Thermobifida halotolerans sp. nov., isolated from a salt mine sample, and emended description of the genus *Thermobifida*

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The actinomycete strain YIM 90462^T was isolated from a salt mine sample collected from Yunnan province, south-west China. Comparative 16S rRNA gene sequence analysis demonstrated that the isolate, which had sequence similarities of more than 97 % to *Thermobifida alba*, *Thermobifida fusca* and *Thermobifida cellulosilytica*, was a candidate member of the genus *Thermobifida*. Subsequently, a polyphasic approach was used to clarify the taxonomic position of strain YIM 90462^T. Its diagnostic diamino acid was meso-diaminopimelic acid and whole-cell sugars contained galactose, xylose and glucose. The predominant menaquinones were MK-10(H₈) and MK-10(H₉). The major fatty acids were iso-C_{16:0} and anteiso-C_{17:0}. Results of DNA–DNA hybridizations and physiological and biochemical tests enabled genotypic and phenotypic differentiation of strain YIM 90462^T from the three *Thermobifida* species with validly published names. Consequently, it is proposed that strain YIM 90462^T should be recognized as a representative of a novel species of *Thermobifida*, *Thermobifida halotolerans* sp. nov. The type strain is YIM 90462^T (=KCTC 19123^T =DSM 44931^T). In addition, an emended description of the genus *Thermobifida* is presented.

The establishment of the genus *Thermobifida* (Zhang *et al.*, 1998) was based on the reclassification of *Thermobifida alba* and *Thermobifida fusca*, which were formerly classified as *Thermomonospora* species (Cross & Goodfellow, 1973; McCarthy & Cross, 1984). Later, *Thermobifida cellulosilytica* was described by Kukolya *et al.* (2002). All three *Thermobifida* species were isolated from manure heaps, composts or damp stored hay and present outstanding cellulose decomposition abilities. *T. fusca* YX is used as a model organism for studies of aerobic, thermophilic,

cellulolytic bacteria and a complete genome is also available for this strain (Wilson, 2004; Lykidis *et al.*, 2007).

Strain YIM 90462^T was isolated from a salt mine sample, collected from Yunnan province, south-west China, after 3 weeks incubation at 45 °C on modified ISP medium 4 containing 10 % NaCl (w/v). In view of its thermotolerant and halotolerant capabilities, this strain was studied further and its phylogenetic position was elucidated. Extraction of genomic DNA and amplification of the 16S rRNA gene were done as described by Li *et al.* (2007). The nearly complete 16S rRNA gene sequence of strain YIM 90462^T was determined and BLAST (Altschul *et al.*, 1997) searches revealed that it possessed significant sequence similarities to those of *Thermobifida* species. Sequence similarities were determined by using CLUSTAL_X (Thompson *et al.*, 1997). Strain YIM 90462^T showed high sequence similarities to *T. alba* DSM 43795^T (98.2 %), *T. cellulosilytica* DSM 44535^T (97.8 %) and *T. fusca* DSM 43792^T (97.0 %). Given the

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Abbreviations: DAP, diaminopimelic acid; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PME, phosphatidylmethylethanolamine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 90462^T is EU250489.

Table 1. Differential phenotypic properties of strain YIM 90462^T and the type strains of *Thermobifida* species

Strains: 1, YIM 90462^T; 2, *T. alba* DSM 43795^T; 3, *T. fusca* DSM 43792^T; 4, *T. cellulosilytica* DSM 44535^T. All tested strains grow well in the absence of NaCl and at 45 °C and pH 7–8. All strains contain the phospholipids DPG, PME, PC, PI, PG and PL. All are positive for degradation of Tweens 20 and 80 and hydrolysis of starch and cellulose; melanin and H₂S production, gelatin liquefaction and urease production are negative. Cellobiose, D-galactose, D-glucose, raffinose, sucrose and D-xylose are utilized as sole carbon sources for growth; *myo*-inositol and sorbitol are not utilized.

Characteristic	1	2	3	4
Aerial mycelium on ISP2	—	—	—	+
Temperature range for growth (°C)	20–50	20–50	28–55	28–55
pH range for growth	6–9	6–9	6–9	6–10
Maximum NaCl concentration (% w/v)	10	3	5	3
Milk coagulation	—	+	+	+
Milk peptonization	—	+	+	+
Nitrate reduction	+	—	—	—
Carbon source utilization				
D-Arabinose	+	+	—	—
D-Fructose	—	—	+	+
Glycerol	+	+	+	—
Lactose	—	—	+	+
Maltose	—	—	+	+
D-Mannose	—	—	+	+
L-Rhamnose	—	+	—	+
D-Ribose	—	—	—	+
L-Sorbose	—	+	—	—
Trehalose	+	—	+	+
Xylitol	—	+	—	—
Presence of PE	+	—	+	+
Menaquinones (>5 %)	10(H ₄) (11.1 %), 10(H ₆) (30.0 %), 10(H ₈) (41.1 %)	10(H ₆) (28.6 %), 10(H ₈) (41.7 %), 11(H ₆) (7.5 %), 11(H ₈) (9.7 %)	10(H ₆) (9.6 %), 10(H ₈) (45.3 %), 11(H ₆) (5.2 %), 11(H ₈) (17.2 %), 12 (15.8 %)	10(H ₆) (22.6 %), 10(H ₈) (58.2 %), 11(H ₈) (10.5 %)
Major fatty acids (>10 %)*	i-C _{16:0} (51.1 %), ai-C _{17:0} (11.7 %)	i-C _{16:0} (24.4 %), ai-C _{17:0} (29.1 %)	i-C _{16:0} (38.5 %), ai-C _{17:0} (15.2 %)	i-C _{16:0} (28.7 %), i-C _{17:0} (26.1 %), ai-C _{17:0} (18.3 %), i-C _{18:0} (12.2 %)
DNA G + C content (mol%)	69.0	71.2	66.2	70.4

