Salinicoccus qingdaonensis sp. nov., isolated from coastal seawater during a bloom of green algae

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A novel Gram-stain-positive, white-pigmented, non-motile, non-sporulating, catalase- and oxidase-positive, strictly aerobic coccus, designated strain ZXM223^T, was isolated from a seawater sample collected from the coast of Qingdao, PR China, during a green algal bloom. It grew at pH 6.0–10.5 and 0–25.0 % (w/v) NaCl, with optimum growth at pH 8.5 and 3.0 % (w/v) NaCl. Growth occurred at 16–42 °C (optimum at 28 °C). The major fatty acids were anteiso-C_{15:0} and iso-C_{15:0}. Menaquinone 6 (MK-6) was the major respiratory quinone. The polar lipids were phosphatidylglycerol, three unidentified phospholipids and two unknown glycolipids. The peptidoglycan type was L-Lys–Gly_{5–6}. The genomic DNA G + C content was 43.5 mol%. Phylogenetic analysis of the 16S rRNA gene sequence placed strain ZXM223^T within the genus *Salinicoccus*, with sequence similarity of 92.2–97.1 % between ZXM223^T and the type strains of this genus. The closest relatives were *Salinicoccus kunmingensis* YIM Y15^T, '*S. salitudinis*' YIM-C678 and *S. alkaliphilus* T8^T. The DNA–DNA relatedness between strain ZXM223^T and *S. kunmingensis* CGMCC 1.6302^T and '*S. salitudinis*' CGMCC 1.6299 (=YIM-C678) was 37 ± 3 and 30 ± 2 %, respectively. The phenotypic, chemotaxonomic and phylogenetic characteristics and low DNA–DNA relatedness support the proposal of a novel species of the genus *Salinicoccus*, *Salinicoccus qingdaonensis* sp. nov., with the type strain ZXM223^T (=LMG 24855^T =CGMCC 1.8895^T).

Salinicoccus roseus was the first species of the genus *Salinicoccus* to be described, by Ventosa *et al.* (1990). The genus was defined as containing Gram-positive, moderately halophilic, non-motile, non-spore-forming, heterotrophic cocci. Microbes belonging to the genus *Salinicoccus* have generally been found in salty environments, such as salt mines, salted fish and fermented seafood. At the time of writing, the genus contained 12 species with validly published names: *S. roseus* (type species; Ventosa *et al.*, 1990), *S. hispanicus* (Ventosa *et al.*, 1992), *S. alkaliphilus* (Zhang *et al.*, 2002), *S. jeotgali* (Aslam *et al.*, 2007), *S. kunmingensis* (Chen *et al.*, 2007), *S. luteus* (Zhang *et al.*, 2007), *S. salsiraiiae* (França *et al.*, 2006), *S. siamensis* (Pakdeeto *et al.*, 2007), *S. halodurans* (Wang *et al.*, 2008), *S. iranensis* (Amoozegar *et al.*, 2008), *S. albus* (Chen *et al.*, 2009) and *S. carniancri* (Jung *et al.*, 2010); the name '*Salinicoccus salitudinis*' (Chen *et al.*, 2008) has not been validly published. In a study of the microbial diversity in a coastal area of Qingdao where massive green algal blooms occur, a halophilic strain, ZXM223^T, was isolated from a seawater sample (collection location 36.047° N 120.341° E). The phenotypic, chemotaxonomic and genotypic characteristics of strain ZXM223^T

indicated that it represented a novel species of the genus *Salinicoccus*.

The seawater sample was collected at a depth of 1 m during a bloom of the green alga *Enteromorpha prolifera*. The sample was serially diluted 10-fold to 10^{−6} dilution with sterile seawater and spread on marine agar 2216 (MA; Difco) and then incubated at 28 °C for 7 days. A white colony was picked from a plate and, after isolation and purification, the isolate was named strain ZXM223^T. The strain was maintained in 0.85 % (w/v) NaCl with 15 % (v/v) glycerol at −80 °C. Unless otherwise specified, all studies were performed on MA at 28 °C.

