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ORIGINAL PAPER

Meiothermus roseus sp. nov., a thermophilic bacterium isolated from a geothermal area

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Abstract Two closely related thermophilic bacterial strains, designated YIM 71031^T and YIM 71039, were isolated from a hot spring in Tengchong county, Yunnan province, south-western China. The novel isolates were observed to be Gram-negative, aerobic, rod-shaped and yellow-pigmented bacteria. The strains were found to be able to grow at 37–65 °C, pH 6.0–9.0 and with a NaCl tolerance up to 1.0 % (w/v). Phylogenetic analysis based on 16S rRNA gene sequences placed these two isolates in the genus *Meiothermus*. They were found to be closely related to *Meiothermus timidus* DSM 17022^T (98.6 % similarity), and formed a cluster with this species. The predominant menaquinone was identified as MK-8 and the major fatty acids (>10 %) as anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$ and $C_{16:0}$. The genomic DNA G+C contents of strains YIM 71031^T and YIM 71039 were determined to be 64.0 and 65.4 mol%, respectively. DNA–DNA hybridizations showed low values between strains YIM 71031^T and YIM 71039 and their closely related neighbour *M. timidus* DSM 17022^T. Morphological phylogenetic and chemotaxonomic results suggest that strains YIM 71031^T and YIM 71039 are representatives of a new species within the genus *Meiothermus*, for which the name *Meiothermus roseus* sp. nov. is proposed. The type strain is YIM 71031^T (=KCTC 42495^T =NBRC 110900^T).

Hong Ming and Yan-Yan Duan contributed equally to this study.

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Introduction

Members of the genus *Meiothermus* originally belonged to the genus *Thermus* and were referred to as the "low temperature" group based on their optimum growth temperature (Loginova et al. 1984; Tenreiro et al. 1995). Later, Nobre et al. (1996a) found that these members formed a separate lineage from the species of the genus *Thermus* based on 16S rRNA gene sequences. Therefore they proposed a new genus *Meiothermus*, distinguished from the genus *Thermus* by their low optimum growth temperature, red or yellow-pigmented colonies and the presence of moderate levels of 2-hydroxy fatty acids. Subsequently, eight species of the genus *Meiothermus* were isolated from geyser or hot springs world-wide, including *Meiothermus cerbereus* (Chung et al. 1997), *Meiothermus taiwanensis* (Chen et al. 2002), *Meiothermus timidus* (Pires et al. 2005), *Meiothermus rufus* and *Meiothermus granaticius* (Albuquerque et al. 2009, 2010), *Meiothermus cateniformans* (Zhang et al. 2010), *Meiothermus hypogaeus* (Mori et al. 2012) and *Meiothermus terrae* (Yu et al. 2014). The species of the genus *Meiothermus* have low growth temperature ranges from 35 to 68 °C whereas other members of the family *Thermaceae* grow at 40–83 °C. All of the species in this genus share chemotaxonomic characteristics, such as having MK-8 as the predominant menaquinone, iso- and anteiso-branched fatty acids as major fatty acids and an uncharacterised phospholipid and glycolipids as major polar lipids (da Costa et al. 2006).

During a study on thermophilic bacteria from hot springs, strains YIM 71031^T and YIM 71039 were isolated from a hot spring in Tengchong county, Yunnan province, south-western China. A preliminary phylogenetic analysis based on 16S rRNA gene sequences indicated that strains YIM 71031^T and YIM 71039 might be candidates for inclusion in a novel species belonging to the genus *Meiothermus*. The aim of the present study was to determine the taxonomic status of these two isolates, by using a polyphasic approach. The two strains were characterised by phenotypic, chemotaxonomic and phylogenetic analysis. The results of phenotypic, chemotaxonomic and phylogenetic analyses indicated that strains YIM 71031^T and YIM 71039 represent a novel species in the genus *Meiothermus*, here named *Meiothermus roseus* sp. nov.