*i, iso; ai, anteiso.

Fig. 1. Phylogenetic dendrogram obtained by neighbour-joining analysis based on 16S rRNA gene sequences showing the position of strain YIM 90462^T among phylogenetic neighbours. Bootstrap values (expressed as a percentage of 1000 replications) are given at nodes. *Thermomonospora chromogena* JCM 6244^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

high 16S rRNA gene sequence similarities, all four strains were tested together in this study.

Cultural characteristics of strain YIM 90462^T and the other three *Thermobifida* type strains were observed using ISP media 2, 3, 4 and 5, Czapek's agar, potato agar and nutrient agar. The colours of the aerial and substrate mycelia were determined by the ISCC-NBS colour charts (standard sampled no. 2106) (Kelly, 1964). Cultural characteristics were similar among the four tested strains. All of them grew on most media tested, apart from Czapek's agar. They grew well on ISP2, potato agar and nutrient agar plates, but poorly on ISP media 3, 4 and 5. In addition, they all produced white aerial mycelium on nutrient agar medium. However, there were some differences between the strains. Isolate YIM 90462^T produced aerial mycelium only on nutrient agar medium, just like *T. alba* and *T. fusca*, but *T. cellulosilytica* also produced aerial mycelium on ISP2 (Table 1). Morphological features of YIM 90462^T were examined by scanning electron microscopy (XL30, ESEM-TMP; Philips) of 2-week-old cultures grown on nutrient agar medium. Morphological features of YIM 90462^T are consistent with those of members of the genus *Thermobifida* described previously (Kukolya *et al.*, 2002; Kroppenstedt & Evtushenko, 2006). Substrate mycelium was well developed, but not fragmented. Single, smooth-ridged spores were produced on the tips of repeatedly branched sporophores to form dense spore clusters on the aerial mycelium.

The physiological and biochemical characteristics of the four strains were tested. Growth was assessed on ISP2 agar plates at various temperatures [0, 4, 10, 15, 20, 28–37 (at 1 °C intervals), 40, 45, 50, 55, 60 and 65 °C]. The ability of the strains to grow at various NaCl concentrations (0–15 %, w/v, at intervals of 0.5 %) was examined using ISP2 as basal medium. The pH range and optimum for growth were tested from pH 4 to 10 following the method described by Xu *et al.* (2005). Carbohydrate utilization was tested by using ISP medium 9 (Shirling & Gottlieb, 1966) as basal medium. Each filter-sterilized compound was added to the medium to give a final concentration of 1.0 % (w/v). Detailed characteristics are given in Table 1 and in the species description. It was noticed that isolate YIM 90462^T could tolerate up to 10 % (w/v) NaCl. The outstanding cellulose-decomposing ability is the major characteristic feature of strains assigned to the genus *Thermobifida* (Kukolya *et al.*, 2002). Strain YIM 90462^T also showed high cellulase activity on crystalline cellulose and the clearing zones that were observed on CM-cellulose-containing agar plates indicated the presence of a complete cellulose-degrading enzyme system. In addition, there was some evidence that strain YIM 90462^T may be useful in enzymological studies (data not shown).

Chemotaxonomic properties (Table 1), including menaquinones (Collins *et al.*, 1977; Groth *et al.*, 1997), phospholipids (Minnikin *et al.*, 1979; Collins & Jones, 1980), whole-cell fatty acid pattern (Kroppenstedt, 1985), cell-wall amino acids and whole-cell sugars (Staneck &

Roberts 1974), were analysed by standard methods. All four tested strains contained *meso*-diaminopimelic acid (*meso*-DAP) as the diagnostic diamino acid in the cell wall and galactose, xylose and glucose as whole-cell sugars. The phospholipids comprised diphosphatidylglycerol (DPG), phosphatidylmethylethanolamine (PME), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG) and an unknown phospholipid. Strain YIM 90462^T, *T. fusca* DSM 43792^T and *T. cellulosilytica* DSM 44535^T also contained phosphatidylethanolamine (PE). The predominant menaquinones were MK-10(H₆) and MK-10(H₈). The fatty acid profiles differed among the four strains, but the major components were iso-C_{16:0} and anteiso-C_{17:0}.

