Salinicoccus iranensis sp. nov., a novel moderate halophile

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A novel moderately halophilic, Gram-positive bacterium, designated strain QW6^T, was isolated from textile industry wastewater in Qom, Iran. Strain QW6^T was strictly aerobic, non-motile, non-sporulating and oxidase- and catalase-positive. It grew at salinities of 1–25 % (w/v) NaCl, showing optimal growth at 7.5–10.0 % (w/v). Growth occurred at 5.0–45.0 °C and over the pH range 6.5–10.0; growth was optimal at 35 °C and pH 7.5. Phylogenetic analysis based on 16S rRNA gene sequence comparisons indicated that strain QW6^T is a member of the genus *Salinicoccus*. The organism possessed Lys- and Gly-containing peptidoglycan. Strain QW6^T had iso-C_{15:0} and anteiso-C_{15:0} as the predominant fatty acids and MK-6 as the major respiratory lipoquinone. The chemotaxonomic profile of this organism was consistent with its classification in the genus *Salinicoccus*. The DNA G + C content of strain QW6^T was 54.4 mol%. On the basis of phenotypic characteristics, 16S rRNA gene sequence analysis and DNA–DNA relatedness of less than 50 % to species of the genus *Salinicoccus*, it is proposed that strain QW6^T (=DSM 18903^T=CCM 7448^T) should be placed in the genus *Salinicoccus* as the type strain of a novel species, *Salinicoccus iranensis* sp. nov.

The genus *Salinicoccus* accommodates aerobic, moderately halophilic bacteria and, at the time of writing, comprised eight species with validly published names: *Salinicoccus roseus* and *Salinicoccus hispanicus* (Ventosa *et al.*, 1990, 1992), *Salinicoccus alkaliphilus* (Zhang *et al.*, 2002), *Salinicoccus salsiriae* (França *et al.*, 2006), *Salinicoccus jeotgali* (Aslam *et al.*, 2007), *Salinicoccus luteus* (Zhang *et al.*, 2007), *Salinicoccus siamensis* (Pakdeeto *et al.*, 2007) and *Salinicoccus kunmingensis* (Chen *et al.*, 2007).

The textile industry produces large quantities of wastewater containing pollutants such as azo dyes and toxic oxyanions that are of major environmental concern. Moderately halophilic bacteria can play an important role in bio-remediation and transformation of these toxic compounds in such polluted saline environments. In this study, a toxic-oxyanion-tolerant, moderately halophilic, aerobic, Gram-positive coccus, designated strain QW6^T, isolated from wastewaters of the textile industry in Qom, Iran, is described on the basis of a polyphasic study. The salinity of the wastewater samples from Qom region was determined to be about 3 % (w/v, as total salt) and the

pH value of the sample was 7.9. Aliquots of the sample were added to 10 % MH medium (Ventosa *et al.*, 1982) supplemented with sea salts solution as follows (% w/v): NaCl, 8.1; MgCl₂, 0.7; MgSO₄, 0.96; CaCl₂, 0.036; KCl, 0.2; NaHCO₃, 0.006; NaBr, 0.0026; proteose-peptone no. 3, 0.5; yeast extract, 1.0, and glucose, 0.1. The pH was adjusted to 7.5 with 1 M KOH prior to autoclaving. Incubation was carried out at 35 °C under aerobic conditions on an orbital shaking incubator (orbital incubator SI 50; Stuart Scientific) at 150 r.p.m. After 2 days incubation, cultures were streaked on agar plates and gave rise to different types of colonies. Pure cultures were isolated after repeated restreaking.

A pure culture of strain QW6^T was grown and maintained in 10 % MH medium solidified by adding 15 g agar (M593 agar) (<http://www.dsmz.de/media/med593.htm>).

Incubation was carried out on a shaker at 150 r.p.m. and 35 °C. Morphological, physiological and biochemical characteristics of the isolate were studied in 10 % MH medium at pH 7.5 and 35 °C, unless stated otherwise.

Reference strains *S. roseus* DSM 5351^T, *S. hispanicus* DSM 5352^T, *S. alkaliphilus* DSM 16010^T, *S. salsiriae* DSM 19496^T and *S. kunmingensis* DSM 17847^T were obtained from the DSMZ.

Abbreviation: SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain QW6^T is DQ767692.