Materials and methods

Strains and culture conditions

Geothermally heated sediment with a few water mixture samples were collected from a hot spring. The sediment sample was sandy, a black-green colour with bacterial mats, and the temperature and pH was 67 °C and 7.0, respectively. For the isolation of thermophilic bacteria, 2 g of sediment samples collected from the hot spring were taken into flasks with 18 ml sterile hot spring water and several glass beads. After mixing, samples were shaken at 50 °C with 200 rpm min^{-1} for 1 h, 1 ml of the mixtures were then diluted 10 and 100 fold with sterile water and 0.2 ml of the diluted samples were spread on R2A medium (BD; Becton, Dickinson and Company), *Thermus* medium (Williams and da Costa 1992) and modified T5 medium (tryptone 0.5 g, yeast extract 2 g, glucose 1 g, lotus root starch 1 g, $CaSO_4$, 0.04 g, 0.01 M Fe citrate, 0.5 ml, KH_2PO_4 0.01 g, K_2HPO_4 0.01 g, $NaHCO_3$ 0.1 g, NaCl 0.2 g, $CaCl_2$ 0.02 g, $MgSO_4$ 0.02 g, water 1000 ml, agar 20 g, pH 7.2). These isolation plates were incubated at 45, 50 and 60 °C for 7 days. Strains YIM 71031^T and YIM 71039 were selected on modified T5 medium at 50 °C. These two strains were purified and cultured on modified T5 medium adjusted to pH 7.2 at 50 °C. The pure cultures were preserved as glycerol suspensions (20 %, v/v) at −80 °C. The type strain *M. timidus* DSM 17022^T was obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures) and used as a reference strain in the present study cultured under comparable conditions.

Phenotypic characteristics

For observation of phenotypic characteristics, strains YIM 71031^T and YIM 71039 were cultured on *Thermus*, R2A and modified T5 media adjusted to pH 7.2 at 50 °C unless mentioned. Growth and microscopic morphological characterisation were performed by light microscopy (Olympus BH2, Olympus) and scanning electron microscopy (XL30 ESEM-

TMP, Philips-FEI). The procedure for SEM sample preparation was described previously by Ming et al. (2014). Gram-staining was carried out using 3 % KOH by the non-staining method (Buck 1982). Cell motility was studied depending on turbidity development throughout a tube of semi-solid medium (Leifson 1960).

Growth at different temperatures was tested at over a range of 4, 15, 28, 30, 37, 40, 45, 50, 55, 60, 65, 70 and 75 °C described by Chung et al. (2000) and Bjornsdottir et al. (2009). The tolerance of sodium levels for growth was observed in the presence of 0–5 % (w/v) NaCl concentration (0, 0.5, 1.0, 1.5 2, 2.5, 3.0, 4.0 and 5.0 % w/v, respectively). The pH for growth was tested from 4.0 to 10.5 (at intervals of 0.5 pH units) at 50 °C for 7 days in *Thermus* medium broth, by using the buffer system as described by Ming et al. (2014). The ability to utilise single carbon sources was tested in a minimal medium composed of *Thermus* basal salts with yeast extract (0.1 g per liter) supplemented with 2 g/l filter sterilized (0.22 μm, Millipore) test carbon source. Nitrogen source utilisation was observed in the minimal *Thermus* medium described above supplemented with 2 g/l filter sterilised (0.22 μm, Millipore) test compounds. Determination of oxidase activity was carried out by using 1 % (w/v) tetramethyl *p*-phenylenediamine as described by Kovacs (1956). Catalase activity was tested using 3 % (w/v) H_2O_2 by assessing bubble production as the positive result. Tween (20, 40, 60 and 80) degradation, H_2S production and nitrate reduction, hydrolysis of gelatin, starch, xylan, aesculin, pectin and cellulose, activity of urea, milk peptonization and coagulation were observed as described by Smibert and Krieg (1994), Gonzalez et al. (1978) and MacFaddin (1980). Antibiotic susceptibility was observed by the disc (Himedia) diffusion plate method (Groth et al. 2004). API galleries (API 20NE and API ZYM) were used to determined metabolic properties and some enzyme activities according to the instructions of the manufacturer (bioMérieux, France). The Biolog Gen III microplate™ system was used for carbon source utilisation and chemical sensitivity tests according to the manufacturer's instructions.

Chemotaxonomy

Biomass for chemical and molecular studies of strains YIM 71031^T, YIM 71039 and *M. timidus* DSM 17022^T were obtained by cultivation on *Thermus* medium adjusted to pH 7.2 at 50 °C for 3 days. Cells were checked for purity by spreading on *Thermus* agar plates, harvested and washed twice with distilled water by centrifugation and then freeze-dried.

