

Marinococcus halotolerans sp. nov., isolated from Qinghai, north-west China

Wen-Jun Li,¹ Peter Schumann,² Yu-Qin Zhang,¹ Guo-Zhong Chen,¹ Xin-Peng Tian,¹ Li-Hua Xu,¹ Erko Stackebrandt² and Cheng-Lin Jiang¹

Correspondence

Wen-Jun Li
wjli@ynu.edu.cn or
lihxu@ynu.edu.cn

¹The Key Laboratory for Microbial Resources of Ministry of Education, Yunnan Institute of Microbiology and Laboratory for Conservation and Utilization of Bio-Resources, Yunnan University, Kunming, Yunnan, 650091, P. R. China

²DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany

An aerobic bacterium was isolated from saline soil located in Qinghai, north-west China. The bacterium, designated YIM 70157^T, was investigated using a polyphasic taxonomic approach. The Gram reaction of the organism was positive. Comparative 16S rRNA gene sequence analysis demonstrated the isolate to be a member of the genus *Marinococcus*, the closest phylogenetic neighbour of the unknown bacterium being *Marinococcus halophilus* DSM 20408^T with a similarity of 99.4%. The peptidoglycan type of YIM 70157^T was A1 γ , with meso-diaminopimelic acid as diagnostic diamino acid. The major fatty acids were ai-C_{15:0}, ai-C_{17:0} and i-C_{16:0}. The menaquinones were MK-7 and MK-6. The phospholipids were diphosphatidylglycerol and phosphatidylinositol. The G + C content of total DNA was 48.5 mol%. On the basis of phylogenetic and phenotypic evidence and DNA–DNA hybridization data, this isolate should be classified as a novel species of *Marinococcus*, for which the name *Marinococcus halotolerans* sp. nov. is proposed. The type strain is YIM 70157^T (=DSM 16375^T=KCTC 19045^T).

The genus *Marinococcus* was proposed to accommodate two species, *Marinococcus albus* and *Marinococcus halophilus*; both species were formerly classified as *Planococcus* species (Novitsky & Kushner, 1976; Hao *et al.*, 1984). They have meso-diaminopimelic acid in the cell wall, a DNA G + C content ranging between 43.9 and 46.6 mol%, a menaquinone system with MK-7, grow at a concentration of 20% NaCl, and are motile cocci. A third *Marinococcus* species, *Marinococcus hispanicus*, has been transferred to the genus *Salinicoccus* (Ventosa *et al.*, 1992). Therefore, at the time of writing, there are only two *Marinococcus* species with validly published names. *M. halophilus* is the predominant coccus found in most hypersaline environments (Ventosa *et al.*, 1983; Márquez *et al.*, 1992).

During our investigations of the extremophilic microbial flora of China (Tang *et al.*, 2003; Li *et al.*, 2004a, b, 2005a, b), strain YIM 70157^T was recovered and characterized by a combination of genotypic and phenotypic methods. It was evident that the isolate was sufficiently distinct from the two species of the genus *Marinococcus* to warrant the description of a novel species, for which the name *Marinococcus halotolerans* sp. nov. is proposed.

Strain YIM 70157^T was isolated from a hypersaline soil sample, collected from Qinghai, north-west China, by using the dilution plating method. Detailed information about the saline soils and salt lakes in Qinghai Province was given by Zhang *et al.* (1987). Modified medium SG (Sehgal & Gibbons, 1960) used for selective isolation contained the following (g l⁻¹): Casamino acids (7.5), yeast extract (10.0), trisodium citrate (3.0), NaCl (2.0), KCl (2.0), MgCl₂.6H₂O (250), MgSO₄.7H₂O (1.0), FeSO₄.7H₂O (0.05) and MnSO₄.7H₂O (0.0002). MgCl₂.6H₂O was sterilized separately and then added to the medium. The plate was incubated at 28 °C for 2 weeks. The isolate was maintained on modified SG agar slants that contained 10% (w/v) MgCl₂.6H₂O at 4 °C and as glycerol suspensions (20%, w/v) at –20 °C. Biomass for chemical and molecular systematic studies was obtained from enrichment agar plates of modified SG agar medium incubated at 28 °C for about 4–5 days.

