Salinicoccus albus sp. nov., a halophilic bacterium from a salt mine

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A novel Gram-positive-staining, catalase- and oxidase-positive, non-motile, non-spore-forming coccus, designated YIM-Y21^T, was isolated from a salt mine in Yunnan, south-west China. The strain was moderately halophilic, facultatively alkaliphilic and obligately aerobic. Colonies were white. Growth occurred with 1.0–30.0 % NaCl (optimum, 10.0 % NaCl), at pH 6.0–10.0 (optimum, pH 8.5) and at 5–40 °C (optimum, 25 °C). The major amino acid constituents of the cell wall were glycine and lysine. The major cellular fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{15:0}. MK-6 was the predominant respiratory quinone, with MK-7 present in minor amounts. The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol and an unidentified phospholipid. The DNA G + C content was 46.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain YIM-Y21^T was most closely related to the type strains of the nine recognized species of the genus *Salinicoccus*. The sequence similarities between the isolate and the type strains of members of this genus were in the range of 90.6–93.8 %, which indicated that strain YIM-Y21^T is a representative of a novel species within the genus *Salinicoccus*. Together with the phenotypic characteristics and chemotaxonomic differences, these results support the proposal of a novel species of the genus *Salinicoccus*, *Salinicoccus albus* sp. nov., with YIM-Y21^T (=CCTCC AA 207024^T =DSM 19776^T =KCTC 13192^T) as the type strain.

The genus *Salinicoccus* was proposed by Ventosa *et al.* (1990), and was defined as containing Gram-positive, catalase- and oxidase-positive, non-motile, non-spore-forming cocci, with MK-6 as the predominant respiratory quinone and a cell-wall peptidoglycan type of L-Lys–Gly₅. At the time of writing, the genus comprised nine species with validly published names: *Salinicoccus roseus* and *S. hispanicus* (Ventosa *et al.*, 1990, 1992), *S. alkaliphilus* (Zhang *et al.*, 2002), *S. salsiriae* (Franca *et al.*, 2006), *S. jeotgali* (Aslam *et al.*, 2007), *S. luteus* (Zhang *et al.*, 2007), *S. siamensis* (Pakdeeto *et al.*, 2007), *S. kunmingensis* (Chen *et al.*, 2007a) and *S. iranensis* (Amoozegar *et al.*, 2008). In a

recent study of the microbial diversity in the ancient salt deposit of the Yipinglang salt mine (25° 18' N 101° 54' E) in Yunnan, south-west China (Chen *et al.*, 2007a, b), a moderately halophilic, facultatively alkaliphilic strain, YIM-Y21^T, was isolated from a subterranean brine sample. Polyphasic taxonomic study of the phenotypic, chemotaxonomic and phylogenetic position of strain YIM-Y21^T indicate that this strain is a representative of a novel species of the genus *Salinicoccus*.

A brine sample (salt-saturated, pH 6.8) was collected from the Yipinglang salt mine at a depth of about 200 m. Serial 1 : 10 dilutions of the sample were plated on Difco marine agar 2216 (MA; pH 7.5) supplemented with 0–30.0 % NaCl at 28 °C for 7–28 days. A white colony was picked from a plate of MA supplemented with 10.0 % NaCl (MA10). After primary isolation and purification, the isolate, named strain YIM-Y21^T, was preserved both on MA10 slants at 4 °C and in Difco marine broth 2216 (MB) supplemented

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM-Y21^T is EF177692.

A supplementary table of the fatty acid compositions of strain YIM-Y21^T and other members of the genus *Salinicoccus* is available with the online version of this paper.