Cell morphology was examined by light microscopy and scanning electron microscopy (SEM) using cells from exponentially growing cultures. Strain QW6^T was prepared for SEM according to the method of Bozzola & Russell (1999) and specimens were observed on a Zeiss DSM 960 EM. Gram staining was performed by the Burke method (Murray *et al.*, 1994) and the result was confirmed by the KOH test (Baron & Finegold, 1990). Motility was analysed by the wet-mount method (Murray *et al.*, 1994). Catalase, oxidase and urease activities, nitrate reduction, hydrolysis of aesculin and production of indole were tested as recommended by Smibert & Krieg (1994). Hydrolysis of Tween 80 was examined as described by Harrigan & McCance (1976). Methyl red and Voges–Proskauer tests were done as recommended by Smibert & Krieg (1994). Determination of acid production from carbohydrates, as well as utilization of carbon and nitrogen sources, was performed as recommended by Ventosa *et al.* (1982).

Antibiotic susceptibility tests were performed on Mueller–Hinton agar plus 10 % (w/v) sea salts seeded with a bacterial suspension containing 1.5×10^6 c.f.u. ml⁻¹ using discs (HiMedia) impregnated with various antibiotics. The plates were incubated at 35 °C for 48 h and the inhibition zone was interpreted according to the manufacturer's manual. To determine the optimal temperature and pH for growth of the strain, broth cultures were incubated at temperatures of 5–55 °C at intervals of 5 °C and at pH 5–11 at intervals of 0.5 pH units. pH values below 6, pH 6–9 and pH values above 9 were obtained using sodium acetate/acetic acid, Tris/HCl and glycine/sodium hydroxide buffers, respectively. Growth at different salt contents (0, 2.5, 5, 7.5, 10, 15, 20, 25 and 30 %, w/v) was tested on MH medium at pH 7.5. Growth was monitored by turbidity at OD₆₀₀ using a spectroscopic method (model UV-160 A; Shimadzu). Other physiological and biochemical tests were performed as described previously (Mata *et al.*, 2002; Quesada *et al.*, 1984). As the habitat of the isolate was polluted by toxic compounds such as tellurite, selenite and selenate oxyanions, the resistance of strain QW6^T to these oxyanions was measured by the agar dilution method of Washington & Sutter (1980). Melted nutrient agar (20 ml) plus various concentrations of tellurite (0.1–40 mM), selenite (1 mM to 1 M) and selenate (1 mM to 1.2 M) were poured separately into 8 cm plates. Then, 10 µl bacterial suspension (1.5×10^8 c.f.u. ml⁻¹) was inoculated onto each plate using a sampler followed by incubation at 35 °C for 7 days. Minimum inhibitory concentrations for tellurite, selenite and selenate oxyanions were determined. Each plate was prepared in triplicate.

Preparation and hydrolysis of the cell wall was carried out using the method of Schleifer (1985) and the interpeptide bridge in the cell-wall peptidoglycan was analysed by using the method described by Schleifer & Kandler (1972). Cell-wall hydrolysates were separated by one- or two-dimensional chromatography on cellulose thin-layer plates (Merck).

Isoprenoid quinone analysis was carried out as described by Monciardini *et al.* (2003). Cellular fatty acids were

extracted from cells of strain QW6^T, *S. roseus* DSM 5351^T, *S. hispanicus* DSM 5352^T, *S. alkaliphilus* DSM 16010^T and *S. salsiriae* DSM 19496^T that had been cultivated on 10 % MH medium at 37 °C for 1 day according to Stead *et al.* (1992) and were analysed by GC (Groth *et al.*, 1996).

For determination of DNA base composition and DNA–DNA hybridization, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). The DNA G+C content was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989). DNA–DNA hybridization was carried out by the Identification Service of the DSMZ, as described by De Ley *et al.* (1970) incorporating the modifications described by Huß *et al.* (1983) using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian).

Genomic DNA of the isolate was extracted with a Genelute DNA extraction kit (Sigma) by following the recommended procedure of the manufacturer.

The 16S rRNA gene of the isolate was amplified using universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3'). The amplification was performed by initial denaturation at 95 °C for 5 min followed by: 10 cycles of 93 °C for 1 min, 63 °C for 1 min, 71 °C for 1.5 min; 20 cycles of 93 °C for 1 min, 67 °C for 1 min, 71 °C for 2 min; and a final extension at 71 °C for 5 min. The purified PCR product was sequenced in both directions using an automated sequencer by the SeqLab Laboratory (Göttingen, Germany).

Phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein, 1993) and MEGA version 3

Fig. 1. Scanning electron micrograph of cells of *Salinicoccus iranensis* sp. nov. QW6^T from an exponentially growing culture. Bar, 2 µm.

(Kumar *et al.*, 2004) after multiple alignment of data available from public databases by CLUSTAL_X (Thompson *et al.*, 1997).

Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969) and clustering was performed using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings (Felsenstein, 1985).

Cells of the novel isolate were Gram-positive, non-spore-forming, non-motile cocci that were strictly aerobic and occurred singly, in pairs, in tetrads or in clumps (Fig. 1). Colonies were round, smooth, entire, opaque and approximately 2 mm in diameter forming an orangish pink,

non-diffusible pigment after 2 days at 35 °C on 10 % MH medium. Strain QW6^T grew at sea salts concentrations in the range 1–25 % (w/v) in MH medium. Growth did not occur at concentrations of sea salts greater than 25 % (w/v). The temperature range for growth was 5–45 °C and the pH range was 6.5–10.0. Optimal growth occurred in MH medium supplemented with 7.5–10 % (w/v) sea salts at 35 °C and pH 7.5. The novel isolate was resistant to potassium tellurite, sodium selenite and sodium selenate up to concentrations of 12, 600 and 1000 mM, respectively.

It must be emphasized that the novel isolate not only tolerated tellurite and selenite, but also reduced these oxyanions. Reduction of tellurite and selenite by this strain resulted in black and dark-red colonies on agar plates due to

Table 1. Comparison of *Salinicoccus iranensis* sp. nov. with members of the genus *Salinicoccus*

Species: 1, *S. iranensis* sp. nov. strain QW6^T; 2, *S. roseus* DSM 5351^T; 3, *S. hispanicus* DSM 5352^T; 4, *S. alkaliphilus* DSM 16010^T; 5, *S. salsiriae* LMG 22840^T; 6, *S. jeotgali* KCTC 13030^T; 7, *S. kunmingensis* DSM 17847^T. Data from Ventosa *et al.* (1990, 1992), Zhang *et al.* (2002), Aslam *et al.* (2007), França *et al.* (2006), Chen *et al.* (2007) and this study. ND, No data; D, differs among strains.

Characteristic	1	2	3	4	5	6	7
Cell diameter (µm)	0.8–1.0	1.0–2.5	1.0–2.5	0.5–0.8	1.0–2.5	1.0–2.0	0.8–1.2
Pigmentation	Orangish pink	Pink–red	Reddish orange	Pinkish	Pink–red	Orange	Yellow
Growth in NaCl (% w/v):							
Range	1.0–25.0	0.5–25.0	0.9–25.0	0–25.0	0–22.0	0.5–15.0	0.5–25.0
Optimum	7.5–10.0	10	10	10	4	5	8–10
Growth pH:							
Range	6.5–10	5.0–9.0	6.0–9.0	6.5–11.5	6.5–9.5	6.5–11.0	6.0–10.0
Optimum	7.5	7.5	8	9.5	8	7	8
Growth temperature (°C):							
Range	5.0–45.0	15.0–37.0	15.0–40.0	10.0–49.0	20.0–45.0	20.0–30.0	4.0–45.0
Optimum	35	37	37	32	37	30	37
Acid production from:							
Fructose	+	–	+	–	+	+	+
Galactose	–	–	+	–	–	–	–
D-Glucose	+	–	+	+	+	+	–
Glycerol	–	–	+	–	+	+	–
Maltose	–	–	+	–	+	+	–
D-Mannitol	+	–	+	+	–	–	+
Sucrose	–	–	D	–	–	–	+
Hydrolysis of:							
Aesculin	–	–	+	+	–	+	+
Casein	–	+	–	–	+	–	–
Gelatin	–	+	+	–	+	–	–
Starch	–	+	–	–	–	–	+
Tween 80	–	+	–	–	ND	–	+
Nitrate reduction	+	–	+	+	+	+	+
Indole test	+	–	–	–	–	+	–
H ₂ S production	–	–	–	–	ND	+	–
Methyl red test	–	–	+	–	–	+	–
Urease	–	–	+	+	–	–	–
Tellurite resistance (mM)*	12	1	4	6	1	ND	0.5
Selenite resistance (mM)*	600	200	600	800	200	ND	400
MK-7	+	+	+	–	ND	+	+
DNA G + C content (mol%)	54.4	51.2	45.6–49.3	49.6	46.2	47	46.2

*The results of these tests were from our laboratory under identical conditions.

the accumulation of intracellular crystals of black elemental Te and red, amorphous elemental Se, respectively.