Quinones were extracted from lyophilized cells as described by Collins et al. (1977) and Minnikin et al. (1984) and then purified and analysed by HPLC (Tamaoka et al. 1983; Hu et al. 2001). Polar lipids were extracted and examined by two-dimensional thin-layer chromatography (TLC) method on silica gel G 60 plates (Merck; Germany). The profile of the lipids was identified using previously described procedures (Collins and Jones 1980; Minnikin et al. 1979, 1984). Cells for cellular fatty acids analysis were obtained by culturing strains YIM 71031^T, YIM 71039 and *M. timidus* DSM 17022^T in *Thermus* and modified T5 media for 3 days. The cellular fatty acids were extracted, methylated and analysed following the instructions of version 6.1 of the Sherlock Microbial Identification System (MIDI) and the TSBA6 databse (Sasser 1990).

The genomic DNA G+C contents of the novel strains were determined by HPLC after enzymatic degradation (Mesbah et al. 1989), with *Escherichia coli* DH5α as the reference strain.

Molecular analysis

Genomic DNA of strains YIM 71031^T and YIM 71039 was extracted, and the 16S rRNA genes were amplified and sequenced by using the universal bacterial primers, as described by Li et al. (2007). The amplicons were purified by using a PCR purification kit (Sangon Biotech, China). The obtained almost complete 16S rRNA gene sequences of the novel strains were compiled using the SeqMan program (DNAStar software). To determine the phylogenetic relationships of strains YIM 71031^T and YIM 71039, the 16S rRNA gene sequences of species in genus *Meiothermus* were retrieved from a BLAST search (Altschul et al. 1990) and the EzTaxon-e server database (Kim et al. 2012). Multiple sequence alignment was performed using the CLUSTAL_X software package (Thompson et al. 1997). Evolutionary distances were calculated by use of the Kimura two-parameter model (Kimura 1980, 1983). Phylogenetic trees were constructed with the neighbour-joining (Saitou and Nei 1987), maximum-parsimony (Fitch

1971) and maximum-likelihood (Felsenstein 1981) tree-making algorithms. Phylogenetic and molecular evolutionary analyses were carried out by using the software packages MEGA version 6.0 (Tamura et al. 2013) and PHYML (Guindon and Gascuel 2003). The topology of each tree with 1000 replications was evaluated by bootstrap analysis (Felsenstein 1985). The 16S rRNA gene from strain *Thermus scotoductus* SE-1^T (AF032127) was used as outgroup. DNA–DNA hybridizations between strains YIM 71031^T, YIM 71039 and *M. timidus* DSM 17022^T were performed at the optimal hybridization temperature (46 °C) using the fluorometric micro-well method (Ezaki et al. 1989; Christensen et al. 2000). The DNA probes labeled with photobiotin (A1935; Sigma) and 96-well microdilution plates (Greiner BioOne) were used; each sample was set with eight replications.

Results and discussion

Phenotypic characteristics

Strains YIM 71031^T and YIM 71039 were both found to grow well on *Thermus*, R2A and modified T5 media. Colonies of the two strains were observed to be regular circular, opaque, convex and yellow-pigmented on all tested media, and rose-coloured diffusible pigment was produced on R2A medium. Cells of strains YIM 71031^T and YIM 71039 were observed to be Gram-negative, aerobic and non-motile. Cells were found to be rod shaped with 0.3–0.5 μm diameter and 2.0–4.0 μm length, and no spores were formed (Fig. S1). Strains YIM 71031^T and YIM 71039 were found to grow at 37–65 °C and pH 6.0–8.0, with optimal growth at 50 °C and pH 7.0–7.5, and at low NaCl concentrations (up to 1.0 % w/v). The strains were found to be positive for catalase and urease activities, milk coagulation and peptonization. Oxidase activity was found to be negative. The strains can hydrolyse gelatin, pectin and aesculin. The degradation of xylan, cellulose, starch, Tweens 20, 40, 60 and 80 was found to be negative. Nitrate was found to be reduced and H_2S not to be produced. The strains were observed to be resistant to amikacin (30 μg/disc), cefurosime sodium (30 μg), ciprofloxacin (5 μg), chloroamphenicol (30 μg), erythromycin (5 μg), gentamicin (10 μg), norfloxacin (10 μg), novobiocin (5 μg), penicillin (10 IU), piperacillin (100 μg), tetracycline (30 μg) and vancomycin (30 μg) but sensitive to ethylhydrocupreine (5 μg), oxacillin (1 μg), polymyxin B (300 IU) and sulfamethoxazole/trimethoprim (23.75/1.25 μg). Phenotypic properties useful for distinguishing strains YIM 71031^T and YIM 71039 from related members of the genus *Meiothermus* are shown in Table 1. The detailed physiological characteristics of strains YIM 71031^T and YIM 71039 are given in the species description below.

