Myceligenerans halotolerans sp. nov., an actinomycete isolated from a salt lake, and emended description of the genus *Myceligenerans*

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A halotolerant actinomycete strain, designated XJEEM 11063^T, was isolated from a salt lake in Xinjiang province, north-western China. Strain XJEEM 11063^T grew at pH 6.0-8.0 (optimal growth at pH 7.0), between 10 and 40 °C (optimal growth at 28-37 °C) and at salinities of 0-10% (w/v) NaCl (optimal growth at 0-5%, w/v). The peptidoglycan type was A4 α , and the whole-cell hydrolysates contained glucose, mannose and arabinose. The major fatty acids were anteiso-C_{15:0}, iso-C_{15:0} and anteiso-C_{17:0}. MK-9(H₄) was the predominant menaquinone and the polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, two unknown phospholipids and three unknown glycolipids. The genomic DNA G+C content was 71.8 mol%. The chemotaxonomic properties supported the affiliation of strain XJEEM 11063^T to the genus *Myceligenerans*. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the organism was most closely related to Myceligenerans xiligouense XLG9A10.2^T (98.3%) and Myceligenerans crystallogenes DSM 17134^T (97.0%). However, it had relatively low values for DNA-DNA relatedness with the above strains (56.0 % and 47.5 %, respectively). Thus, on the basis of the results from this study, a novel species, *Myceligenerans halotolerans* sp. nov., is proposed. The type strain is XJEEM 11063^T (=DSM 21949^T=CCTCC AA 208063^T).

The genus *Myceligenerans* was described by Cui *et al.* (2004). At the time of writing, this genus comprises only two species with validly published names: *Myceligenerans xiligouense* (Cui *et al.*, 2004) and *Myceligenerans crystallogenes* (Groth *et al.*, 2006). Members of the genus *Myceligenerans* are Gram-positive and aerobic. Chemotaxonomically, members

of the genus *Myceligenerans* contain L-Lys–L-Thr–D-Glu in the cell-wall peptidoglycan (variation A4 α), MK-9(H₄) and MK-9(H₆) as the predominant menaquinones and anteiso-C_{15:0} and iso-C_{15:0} as the major fatty acids and have DNA G+C contents of 72–72.3 %.

Strain XJEEM 11063^T was isolated from a soil sample collected from the Qijiaojing salt lake (GPS coordinates 43° 26′ 52″ N 91° 29′ 18″ E) after 2 weeks of incubation at 37 °C on glucose-tryptone-yeast (GTY) medium with 5% (w/v) NaCl (Tang *et al.*, 2010). The strain was maintained on tryptic soy agar slants at 4 °C and as suspensions of mycelial fragments in glycerol (20%, v/v) at -80 °C. Biomass for chemical and molecular studies was obtained

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Abbreviation: ISP, International Streptomyces Project.

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A supplementary table and a supplementary figure are available with the online version of this paper.

by cultivation in shaken flasks (about 150 r.p.m.) using tryptic soy broth at 37 $^\circ\mathrm{C}$ for a week.

Cultural characteristics were determined after 3-4 weeks by methods used in the International Streptomyces Project (ISP) (Shirling & Gottlieb, 1966). The colour of substrate and aerial mycelia and any soluble pigments produced were determined by comparison with chips from the ISCC-NBS colour charts (Kelly, 1964). Strain XJEEM 11063^T grew on ISP 4 agar, nutrient agar, Czapek's agar and ISP 2 agar, but not on potato agar or oatmeal agar (ISP 3). Moreover, only sparse aerial mycelium was produced on ISP 4 agar. Aerial and substrate mycelia were white-yellow on ISP 4 and tryptic soy agar. Weak diffusible pigments were produced on Czapek's agar. Morphological characteristics of strain XJEEM 11063^T were observed by light microscopy (model BH 2; Olympus) and scanning electron microscopy (JSM5600LV; JEOL) after 15 days of growth on tryptic soy agar medium. The morphological features of strain XJEEM 11063^T were consistent with those of the recognized members of the genus Myceligenerans. The substrate mycelium was well developed and spore-like cells occurred in the substrate mycelium.

Growth was tested at 4, 10, 15, 20, 28, 37, 40, 45 and 55 °C on tryptic soy agar. For NaCl tolerance experiments, tryptic sov agar was used as the basal medium. The following NaCl concentration range was used: 0-30 % (w/v), at intervals of 1%. The pH growth range was investigated between pH 4.0 and pH 10.0 at intervals of 1 pH unit, using the following buffer systems: pH 4.0-5.0, 0.1 M citric acid/ 0.1 M sodium citrate; pH 6.0-8.0, 0.1 M KH₂PO₄/0.1 M NaOH; pH 9.0-10.0, 0.1 M NaHCO₃/0.1 M Na₂CO₃. M. xiligouense XLG9A10.2^T and M. crystallogenes DSM 17134^T were used as reference strains for the phenotypic tests. The media and procedures used for the determination of physiological features were those described by Williams et al. (1989). Metabolic properties and enzyme activities were determined by means of the API 50 CHB and API ZYM systems (bioMérieux) according to the manufacturer's instructions. Antibiotic susceptibility was determined by the method of Williams (1967). Oxidation of 95 different substrates was tested using Biolog GP2 microplates (Biolog Inc.) within the density range specified by the manufacturer (20% turbidity). Anaerobic growth was determined using the GasPak Anaerobic System (BBL) according to the manufacturer's instructions. The organism could be distinguished from recognized members of the genus Myceligenerans using phenotypic properties (Table 1 and Supplementary Table S1 in IJSEM Online).

