Zhihengliuella halotolerans gen. nov., sp. nov., a novel member of the family *Micrococcaceae*

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The actinobacterial strain YIM 70185^T was isolated from a saline soil sample collected from Qinghai province, north-west China, and subjected to a taxonomic investigation. Phylogenetic analysis based on 16S rRNA gene sequences revealed 93.5–96.4 % similarity to members of related genera in the family *Micrococcaceae*. In the phylogenetic dendrogram based on 16S rRNA gene sequence analysis, strain YIM 70185^T formed a separate clade next to the genera *Micrococcus* and *Citricoccus* within the family *Micrococcaceae*. The peptidoglycan type was A4α, L-Lys–L-Ala–L-Glu. Cell-wall sugars contained glucose and tyvelose. The polar lipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, an unknown phospholipid and an unknown glycolipid. The menaquinones were MK-9, MK-10 and MK-8 (molar ratio 5 : 2 : 1). The major fatty acids were ai-$C_{15:0}$ and i-$C_{15:0}$ and the DNA G+C content was 66.5 mol%. These chemotaxonomic profiles supported the assignment of strain YIM 70185^T to a novel genus within the family *Micrococcaceae*. The name *Zhihengliuella halotolerans* gen. nov., sp. nov. is proposed. The type strain of *Zhihengliuella halotolerans* is YIM 70185^T (=DSM 17364^T=KCTC 19085^T).

Strain YIM 70185^T was isolated by using the dilution plating method on marine agar (Difco 2216; MA) supplemented with 15 % NaCl (w/v). The strain was maintained on MA slants at 4 °C and as 20 % (w/v) glycerol suspensions at −20 °C. Biomass for chemical and molecular studies was obtained by cultivation in shaken flasks (about 150 r.p.m.) using marine broth (Difco 2216; MB) at 28 °C for 1 week.

Gram staining and the KOH lysis test were carried out according to Gram (1884) and Cerny (1978), respectively. Morphology and motility were examined by light microscopy (model BH2; Olympus) and electron microscopy (JEM-1010 electron microscope; JEOL) using cells from exponentially growing cultures. For transmission electron microscopy observation, cells were negatively stained with 1 % (w/v) phosphotungstic acid after air-drying. Colony morphology was observed on MA or ISP5 medium (Shirling & Gottlieb, 1966) containing 10 % NaCl and trypticase soy agar (TSA) containing 10 % NaCl after incubation at 28 °C for 2 days. The colony colour was determined with the ISCC–NBS colour charts (Kelly, 1964). Growth was tested at 4, 10, 28, 30, 37, 40, 45 and 55 °C on TSA medium containing 10 % NaCl. The ability of the strain to grow at different pHs and NaCl concentrations was examined according to Tang *et al.* (2003) except that MB was used instead of ISP5 as the basic medium. Metabolic properties were determined using API ID 32E test kits (bioMérieux) according to the manufacturer's instructions.

Strain YIM 70185^T was aerobic and stained Gram-positive. Short, rod-shaped cells (0.6–1.0 × 1.5–2.0 μm) were observed, but neither flagella nor spores were found. Pale-yellow colonies with a smooth surface and a maximum diameter of 1 mm were formed on MA or TSA containing 10 % NaCl after incubation for 48 h at 28 °C. Strain YIM 70185^T grew well at 28 and 30 °C, but slowly at 4 and 45 °C. Growth was observed at initial pH values between 6 and 10 and on TSA containing 0–25 % NaCl. The strain grew optimally at pH 8.0–9.0 and in the presence of 10 % NaCl. The detailed physiological and biochemical characteristics of the strain are given in the species description.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 70185^T is DQ372937.