Chemotaxonomic characteristics

The predominant menaquinone of both novel isolates was identified as MK-8. The cellular fatty acids of strain YIM 71031^T (>5 %) were identified as anteiso-$C_{15:0}$ (21.6 %), iso-$C_{15:0}$ (15.8 %), anteiso-$C_{17:0}$ (13.5 %), iso-$C_{16:0}$ (13.1 %), $C_{16:0}$ (10.6 %) and iso-$C_{17:0}$ (7.8 %). The cellular fatty acids of strain YIM 71039 were identified as anteiso-$C_{15:0}$ (12.2 %), iso-$C_{15:0}$ (15.4 %), anteiso-$C_{17:0}$ (12.6 %), iso-$C_{16:0}$ (10.9 %), $C_{16:0}$ (11.7 %) and iso-$C_{17:0}$ (13.6 %). Similar to the genus *Thermus*, iso- and anteiso-branched $C_{15:0}$ and $C_{17:0}$ fatty acids are the predominant fatty acids of most strains of the genus *Meiothermus* (Nobre et al. 1996b; da Costa et al. 2006). In the present study, strains YIM 71031^T and YIM 71039 were found to not only contain anteiso-$C_{15:0}$ and iso-$C_{15:0}$ as the major fatty acids but also possessed iso-$C_{16:0}$ and $C_{16:0}$ reached levels >10 % of the total fatty acids. Among the present validly named species of the genus *Meiothermus*, only *M. hypogaeus* AZM34c11^T had the similar results. Moreover, both strains YIM 71031^T and YIM 71039 were found to contain <1 % hydroxy fatty acids, similar to *M. rufus* AZM34c11^T, *M. granaticius* AF-68^T and *M. hypogaeus* AZM34c11^T. Modified T5 medium is widely used in the isolation and cultivation of thermophiles from extreme thermal ecological niches such as geothermal areas or hot springs, and many thermophilic bacteria and actinobacteria can grow well on this medium when they cannot be cultured on *Thermus* medium. Strains YIM 71031^T and YIM 71039 exhibited similar fatty acid profiles regardless of which medium was used. Detailed fatty acid profiles of strains YIM 71031^T and YIM 71039 are given in Table S1. The polar lipids profiles of the two strains were found to be comprised of one major unidentified phospholipid and two glycolipids (Fig. S2).

The genomic DNA G+C content of strains YIM 71031^T and YIM 71039 were 64.0 and 65.4 mol%,

Table 1 Differential physiological characteristics among strains YIM 71031^T, YIM 71039 and their close neighbour in the genus *Meiothermus*

	YIM 71031^T	YIM 71039	*M. timidus* DSM 17022^T
Optimum growth temperature (°C)	50	50	55–60
H_2S production	−	−	+
Milk peptonization	+	+	−
Starch	−	−	+
Tween 20	−	−	+
Assimilation of			
N-acetyl-glucosamine	−	+	−
Activities of			
Arginine dihydrolase	+	+	−
α-Chymotrypsin	+	+	−
N-acetyl-β-glucosaminidase	−	+	−
Utilization of			
Formic acid	−	+	−
α-Hydroxy-butyric acid	+	−	+
D-Raffinose	+	−	+
L-Rhamnose	+	+	−
D-Saccharic acid	−	−	+
D-Sorbitol	+	−	+
Sucrose	−	−	+
D-Trehalose	−	−	+
L-Alanine	−	−	+
L-Arginine	+	−	+
L-Cystine	−	−	+
Glycine	−	−	+
L-Lysine	−	−	+
L-Methionine	−	−	+
L-Phenylalanine	−	−	+
L-Pyroglutamic acid	−	−	+
L-Serine	−	−	+
L-Tyrosine	−	−	+
DNA G+C content (mol%)	64.0	65.4	65.1[a]

Data were obtained during this study under identical growth conditions

[a] Date from Pires et al. (2005)

respectively, which is consistent with the DNA G+C contents of members of the genus *Meiothermus* (60.8–69.6 mol%).