The morphology and motility of cells grown for 10–48 h on modified SG agar were examined by using light microscopy (model BH 2; Olympus) and transmission electron microscopy (model H-800; Hitachi). For transmission electron microscopy observation, cells were negatively stained with 1% (w/v) phosphotungstic acid, after air drying. Observation of flagella was also performed using the Leifson flagella staining method (Leifson, 1960). Gram staining was carried out using the standard Gram reaction combined with the

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KOH lysis test method (Cerny, 1978). The colony colour of the isolate grown on modified SG agar medium was determined by comparing the cultures with the most suitable colour chips from the ISCC-NBS colour charts (Kelly, 1964). Growth at different temperatures, salt (NaCl, KCl and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) concentrations and pH values was investigated as described by Tang *et al.* (2003), except that modified SG was used as the basic medium. Metabolic properties were determined using the API ID 32E test kits (bioMérieux) according to the manufacturer's instructions. Other physiological and biochemical tests were performed as described previously (Li *et al.*, 2004a, b, 2005a, b).

Peptidoglycan was purified and the cell-wall 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 diaminopimelic acid isomer was identified by using the method of Rhuland *et al.* (1955). Analyses of polar lipids and menaquinones were performed according to published procedures (Groth *et al.*, 1999). Analysis of the whole-cell fatty acid pattern was performed according to previously described methods (Miller, 1982) using the MIDI system (Microbial ID).

Extraction and amplification of genomic DNA for 16S rRNA gene sequence analysis were carried out as described previously (Xu *et al.*, 2003). The DNA G + C content of strain YIM 70157^T was determined by reverse-phase HPLC of nucleosides according to Mesbah *et al.* (1989). DNA-DNA hybridization was carried out by applying the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) under optimal hybridization conditions.

Multiple alignments with sequences of a broad selection of related species of the order *Bacillales* and calculations of levels of sequence similarity were carried out using CLUSTAL X (Thompson *et al.*, 1997). A phylogenetic tree (Fig. 1) was reconstructed using the neighbour-joining method of Saitou & Nei (1987) from K_{nuc} values (Kimura, 1980, 1983). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985), with 1000 replicates.

Cells of strain YIM 70157^T were aerobic, Gram-positive, non-spore-forming, motile and spherical; each cell possessed

a single polar flagellum and was about 1.0–1.2 µm in diameter (not shown). Colonies of YIM 70157^T were orange, circular, lubricious and opaque on most agar media tested. The strain grew optimally in modified SG medium at 28 °C at pH 7.0–7.5 and in the presence of 10.0% (w/v) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ can also be substituted by NaCl or KCl); the concentration ranges of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, NaCl and KCl for growth were 0–20, 0–25 and 0–25%, respectively. Strain YIM 70157^T could utilize maltose, mannitol, glucose, mannose, fructose, cellobiose, salicin, acetamide, galactose, xylose and dextrin as carbon sources, but could not use adonitol, arabinose, arabitol, rhamnase, inositol or sorbitol. Acid was produced only from aesculin, glucose and mannitol. Other physiological properties are given in detail in Table 1 and in the species description.

The peptidoglycan type of strain YIM 70157^T was A1 γ based on *meso*-diaminopimelic acid. The phospholipids were phosphatidylinositol and diphosphatidylglycerol. The menaquinones were MK-7 (91.6%) and MK-6 (8.4%). The major fatty acids were ai-C_{15:0} (37.40%), ai-C_{17:0} (21.11%) and i-C_{16:0} (9.07%); the complete profile of cellular fatty acids is given in detail in the species description.

