Salinicoccus kunmingensis sp. nov., a moderately halophilic bacterium isolated from a salt mine in Yunnan, south-west China

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A novel yellow-pigmented, non-motile, non-sporulating, catalase- and oxidase-positive, obligately aerobic, moderately halophilic, facultatively alkaliphilic Gram-positive coccus, strain YIM Y15^T, was isolated from a brine sample from a salt mine in Yunnan, south-west China. Strain YIM Y15^T grew in the presence of 0.5–25 % (w/v) NaCl and at pH 6.0–10.0, with optimum growth at 8–10 % (w/v) NaCl and pH 8.0. It grew at 4–45 °C, with optimum growth at 37.0 °C. The major cellular fatty acids were anteiso-C_{15:0} and iso-C_{15:0}. Menaquinone 6 (MK-6) was the major respiratory quinone. The cell wall contained Lys and Gly. The DNA G+C content was 46.2 mol%. Phylogenetic analyses based on 16S rRNA gene sequence comparisons revealed that strain YIM Y15^T was a member of the genus *Salinicoccus*, with low 16S rRNA gene sequence similarity to the type strains of the five described species of the genus, *Salinicoccus alkaliphilus* JCM 11311^T (sequence similarity 96.0 %), *Salinicoccus roseus* DSM 5351^T (94.9 %), *Salinicoccus jeotgali* KCTC 13030^T (94.8 %), *Salinicoccus salsiriae* LMG 22840^T (94.8 %) and *Salinicoccus hispanicus* DSM 5352^T (94.7 %). Together with the phenotypic differences, these results supported the proposal of a novel species of the genus *Salinicoccus*, *Salinicoccus kunmingensis* sp. nov., with YIM Y15^T (=DSM 17847^T =CGMCC 1.6302^T) as the type strain.

Various kinds of moderately/extremely halophilic bacteria and archaea have been isolated from ancient salt deposits, in which several new taxa were identified (Dombrowski, 1966; Grant *et al.*, 1998; Norton & Grant, 1988; Norton *et al.*, 1993; Vreeland & Powers, 1999; Vreeland *et al.*, 2000; McGenity *et al.*, 2000). In a recent study of the microbial diversity of the ancient salt deposit of the Yipinglang salt mine (25° 18' N 101° 54' E) in Yunnan, south-west China, a moderately halophilic, facultatively alkaliphilic strain, YIM Y15^T, was isolated from a brine sample. Polyphasic taxonomic study of the phenotypic, chemotaxonomic and genotypic characteristics and phylogenetic position of strain YIM Y15^T indicated that this strain represented a novel species of the genus *Salinicoccus*.

Brine, saline soil and halite samples were collected from the Yipinglang salt mine at a depth of about 200 m. Serial dilutions (1:10) of the samples were plated on Difco marine agar 2216 (MA; pH 7.2) supplemented with 0–20 % (w/v) NaCl and ISP medium 2 agar (Shirling & Gottlieb, 1966) at 28 °C for 7–28 days. A yellow-pigmented colony, named strain YIM Y15^T, was picked from a brine plate of MA. Unless otherwise indicated, morphological and physiological studies were performed with cells grown on MA supplemented with 8 % (w/v) NaCl at pH 8.0 and 37 °C and on some other media as controls, e.g. nutrient agar, tryptic soy agar (TSA; BBL) and ISP medium 2 agar. The strain was maintained both on agar slants of MA supplemented with 8 % (w/v) NaCl (pH 8.0) at 4 °C and in Difco marine broth (MB) supplemented with 20 % (v/v) glycerol at –80 °C.

Cell morphology was examined by light microscopy (model BH 2; Olympus). Gram staining was carried out

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM Y15^T is DQ837380.

using the standard Gram reaction combined with the KOH lysis test method (Gregersen, 1978). Anaerobic growth was determined using the GasPak Anaerobic System (BBL) according to the manufacturer's instructions. Motility was observed on half-strength MA, solidified with 0.3 % agar, under high-moisture conditions and in a hanging-drop preparation under a $\times 100$ objective lens with oil immersion.