Molecular analysis

The almost-complete 16S rRNA gene sequences of strains YIM 71031^T (1530 nt, GenBank accession number KP232921) and YIM 71039 (1533 nt, GenBank accession number KP232922) were determined. Comparison of the sequences with the corresponding 16S rRNA gene sequences retrieved from the GenBank/EMBL/DDBJ databases clearly demonstrated that strains YIM 71031^T and YIM 71039 belong to the genus *Meiothermus*. Phylogenetic analyses (Fig. 1) showed that strains YIM 71031^T and YIM 71039 form a monophyletic lineage (99.0 % similarity). The novel strains were found to be closely related to *M. timidus* DSM 17022^T (98.6 % similarity) and formed a well separated branch with this type strain. This branch was also supported in the maximum-parsimony phylogenetic tree and maximum-likelihood phylogenetic tree (Figs. S3–S4). Strains YIM 71031^T and YIM 71039 displayed low sequence similarity (<94.2 %) to all other currently recognised members of the genus *Meiothermus*.

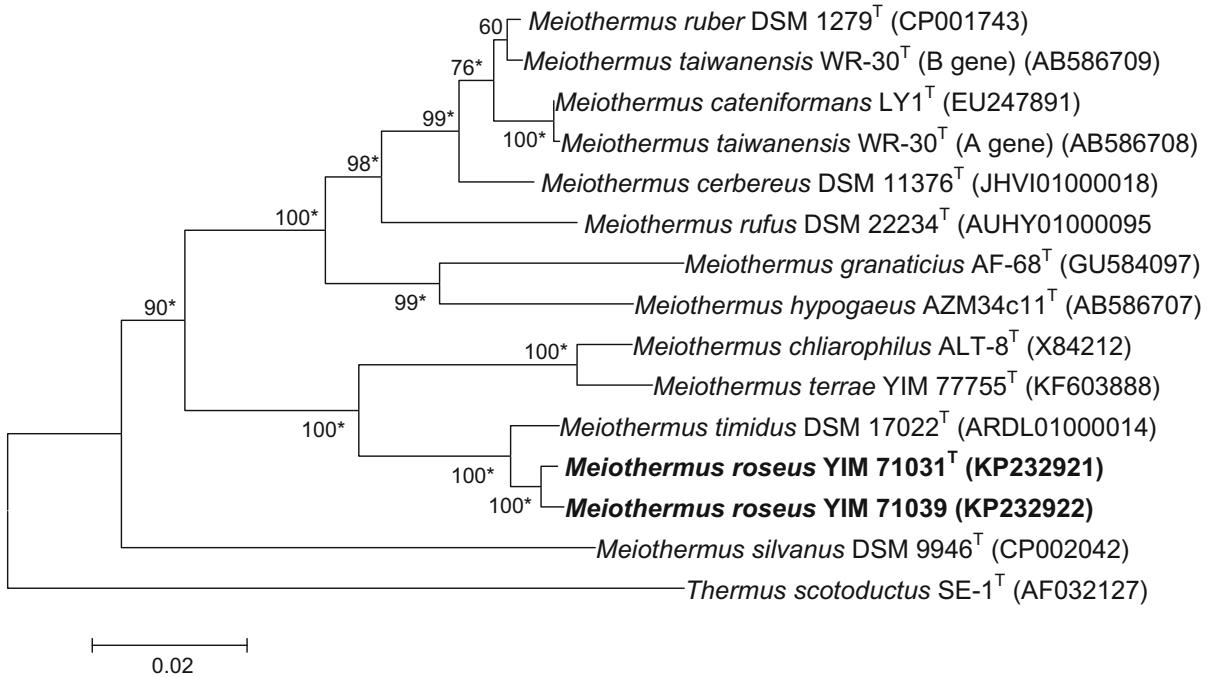

Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain YIM 71031^T and YIM 71039 among members of the genus *Meiothermus*. Numbers at branch points refer to bootstrap percentages. Only bootstrap values above 50 % based on 1000 resampling are shown at branch points. *Asterisks* indicate branches that were also recovered using maximum-parsimony and maximum-likelihood tree. *Bar* 0.02 substitutions per nucleotide position. The 16S rRNA gene from strain *Thermus scotoductus* SE-1^T (AF032127) was used as outgroup

The DNA–DNA relatedness between strains YIM 71031^T and YIM 71039 was 89.9 ± 2.3 %. The high DNA–DNA hybridization value supports the conclusion that these two novel strains belong to the same species, which is consistent with the phylogenetic analysis and their physiological and biochemical characteristics. The levels of DNA–DNA relatedness between strain YIM 71031^T and strain YIM 71039 and *M. timidus* DSM 17022^T were 53.9 ± 2.5 and 50.7 ± 3.1 %, respectively, values which are significantly lower than the threshold value of 70 % for prokaryotic species delineation (Wayne et al. 1987). Therefore, these data suggest that strains YIM 71031^T and YIM 71039 represent a separate species of the genus *Meiothermus*.