Detailed physiological and biochemical characteristics of the strain are shown in Table 1 and in the species description.

Phenotypic data are compatible with assignment of strain QW6^T to the genus *Salinicoccus* (Ventosa *et al.*, 1990, 1992; Zhang *et al.*, 2002, 2007; França *et al.*, 2006; Aslam *et al.*, 2007; Pakdeeto *et al.*, 2007; Chen *et al.*, 2007).

The almost complete 16S rRNA gene sequence (1499 bp) of strain QW6^T was determined. Alignments of this sequence with sequences belonging to type strains of the halophilic cocci available from GenBank showed that the closest relative of strain QW6^T was *S. roseus* DSM 5351^T, with a sequence similarity of 96.4%. The sequence similarities of strain QW6^T to *S. hispanicus* DSM 5352^T, *S. luteus* YIM 70202^T, *S. jeotgali* KCTC 13030^T, *S. salsiraiiae* LMG 22840^T, *S. siamensis* PN1-2^T, *S. alkaliphilus* DSM 16010^T and *S. kunmingensis* YIM Y15^T were 95.0, 94.9, 94.5, 94.5, 94.1, 94.0 and 92.9%, respectively. The level of 16S rRNA gene sequence similarity between strain QW6^T and other Gram-positive cocci was less than 91%. The phylogenetic tree constructed by the neighbour-joining method is shown in Fig. 2.

The G+C content of strain QW6^T was 54.4 mol%. The major isoprenoid quinone of strain QW6^T was MK-6, although MK-7 and MK-5 were present in trace amounts. The cell-wall murein type was L-Lys-Gly₅. Cells of strain QW6^T and of the type strains of *S. roseus*, *S. hispanicus*, *S.*

alkaliphilus and *S. salsiraiiae* were cultivated under identical conditions for comparison of the cellular fatty acid content. These data are compiled in Table 2 together with the data for *S. jeotgali* (Aslam *et al.*, 2007). The fatty acid content of *S. jeotgali* was very similar to those of the other type strains, even though the cells were grown under different conditions. Strain QW6^T had iso-C_{15:0} and anteiso-C_{15:0} as major fatty acids; most minor fatty acids were similar to those of the type strains of members of the genus *Salinicoccus*, except that strain QW6^T had a remarkably higher C_{20:0} content.

Based on sequence dissimilarity values (>3%), the novel isolate did not belong to any other described species and was likely to represent a novel species of the genus *Salinicoccus* (Stackebrandt & Goebel, 1994).

DNA–DNA hybridization experiments revealed low levels of relatedness between strain QW6^T and *S. roseus* DSM 5351^T (40.3%), *S. hispanicus* DSM 5352^T (1.3%) and *S. alkaliphilus* DSM 16010^T (19.6%). According to Wayne *et al.* (1987), less than 70% DNA–DNA relatedness is considered to be the threshold value for the delineation of genospecies, so the values are low enough to separate strain QW6^T from these three species.

The phylogenetic distinctiveness and low DNA–DNA relatedness values, together with several phenotypic differences (physiological and biochemical, as well as DNA G+C content; Table 1), indicate that strain QW6^T represents a novel species of the genus *Salinicoccus*, for which the name *Salinicoccus iranensis* sp. nov. is proposed.

Fig. 2. Neighbour-joining tree showing the relationship between *Salinicoccus iranensis* sp. nov. QW6^T and related taxa. Bootstrap values greater than 50% are indicated. Bar, 2% estimated sequence divergence.

Description of *Salinicoccus iranensis* sp. nov.

Salinicoccus iranensis (i.ran.en'sis. N.L. masc. adj. *iranensis* from Iran, where the organism was isolated).