Cells of strain YIM Y15^T were Gram-positive cocci, approximately 0.8–1.2 μm in diameter, occurring singly and in pairs, tetrads or clumps, and strictly aerobic, non-motile and non-sporulating. Colonies were circular, convex, yellow-pigmented and non-translucent with shiny, glistening surfaces and entire margins, 2–3 mm in diameter after incubation for 3 days at 37 °C on MA supplemented with 8 % (w/v) NaCl. No diffusible pigments were produced on any media tested.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of PCR products were carried out as described by Cui *et al.* (2001). Electrophoresis of sequencing reaction products was done by using a Beckman CEQ 2000 sequencer according to the manufacturer's protocol. The resulting 16S rRNA gene sequence was compared to sequences obtained from the GenBank database to find the most closely related species. Phylogenetic analyses were performed using the software package MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignment of sequence data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (corrected by Kimura's two-parameter model; Kimura, 1980) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and parsimony (Kluge & Farris, 1969) trees (not shown) were generated using treeing algorithms contained in the PHYLIP package (Felsenstein, 1993). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985).

The almost-complete 16S rRNA gene sequence (1493 bp) of strain YIM Y15^T was determined. A neighbour-joining tree (Fig. 1) showed that strain YIM Y15^T was closely related to the type strains of the five recognized members of the genus *Salinicoccus* Ventosa *et al.* 1990, and the six strains formed a distinct clade in the phylogenetic tree with significant bootstrap support (99 %), in which strain YIM Y15^T and *Salinicoccus alkaliphilus* JCM 11311^T (Zhang *et al.*, 2002) formed a distinct subclade (Fig. 1). The values of 16S rRNA gene sequence similarity between strain YIM Y15^T and the five closest relatives were 96.0 % (*S. alkaliphilus* JCM 11311^T), 94.9 % (*Salinicoccus roseus* DSM 5351^T; Ventosa *et al.*, 1990), 94.8 % [*Salinicoccus jeotgali* KCTC 13030^T (Aslam *et al.*, 2007); *Salinicoccus salsiriae* LMG 22840^T (França *et al.*, 2006)] and 94.7 % (*Salinicoccus hispanicus* DSM 5352^T; Marquez *et al.*, 1990; Ventosa *et al.*, 1992). It has been suggested that bacterial strains with less than 97 % 16S rRNA gene sequence

Fig. 1. Phylogenetic dendrogram based on 16S rRNA gene sequences and constructed using the neighbour-joining method showing the phylogenetic positions of strains YIM Y15^T and related taxa. Labels 'm' and 'p' indicate branches that were also found with the maximum-likelihood (Felsenstein, 1981) or parsimony (Kluge & Farris, 1969) algorithms; asterisks indicate branches that were recovered with all three methods. Numbers at nodes indicate bootstrap values (>50 %) based on neighbour-joining analyses of 1000 resampled datasets. Bar, 2 substitutions per 100 nucleotides.

identity are members of different genomic species (Stackebrandt & Goebel, 1994). It is therefore evident from the phylogenetic data that strain YIM Y15^T represents a previously unknown species of the genus *Salinicoccus*.