In conclusion, chemotaxonomic characteristics (major fatty acids, polar lipids and predominant menaquinones) and phylogenetic trees demonstrated that strains YIM 71031^T and YIM 71039 have many properties in common with species in the genus *Meiothermus*. However, the strains YIM 71031^T and YIM 71039 possess a few physiological and biochemical properties and low DNA–DNA relatedness values that distinguish them from other described *Meiothermus* species (see Table 1 and the species description). Therefore, based on the phenotypic, phylogenetic and chemotaxonomic results, we conclude that strains YIM 71031^T and YIM 71039 represent a novel species of the genus *Meiothermus*, for which the name *Meiothermus roseus* sp. nov. is proposed.

Description of *Meiothermus roseus* sp. nov.

Meiothermus roseus (ro'se.us. L. masc. adj. *roseus*, rose coloured, rosy, as a rose-coloured diffusible pigment is produced on R2A medium).

Cells are Gram-negative, aerobic, non-motile. The rod shaped cells are 0.3–0.5 μm in diameter and 2.0–4.0 μm in length, and no spores are formed. Good growth occurs on *Thermus*, R2A and modified T5 media. Colonies are regular circular, opaque, convex and yellow-pigmented on all tested media. A rose-coloured diffusible pigment is produced on R2A

medium. Grows at 37–65 °C (optimal 50 °C). The pH range for growth is 6.0–8.0 (optimal pH 7.0–7.5) and the NaCl concentration range for growth is of 0–1 % (w/v). Activities of catalase and urease are positive but oxidase is negative. Positive for milk coagulation and peptonization, hydrolysis of gelatin, aesculin and pectin, negative for degradation of Tweens 20, 40, 60 and 80, cellulose, xylan and starch. Nitrate is reduced and H_2S is not produced. Can utilise acetoacetic acid, D-cellobiose, dextrin, D-fructose, D-fructose-6-PO_4, D-fucose, L-fucose, D-galactose, D-galacturonic acid, L-galactonic acid lactone, gentiobiose, α-D-glucose, D-glucuronic acid, α-hydroxybutyric acid, α-keto-butyric acid, α-keto-glutaric acid, α-D-lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, 3-methyl glucose, methyl pyruvate, D-raffinose, L-rhamnose, D-sorbitol, stachyose and D-turanose but not acetic acid, γ-amino-butryric acid, D-arabitol, bromo-succinic acid, citric acid, formic acid, D-gluconic acid, D-glucose-6-PO_4, glycerol, *p*-hydroxy-phenylacetic acid, β-hydroxy-D,L-butyric acid, inosine, L-lactic acid, D-lactic acid methyl ester, L-malic acid, D-malic acid, β-methyl-D-glucoside, mucic acid, myo-inositol, propionic acid, quinic acid, D-saccharic acid, D-salicin, sucrose or D-trehalose as the sole carbon source. Able to use L-alanine, L-arginine, glucuronamide, L-glutamic acid, glycyl-L-proline and L-histidine as nitrogen source but not D-aspartic acid, L-aspartic acid, L-cystine, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, glycine, L-isoleucine, L-lysine, *N*-acetyl-β-D-mannosamine, L-methionine, *N*-acetyl neuraminic acid, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, L-tryptophan, L-tyrosine or L-valine. Activities of acid phosphatase, alkaline phosphatase, arginine dihydrolase, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), α-galactosidase, β-galactosidase and β-glucosidase, α-glucosidase, β-glucuronidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase are positive but activities of β-fucosidase, *N*-acetyl-β-glucosaminidase, lipase (C14) or α-mannosidase are negative. Positive results for assimilation of D-glucose, L-arabinose, D-mannitol, maltose and D-mannose but negative results for indole production, glucose acidification, assimilation of adipate, caprate, *N*-acetyl-glucosamine, phenylacetate, potassium gluconate or trisodium citrate. The predominant menaquinone is MK-8. The major cellular fatty acids consist of anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$ and $C_{16:0}$. The polar lipid profile contains a major unidentified phospholipid and two glycolipids. The genomic DNA G+C content of the type strain is 64.0 mol%.

The type strain is YIM 71031^T (=KCTC 42495^T =NBRC 110900^T), which was isolated from a hot spring in Tengchong county, Yunnan province, southwestern China. The GenBank accession numbers for the 16S rRNA gene sequences of strains YIM 71031^T and YIM 71039 are KP232921 and KP232922, respectively.

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