A purified cell-wall preparation was obtained after disruption of cells by shaking with glass beads and subsequent trypsin digestion by the method of Schleifer (1985). Amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates, using the solvent systems of Schleifer & Kandler (1972). The N-terminal amino acid of the interpeptide bridge was determined by dinitrophenylation, as described by Schleifer (1985). Molar ratios of amino acids were determined by GC and GC-MS of *N*-heptafluorobutyryl amino acid isobutyl esters (MacKenzie, 1987). Analysis of enantiomers of peptidoglycan amino acids was performed by GC of *N*-pentafluoropropionyl amino acid isopropyl esters (Frank *et al.*, 1980) on an L-Chirasil–Val column (Macherey-Nagel) as described by Groth *et al.* (1997). Sugar analysis of the purified cell wall followed procedures described by Staneck & Roberts (1974). Polar lipids were extracted, examined by two-dimensional TLC and identified by using published procedures (Minnikin *et al.*, 1979; Collins & Jones, 1980). Menaquinones were isolated by using the method of Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1997). Analysis of the whole-cell fatty acid pattern followed the instructions of the MIDI System (Microbial ID) (Kroppenstedt, 1985) by using exponential phase cultures.

The peptidoglycan type of strain YIM 70185^T was A4α, L-Lys–L-Ala–L-Glu. Glucose and tyvelose were detected in the purified cell wall. The polar lipids contained phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, an unknown phospholipid and an unknown glycolipid. The menaquinones were MK-9, MK-10, MK-8 (5 : 2 : 1). The major cellular fatty acids were ai-$C_{15:0}$ (63.9 %) and i-$C_{15:0}$ (15.0 %); the complete profile of cellular fatty acids is given in detail in the species description.

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were done as described by Xu *et al.* (2003). Multiple alignments with sequences of the most closely related actinobacteria and calculations of levels of sequence similarity were carried out using CLUSTAL X (Thompson *et al.*, 1997). A phylogenetic tree was constructed using the neighbour-joining method of Saitou & Nei (1987) from K_{nuc} values (Kimura, 1980) using MEGA version 2.1 (Kumar *et al.*, 2001). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Phylogenetic analysis of the 16S rRNA gene sequence (1475 bp) of strain YIM 70185^T revealed relatively remote relatedness to members of the family *Micrococcaceae* (similarities ranging from 93.5 to 96.4 %), forming a distinct subclade within the radiation of this family (Fig. 1). Patterns of selected 16S rRNA gene signature nucleotides defined for the family *Micrococcaceae* (Stackebrandt *et al.*, 1997; Stackebrandt & Schumann, 2000) were consistent with nucleotides determined for the 16S rRNA gene sequence of strain YIM 70185^T except that G, C–G and U–C were determined at positions 640, 839 : 847 and 1025 : 1036, respectively. Positions 502 : 543 and 1310 : 1327, given as R–Y by Stackebrandt & Schumann (2000), were determined as G–C. The G+C content of the DNA was determined to be 66.5 mol% by reverse-phase HPLC of nucleosides according to Mesbah *et al.* (1989).

Chemotaxonomic characteristics that differentiate strain YIM 70185^T from representatives of its closest phylogenetic neighbours detected by 16S rRNA gene sequence analysis are given in Table 1.

Although some species of the '*Arthrobacter nicotianae* group', e.g. *Arthrobacter nicotianae*, *Arthrobacter protophormiae*, *Arthrobacter uratoxydans* (Stackebrandt *et al.*, 1983), *Arthrobacter rhombi* (Osorio *et al.*, 1999), *Arthrobacter bergerei* and *Arthrobacter arilaitensis* (Irlinger *et al.*, 2005), possess A4α peptidoglycan with the interpeptide bridge Ala–Glu, they have different cell-wall sugar compositions, quinone systems or DNA G+C contents compared with those of strain YIM 70185^T.

Tyvelose has been reported to be present in the cell walls of only a few members of the family *Microbacteriaceae*, suborder *Micrococcineae*, such as *Agromyces cerinus* subsp. *cerinus* (Zgurskaya *et al.*, 1992) and *Agrococcus citreus* (Wieser *et al.*, 1999). The presence of tyvelose differentiates the new isolate from other taxa in the family *Micrococcaceae*, although the cell-wall sugars have been studied in only a limited set of species so far.