Growth was tested at different temperatures (4, 10, 15, 20, 25, 30, 37, 45 and 50 °C) on MA supplemented with 8 % (w/v) NaCl and at different pH values (5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0) in MB supplemented with 8 % (w/v) NaCl. For pH tolerance experiments, the following buffer solutions were used: pH 5.0, citric acid/sodium citrate; pH 6.0, 7.0, 8.0, NaOH/KH₂PO₄; pH 9.0, 10.0, Na₂CO₃.10H₂O/NaHCO₃; pH 11.0, Na₂HPO₄.10H₂O/NaOH. Tolerance of/requirement for salts was determined on MA containing 0–30 % (w/v) NaCl, MgCl₂ or KCl. For the catalase test, the evolution of bubbles upon addition of 3 % H₂O₂ solution to cells was observed under a stereoscopic microscope. Oxidase activity was tested by the oxidation of 1 % (w/v) tetramethyl- β -phenylenediamine (bioMérieux). Hydrolysis of polymers, urease activity, nitrate reduction and Voges–Proskauer and methyl red tests were determined as described previously (Cowan & Steel, 1965; Gerhardt *et al.*, 1981; Ventosa *et al.*, 1982). Substrate utilization as sole carbon and energy sources, the fermentation/oxidation profile, acid production, activities of constitutive enzymes and some other physiological characteristics were examined using API 20E, API 20NE, API 50CH (with API 50 CH B/E medium) and API ZYM strips (bioMérieux) according to the manufacturer's instructions. Additionally, Biolog GP2 MicroPlates and the MicroLog computer software (Biolog) were used. All suspension media were supplemented with 2 % (w/v)

NaCl. Antibiotic resistance was determined by inoculating bacterial suspension on MA at 37 °C and applying Antibiotics-Sensitabs (Tiantan Medicine Co.). Inhibition zones were measured after 5 days incubation and interpreted according to the manufacturer's manual.

Strain YIM Y15^T was catalase- and oxidase-positive, with a wide growth temperature range of 4–45 °C (optimum 37 °C). It was moderately halophilic, as the optimum NaCl concentration for growth was between 8 and 10 %, with a NaCl concentration range for growth of 0.5–25 % (w/v) (Kushner, 1993). Good growth occurred on MA (containing 19.5 g NaCl l⁻¹) and TSA (containing 5.0 g NaCl l⁻¹), but there was no growth on nutrient agar or ISP 2. Strain YIM Y15^T was a facultatively alkaliphilic organism with a

growth range of pH 6.0–10.0, and optimum growth was observed at pH 8.0. The results of other phenotypic tests are listed in the species description and in Table 1.

Isolation of the cell-wall fraction and preparation of the hydrolysate of the cell wall were carried out using the method of Schleifer (1985). The amino acid composition of the cell-wall hydrolysate was determined by using TLC as described by Staneck & Roberts (1974). Isoprenoid quinones were analysed by HPLC as described by Groth *et al.* (1996). For isoprenoid quinone analyses, the organism was inoculated in MB supplemented with 8 % (w/v) NaCl in flasks on a rotary shaker at 200 r.p.m. The biomass was harvested by centrifugation, washed twice with distilled water and freeze-dried. The fatty acid

Table 1. Differentiating characteristics of strain YIM Y15^T from its closest phylogenetic relatives

Strains: 1, YIM Y15^T (data from this study); 2, *S. alkaliphilus* JCM 11311^T (Zhang *et al.*, 2002); 3, *S. roseus* DSM 5351^T (Ventosa *et al.*, 1990); 4, *S. hispanicus* DSM 5352^T (Marquez *et al.*, 1990; Ventosa *et al.*, 1992); 5, *S. jeotgali* KCTC 13030^T (Aslam *et al.*, 2007); 6, *S. salsiriae* LMG 22840^T (França *et al.*, 2006). +, Positive; –, negative; ND, no data.