with 20 % glycerol at -80°C . The reference strain *S. kunmingensis* YIM Y15^T, which was employed as a control in phenotypic tests, was obtained from the collection of the Yunnan Institute of Microbiology. Unless otherwise indicated, morphological and physiological studies were performed with cells grown on MA10 (pH 8.5) at 25°C . Cell morphology was examined with a light microscope (BH 2; Olympus) as well as with a transmission electron microscope (H-800; Hitachi). Gram staining was carried out by using the standard Gram reaction (Doetsch, 1981) combined with the KOH lysis test (Gregersen, 1978). Growth was tested at various temperatures (5 – 55°C , in increments of 5°C) on MA10 and at different pH values (5.0 – 11.0 , in increments of 0.5 pH units) on MA10 as well as in MB supplemented with 10.0% NaCl. For pH endurance experiments, the following buffer systems were used: pH 5.0 , 0.1 M citric acid/ 0.1 M sodium citrate; pH 6.0 – 8.0 , 0.1 M KH_2PO_4 / 0.1 M NaOH; pH 9.0 – 10.0 , 0.1 M NaHCO_3 / 0.1 M Na_2CO_3 ; pH 11.0 , 0.1 M Na_2HPO_4 / 0.1 M NaOH. Tolerance of and requirement for salts were determined in nutrient broth (Difco) (NB; 3.0 g beef extract and 5.0 g peptone l^{-1}) at various concentrations of NaCl, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ or KCl (0.5% and 1.0 – 33.0% in increments of 1.0%) supplemented with modified artificial seawater [containing (l^{-1}) 5.94 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.64 g KCl and 1.3 g CaCl_2] (Lim *et al.*, 2005), with trypticase soy agar (TSA; Difco) and ISP medium 2 agar (Shirling & Gottlieb, 1966) as controls. Methyl red and Voges–Proskauer tests and determination of aesculin hydrolysis, indole and H_2S production, and nitrate and nitrite reduction were performed as recommended by Smibert & Krieg (1994). Hydrolysis of casein, DNA, gelatin, starch, Tweens 20, 40, 60 and 80 and urease activity were determined as described by Cowan & Steel (1965). Observation of motility and tests for antibiotic susceptibility, anaerobic growth and catalase and oxidase activities were performed as described by Chen *et al.* (2007a). Other enzyme activities were assayed by using API ZYM strips (bioMérieux) according to the manufacturer's instructions. Acid production from carbohydrates and utilization of carbon and nitrogen sources were determined as recommended by Ventosa *et al.* (1982), as well as by employing the API 50CH system (bioMérieux) and GP2 MicroPlates (Biolog) according to the manufacturers' instructions. All suspension media were supplemented with 10.0% NaCl and artificial seawater and incubated at 25°C . The results of the phenotypic tests are given in the species description and in Table 1.

DNA was isolated according to Hopwood *et al.* (1985) and the G+C content was determined by using the HPLC method (Mesbah *et al.*, 1989). Genomic DNA extraction, PCR-mediated amplification of 16S rRNA gene and purification of PCR products were done as described previously (Cui *et al.*, 2001). Phylogenetic analysis was performed by using the software package MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of sequence data with 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 by using the algorithms contained in the PHYLIP package (Felsenstein, 2002). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985).

The DNA G+C content of strain YIM-Y21^T was $46.1\text{ mol}\%$. The almost-complete 16S rRNA gene sequence (1478 bp) of the strain was determined. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the novel isolate belonged to the family *Staphylococcaceae* and was closely related to the type strains of the nine recognized species of the genus *Salinicoccus* (Ventosa *et al.*, 1990). The ten sequences formed a distinct cluster in the phylogenetic tree, in which strain YIM-Y21^T occupied a distinct sub-branch at the periphery of the cluster, supported by a significant bootstrap resampling value (99%) (Fig. 1). The values of sequence similarity between the isolate and the type strains of members of the genus *Salinicoccus* were in the range of 90.6% (*S. iranensis* QW6^T) to 93.8% (*S. kunmingensis* YIM Y15^T). Species of the genus *Jeotgalicoccus* (Yoon *et al.*, 2003; Hoyle *et al.*, 2004) were the next closest relatives, but they formed a quite separate and robust cluster (Fig. 1). It is therefore evident from the phylogenetic data that strain YIM-Y21^T could be a representative of a previously unknown species of the genus *Salinicoccus*.

Isolation of the cell-wall fraction and preparation of the cell-wall hydrolysate were carried out by using the method of Schleifer (1985). The amino acid composition of the cell-wall hydrolysate was determined using TLC as described by Stanek & Roberts (1974). Polar lipids were extracted by the method of Minnikin *et al.* (1979) and identified by separating with 2D TLC and spraying the chromatogram with specific reagents (Collins & Jones, 1980). Isoprenoid quinones were analysed by HPLC as described by Groth *et al.* (1996). The fatty acid composition was determined as described by Sasser (1990) using the Microbial Identification System (MIDI; Microbial ID) with cells grown in MB supplemented with 10.0% NaCl (pH 8.5) in flasks on a rotary shaker at 200 r.p.m. and 25°C for 3 days. The chemotaxonomic data for strain YIM-Y21^T were compatible with its assignment to the genus *Salinicoccus*. The major amino acid constituents of the cell-wall hydrolysate were glycine and lysine, which are compatible with the peptidoglycan type of L-Lys–Gly₅ described for this genus (Ventosa *et al.*, 1990). The fatty acid profile of strain YIM-Y21^T was similar to those of the type strains of the genus *Salinicoccus* (Supplementary Table S1, available in IJSEM Online). Major fatty acids of this strain were anteiso- $\text{C}_{15:0}$ (42.6%), anteiso- $\text{C}_{17:0}$ (15.4%) and iso- $\text{C}_{15:0}$ (13.7%). Menaquinone 6 (MK-6) (69.2%) and MK-7 (30.8%) were the respiratory quinones. The polar lipids of the strain consisted of diphosphatidylgly-