Cell morphology was observed by light microscopy (Olympus CX) and transmission electron microscopy (JEOL JEM-1200EX), with cells grown for 24 h at 28 °C on MA. Gram staining was carried out using the standard Gram reaction (Beveridge *et al.*, 2007). Flagellation was examined by using both light microscopy after Leifson staining according to Beveridge *et al.* (2007) and transmission electron microscopy after negative staining with 1 % (w/v) phosphotungstic acid. Motility was detected by observation of growth on marine broth 2216 (MB; Difco) solidified with 0.3 % agar. Anaerobic growth was determined after incubation in a glass vacuum desiccator on MA for 7 days at 28 °C, with the reaction of 10 g gallic acid and 100 ml 10 % NaOH used to consume oxygen. Capsule

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ZXM223^T is FJ436724.

A supplementary alignment file, four supplementary figures and a supplementary table are available with the online version of this paper.

staining, endospore formation, catalase and oxidase activities and degradation of casein, gelatin, starch and Tweens 80 and 20 were studied by using standard protocols (Tindall *et al.*, 2007), with suitably modified MA or MB. Poly- β -hydroxybutyrate (PHB) accumulation was observed with a fluorescence microscope (Olympus BH2) using Nile blue A as a fluorescent stain (Ostle & Holt, 1982).

The NaCl tolerance range and optimum for growth were examined in MB in the absence of NaCl and MB containing NaCl at 0.5, 1, 1.5, 2, 3, 6, 9, 12, 15, 20, 23, 24 and 25 % (w/v) in a constant-temperature shaker. The growth temperature range was determined on MA plates at 0, 4, 10, 16, 25, 28, 32, 37, 40, 42, 45 and 50 °C. The pH tolerance range and optimum for growth were tested in MB prepared at pH 5.0–11.0 (at intervals of 0.5 pH units) and pH 12.0 by monitoring changes in OD₆₀₀ with time at 28 °C. These buffers were used in pH tolerance experiments: pH 5.0–5.5, 0.1 M acetic acid/0.1 M sodium acetate; pH 6.0–8.0, 0.1 M KH₂PO₄/0.1 M NaOH; pH 8.5, 0.05 M Tris/HCl; pH 9.0–10.5, 0.1 M NaHCO₃/0.1 M Na₂CO₃; pH 11.0, 0.05 M Na₂HPO₄/0.1 M NaOH; pH 12.0, 0.2 M KCl/0.2 M NaOH. Susceptibility to antibiotics was determined by inoculating bacterial suspension on MA at 28 °C and applying antibiotics discs. Inhibition zones were measured after 2 days of incubation and interpreted according to the manufacturer's manual (Hangzhou Microbial Reagent Co.). Activities of constitutive enzymes, the fermentation/oxidation profile, acid production and substrate utilization as sole carbon and energy sources were determined twice by using API 20E, API 20NE, API 50CH (with API 50 CH B/E medium) and API ZYM strips (bioMérieux) and Biolog GP and GN MicroPlates according to the manufacturer's instructions; sterile 3 % (w/v) NaCl was used to prepare the inocula. *S. kunmingensis* CGMCC 1.6302^T and '*S. salitudinis*' CGMCC 1.6299 (=YIM-C678), obtained from the China General Microbiological Culture Collection Center, were used as reference strains.

For quantitative analysis of cellular fatty acid compositions, respiratory quinones and polar lipids, cell mass was harvested from MB after incubation at 28 °C for 48 h, while strains were in their stationary phase, and freeze-dried. The analyses were then carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and Dr Brian Tindall (DSMZ). Wet biomass was well suspended in 2-propanol/water (1:1, v/v) for peptidoglycan structure analysis, which was also carried out by the Identification Service of the DSMZ. Peptidoglycan structure and amino acid composition were analysed according to Schleifer & Kandler (1972) and MacKenzie (1987).