Characteristic	1	2	3	4	5	6
Colony pigmentation	Yellow	Pinkish	Pink–red	Reddish orange	Orange	Pink–red
Temperature for growth (°C)						
Range	4–45	10–49	15–37	15–40	20–30	20–45
Optimum	37	32	37	37	30	37
NaCl for growth (%; w/v)						
Range	0.5–25.0	0–25.0	0.5–25.0	0.9–25.0	0.5–15	0–22
Optimum	8–10	10	10	10	5	4
pH for growth						
Range	6.0–10.0	6.5–11.5	5.0–9.0	6.0–9.0	6.5–11.0	6.5–9.5
Optimum	8.0	9.5	7.5	8.0	7.0	8.0
Hydrolysis of:*						
Aesculin	+	–†	–	+	ND	–
Casein	–	–	+	–	–	+
Gelatin	–	–	+	+	–	+
Starch	+	–	+	–	–	–
Tween 80	+	–	+	–	–	ND
Methyl red test*	–	–	–	+ / –†	+	–
Nitrate reduction*	+	+	+ / –†	+	+	+
Urease*	–	+ / –†	–	+ / –†	–	–
Acid production from:						
D-Galactose	–	–	–	+	–	–
D-Glucose	–	+	–	+	+	+
D-Fructose	+	–	–	+	+	+
Glycerol	–	–	–	+	+	+
Maltose	–	–	–	+	+	+
D-Mannitol	+	ND	–	+	–	–
Sucrose	+	–	–	+	–	–
Major fatty acids	ai-C _{15:0} , i-C _{15:0} , i-C _{16:0} , i-C _{17:0}	ai-C _{15:0} , i-C _{15:0} , i-C _{16:0} , ai-C _{17:0}	ai-C _{15:0} , i-C _{15:0} , ai-C _{17:0} , i-C _{16:0}	i-C _{15:0} , ai-C _{15:0} , C _{16:0} , i-C _{16:0}	ai-C _{15:0} , i-C _{15:0} , C _{16:0} , ai-C _{17:0}	ai-C _{15:0} , i- C _{15:0} , i-C _{17:0} , ai-C _{17:0}
Presence of MK-7*	+ (1.2 %)	–	+ (0.8 %)†	+ (trace)†	+ (trace)	ND
DNA G + C content (mol%)	46.2	49.6	51.2	45.7	47.0	46.2

*Unless otherwise indicated, results of these tests for *S. alkaliphilus* JCM 11311^T, *S. roseus* DSM 5351^T and *S. hispanicus* DSM 5352^T were confirmed in parallel tests with strain YIM Y15^T under identical conditions. The Voges–Proskauer test was negative for all four strains in the parallel test.

†Results from this study.

composition was determined as described by Sasser (1990) using the Microbial Identification System (MIDI; Microbial ID) with cells grown on MA supplemented with 8% (w/v) NaCl for 3 days at 37 °C. DNA was isolated according to Hopwood *et al.* (1985) and its G+C content was determined by the thermal denaturation method (Mandel & Marmur, 1968) with a Shimadzu UV-visible spectrophotometer (UV1601).

Chemotaxonomic data for strain YIM Y15^T are compatible with its assignment to the genus *Salinicoccus*. The major amino acid constituents of the cell-wall hydrolysate were glycine and lysine, which is compatible with the murein type of L-Lys-Gly₅ described for the genus *Salinicoccus* (Ventosa *et al.*, 1990). The strain studied had menaquinone 6 (MK-6) (98.8%) and MK-7 (1.2%) as its respiratory quinones. The fatty acid profiles of strain YIM Y15^T and related type strains are given in Table 2. The major fatty acids of the strain studied were iso- and anteiso-branched fatty acids [ai-C_{15:0} (28.4%), i-C_{15:0} (23.1%), ai-C_{17:0} (5.8%), i-C_{16:0} (5.8%) and i-C_{17:0} (5.7%)]. The DNA G+C content of strain YIM Y15^T was 46.2 mol%.

The yellow pigmentation and wide growth temperature range (4–45 °C) of strain YIM Y15^T, together with the presence of MK-7 (1.2%) in minor amounts and some biochemical characteristics, such as the ability to hydrolyse aesculin and Tween 80 and acid production from D-fructose, D-mannitol and sucrose, differed markedly from the five described *Salinicoccus* species (Table 1).

On the basis of the phylogenetic, chemotaxonomic and phenotypic results presented above, we propose that strain YIM Y15^T represents a novel species of the genus *Salinicoccus*, *Salinicoccus kunmingensis* sp. nov.

Description of *Salinicoccus kunmingensis* sp. nov.