A purified cell-wall preparation was obtained and hydrolysed as described by Schleifer & Kandler (1972). *M. xiligouense* XLG9A10.2^T was used as a reference strain for cell-wall amino acid analysis. Amino acids in cell-wall hydrolysates were analysed by precolumn derivatization with o-phthalaldehyde (OPA) by HPLC (Tang *et al.*, 2009a). Cell-wall sugars were detected by precolumn derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) by HPLC

Table 1. Differential phenotypic characteristics between strain XJEEM 11063^{T} and its closest relatives in the genus *Myceligenerans*

Strains: 1, XJEEM 11063^T (data from this study); 2, *M. xiligouense* XLG9A10.2^T (data from Cui *et al.*, 2004); 3, *M. crystallogenes* DSM 17134^T (Groth *et al.*, 2006). +, Positive; –, negative; (+), weakly positive. All strains are positive in tests for the decomposition of aesculin, gelatin and starch, for the peptonization of milk and for the activity of esterase (C4), esterase lipase (C8), α -glucosidase and β -glucosidase. All strains are susceptible to ciprofloxacin (5 µg) and vancomycin (30 µg) and resistant to norfloxacin (10 µg). None of the strains decomposes hypoxanthine or shows activity for α -chymotrypsin, β -glucuronidase, α -mannosidase or α -fucosidase.

1	2	3
White to	Yellow	White to
cream		cream
0-10	2-17.5	0–5
+	_	+
—	-	+
—	-	+
_	+	+
_	+	+
_	+	+
+	_	_
+	_	_
10-40	4-50	10-40
_	+	+
_	+	+
_	+	+
_	+	+
_	+	+
_	+	+
—	+	+
_	(+)	_
+	_	+
+	_	+
	1 White to cream 0–10 + - - - + + + 10–40	I 2 White to Yellow cream 0-10 0-10 2-17.5 + - - - - +

*Data determined in this study.

(Tang *et al.*, 2009b). Polar lipids were extracted and examined by two-dimensional TLC and identified using previously described procedures (Minnikin *et al.*, 1984). Menaquinones were isolated according to Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt, 1982). For fatty acid analysis, cells of strain XJEEM 11063^T were cultured on tryptic soy agar (TSA; BD) at 37 °C for 4 days. Cellular fatty acid analysis was performed as described by Sasser (1990) using the Microbial Identification System (MIDI). Strain XJEEM 11063^T contained glucose, mannose and arabinose as the major cell-wall sugars. The peptidoglycan of strain XJEEM 11063^T contained the amino acids Lys,

Thr, Ala and Glu (the same as *M. xiligouense* XLG9A10.2^T) and belonged to type A4a: L-Lys-L-Thr-D-Glu [variation A11.57, according to DSMZ (2001)]. The polar lipids were diphosphatidvlglvcerol, phosphatidvlglvcerol, phosphatidvlinositol, phosphatidylinositol mannosides, two unknown phospholipids and three unknown glycolipids (see Supplementary Fig. S1 in IJSEM Online). The predominant menaquinone was MK-9 (H₄) (90%) and minor amounts of MK-9, MK-9(H₂) and MK-9(H₆) were detected. Strain XJEEM 11063^T had a cellular fatty acid profile that contained major amounts of branched fatty acids. The fatty acids were anteiso- $C_{15:0}$ (41.3%), iso- $C_{15:0}$ (31.4%), anteiso-C_{17:0} (19.1%), iso-C_{17:0} (3.7%), iso-C_{16:0} (2.2%) and $C_{16:0}$ (1.4%). The chemotaxonomic data for strain XJEEM 11063^T were consistent with its assignment to the genus Myceligenerans.

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were performed as described by Li et al. (2007). Multiple alignments with sequences of strains closely related to the genus Myceligenerans and calculations of levels of sequence similarity were conducted using EzTaxon server 2.0 (Chun et al., 2007). Phylogenetic analyses were performed using three tree-making algorithms: the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was reconstructed using the neighbour-joining method of Saitou & Nei (1987) from K_{nuc} values (Kimura, 1980) using MEGA version 4.0 (Tamura et al., 2007). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. The method of Marmur (1961) was used to prepare genomic DNA of strain XJEEM 11063^T for measurement of the G+C content. The G+C content was determined by reverse-phase HPLC of nucleosides according to Mesbah et al. (1989). Levels of DNA-DNA relatedness were determined according to the optical renaturation method (De Ley et al., 1970; Huß et al., 1983; Jahnke, 1992).

