et al.* (1984) and analysed by HPLC (Kroppenstedt, 1982) with *Pseudonocardia antarctica* DSM 44749^T [predominant menaquinone is MK-8(H₄)] and *Bacillus psychrodurans* DSM 11713^T (predominant menaquinones are MK-8 and MK-7) as reference strains. For fatty acid analysis, strain YIM 49065^T was prepared by scraping cells from TSA that had been incubated for 5 days at 28 °C. *I. calvum* DSM 43043^T and *H. oryzae* DSM 22331^T were also grown under the same conditions until they reached exponential phase. Analysis was carried out by using the Sherlock Microbial Identification System (MIDI) according

Table 1. Characteristics that differentiate strain YIM 49065^T from its nearest phylogenetic neighbours

All data were obtained from this study. +, Positive; –, negative.

Characteristic	YIM 49065 ^T	<i>I. calvum</i> DSM 43043 ^T	<i>H. oryzae</i> DSM 22331 ^T
pH range	6–8	6–8	5–11
Temperature range (°C)	20–37	20–40	8–40
Aerial mycelium	White	None	None
Substrate mycelium	White, pale orange-yellow	Whitish, cream-whitish	Pale yellow
Degradation of gelatin	+	–	–
Utilization as sole carbon source			
Cellobiose	–	+	–
Glucose	+	–	+
Inositol	–	–	+
Mannitol	–	–	+
Raffinose	–	+	–
L-Rhamnose	–	+	–
D-Ribose	+	–	–
Sorbitose	–	–	+
Trehalose	–	–	+
Major fatty acids (%; >10%)	iso-C _{14:0} (11.3), iso-C _{15:0} (43.3), iso-C _{16:0} (13.0)	iso-C _{15:0} (37.6), iso-C _{16:0} (13.1)	iso-C _{14:0} (14.7), iso-C _{15:0} (41.8), iso-C _{16:0} (18.7)
Whole cell-sugars	Galactose, mannose, glucose, arabinose	Galactose, mannose, glucose	Galactose, mannose, glucose, ribose
DNA G + C content (mol%)	69.6	68.8	71.2

to the manufacturer's instructions. Fatty acid methyl esters were then analysed by GC (Agilent Technologies 7890A GC System) by using the Microbial Identification software package (Sherlock Version 6.1; MIDI database TSA6).

The chemotaxonomic data showed some significant similarities among strains YIM 49065^T, *I. calvum* DSM 43043^T and *H. oryzae* DSM 22331^T, even though there was some diversity. The whole cell sugars of strain YIM 49065^T were galactose, mannose, arabinose and glucose. The polar lipids of the three strains comprised diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannoside, with an unknown ninhydrin-positive phosphoglycolipid and two unknown lipids (Fig. S2). Interestingly, the predominant menaquinone was detected as MK-8(H₄) for strains YIM 49065^T, *I. calvum* DSM 43043^T and *H. oryzae* DSM 22331^T (Fig. S3). This result differs from previous reports that the predominant menaquinone of *I. calvum* was MK-8 (Collins *et al.*, 1984; Kalakoutskii, 1989; Kageyama *et al.*, 2007). The main reason why *H. oryzae* was proposed as a new genus in the family *Intrasporangiaceae* was that the predominant menaquinone of *H. oryzae* DSM 22331^T was determined as MK-8(H₄) by Kageyama *et al.* (2007). Given that all other representatives of the family possess totally MK-8(H₄) as the characteristic menaquinone (Stackebrandt & Schumann, 2006; Wang *et al.*, 2009), MK-8(H₄) should be the characteristic menaquinone not only of the other members of the genus *Intrasporangium*, but also of the family *Intrasporangiaceae*. The cellular fatty acid profile for the novel strain consisted predominantly of branched fatty

acids, including (>10% of total fatty acids) iso-C_{14:0} (11.3%), iso-C_{15:0} (43.3%) and iso-C_{16:0} (13.0%). This profile is similar to those of the closely related species *I. calvum* and *H. oryzae*, but there were differences in the proportions of each fatty acid (Table 1). The fatty acid profiles of strains YIM 49065^T, *I. calvum* DSM 43043^T and *H. oryzae* DSM 22331^T are given in Table S1.

Multiple alignments and sequence evolutionary distance calculations were carried out by using CLUSTAL X software (Thompson *et al.*, 1997), and the positions with gaps were excluded before final analysis. Phylogenetic analyses were performed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods contained in MEGA version 4 (Tamura *et al.*, 2007). Maximum-likelihood analysis was performed by using PhyML (Guindon & Gascuel, 2003). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. The 16S rRNA gene sequence of strain YIM 49065^T shared the highest pairwise similarity with *I. calvum* DSM 43043^T (98.8%) and *H. oryzae* KV-657^T (98.6%), while *I. calvum* and *H. oryzae* exhibited 98.7% similarity to each other. This result was confirmed by using MEGA version 4 with pairwise deletion for gaps; however, the similarity value between *I. calvum* and *H. oryzae* was reported as 97.6% by Kageyama *et al.* (2007), determined with gaps. The phylogenetic tree reconstructed by using the neighbour-joining method suggested that strain YIM 49065^T formed a stable clade with *I. calvum* and *H. oryzae*, being well separated from the other genera of family *Intrasporangiaceae* (Fig. 1). This relationship was supported by maximum-parsimony

Fig. 1. Neighbour-joining tree derived from aligned 16S rRNA gene sequences, showing the position of YIM 49065^T among its phylogenetically closest neighbours. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Bar, 0.005 substitutions per nucleotide position.

and maximum-likelihood tree-making algorithms with high bootstrap value (Figs S4 and S5).