Salinicoccus kunmingensis (kun.ming.en'sis. N.L. masc. adj. *kunmingensis* pertaining to Kunming, a city in south-west China, near which the sample from which the type strain was isolated was collected).

Cells are Gram-positive, non-motile, non-sporulating, catalase- and oxidase-positive, obligately aerobic cocci (0.8–1.2 µm) that occur singly or in pairs, tetrads or clumps. Colonies are circular, convex, yellow-pigmented and non-translucent with shiny, glistening surfaces and entire margins, 2–3 mm in diameter after 3 days on MA supplemented with 8% (w/v) NaCl at pH 8.0 and 37 °C. No diffusible pigments are produced on any media tested. Growth occurs at 4–45 °C (optimum 37 °C) and pH 6.0–10.0 (optimum pH 8.0). Moderately halophilic, with growth at 0.5–25% (w/v) NaCl [optimum 8–10% (w/v) NaCl]. Also grows well on MA supplemented with 2–15% (w/v) MgCl₂ or KCl instead of NaCl. Positive for hydrolysis of aesculin, starch and Tween 80, but negative for hydrolysis of casein, chitin, gelatin and Tween 20. Nitrate is reduced to nitrite. Voges–Proskauer and methyl red tests

Table 2. Fatty acid composition of strain YIM Y15^T and members of the genus *Salinicoccus*

Strains: 1, YIM Y15^T (data from this study); 2, *S. alkaliphilus* JCM 11311^T (data from Zhang *et al.*, 2002); 3, *S. roseus* DSM 5351^T; 4, *S. hispanicus* DSM 5352^T (data in columns 3 and 4 from Yoon *et al.*, 2003); 5, *S. jeotgali* KCTC 13030^T (Aslam *et al.*, 2007); 6, *S. salsiriae* LMG 22840^T (França *et al.*, 2006). Values are percentages of total fatty acids. —, Not detected; ND, no data. Other components in the fatty acid pool of strain YIM Y15^T (%) are iso-C_{15:1} at 5 (0.9), iso-C_{15:1} I (0.5), anteiso-C_{15:1} A (0.3), C_{17:0} (0.2) and iso-C_{17:1} I (0.6).

Fatty acid	1	2	3	4	5	6
Saturated fatty acids						
C _{14:0}	1.9	1.6	—	0.5	4.4	0.8 ± 0.2
C _{15:0}	0.7	—	0.7	0.5	—	—
C _{16:0}	3.2	1.5	1.0	2.7	10.3	2.9 ± 0.4
C _{17:0} 3-OH	—	—	0.5	—	—	ND
C _{18:0}	2.1	—	—	1.5	—	0.7 ± 0.1
C _{20:0}	0.2	1.3	—	—	—	2.1 ± 0.5
Unsaturated fatty acids						
C _{16:1} ω7c alcohol	3.4	5.8	0.6	0.8	4.1	—
C _{16:1} ω11c	2.7	2.2	—	—	3.6	—
C _{18:1} ω5c	—	—	0.7	—	—	ND
C _{18:1} ω7c	0.4	—	—	0.7	—	ND
C _{18:1} ω9c	0.5	—	—	0.5	—	ND
Branched fatty acids						
iso-C _{13:0} 3-OH	1.0	—	—	—	—	ND
iso-C _{14:0}	2.9	4.4	0.6	0.5	3.2	1.0 ± 0.1
iso-C _{15:0}	23.1	22.3	12.5	20.8	22.8	26.6 ± 2.2
anteiso-C _{15:0}	28.4	27.6	32.9	35.9	32.4	35.3 ± 0.7
iso-C _{16:0}	5.8	10.1	4.3	2.5	4.1	3.5 ± 0.6
iso-C _{17:0}	5.7	3.3	6.2	8.8	—	9.5 ± 0.8
iso-C _{17:1} ω10c	4.7	4.0	7.3	3.5	—	3 ± 0.3
iso-C _{17:0} 3-OH	—	—	1.0	—	—	ND
anteiso-C _{17:0}	5.8	8.9	13.5	11.9	6.2	8.5 ± 0.6
iso-C _{18:0}	0.5	—	0.6	—	—	ND
iso-C _{19:0}	0.7	2.8	2.3	2.5	—	3.0 ± 0.1
anteiso-C _{19:0}	1.0	1.9	5.8	1.7	—	1.0 ± 0.2
iso-C _{20:0}	—	1.5	—	—	—	—
Summed feature 4*	—	—	0.8	0.6	—	ND
Unknown†	2.8	0.9	1.4	1.2	4.3	2.3 ± 0.3