For the quinone system, *Renibacterium salmoninarum* and some *Arthrobacter* species, such as *Arthrobacter gangotriensis* (Gupta *et al.*, 2004), have predominant menaquinones similar to those of the new isolate, but they differ in other chemotaxonomic characteristics such as peptidoglycan structure, cell-wall sugars, major fatty acids and DNA G+C content (Table 1).

16S rRNA gene sequence analysis, chemotaxonomic properties and the profile of metabolic properties (see species description) revealed that strain YIM 70185^T represents a new genus and a novel species within the family *Micrococcaceae*, for which the name *Zhihengliuella halotolerans* gen. nov., sp. nov. is proposed.

Description of *Zhihengliuella* gen. nov.

Zhihengliuella [Zhi.heng.li.u.el'la. N.L. fem. dim. n. *Zhihengliuella* named after Zhi-Heng Liu (1940–), a Chinese microbiologist who devotes himself to the study of actinomycete taxonomy].

Gram-positive, mesophilic and aerobic. Cells are non-motile, non-spore-forming, short rods (0.6–1.0 × 1.5–2.0 μm). Catalase-positive and oxidase-negative. The peptidoglycan type is A4α, L-Lys–L-Ala–L-Glu. The predominant menaquinones are MK-9 and MK-10; MK-8 occurs in smaller amounts. The major fatty acids are ai-$C_{15:0}$ and i-$C_{15:0}$. The major polar lipids are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylinositol. The G+C content of genomic DNA is about 66 mol%.