Comparison of the almost-complete 16S rRNA gene sequence (1453 nucleotide positions) of strain YIM 70157^T with homologous sequences of a wide range of related type strains revealed that the closest phylogenetic relatedness is to *M. halophilus* DSM 20408^T (99.4% sequence similarity). The heterogeneity with respect to the second *Marinococcus* type strain, *M. albus* DSM 20748^T, was significantly higher (only 89.5% sequence similarity). A distance-matrix dendrogram is presented in Fig. 1. The 20.8% DNA-DNA relatedness determined between strain YIM 70157^T and *M. halophilus* DSM 20408^T was significantly lower than the 70% value considered to be the recommended threshold value for the delineation of genomic species (Wayne *et al.*, 1987).

The result of the 16S rRNA gene sequence comparisons clearly demonstrated that strain YIM 70157^T is a member of the genus *Marinococcus*. Similarities in some phenotypic characteristics with respect to the only two type strains further support the inclusion of strain YIM 70157^T in the

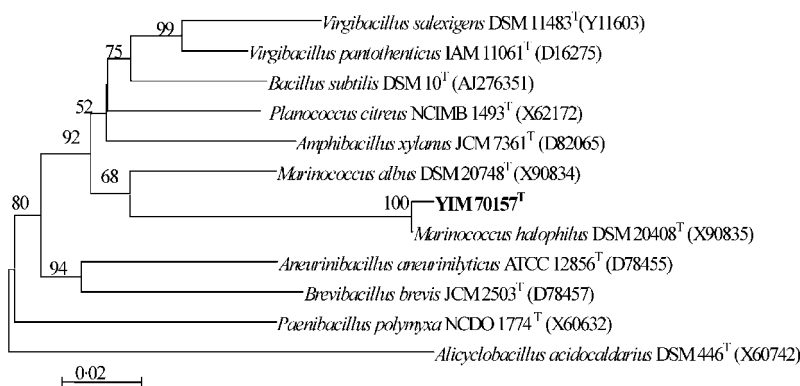


Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequences, showing the position of strain YIM 70157^T among phylogenetic neighbours. Numbers on branch nodes are bootstrap values (1000 resamplings). The sequence of *Alicyclobacillus acidocaldarius* DSM 446^T was used as a root. Bar, 1% sequence divergence.

Table 1. Differential phenotypic characteristics among strains YIM 70157^T, *M. halophilus* DSM 20408^T and *M. albus* DSM 20748^T

All three strains are aerobic, Gram-positive, non-spore-forming, motile cocci with flagella. All have the *meso*-diaminopimelic acid cell wall type and have MK-7 as the major menaquinone system. All are catalase-positive, give negative results in the methyl red and Voges–Proskauer tests, and are negative for melanin production, H₂S and indole production; Tweens 20 and 80 and starch are not decomposed. All are β -glucosidase-, β -galactosidase- and lipase-positive, but negative for arginine dihydrolase, ornithine decarboxylase, L-aspartic arylamidase and growth in cellulose. All utilize maltose, mannitol, glucose, mannose, fructose, salicin, acetamide and galactose as sole carbon sources, but do not utilize adonitol, arabinose, arabitol, inositol, rhamnose or sorbitol. Data for *M. halophilus* DSM 20408^T and *M. albus* DSM 20748^T are from Hao *et al.* (1984). Symbols: +, positive; –, negative.

