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Halococcus qingdaonensis sp. nov., a halophilic archaeon isolated from a crude sea-salt sample

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Abstract

A Gram-negative, extremely halophilic, coccoid archaeal strain, CM5^T, was isolated from a crude sea-salt sample collected near Qingdao, China. The organism grew optimally at 35–40 °C and pH 6.0 in the presence of 20 % (w/v) NaCl. Its colonies were red in colour and it could use glucose as a sole carbon source for growth. The 16S rRNA gene sequence of CM5^T was most closely related to those of *Halococcus* species. Its pattern of antibiotic susceptibility was similar to those of other described *Halococcus* species. Biochemical tests revealed no sign of H_2S production or gelatin liquefaction. The main polar lipids of strain CM5^T were phosphatidylglycerol, phosphatidylglycerol methylphosphate and sulfated diglycosyl diether. No phosphatidylglycerol sulfate was present. The DNA G+C content of strain CM5^T was 61.2 mol% and it gave DNA–DNA reassociation values of 33.7, 57.1 and 29.6 %, respectively, with *Halococcus salifodinae* DSM 8989^T, *Halococcus dombrowskii* DSM 14522^T and *Halococcus morrhuae* ATCC 17082^T. Based on its morphological and chemotaxonomic properties and phylogenetic analysis of 16S rRNA gene sequence data, we propose that CM5^T should be classified within a novel species, *Halococcus qingdaonensis* sp. nov., with strain CM5^T (=CGMCC 1.4243^T=JCM 13587^T) as the type strain.

The extremely halophilic bacteria are defined as microorganisms that grow best in media containing 2.5–5.2 M (saturated) NaCl (Kushner & Kamekura, 1988) and include members of both the *Archaea* and the *Bacteria*, among them aerobic halophilic archaea that require at least 12 % (2 M) NaCl for growth, which are classified within the family *Halobacteriaceae*. In recent years, many halobacterial strains have been isolated and described within novel species. The number of genera within this family has increased to 22 (http://www.bacterio.cict.fr/classifgenerafamilies.html#Halobacteriaceae). *Halococcus* was the second genus after *Halobacterium* to be classified within the family *Halobacteriaceae* (Skerman *et al.*, 1980). Currently, there are five recognized species in this genus (Larsen, 1989; Stan-Lotter *et al.*, 2002; Garrity *et al.*, 2004; Goh *et al.*, 2006). Here we report the isolation and taxonomic analysis of a strain representing a novel *Halococcus* species from a crude sea-salt sample collected near Qingdao in eastern China.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CM5^T is AY243109.

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CM medium (Seghal & Gibbons, 1960) with 20 % NaCl at pH 7.0 was used for halobacterial enrichment and growth. The sample was firstly inoculated in liquid medium and cultured on a shaker (120 r.p.m.) at 37 °C in the dark until turbid, and then streaked onto solid medium to produce single colonies. Streaking was repeated several times to obtain pure single colonies. Purified strains were cultured and maintained in liquid or on solid ATCC213 medium with 18 % NaCl at pH 6.0 in the dark.

Seven days after being cultured in liquid medium, living cells were observed under a phase-contrast microscope. Gram staining was performed according to Dussault (1955) and electron microscopy was used to reveal the detailed morphology. Samples were fixed in 5 % (v/v) glutaraldehyde (in 0.2 M phosphate buffer, pH 7.2) for 2 h on ice, washed three times in phosphate buffer and subsequently post-fixed for 1 h in 2 % osmium tetroxide. The pellet was dehydrated with propanol and embedded in resin. Embedding was done according to the protocol of Spurr (1969). Ultrathin sections were prepared with an LKB ultramicrotome and double stained with uranyl acetate and lead citrate. After air-drying, the samples were examined with an electron microscope (JEM-1230). For negative staining, liquid culture of cells at the exponential growth phase was allowed to dry on the grids and stained with 2 % phosphotungstic acid.