Table 1. Differentiating characteristics of strain YIM-Y21^T and the type strains of other *Salinicoccus* species

Strains: 1, YIM-Y21^T (data from this study); 2, *S. kunmingensis* YIM Y15^T (Chen *et al.*, 2007a); 3, *S. alkaliphilus* T8^T (unless indicated, data from Zhang *et al.*, 2002); 4, *S. roseus* DSM 5351^T (Ventosa *et al.*, 1990); 5, *S. hispanicus* DSM 5352^T (Marquez *et al.*, 1990; Ventosa *et al.*, 1992); 6, *S. jeotgali* S2R53-5^T (Aslam *et al.*, 2007); 7, *S. salsiraiiae* RH1^T (Franca *et al.*, 2006); 8, *S. siamensis* PN1-2^T (Pakdeeto *et al.*, 2007); 9, *S. luteus* YIM 70202^T (Zhang *et al.*, 2007); 10, *S. iranensis* QW6^T (Amoozegar *et al.*, 2008). +, Positive; –, negative; tr, trace amount; ND, no data available. All strains possess MK-6 as the predominant respiratory quinone.

Characteristic	1	2	3	4	5	6	7	8	9	10
Colony colour	White	Yellow	Pinkish	Pink–red	Reddish orange	Orange	Pink–red	Orange	Orange	Orange–pink
Growth temperature (°C)										
Range	5–40	4–45	10–49	15–37	15–40	20–30	20–45	15–45	4–45	5.0–45.0
Optimum	25	37	32	37	37	30	37	37	30	35
Growth in NaCl (%)										
Range	1.0–30.0	0.5–25	0–25	0.5–25	0.9–25	0.5–15	0–22	1.5–25	1–25	1.0–25.0
Optimum	10	8–10	10	10	10	5	4	10	10	7.5–10.0
Growth pH										
Range	6.0–10.0	6.0–10.0	6.5–11.5	5.0–9.0	6.0–9.0	6.5–11.0	6.5–9.5	6–9	7.0–11.0	6.5–10
Optimum	8.5	8.0	9.5	7.5	8.0	7.0	8.0	8.5	8.0–9.0	7.5
Hydrolysis of:										
Aesculin	–	+	+	–	+	+	–	–	+	–
Casein	–	–	–	+	–	–	+	–	–	–
Gelatin	–	–	–	+	+	–	+	–	–	–
Starch	–	+	–	+	–	–	–	–	–	–
Tween 80	+	+	–	+	–	–	ND	–	–	–
Nitrate reduced to nitrite	+	+	+	+	+	+	+	–	+	+
Indole production	–	–	–	–	–	+	–	–	–	+
Methyl red test	–	–	–	–	+	+	–	–	–	–
Urease	–	–	+	–	+	–	–	–	–	–
Voges–Proskauer test	+	–	–	–	–	–	–	–	–	–
Acid production from:										
D-Fructose	+	+	–	–	+	+	+	+	–	+
D-Galactose	–	–	–	–	+	–	–	–	–	–
D-Glucose	+	–	+	–	+	+	+	+	+	+
Glycerol	–	–	–	–	+	+	+	+	ND	–
Maltose	–	–	–	–	+	+	+	–	+	–
D-Mannitol	+	+	+	–	+	–	–	–	ND	+
Sucrose	–	+	–	–	+	–	–	–	+	–
Presence of MK-7 (%)†	30.8	1.2	–	0.8*	tr*	tr	ND	ND	+	tr
DNA G + C content (mol%)	46.1	46.2	49.6	51.2	45.7	47.0	46.2	46.0	49.7	46.2

*Data from Chen *et al.* (2007a).

†Percentages of total respiratory quinones are given where available.

cerol, phosphatidylglycerol and an unidentified phospholipid.

The results of phylogenetic analysis and chemotaxonomic studies supported the view that strain YIM-Y21^T should be assigned to the genus *Salinicoccus*. However, the white colony colour of strain YIM-Y21^T, the strain's ability to tolerate up to 30.0% NaCl and the comparatively low optimum temperature (25 °C) for growth, as well as the positive result of Voges–Proskauer test, the significant amount of MK-7 (Table 1) and the high 16S rRNA gene sequence divergence (more than 6%) from those of the other members of the genus, differentiated strain YIM-Y21^T markedly from the type strains of the nine recognized *Salinicoccus* species. In

conclusion, phylogenetic analysis based on 16S rRNA gene sequence data, its phenotypic distinctiveness and the chemotaxonomic data suggest that strain YIM-Y21^T represents a novel species of the genus *Salinicoccus*, for which the name *Salinicoccus albus* sp. nov. is proposed.