Genomic DNA was extracted and purified according to standard methods (Ausubel *et al.*, 1995). The 16S rRNA gene was amplified by PCR with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR products were purified and sequenced by Shanghai Biosune (Shanghai, PR China) with an Applied Biosystems automatic sequencer (ABI

3730). The 16S rRNA gene sequence of strain ZXM223^T was compared and aligned with available sequences in the GenBank database by BLAST, and was also compared with those in the EzTaxon public database (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Phylogenetic analysis was performed using the MEGA version 4.0 software package (Tamura *et al.*, 2007) after multiple alignment of sequence data by CLUSTAL W (Thompson *et al.*, 1997). The sequence alignment is available as Supplementary File S1 (available in IJSEM Online). Phylogenetic trees were established with the neighbour-joining, maximum-parsimony and minimum-evolution DNA distance programs in the MEGA package (Tamura *et al.*, 2007). Bootstrap values were determined based on 1000 replications.

The DNA G + C content was determined by the method of Mesbah *et al.* (1989) using reversed-phase HPLC. DNA–DNA hybridization experiments were performed with genomic DNA from strain ZXM223^T, *S. kunmingensis* CGMCC 1.6302^T and '*S. salitudinis*' CGMCC 1.6299 using a method described previously (Liu & Shao, 2005). The hybridization temperature was 42 °C and the formamide concentration was 50 %. The DIG High Prime DNA Labelling and Detection Starter kit II (Roche) was used in this study.

Colonies of strain ZXM223^T were circular, convex, white-pigmented and 1–2 mm in diameter after incubation for 3 days at 28 °C on MA. No diffusible pigments were produced. Cells of strain ZXM223^T were Gram-stain-positive cocci, approximately 0.4–0.6 µm in diameter (Supplementary Fig. S1), occurring singly and in pairs, tetrads or clumps, and were strictly aerobic, non-motile, non-sporulating, negative for PHB accumulation and positive for presence of a capsule, with no flagella present. Strain ZXM223^T grew at 16–42 °C and grew over wide ranges of NaCl concentration (0–25.0 %, w/v) and pH (pH 6.0–10.5). Optimal growth was observed at 28 °C, 3.0 % (w/v) NaCl and pH 8.5. The major fatty acids were anteiso-C_{15:0} (33.7 %), iso-C_{15:0} (19.6 %), anteiso-C_{17:0} (9.2 %), iso-C_{17:0} (9.0 %) and C_{16:0} (8.7 %). The fatty acid profiles of strain ZXM223^T and *S. kunmingensis* CGMCC 1.6302^T are given in Supplementary Table S1. Strain ZXM223^T had menaquinone 6 (MK-6) (100 %) as its respiratory quinone. Phosphatidylglycerol, three unidentified phospholipids and two unknown glycolipids were the major polar lipid components of strain ZXM223^T (Supplementary Fig. S2), which differed from the major polar lipids of the few other species in the genus for which polar lipid analysis has been reported (Table 1). The chemotaxonomic features are in general agreement with those of other members of *Salinicoccus*. Other phenotypic characteristics are listed in Table 1 and the species description.

The almost-complete 16S rRNA gene sequence (1408 bp) of strain ZXM223^T was determined. Sequence similarity calculations indicated that the closest relatives of strain ZXM223^T were *S. kunmingensis* YIM Y15^T (97.1 % sequence similarity), '*S. salitudinis*' YIM-C678 (96.8 %

Table 1. Differentiating characteristics of strain ZXM223^T and its closest phylogenetic relatives