Halobacterial growth was determined by measuring optical density at 460 nm at intervals during growth of the liquid culture. The effect of different concentrations of NaCl on growth of strain CM5^T was tested in liquid ATCC213 medium. After 7 days of incubation, the optical density at 460 nm was measured. Susceptibility of CM5^T to the antibiotics ampicillin, tetracycline, hygromycin, kanamycin, streptomycin, rifampicin, bacitracin, penicillin, chloramphenicol, neomycin and erythromycin (all from Sigma) was tested by placing 6 mm diameter discs containing 20 μg antibiotic on agar plates followed by 7 days incubation at 37 °C. Sensitivity was deemed strong when the diameter of the zone of inhibition was >15 mm (i.e. 4.5 mm beyond the antibiotic disc) and moderate between 6 and 15 mm (1–4.5 mm beyond the antibiotic disc).

Physiological and biochemical tests were performed according to Gibbons (1974) and Tian *et al.* (1997). Anaerobic growth of CM5^T with nitrate as the electron acceptor was tested as described by Mancinelli & Hochstein (1986) and growth with DMSO and fermentation of L-arginine as the electron acceptor were tested as described by Oren *et al.* (1997) and Oren & Trüper (1990), in closed tubes fully filled with the growth medium and held in the dark for more than 1 month which were then compared with growth on media without the test compounds.

Cells for pigment determination were collected by centrifugation and washed twice with 25 % NaCl. They were extracted with a 1 : 1 (v/v) mixture of acetone and methanol for 1 h. After centrifugation, the absorption spectrum of the supernatant was determined. Polar lipids were extracted from 300 mg freeze-dried cells using the method described by Tindall (1990). They were further purified by extraction with chloroform/methanol/0.3 % NaCl (1 : 2 : 0.8, by vol.) and separated by two-dimensional silica-gel TLC (Ross *et al.*, 1985). Polar-lipid extracts were spotted onto the corner of a 10×10 cm thin-layer silica-gel plate (60F$_{254}$; Merck). The first direction was developed in chloroform/methanol/water (65 : 25 : 4, by vol.) and the second in chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.). Total lipids and specific functional groups were detected using phosphomolybdic acid, molybdenum blue, α-naphthol and Bial's reagent (orcinol ferric chloride spray reagent) (Stan-Lotter *et al.*, 1999, 2002). The equivalence of spots was determined by co-chromatography of extracts of known haloarchaea in two dimensions and by comparison with published data.

The DNA G+C content of strain CM5^T was determined using the thermal denaturation method of Marmur & Doty (1962) with *Escherichia coli* JM105 as a control. We used the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) to perform DNA–DNA hybridization experiments. The three most closely related strains, *Halococcus dombrowskii* DSM 14522^T, *Halococcus morrhuae* ATCC 17082^T and *Halococcus salifodinae* DSM 8989^T, were used as reference strains.

PCR amplification of 16S rRNA genes was performed according to Wang *et al.* (2000) after total DNA extraction by using primers 5′-ATTCCGGTTGATCCTGCCGGA-3′ (primer 1; positions 6–25, according to *E. coli* numbering) and 5′-AGGAGGTGATCCAAGCCGCAG-3′ (primer 2; positions 1540–1521) with 36 cycles of denaturing (94 °C, 1 min), annealing (52 °C, 1 min) and extension (72 °C, 3 min). The PCR product was ligated to the T-vector and transformed into *E. coli* DH10 for purification and sequencing. Sequences used for comparison with the 16S rRNA gene sequence from strain CM5^T were obtained from GenBank by using the BLASTN program and the sequence was aligned with closely related 16S rRNA gene sequences with CLUSTAL X software program version 1.83 (Thompson *et al.*, 1997). The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) in MEGA program version 3.1 (Kumar *et al.*, 2001, 2004). Confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Cells of strain CM5^T were coccoids, 0.6–1.5 μm in diameter (Fig. 1). They were non-motile and often arranged in doublets or tetrads; they stained Gram-negative and did not lyse in distilled water. The colonies were red in colour, wet and smooth-surfaced with clear edges and reached 0.5 mm in size after 7 days of culture at 37 °C on ATCC213 medium. Strain CM5^T could grow at pH 4.0–9.0, with optimal growth at pH 6.0. It required at least 10 % NaCl for growth and 18 % was the optimum. The optimal Mg^{2+} concentration was 40 mM. The permissive temperature for growth was between 26 and 45 °C, with optimum growth between 35 and 40 °C.