The results of 16S rRNA gene sequence comparisons clearly demonstrated that strain XJEEM 11063^T belonged to the genus *Myceligenerans*. The relationship between the novel strain and its nearest phylogenetic relatives is shown in Fig. 1.

The 16S rRNA gene sequence similarities between strain XJEEM 11063^T and the type strains of *M. xiligouense* and *M.* crystallogenes were 98.3 % and 97.0 %, respectively. In the phylogenetic tree based on the neighbour-joining algorithm. strain XJEEM 11063^T formed a monophyletic clade with M. xiligouense and M. crystallogenes supported by a bootstrap value of 100 % (Fig. 1). The topologies of the phylogenetic trees built using the maximum-likelihood and maximumparsimony algorithms were similar to those of the tree constructed by neighbour-joining analysis (data not shown). The G+C content of the DNA was 71.8 mol%. According to DNA-DNA hybridization experiments, strain XJEEM 11063¹ showed DNA–DNA relatedness values of 56 \pm 3 % and 47.5 \pm 5% to M. xiligouense XLG9A10.2^T and M. crystallogenes DSM 17134^{T} , respectively. These values were well below the 70% cut-off point recommended for the assignment of strains to the same genomic species (Wayne et al., 1987).

Therefore, on the basis of phenotypic and phylogenetic differences and DNA–DNA hybridization tests, strain XJEEM 11063^T should be classified as representing a novel species in the genus *Myceligenerans*, for which the name *Myceligenerans halotolerans* sp. nov. is proposed. Additionally, as a consequence of the newly reported characteristics that were not included in the original description of the genus *Myceligenerans*, the description of the genus *Myceligenerans*, the description of the genus (Cui *et al.*, 2004) is emended.

Emended description of the genus *Myceligenerans* Cui *et al.* 2004

The genus is as described by Cui *et al.* (2004), with the following emendations. Aerial mycelium is absent or sparse. Cell-wall sugars are glucose and mannose. The predominant menaquinone is MK-9(H₄); cells contain phosphatidyl-glycerol and diphosphatidylglycerol. The DNA G+C content of members of the genus is 71.8–72.3 %.

Description of Myceligenerans halotolerans sp. nov.

Myceligenerans halotolerans (ha.lo.to'le.rans. Gr. n. *hals halos* salt; L. pres. part. *tolerans* tolerating, enduring; N.L. part. adj. *halotolerans* salt-tolerating).



Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequences, showing the position of strain XJEEM 11063^{T} and its most closely related phylogenetic neighbours. Filled circles indicate that the corresponding nodes were also found in the trees generated with both maximum-parsimony and maximum-likelihood algorithms. Numbers on branch nodes are bootstrap values (1000 resamplings, only values >50% are given). The sequence of *Arthrobacter globiformis* DSM 20124^T (GenBank accession no. X80736) was used as an outgroup. Bar, 1% sequence divergence.

Aerobic, Gram-positive. Substrate mycelia are welldeveloped and fragment. Aerial mycelia are sparse on ISP 4 agar medium. Temperature, pH and NaCl tolerance ranges for the type strain are 10-40 °C, pH 6.0-8.0 and 0-10% (w/v), respectively. Optimal growth occurs at 28-37 °C, pH 7.0 and 0-5 % (w/v) NaCl. Aesculin, starch and Tweens 40 and 60 are degraded, but casein, cellulose, chitin, Tweens 20 and 80 and urea are not. Tests for gelatin liquefaction, nitrate reduction and milk peptonization and coagulation are positive. Tests for H₂S and melanin production are negative. In the API ZYM system, esterase (C4), esterase lipase (C8), α -glucosidase and β -glucosidase are positive, but alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are negative. Acid is produced from aesculin and trehalose, but not from all other carbon sources (API 50 CHB). Oxidation results for 95 different substrates (Biolog GP2) are listed in Supplementary Table S1 in IJSEM Online. Sensitive to the following antibiotics (µg): amoxicillin (10), amikacin (30), ampicillin (10), ciprofloxacin (5), netilmicin (10), novobiocin (5), penicillin (10 U), sulfamethoxazole/trimethoprim (23.75/1.25), tetracycline (30), tobramycin (10) and vancomvcin (30). Resistant to clindamycin (2 µg), erythromycin (15 µg) and norfloxacin (10 µg). The peptidoglycan type is A4 α , and the whole-cell hydrolysates contain glucose, mannose and arabinose. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, two unknown phospholipids and three unknown glycolipids. The predominant menaquinone is MK-9(H₄). Major cellular fatty acids are anteiso-C15:0, iso-C15:0 and anteiso- $C_{17 \cdot 0}$.

The type strain, XJEEM 11063^{T} (=DSM 21949^{T} =CCTCC AA 208063^T), was isolated from a salt lake in Xinjiang, north-western China. The G+C content of the DNA of the type strain is 71.8 mol%.

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