Strains: 1, strain ZXM223^T (data from this study); 2, *S. kunmingensis* YIM Y15^T (unless indicated, data from Chen *et al.*, 2007); 3, '*S. salitudinis*' YIM-C678 (Chen *et al.*, 2008); 4, *S. alkaliphilus* T8^T (Zhang *et al.*, 2002); 5, *S. jeotgali* S2R53-5^T (Aslam *et al.*, 2007); 6, *S. siamensis* PN1-2^T (Pakdeeto *et al.*, 2007); 7, *S. roseus* DSM 5351^T (Ventosa *et al.*, 1990); 8, *S. salsiraiiae* RH1^T (França *et al.*, 2006); 9, *S. hispanicus* DSM 5352^T (Ventosa *et al.*, 1992); 10, *S. carnicancri* Crm^T (Jung *et al.*, 2010); 11, *S. albus* YIM-Y21^T (Chen *et al.*, 2009); 12, *S. luteus* YIM 70202^T (Zhang *et al.*, 2007). +, Positive; –, negative; tr, trace; ND, no data available. All strains are positive for Gram staining and catalase and oxidase activities and negative for spore formation and motility.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Colony pigmentation*	WH	Y	PY	PH	O	O	P–R	P–R	RO	I	WH	O
Cell diameter (µm)	0.4–0.6	0.8–1.2	0.9–1.5	0.5–0.8	1.0–2.0	0.6–0.8	1.0–2.5	1.0–2.5	1.0–2.0	1.0–2.5	0.6–1.1	0.9
Temperature for growth (°C)												
Range	16–42	4–45†	4–37†	10–49	20–30	15–45	15–37	20–45	15–40	4–45	5–40	4–45
Optimum	28	37†	25†	32	30	37	37	37	37	30–37	25	30
NaCl concentration for growth (% w/v)												
Range	0–25.0	0.5–25.0	1.0–25.0	0–25.0	0.5–15.0	1.5–25.0	0.5–25.0	0–22.0	0.9–25.0	0–20.0	1.0–30.0	1.0–25.0
Optimum	3.0	8.0–10.0	10.0–12.0	10.0	5.0	10.0	10.0	4.0	10.0	12.0	10.0	10.0
pH for growth												
Range	6.0–10.5	6.0–10.0	6.0–11.0	6.5–11.5	6.5–11.0	6.0–9.0	5.0–9.0	6.5–9.5	6.0–9.0	6.0–11.0	6.0–10.0	7.0–11.0
Optimum	8.5	8.0	8.0	9.5	7.0	8.5	7.5	8.0	8.0	7.0–8.0	8.5	8.0–9.0
H ₂ S production	–	–†	–†	–	+	ND	–	ND	–	–	–	–
Indole production	–	–†	–†	–	+	–	–	ND	–	ND	–	–
Methyl red test	–	–†	–†	–	+	–	–	–	+ / –‡	–	–	–
Nitrate reduction	+	+†	+†	+	+	–	+ / –‡	+	+	–	+	+
Urease	–	–†	–†	+ / –‡	–	–	–	–	+ / –‡	+	–	–
Voges–Proskauer test	+	–†	–†	–	–	–	–	–	–	–	+	–
Hydrolysis of:												
Aesculin	+	+†	+†	+ / –‡	+	–	–	–	+	+	–	+
Casein	–	–†	–†	–	–	–	+	+	–	+	–	–
Gelatin	–	–†	–†	–	–	–	+	+	+	–	–	–
Starch	–	+†	–†	–	–	–	+	–	–	–	–	–
Tween 80	+	+†	–†	–	–	–	+	ND	–	–	+	–
Tween 20	–	+†	–†	–	ND	ND	ND	ND	ND	ND	–	ND
Acid production from:												
D-Fructose	+	+	–	–	+	+	–	+	+	+	+	–
D-Galactose	–	–	–	–	–	–	–	–	+	–	–	–
D-Glucose	+	–	+	–	+	+	–	+	+	+	+	+
Glycerol	+	–	–	–	+	+	–	+	+	+	–	ND
Maltose	–	–	–	–	+	–	–	+	+	+	–	+
D-Mannitol	–	+	–	ND	–	–	–	–	+	+	+	ND
Sucrose	–	+	+	–	–	–	–	–	+	+	–	+
Trehalose	+	–	–	+	+	+	–	+	–	+	–	ND
Presence of MK-7 (%)	–	1.2	3.8	–	tr	ND	0.8‡	ND	tr‡	–	30.8	+
Polar lipids												
Diphosphatidylglycerol	–	ND	ND	ND	ND	ND	ND	ND	ND	+	+	ND
Phosphatidylglycerol	+	ND	ND	ND	ND	ND	ND	ND	ND	+	–	ND
Unknown phospholipid(s)	+	ND	ND	ND	ND	ND	ND	ND	ND	–	+	ND
Unknown glycolipid(s)	+	ND	ND	ND	ND	ND	ND	ND	ND	+	–	ND
DNA G + C content (mol%)	43.5	46.2	46.5	49.6	47	46	51.2	46.2	45.7	47.8	46.1	49.7