Pigment determination revealed three absorbance spectrum peaks at 389, 495 and 527 nm for CM5^T, which are characteristic of carotenoids in extremely halophilic archaea. Two-dimensional TLC analysis of the polar lipids in strain CM5^T (Fig. 2) as well as the three reference strains revealed the presence of $C_{20}C_{20}$ and $C_{20}C_{25}$ archaeal core lipids, which were detected by double spots. Glycerol diethers of phosphatidylglycerol and phosphatidylglycerol methylphosphate and sulfated diglycosyl diether were present in strain CM5^T; no phosphatidylglycerol sulfate was present. These patterns were similar to those of *Hcc. morrhuae* DSM 1307^T, *Hcc. salifodinae* DSM 8989^T and *Hcc. dombrowskii* DSM 14522^T. Two unknown phospholipids and one unknown glycolipid were present in strain CM5^T. The overall phospholipid pattern of strain CM5^T was characteristic of members of the genus *Halococcus* (Ross *et al.*, 1985; Wainø *et al.*, 2000).

Growth of strain CM5^T was strongly inhibited by the antibiotics rifampicin and bacitracin and moderately inhibited by neomycin and chloramphenicol. No inhibition was observed when the strain was grown in the presence of erythromycin, ampicillin, kanamycin, streptomycin, tetracycline, hygromycin or penicillin. The susceptibility pattern of strain CM5^T was similar to those of *Hcc. dombrowskii* DSM 14522^T and *Hcc. morrhuae* DSM 1307^T (determined in our experiments), except that the latter strains were moderately susceptible to erythromycin, similar to the result described by Stan-Lotter *et al.* (2002).

Strain CM5^T was distinct from other *Halococcus* species in a number of biochemical properties (Table 1). Unlike *Hcc. morrhuae* and *Halococcus saccharolyticus*, it did not produce H_2S and was negative for gelatin hydrolysis. Nitrate reduction was not detected

with CM5^T. Indole but not organic acids could be produced from sugars. It had catalase, but no oxidase, arginine dihydrolase or urease activity. It could use glucose, galactose, sucrose, inositol, fructose and rhamnose, but not sorbitol, cellobiose, mannitol, dextran or lactose, as sole carbon sources. CM5^T could not grow anaerobically in the presence of nitrate or DMSO or by fermenting L-arginine.

The G+C content of strain CM5^T was 61.2 mol%, similar to the values determined for *Hcc. morrhuae* [62.3 mol% (this study); 61–66 mol% (Larsen, 1989)], *Hcc. dombrowskii* (61.3 mol%; Stan-Lotter *et al.*, 2002) and *Hcc. salifodinae* [62.7 mol% (this study); 62±1 mol% (Denner *et al.*, 1994)]. DNA–DNA reassociation values between strain CM5^T and related type strains were 33.65 % with *Hcc. salifodinae* DSM 8989^T, 57.1 % with *Hcc. dombrowskii* DSM 14522^T and 29.6 % with *Hcc. morrhuae* ATCC 17082^T (each repeated three times). Levels of 70 % or more relatedness can be considered indicative of species levels of similarity (Gutierrez *et al.*, 1989, 1990); hence, strain CM5^T represented a novel species of the genus *Halococcus*.

The full sequence (1476 bases) of the 16S rRNA gene of strain CM5^T was determined. Phylogenetic analysis of strain CM5^T using 16S rRNA gene sequences showed high similarity to *Hcc. morrhuae* ATCC 17082^T (99.3 %) and *Hcc. dombrowskii* H4^T (99.2 %), but less to *Hcc. salifodinae* DSM 8989^T (94 %), *Hcc. saccharolyticus* ATCC 49257^T (93.8 %) and *Halococcus hamelinensis* 100A6^T (93.2 %). Phylogenetic analysis based on neighbour-joining showed that strain CM5^T forms a branch within the *Hcc. morrhuae* lineage (Fig. 3). The genus *Halococcus* appears to contain at least two lineages on the basis of 16S rRNA gene sequence data: one contains *Hcc. salifodinae* and *Hcc. saccharolyticus*, while the other consists of *Hcc. morrhuae* and other coccoid strains (Stan-Lotter *et al.*, 2002).