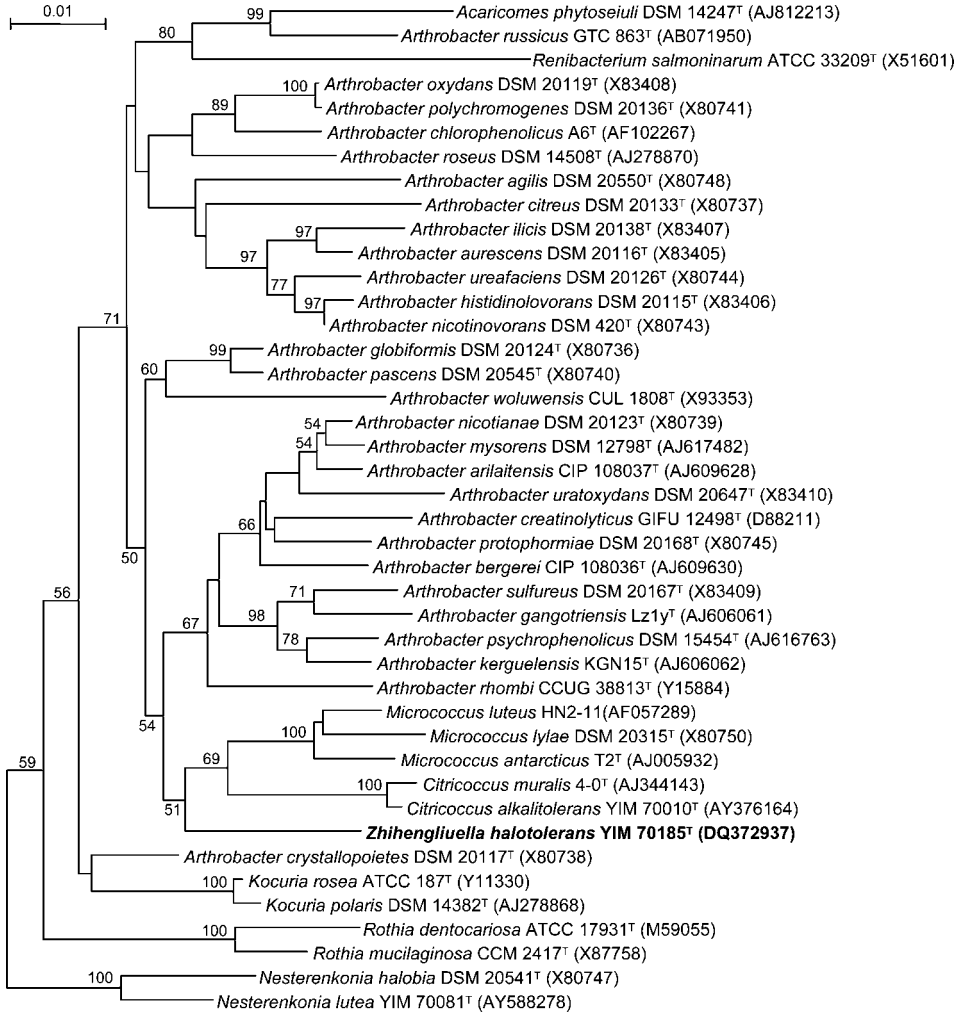

Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequences, showing the position of strain YIM 70185^T among its phylogenetic neighbours. Numbers at branch nodes are bootstrap values (1000 resamplings; only values over 50 % are given). The sequence of *Brevibacterium linens* DSM 20425^T (GenBank accession no. X77451) was used as the root (not shown). Bar, 1 % sequence divergence.

16S rRNA gene sequence similarity reveals membership of the family *Micrococcaceae*. The type species is *Zhihengliuella halotolerans*.

Description of *Zhihengliuella halotolerans* sp. nov.

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

In addition to the characteristics that define the genus, the species has the following characteristics. The pale-yellow colonies are circular, opaque and approximately 1.0 mm in diameter after 24 h at 28 °C. Optimum growth occurs at pH 8.0–9.0 and at 28–30 °C with 10 % NaCl. Negative for ornithine decarboxylase, urease, *N*-acetylglucosaminidase, β-galactosidase, α-glucosidase, gelatin liquefaction, methyl red and Voges–Proskauer tests and nitrate reduction, but positive for arginine dihydrolase, lysine decarboxylase, lipase, β-glucosidase, α-galactosidase, ammonia production, starch, hydrolysis of Tweens 20 and 80 and milk peptonization. Malonate is utilized and acid is produced from maltose, glucose, sucrose, L-arabinose and trehalose. The cell wall contains glucose and tyvelose. Polar lipids include an unknown phospholipid and an unknown glycolipid. The menaquinones are MK-9, MK-10, MK-8 (5 : 2 : 1). The cellular fatty acid profile contains ai-$C_{15:0}$ (63.9 %), i-$C_{15:0}$ (15.0 %), ai-$C_{17:0}$ (8.5 %), i-$C_{16:0}$ (7.1 %), $C_{16:0}$ (2.5 %), i-$C_{17:0}$ (1.0 %), i-$C_{14:0}$ (0.8 %), $C_{14:0}$ (0.8 %), $C_{15:0}$ (0.2 %) and ai-$C_{15:1}$ (0.2 %). The G+C content of the DNA of the type strain is 66.5 mol%.

Table 1. Differential chemotaxonomic characteristics of the genus *Zhihengliuella* and related genera of the family *Micrococcaceae*

All taxa contain L-Lys as the diamino acid of the peptidoglycan. Data for reference genera were taken from Stackebrandt & Schumann (2000), Liu *et al.* (2000) and Wieser *et al.* (2002) (*Micrococcus*), Keddie *et al.* (1986), Koch *et al.* (1995), Stackebrandt *et al.* (1995) and recent publications (*Arthrobacter globiformis* group), Collins & Kroppenstedt (1983), Keddie *et al.* (1986), Koch *et al.* (1995), Stackebrandt *et al.* (1995), Gupta *et al.* (2004), Margesin *et al.* (2004) and Chen *et al.* (2005) (*Arthrobacter nicotianae* group), Stackebrandt & Schumann (2000) and W.-J. Li *et al.* (2006) (*Kocuria*), Stackebrandt *et al.* (1995), Collins *et al.* (2002), W.-J. Li *et al.* (2004, 2005b) (*Nesterenkonia*), Sanders & Fryer (1980) (*Renibacterium*), Bergan & Kocur (1982), Gerencser & Bowden (1986), Collins *et al.* (2000), Fan *et al.* (2002) and Y. Li *et al.* (2004) (*Rothia*), Altenburger *et al.* (2002) and W.-J. Li *et al.* (2005a) (*Citricoccus*) and Pukall *et al.* (2006) (*Acaricomes*). Menaquinones are exemplified by MK-8(H_2), partially saturated menaquinone with one of eight isoprene units hydrogenated. Fatty acids are exemplified by ai-$C_{15:0}$, 12-methyltetradecanoic acid; i-$C_{15:0}$, 13-methyltetradecanoic acid. Sugars and polar lipids in parentheses vary among species or strains. ND, No data available.