*Summed feature 4 represents iso-C_{17:1} I and/or anteiso-C_{17:1} B, which could not be separated by GLC with the MIDI system.

†Unknown fatty acids with equivalent chain-lengths of 15.665 for *S. alkaliphilus* JCM 11311^T, 15.669 for strain YIM Y15^T, *S. roseus* DSM 5351^T, *S. hispanicus* DSM 5352^T and *S. jeotgali* KCTC 13030^T and 15.670 for *S. salsiriae* LMG 22840^T.

are negative. H₂S and indole are not produced. Cells are resistant to gentamicin (10 µg), kanamycin (30 µg), lincomycin (2 µg), polymyxin B (30 µg) and streptomycin (10 µg), but ampicillin (30 µg), chloramphenicol (30 µg), nalidixic acid (20 µg), novobiocin (30 µg), rifampicin (5 µg) and tetracycline (30 µg) inhibit growth. In API 20NE strips, a positive result is obtained for assimilation of potassium gluconate and negative results are obtained for

assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid and fermentation of glucose. Acid is produced from amygdalin, *N*-acetylglucosamine, D-fructose, inositol, D-lactose, D-mannitol and sucrose in API 50CH strips. In Biolog GP2 plates, the following substrates are used as sole carbon and energy sources: *N*-acetyl-D-glucosamine, amygdalin, L-arabinose, D-cellobiose, D-fructose, D-glucose, glycerol, γ -hydroxybutyric acid, *myo*-inositol, α -D-lactose, D-mannitol, D-mannose, D-psicose, D-raffinose, L-rhamnose, D-ribose, sucrose, trehalose, Tween 40, L-alaninamide and L-asparagine. Constitutive enzymes expressed by the type strain are catalase, cytochrome oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, cystine arylamidase, α -chymotrypsin, α -mannosidase, gelatinase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and valine arylamidase. Arginine dihydrolase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, β -glucuronidase, trypsin, *N*-acetyl- β -glucosaminidase, β -galactosidase/*o*-nitrophenyl- β -D-galactopyranosidase (ONPG), tryptophan deaminase, lysine decarboxylase, β -galactosidase/*p*-nitrophenyl- β -D-galactopyranosidase (PNPG), ornithine decarboxylase, α -fucosidase and urease are not produced. The assumed cell-wall murein type is L-Lys-Gly₅. The predominant respiratory quinone is MK-6, and MK-7 is present in minor amounts. The major cellular fatty acids are anteiso-C_{15:0} (28.4 %) and iso-C_{15:0} (23.1 %). The DNA G + C content of the type strain is 46.2 mol%.

The type strain, YIM Y15^T (=DSM 17847^T =CGMCC 1.6302^T), was isolated from a brine sample collected from the Yipinglang salt mine in Kunming, south-west China.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (NSFC) (30460004, 30660004, 30360004, 30560001 and 30260004), SRF for ROCS (SEM), the Yunnan Provincial Sciences and Technology Department (2005PY01-1, 2004C0002Z and 2006C0006M) and the National Basic Research Program of China (973 Program, no. 2004CB719601). We are grateful to Wen-Dong Zhang, Qin Zhou, Yu-Rong Yang and Li-Xia Duan for their excellent technical assistance. We are grateful to Dr Jean Euzéby for recommending the proper etymology.

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