On the basis of its 16S rRNA gene sequence, G+C content, polar-lipid content, antibiotic sensitivity and other characteristics, the coccoid strain CM5^T was identified as being a halophilic archaeon of the genus *Halococcus*. Unlike *Hcc. morrhuae* and *Hcc. dombrowskii*, CM5^T produced no H_2S, was not sensitive to tetracycline and could use glucose as a sole carbon source. *Hcc. dombrowskii* requires at least 15 % NaCl for growth, and the optimal concentration is 25–30 % (Stan-Lotter *et al.*, 2002), whereas the minimal and optimal NaCl concentrations for growth of strain CM5^T were 10 and 18–20 %, respectively. Strain CM5^T differed from *Hcc. dombrowskii* and *Hcc. morrhuae* with respect to its cellular morphology and arrangement, DNA–DNA reassociation value, susceptibility to some antibiotics and usage of carbohydrates. The values of less than 70 % DNA–DNA reassociation between CM5^T and *Hcc. morrhuae* ATCC 17082^T (29.6 %) and *Hcc. dombrowskii* DSM 14522^T (57.1 %) also support the suggestion to designate CM5^T as representing a novel species. In conclusion, we describe CM5^T as the type strain of a novel species, for which we propose the name *Halococcus qingdaonensis* sp. nov.

Description of *Halococcus qingdaonensis* sp. nov.

Halococcus qingdaonensis (qing.dao.nen'sis. N.L. masc. adj. *qingdaonensis* pertaining to Qingdao, from where the type strain was isolated).

Aerobic, Gram-negative, non-motile cocci, 0.6–1.5 μm in diameter, often occurring as doublets or tetrads. Colonies are red in colour, wet and smooth-surfaced with clear edges, reaching 0.5 mm in size after 7 days of cultivation at 37 °C. The optimum temperature for growth is 35–40 °C; the pH range for growth is 4.0–9.0, with optimum growth at pH 6.0. It requires at least 10 % NaCl for growth and 18 % is the optimum. No lysis is observed in distilled water. The optimal Mg^{2+} concentration is 40 mM. Catalase reaction is positive. It has no oxidase, arginine dihydrolase or urease activity. Gelatin is not liquefied. The reaction

for Tween 80 is negative. Glucose can be used as the sole carbon source for growth. The type strain is susceptible to rifampicin and bacitracin, moderately susceptible to neomycin and chloramphenicol and resistant to erythromycin, ampicillin, kanamycin, streptomycin, tetracycline, hygromycin and penicillin. Main polar lipids are phosphatidylglycerol, phosphatidylglycerol methylphosphate, sulfated diglycosyl diether. No phosphatidylglycerol sulfate is present. The G+C content of the type strain is 61.2 mol%.

The type strain, CM5^T (=CGMCC 1.4243^T=JCM 13587^T), was isolated from a crude sea-salt sample collected near Qingdao in eastern China.

Acknowledgments

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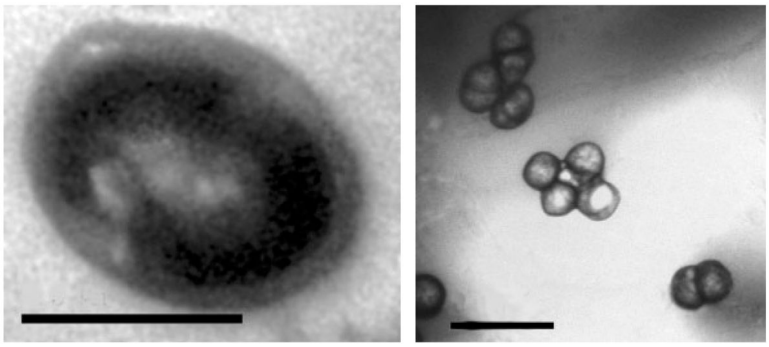

Fig. 1.
Transmission electron micrographs of strain CM5^T. Left, ultrathin section (bar, 0.5 μm); right, negative staining showing doublet/tetrad arrangements (bar, 2 μm).