Cells are Gram-positive cocci, 0.8–1.0 µm in diameter, occurring singly, in pairs, in tetrads or in clumps. Non-motile and strictly aerobic. Endospores are not formed. Colonies are round, smooth, slightly convex and orangish pink in colour; no diffusible pigment is produced. Growth occurs in media containing 1.0–25.0% (w/v) NaCl; 10% (w/v) is optimal for growth. No growth occurs in the absence of NaCl. The pH range for growth is 6.5–10.0 (optimum at pH 7.5). Growth occurs at 5.0–45.0 °C (optimum at 35 °C). Catalase, oxidase, and Simmons' citrate and indole tests are positive. H₂S production and methyl red, Voges–Proskauer and urease tests are negative. Tween 20 is weakly hydrolysed, whereas casein, starch, aesculin, gelatin and Tween 80 are not. Utilizes sucrose, glucose, starch, aesculin, D-mannitol, D-mannose, lactate, dextrin, salicin, inositol and fructose as sole carbon and energy sources. Acid is produced from (+)-D-glucose and

fructose, but not from arabinose, maltose, galactose, glycerol, raffinose, D-melibiose, L-rhamnose, lactose or sucrose. Nitrate is reduced. L-Cysteine, L-proline and L-glycine are utilized as sole nitrogen sources, whereas urea, yeast extract, L-arginine, L-ornithine, L-asparagine and L-phenylalanine are not. Susceptible to carbenicillin (100 µg), cephalothin (30 µg), cefoxitin (30 µg), chloramphenicol (30 µg), cotrimoxazole (25 µg), erythromycin (15 µg), neomycin (30 µg), nitrofurantoin (300 µg) and tetracycline (30 µg); resistant to ampicillin (10 µg), bacitracin (10 U), clindamycin (2 µg), cloxacillin (5 µg), gentamicin (30 µg), novobiocin (5 µg), penicillin G (10 U), polymyxin B (100 U), streptomycin (10 µg) and tobramycin (10 µg); intermediately susceptible to amikacin (30 µg) and kanamycin (30 µg). Other phenotypic features of this strain are shown in Table 1. The cell wall contains peptidoglycan of the L-Lys-Gly₅ type. The major respiratory lipoquinone is MK-6. Predominant fatty acids are iso-C_{15:0} and anteiso-C_{15:0}. The DNA G + C content of the type strain is 54.4 mol%.

The type strain is QW6^T (=DSM 18903^T=CCM 7448^T), isolated from wastewaters of the textile industry in Qom, Iran.

Table 2. Cellular fatty acid content of cells of strain QW6^T and the type strains of *Salinicoccus* species

Strains: 1, strain QW6^T; 2, *S. roseus* DSM 5351^T; 3, *S. alkaliphilus* DSM 16010^T; 4, *S. hispanicus* DSM 5352^T; 5, *S. salsiriae* DSM 19496^T; 6, *S. jeotgali* S2R53-5^T. Values below 0.6% are not shown. All data are from this study (cells cultivated on plates of 10% MH medium containing 8% NaCl at 37 °C for 24 h) apart from data for *S. jeotgali* S2R53-5^T (cells cultivated on saline agar for 2 days; Aslam *et al.*, 2007).

Fatty acid	1	2	3	4	5	6
Saturated fatty acids:						
C _{14:0}	2.9	5.2	1.7	1.5	0.9	4.4
C _{15:0}	1.3	3.1	0.9	0.7		
C _{16:0}	8.8	11.2	3.4	3.4	3.5	10.0
C _{18:0}	2.7	3.3		0.7		
C _{19:0}		0.7				
C _{20:0}	7.2	1.7				
Unsaturated fatty acids:						
C _{16:1} ω7c alcohol	0.9	1.1	2.3	1.3		4.1
C _{16:1} ω11c	1.7	1.2	1.7	0.6		3.6
Branched fatty acids:						
iso-C _{14:0}	4.1	6.8	5.0	3.0	1.0	3.2
iso-C _{15:0}	23.4	20.7	24.6	27.5	17.3	22.8
iso-C _{15:1} AT5				1.3		
anteiso-C _{15:0}	23.6	22.6	31.1	31.8	36.8	32.4
iso-C _{16:0}	3.7	5.5	9.7	3.7	5.1	4.1
iso-C _{17:0}	4.9	3.9	4.6	7.4	7.8	5.0
iso-C _{17:1} ω10c	1.5	0.7	2.4	3.5	5.0	
anteiso-C _{17:0}	4.5	3.9	6.8	6.4	14.2	6.2
iso-C _{19:0}	1.8	1.2	0.8	0.7	1.1	
anteiso-C _{19:0}	0.7	0.7			1.1	
Unknown fatty acid (ECL 15.668)	4.4	5.0	2.6	5.0	4.0	4.3

Acknowledgements

Financial support for this work was provided by the Research Council, University of Tehran. We are grateful to Dr Jean Euzéby for recommending the proper etymology.

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