The G + C content of genomic DNA was determined using the HPLC method as described by Mesbah *et al.* (1989), with DNA prepared according to the method of Marmur (1961). DNA–DNA hybridization was carried out by using photobiotin-labelled probes in microplate wells with five replications as described by Ezaki *et al.* (1989) and He *et al.* (2005). Fluorescence was measured by using a microplate spectrofluorometer (Gemini XPS; Molecular Devices). Reciprocal reactions were performed for every DNA pair. The DNA G + C content of strain YIM 49065^T was 69.6 mol%. The levels of DNA–DNA relatedness between strain YIM 49065^T and its nearest neighbours, *I. calvum* DSM 43043^T and *H. oryzae* DSM 22331^T, were 43.9 % and 52.1 %, respectively (standard deviations were 3.2 % and 5.4 %). *I. calvum* and *H. oryzae* shared a value of 40.6 % (standard deviation 7.1 %). These are lower than the 70 % cut-off point recommended for delineation of a novel species (Stackebrandt & Goebel, 1994), suggesting that strains YIM 49065^T, *I. calvum* DSM 43043^T and *H. oryzae* DSM 22331^T represent different species.

On the basis of phenotypic, genotypic and phylogenetic data presented here, strain YIM 49065^T differs from its closest relatives, *I. calvum* DSM 43043^T and *H. oryzae* DSM 22331^T. It is suggested that strain YIM 49065^T should be recognized as a novel species of the genus *Intrasporangium*, for which we propose the name *Intrasporangium mesophilum* sp. nov. Furthermore, based on chemotaxonomic characteristics and phylogenetic analysis, we propose that *H. oryzae* should be reclassified as *Intrasporangium oryzae* comb. nov. The description of the genus *Intrasporangium* is emended, and differential chemotaxonomic characteristics among the genera of the family *Intrasporangiaceae* are listed in Table S2.

Emended description of the genus *Intrasporangium* Kalakoutskii *et al.* 1967

This emended description is based on that given by Stackebrandt & Schumann (2006), with the following changes and additions. The predominant menaquinone is MK-8(H₄). Major fatty acids (>10 %) are iso-C_{15:0} and iso-C_{16:0}. The polar lipids comprise diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannoside, with an unknown ninhydrin-positive phosphoglycolipid and two unknown lipids. The type species is *Intrasporangium calvum*.

Emended description of *Intrasporangium calvum* Kalakoutskii *et al.* 1967

The description is based on that by Kalakoutskii *et al.* (1967) and Stackebrandt & Schumann (2006) with the following features. Growth is observed in the range pH 6–8 and 20–40 °C. NaCl tolerance range is 0–3 % (w/v). Tests for catalase activity and nitrate reduction are positive; tests for H₂S production, gelatin liquefaction and urea hydrolysis are

negative. Cellobiose, fructose, raffinose, L-rhamnose, sucrose and D-xylose are utilized as sole carbon source, but arabinose, glucose, inositol, lactose, mannitol, D-ribose, sorbose and trehalose are not utilized. The dominant menaquinone, major cellular fatty acids and polar lipid profile are as described in the description for the genus *Intrasporangium*.

The type strain is ATCC 23552^T (=DSM 43043^T = NBRC 12989^T = JCM 3097^T = NRRL B-3866^T = VKM Ac-701^T).

Description of *Intrasporangium oryzae* comb. nov.

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

Basonym: *Humihabitans oryzae* Kageyama *et al.* 2007.

The description is identical to that given by Kageyama *et al.* (2007), supplemented with recent data. Nitrate is reduced, but gelatin is not hydrolysed and H₂S is not produced. Can use fructose, glucose, inositol, mannitol, sorbose, sucrose, trehalose and D-xylose as sole carbon sources, but not arabinose, cellobiose, lactose, raffinose, L-rhamnose or D-ribose. The polar lipids remain as given in the genus description.

The type strain is KV-657^T (=JCM 15924^T =NBRC 101802^T =NRRL B-24470^T), isolated from rice paddy soil in Japan.

Description of *Intrasporangium mesophilum* sp. nov.

Intrasporangium mesophilum [me.so.'phi.lum. Gr. adj. *mesos* middle; Gr. adj. *philos* loving; N.L. neut. adj. *mesophilum* middle (temperature)-loving, mesophilic].

Aerobic, Gram-positive, non-motile actinomycete that forms extensively branched and fragmenting mycelia. Grows well on ISP 2, ISP 3, ISP 5, potato-glucose agar, nutrient agar and Czapek's agar but poorly on ISP 4. No diffusible pigment is produced. Temperature range for growth is 20–37 °C, with optimum growth at 28 °C. Growth occurs at pH 6–8 and in the presence of 0–3 % NaCl. Gelatin is degraded, but casein, cellulose, urea and Tweens 20, 40 and 80 are not. Positive for catalase, reduction of nitrate, and milk coagulation and peptonization, but negative for H₂S and melanin production. Fructose, glucose, D-ribose, sucrose and D-xylose are utilized as sole carbon sources, but arabinose, cellobiose, inositol, lactose, mannitol, raffinose, L-rhamnose, sorbose and trehalose are not. The cell wall contains LL-diaminopimelic acid as the diagnostic diamino acid. Whole-cell hydrolysates contain galactose, mannose, arabinose and glucose. The predominant menaquinone is MK-8(H₄). The major fatty acids (>10 %) are iso-C_{14:0}, iso-C_{15:0} and iso-C_{16:0}. The polar lipids comprise diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside, an unknown ninhydrin-positive phosphoglycolipid and two unknown lipids. The DNA G + C content of the type strain is 69.6 mol%.

The type strain, YIM 49065^T (=DSM 23217^T =CCTCC AA 209077^T), was isolated from the rhizospheric soil of an oil-seed plant, *Jatropha curcas*, collected in Chuxiong, Yunnan province, China.

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