Multiple alignments with sequences of the most closely related actinobacterial strains were carried out using CLUSTAL_X (Thompson *et al.*, 1997). The neighbour-joining phylogenetic tree was reconstructed by using the software package MEGA version 3.1 (Kumar *et al.*, 2004) and evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. Phylogenetic analysis confirmed the placement of strain YIM 90462^T in the genus *Thermobifida* (Fig. 1). Genomic hybridization experiments between strain YIM 90462^T and *T. alba* DSM 43795^T (33.8 %), *T. fusca* DSM 43792^T (50.2 %) and *T. cellulosilytica* DSM 44535^T (35.4 %) were performed by using the method described by He *et al.* (2005) and repeated five times. These values were significantly lower than the 70 % value considered to be the threshold for the delineation of genomic species (Wayne *et al.*, 1987). The DNA G + C contents of strain YIM 90462^T (69.0 mol%), *T. alba* DSM 43795^T (71.2 mol%), *T. fusca* DSM 43792^T (66.2 mol%) and *T. cellulosilytica* DSM 44535^T (70.4 %) were determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989). These data for the G + C contents of members of the genus *Thermobifida* are very close to the results reported by Kukolya *et al.* (2002). The G + C content of *T. fusca* DSM 43792^T was significantly lower than those of other *Thermobifida* species. This result was confirmed by genome analysis of *T. fusca* YX, which revealed that the precise G + C content of *T. fusca* YX was 67.5 mol% (Lykidis *et al.*, 2007).

Similarities in morphological characteristics, cell-wall amino acids, whole-cell sugars and DNA G + C contents between the four strains studied support the inclusion of strain YIM 90462^T within the genus *Thermobifida*. However, the isolate could be distinguished from *T. alba* DSM 43795^T, *T. fusca* DSM 43792^T and *T. cellulosilytica* DSM 44535^T by its fatty acid profile, menaquinones and phospholipids and some physiological and enzymic properties (Table 1). DNA–DNA hybridization confirmed the differentiation between YIM 90462^T and the other three species of *Thermobifida* at the species level. It is clear from the combined genotypic and phenotypic data shown that strain YIM 90462^T should be classified as a representative of a novel species within the genus *Thermobifida*, *Thermobifida halotolerans* sp. nov. As a consequence of

the characteristics of members of the genus *Thermobifida* that were examined in this study, the description of the genus *Thermobifida* needs to be emended.

Emended description of *Thermobifida* Zhang *et al.* 1998

The following features are observed, in addition to those given in the description by Zhang *et al.* (1998). The phospholipid pattern is complex, consisting of DPG, PME, PC, PI, PG and an unknown phospholipid; PE may occur. The predominant menaquinones are MK-10(H₆) and MK-10(H₈). The major fatty acids are iso-C_{16:0} and anteiso-C_{17:0}. Glucose, galactose and xylose are found in whole-organism hydrolysates. The G + C content of the genomic DNA is 66–72 mol%. Phylogenetically, the genus belongs to the family *Nocardiopsaceae*. The type species is *Thermobifida alba* (Locci *et al.* 1967) Zhang *et al.* 1998.

Description of *Thermobifida halotolerans* sp. nov.

Thermobifida halotolerans (ha.lo.to'le.rans. Gr. n. *hals*, *halos* salt; L. part. adj. *tolerans* tolerating; N.L. part. adj. *halotolerans* referring to the ability to tolerate high salt concentrations).

Aerobic, Gram-positive. Grows well on ISP medium 2, potato agar and nutrient agar plates and poorly on ISP media 3, 4 and 5. Does not grow on Czapek's agar. Substrate mycelium is well developed, but not fragmented. Aerial mycelium is only formed on nutrient medium. Produces single, smooth spores on aerial hyphae on the tips of dichotomously branched sporophores. Good growth occurs at 45 °C and pH 7.0–8.0. Positive for nitrate reduction, starch hydrolysis, cellulose hydrolysis and degradation of Tweens 20 and 80, but negative for gelatin liquefaction, melanin production, milk peptonization and coagulation and H₂S and urease production. The following substrates are utilized as sole carbon sources: D-arabinose, cellobiose, D-galactose, D-glucose, glycerol, raffinose, sucrose, trehalose and D-xylose. D-Fructose, *myo*-inositol, lactose, maltose, D-mannose, L-rhamnose, D-ribose, sorbitol, L-sorbose and xylitol are not utilized. The whole-cell sugars are galactose, xylose and glucose. The diagnostic diamino acid is *meso*-DAP. The predominant menaquinones are MK-10(H₆) and MK-10(H₈). Phospholipids are DPG, PG, PE, PME, PC, PI and an unknown phospholipid. The major fatty acids (>10 %) are iso-C_{16:0} (51.1 %) and anteiso-C_{17:0} (11.7 %).

The type strain is YIM 90462^T (=KCTC 19123^T =DSM 44931^T), isolated from a saline soil sample collected from Yunnan, south-west China. The DNA G + C content of the type strain is 69.0 mol%.

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