Characteristic	<i>M. halotolerans</i> YIM 70157 ^T	<i>M. halophilus</i> DSM 20408 ^T	<i>M. albus</i> DSM 20748 ^T
Colony pigmentation (PYGV medium*)	Orange	Yellow–orange	White
pH tolerance	6.5–9.0	6.0–10.0	6.0–10.0
Optimum salinity (%)	10	5–15	5–15
Growth without salt	+	–	–
Acid production from fructose	–	+	–
Nitrate reduction	+	–	+
Carbon-source utilization			
Cellobiose	+	–	–
Dextrin	+	–	–
Mannose	+	–	–
Hydrolysis of:			
Casein	–	+	–
Gelatin	–	+	–
Enzymic properties			
Lysine decarboxylase	–	+	+
α -Maltosidase	+	–	–
α -Galactosidase	–	+	+
Oxidase	–	–	+
Major fatty acids (%)	ai-C _{15:0} (37.4), ai-C _{17:0} (21.1), i-C _{16:0} (9.1), i-C _{17:0} (7.7), i-C _{15:0} (6.9), C _{16:0} (6.7)	ai-C _{15:0} (45), ai-C _{17:0} (30), i-C _{16:0} (14), C _{16:0} (7)	ai-C _{15:0} (64), ai-C _{17:0} (39)
DNA G+C content (mol%)	48.5	46.4	44.9

*Medium described by Staley (1968).

genus *Marinococcus*. However, strain YIM 70157^T differs from *M. halophilus* DSM 20408^T by the ability to utilize mannose, dextrin and cellobiose as sole carbon sources, oxidative acid production from aesculin, and some differences in enzymic properties (Table 1). DNA–DNA hybridization confirmed the differentiation of both strains at the species level. These pieces of evidence show that the novel isolate YIM 70157^T represents a hitherto unknown species of *Marinococcus*, for which the name *Marinococcus halotolerans* sp. nov. is proposed.

Description of *Marinococcus halotolerans* sp. nov.

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

Aerobic, Gram-positive, non-spore-forming, motile, spherical with a single polar flagellum; cell diameter is about

1.0–1.2 μ m. The colony colour on most media tested is orange. Colonies are circular, opaque and approximately 1.5–1.8 mm in diameter after 24 h at 28 °C. The optimum concentration of MgCl₂·6H₂O for growth is 10% (w/v) (MgCl₂·6H₂O can also be substituted by NaCl or KCl). The optimum growth pH and temperature are 7.0–7.5 and 28 °C, respectively. Concentration ranges of MgCl₂·6H₂O, NaCl and KCl for growth are 0–20, 0–25 and 0–25%, respectively. Catalase-positive and oxidase-negative. Positive for nitrate reduction, but negative for gelatin liquefaction, ammonia production, in methyl red and Voges–Proskauer tests, and for milk peptonization and coagulation, growth on cellulose, H₂S and melanin production, casein and starch hydrolysis. The following substrates are utilized: maltose, mannitol, glucose, mannose, fructose, cellobiose, salicin, acetamide, galactose, xylose and dextrin. Adonitol, arabinose, arabitol, rhamnose, inositol and sorbitol are not utilized. Acid is produced from aesculin, glucose and mannitol. The peptidoglycan type is A1 γ (*meso*-diaminopimelic acid directly cross-linked). The phospholipids

contain phosphatidylinositol and diphosphatidylglycerol. The menaquinones are MK-7 (91.6%) and MK-6 (8.4%). The fatty acid profiles contain ai-C_{15:0} (37.4%), ai-C_{17:0} (21.11%), i-C_{16:0} (9.07%), i-C_{17:0} (7.69%), i-C_{15:0} (6.92%), C_{16:0} (6.68%) and small amounts of C_{14:0} (0.54%), C_{15:0} (0.55%), C_{17:0} (0.42%), C_{18:0} (0.67%), i-C_{14:0} (2.4%), i-C_{18:0} (0.76%), i-C_{19:0} (0.12%), ai-C_{19:0} (0.25%), C_{16:1 ω 7c} (0.36%), C_{16:1 ω 11c} (1.16%), C_{18:1 ω 9c} (0.16%), i-C_{17:1 ω 10c} (0.11%) and C_{16:1 ω 7c} alcohol (3.14%). The DNA G+C content is 48.5 mol% (HPLC method).

Isolated from a saline soil sample collected from Qinghai, north-west China. The type strain is YIM 70157^T (=DSM 16375^T =KCTC 19045^T).

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