*I, Ivory; O, orange; P, pink; PH, pinkish; PY, pale yellow; R, red; RO, reddish orange; WH, white; Y, yellow.

†Confirmed in this study in parallel tests for *S. kunmingensis* CGMCC 1.6302^T and '*S. salitudinis*' CGMCC 1.6299 under identical conditions.

‡Data from Chen *et al.* (2007, 2008).

and *S. alkaliphilus* T8^T (96.2%). Phylogenetic analysis indicated that strain ZXM223^T was closely related to the type strains of the genus *Salinicoccus* (Ventosa *et al.*, 1990),

and these strains formed a distinct clade in the phylogenetic tree (Fig. 1) with significant bootstrap support (99%), in which strain ZXM223^T, *S. kunmingensis* YIM

Y15^T and 'S. salitudinis' YIM-C678 formed a distinct subclade (Fig. 1). Trees reconstructed by the maximum-parsimony and minimum-evolution method are shown in Supplementary Figs S3 and S4. The results of our phylogenetic analysis revealed that strain ZXM223^T could represent a previously unknown species of the genus *Salinicoccus*.

The DNA G + C content of strain ZXM223^T was 43.5 mol%. DNA–DNA hybridization experiments showed low DNA–DNA relatedness between strain ZXM223^T and *S. kunmingensis* CGMCC 1.6302^T (37 ± 3 %) and between strain ZXM223^T and 'S. salitudinis' CGMCC 1.6299 (30 ± 2 %), demonstrating their affiliation to separate species in accordance with the cut-off value of 70 % recognized by Wayne *et al.* (1987) for discrimination of bacterial species.

The phylogenetic data and its morphological, biochemical and chemotaxonomic characteristics support the conclusion that strain ZXM223^T should be assigned to the genus *Salinicoccus*. However, the white colony pigmentation, comparatively small colony diameter, low optimum salinity for growth (3 %) and positive Voges–Proskauer test reaction, as well as the ability to hydrolyse Tween 80 and produce acid from glycerol and trehalose, together with the low 16S rRNA gene sequence similarities with species of the genus *Salinicoccus* and the low level of DNA–DNA relatedness, clearly distinguished strain ZXM223^T from other members of the genus. On the basis of its distinct phenotypic and genetic properties, we conclude that strain ZXM223^T represents a novel species of the genus *Salinicoccus*, for which the name *Salinicoccus qingdaonensis* sp. nov. is proposed.

Description of *Salinicoccus qingdaonensis* sp. nov.

Salinicoccus qingdaonensis (qing.dao.nen'sis. N.L. masc. adj. *qingdaonensis* pertaining to Qingdao, a city in the coastal region of east China, near the sampling site from which the type strain was isolated).