Characteristic	*Zhihengliuella*	*Micrococcus*	*Arthrobacter globiformis* group	*Arthrobacter nicotianae* group	*Kocuria*	*Nesterenkonia*	*Renibacterium*	*Rothia*	*Citricoccus*	*Acaricomes*
Interpeptide bridge	L-Ala–L-Glu	Peptide subunit/D-Asp	MCA_{var}*	Ala–Glu, Ser–Glu, Asp/Glu	L-$Ala_{3–4}$	Gly–L-Glu/Glu/Gly–D-Asp	L-Ala–Gly	L-Ala_3, L-Ala, L-Ser, Gly/Gly–Ala	Gly–Glu	Ala_3
Cell-wall sugars†	Tyv, Glc	Gal/Man	Gal, (Glc, Rha, Man)	Glc, (Gal)	Gal, Glc	Rib, Xyl, Ara, (Glc)	Gal, Rha	Gal, Glc, Fru	ND	Gal, Glc
Predominant menaquinone(s)	9, 10	8, 8(H_2)/8(H_2)	8(H_2), 9(H_2)/9(H_2)	8, 9, 10/8, 9/9, 10/9, 10, 11	7(H_2)/8(H_2)/7(H_2), 8(H_2)/8(H_2), 9(H_2)	7, 8/7, 8, 9	9, 10	7/6(H_2), 7	9(H_2)	10(H_2)
Polar lipids‡	DPG, PI, PG, PL, GL	DPG, PI, PG, PL, GL	DPG, PG, PI, DMDG	DPG, PG, PI, DMDG	DPG, PG, (PI, PL, GL)	DPG, PG, (PI, PC, GL)	DPG, GL	DPG, PG, (PI)	DPG, PI, PG, PL, GL	DPG, PG, PI
Major fatty acid(s)	ai-$C_{15:0}$, i-$C_{15:0}$	ai-$C_{15:0}$, i-$C_{15:0}$	ai-$C_{15:0}$, i-$C_{15:0}$, i-$C_{16:0}$, ai-$C_{17:0}$	ai-$C_{15:0}$	ai-$C_{15:0}$/ai-$C_{17:0}$, ai-$C_{15:0}$, i-$C_{15:0}$/ai-$C_{15:0}$, i-$C_{15:0}$	ai-$C_{15:0}$, ai-$C_{17:0}$, i-$C_{16:0}$	ai-$C_{15:0}$, ai-$C_{17:0}$	ai-$C_{15:0}$, ai-$C_{17:0}$, i-$C_{16:0}$	ai-$C_{15:0}$	ai-$C_{15:0}$, ai-$C_{17:0}$,
G+C content (mol%)	66.5	66–76	59–70	55–66	60–75	64–72	52–54	47–60§	63–68	57.7

*Variable monocarboxylic amino acid in the interpeptide bridge.

†Ara, Arabinose; Fru, fructose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Rib, ribose, Tyv, tyvelose; Xyl, xylose.

‡DMDG, Dimannosyldiacylglycerol; DPG, diphosphatidylglycerol; GL, unidentified glycolipid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, unidentified phospholipid.

§G+C contents of 54–59 mol% are reported for the type strains of *Rothia* species in the cited publications.

The type strain is YIM 70185^T (=DSM 17364^T=KCTC 19085^T), isolated from a saline soil sample collected from Qinghai province, north-west China.

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