Description of *Salinicoccus albus* sp. nov.

Salinicoccus albus (al'bus. L. masc. adj. *albus* white, referring to the colony colour).

Cells stain Gram-positive and are non-motile, non-sporulating, obligately aerobic cocci (0.6–1.1 µm) that occur singly or in pairs, tetrads or clumps. Colonies are

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence analysis and constructed using the neighbour-joining method showing the phylogenetic positions of strains YIM-Y21^T and related taxa. p, Branches that were also recovered with the maximum-parsimony (Kluge & Farris, 1969) algorithm; *, branches that were also recovered using the maximum-likelihood (Felsenstein, 1981) and parsimony algorithms. Numbers at nodes indicate bootstrap values ($\geq 50\%$) based on neighbour-joining analysis of 1000 resampled datasets. Bar, 2 substitutions per 100 nt.

white, circular, convex, smooth, non-translucent with entire margins, 1–2 mm in diameter after incubation at 25 °C for 3–5 days on MA supplemented with 10.0 % NaCl. No diffusible pigments are produced. Moderately halophilic and facultatively alkaliphilic, growth occurring with 1.0–30.0 % NaCl (optimum, 10.0 % NaCl) and at pH 6.0–10.0 (optimum, pH 8.5). NaCl cannot be replaced by $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ or KCl. Growth at 5–40 °C, with optimum growth at 25 °C. Positive for hydrolysis of Tweens 40 and 80, but negative for hydrolysis of casein, DNA, aesculin, gelatin, starch and Tweens 20 and 60. H_2S and indole are not produced. Voges–Proskauer test is positive but the methyl red test is negative. Nitrate is reduced to nitrite, but nitrite is not reduced to nitrogen gas. Acid is produced from D-arabinose, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol and D-xylose, but not from D-adonitol, L-arabinose, cellobiose, glycerol, D-lactose, maltose, melezitose, melibiose, raffinose, L-rhamnose, salicin, L-sorbose, sucrose or trehalose. Sole carbon and energy sources are L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol, D-mannitol, D-mannose, raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, trehalose and D-xylose. The following substances are not used as sole sources of carbon or nitrogen: D-adonitol, dulcitol, D-fucose, inulin, D-lactose, maltose, melezitose, melibiose, salicin, adonitol, ethanol, acetate, benzoate, butyrate, citrate, formate, fumarate, D-gluconate, malate, malonate, pyruvate, suberate, succinate, L-alanine, L-arginine, L-asparagine, L-cysteine, L-cystine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-serine, threonine, L-tryptophan and L-valine. With the API 50CH system, the

following organic compounds are metabolized: D-arabinose, D-arabitol, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, potassium 2-ketogluconate, potassium 5-ketogluconate, D-sorbitol and D-xylose. The following substrates are oxidized in the Biolog GP system: D-arabitol, D-fructose, D-mannose, D-propionic acid, L-rhamnose, D-ribose, D-sorbitol and Tweens 40 and 80. Constitutive expression of acid phosphatase, alkaline phosphatase, catalase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and oxidase, but not α -chymotrypsin, cystine arylamidase, α -fucosidase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, β -glucuronidase, α -mannosidase, trypsin, lipase (C14), urease or valine arylamidase. Cells are resistant to gentamicin (10 μg), kanamycin (30 μg), lincomycin (2 μg), polymyxin B (30 μg) and streptomycin (10 μg), but susceptible to ampicillin (30 μg), chloramphenicol (30 μg), nalidixic acid (20 μg), novobiocin (30 μg), rifampicin (5 μg) and tetracycline (30 μg). The major amino acid constituents of the cell-wall hydrolysate are glycine and lysine. MK-6 is the predominant respiratory quinone (69.2 % in the type strain), with MK-7 (30.8 % in the type strain) present in minor amounts. The polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol and one unknown phospholipid. The major cellular fatty acids are anteiso- $\text{C}_{15:0}$, anteiso- $\text{C}_{17:0}$ and iso- $\text{C}_{15:0}$. The G + C content of the DNA of the type strain is 46.1 mol%.

The type strain, YIM-Y21^T (=CCTCC AA 207024^T =DSM 19776^T =KCTC 13192^T), was isolated from a brine sample collected from the Yipinglang salt mine in Yunnan, south-west China.

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