Cells are strictly aerobic, non-motile, non-sporulating cocci (0.4–0.6 µm in diameter) that occur singly or in pairs,

tetrads or clumps. Gram-stain-positive, with a capsule. Catalase- and oxidase-positive and negative for gelatinase and PHB accumulation. Colonies are white, circular, smooth and convex, 1–2 mm in diameter after 3 days of incubation at 28 °C on MA, with no diffusible pigment production. Moderately halophilic, with growth at 0–25.0 % (w/v) NaCl. Growth occurs at pH 6.0–10.5 and at 16–42 °C. Optimum growth at 3.0 % (w/v) NaCl, pH 8.5 and 28 °C. Nitrate is reduced to nitrite. H₂S and indole production and methyl red test are negative, but Voges–Proskauer test is positive. Aesculin and Tween 80 are hydrolysed, but casein, chitin, gelatin, starch and Tween 20 are not. Constitutive enzymes expressed by the type strain are α-Phe–Pro arylamidase, leucine aminopeptidase, L-pyrrolidonyl arylamidase and naphthol-AS-BI-phosphoamidase; acid phosphatase, alkaline phosphatase, arginine dihydrolase, α-chymotrypsin, α-fucosidase, α-galactosidase, α-glucosidase, α-mannosidase, β-alanine arylamidase, β-galactosidase, β-glucuronidase, β-glucosidase, β-xylosidase, γ-glutamyl transferase, cystine aminopeptidase, arginine decarboxylase, esterase (C4), esterase lipase (C8), Glu–Gly–Arg arylamidase, glutamyl arylamidase, glycine arylamidase, L-proline arylamidase, lipase (C14), lysine decarboxylase, N-acetyl-β-galactosaminidase, N-acetyl-β-glucosaminidase, ornithine decarboxylase, trypsin, tyrosine arylamidase, tryptophan deaminase, urease and valine aminopeptidase are not expressed. Acid is produced from D-fructose, D-glucose, trehalose, glycerol, N-acetylglucosamine and potassium 5-ketogluconate. In Biolog GP2 and GN2 plates, the following substrates are used as sole carbon and energy sources: D-fructose, α-D-glucose, D-mannose, D-psicose, trehalose, acetic acid, α-hydroxybutyric acid, L-lactic acid, L-malic acid, succinic acid monomethyl ester, propionic acid, pyruvic acid, succinic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-glutamic acid, L-pyroglutamic acid, L-serine, glycerol, thymidine, uridine, N-acetyl-D-glucosamine, monomethyl succinate, acetic acid, formic acid, α-ketobutyric acid, bromosuccinic acid, succinamic acid, L-asparagine, L-aspartic acid, L-histidine, hydroxy-L-proline, L-ornithine, L-proline, L-threonine and urocanic acid. Cells are susceptible to the following (µg per disc, unless indicated): midecamycin (30), ofloxacin (5),

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence analysis and reconstructed using the neighbour-joining method showing the phylogenetic positions of strain ZXM223^T and related taxa. Branches were also recovered with the maximum-parsimony and minimum-evolution method (see Supplementary Figs S3 and S4). Numbers at nodes indicate bootstrap values (>50 %) based on neighbour-joining analyses of 1000 resampled datasets. Bar, 2 substitutions per 100 nucleotide positions.

ciprofloxacin (5), vancomycin (30), furazolidone (300), chloromycetin (30), clindamycin (2), cefobid (30), amikacin (30), vibramycin (30), minocycline (30), erythromycin (15), penicillin (10), oxacillin (1), ampicillin (10), carbenicillin (100), piperacillin (100), cephalothin IV (30), cephalothin V (30), cephalothin VI (30) and rocephin (30), but resistant to norfloxacin (10), polymyxin B (100 U), compound sulfamethoxazole (75), gentamicin (10), kanamycin (30), neomycin (10), tetracycline (30) and fortum (30). The major fatty acids are anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0}, iso-C_{17:0} and C_{16:0}. MK-6 is the predominant respiratory quinone. The polar lipids consist of phosphatidylglycerol, three unidentified phospholipids and two unknown glycolipids. The cell-wall peptidoglycan type is L-Lys-Gly₅₋₆. The DNA G + C content of the type strain is 43.5 mol%.

The type strain, ZXM223^T (=LMG 24855^T =CGMCC 1.8895^T), was isolated from a seawater sample collected from coastal seawater of Qingdao, east China, during a bloom of the green alga *Enteromorpha prolifera*.

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