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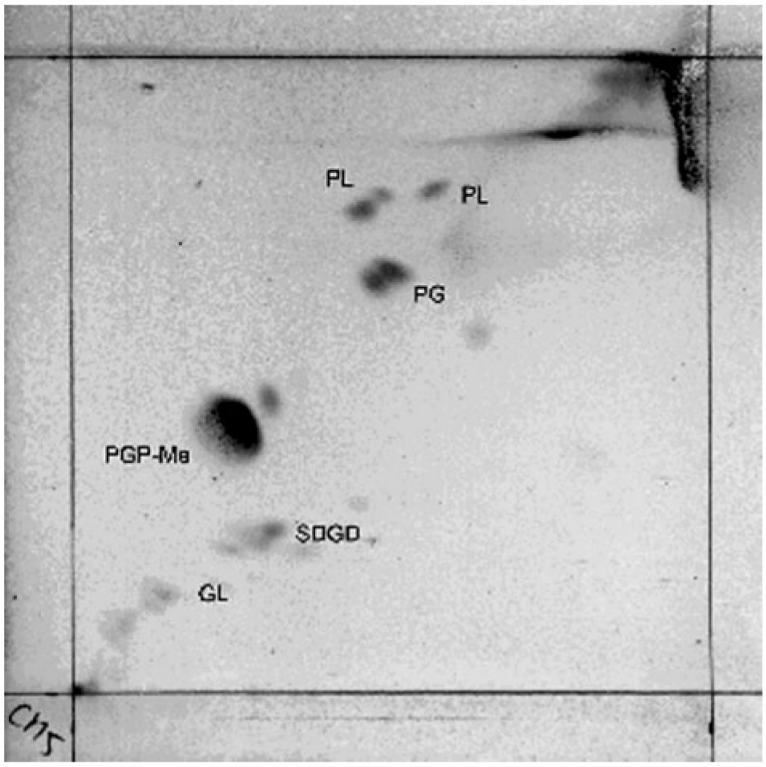

Fig. 2.
Polar lipid pattern of strain CM5^T. PG, Glycerol diether of phosphatidylglycerol; PGP-Me, glycerol diether of phosphatidylglycerol methylphosphate; SDGD, sulfated diglycosyl diether; PL, unidentified phospholipid; GL, unidentified glycolipid.

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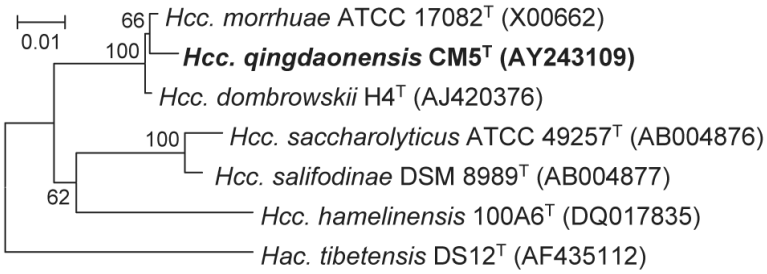

Fig. 3.

Phylogenetic relationships of strain CM5^T and other related taxa, constructed using neighbour-joining method and based on 16S rRNA gene sequences. *Halalkalicoccus tibetensis* DS12^T was used as an outgroup. Bootstrap values are shown as percentages of 1000 replicates. Bar, 0.01 changes per nucleotide position.

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Table 1

Characteristics that differentiate strain CM5^T from type strains of other *Halococcus* species

Strains: 1, strain CM5^T; 2, *Hcc. morrhuae* ATCC 17082^T; 3, *Hcc. dombrowskii* H4^T; 4, *Hcc. salifodinae* DSM 8989^T (data in columns 1–4 determined in this study unless indicated); 5, *Hcc. saccharolyticus* ATCC 49257^T (data from Montero *et al.*, 1989); 6, *Hcc. hamelinensis* 100A6^T (data from Goh *et al.*, 2006). +, Positive reaction or growth; −, no reaction or growth; V, variable; ND, no data available.

Characteristic	1	2	3	4	5	6
Optimum NaCl concentration (% w/v)	18	20–30[a*]	20–30[b]	20–30[c]	20–30	15
pH range for growth	4.0–9.0	5.5–8.0[a]	5.2–8.0[b]	6.8–9.5[c]	6.0–8.0	4.0–9.0
Oxidase	−	+[a]	+	+	+	−
Nitrate reduction	−	+	+	+	+	+
Starch hydrolysis	+	−	−	−	−	+
Gelatin liquefaction	−	+[a]	+	+	V	−
Tween 80 hydrolysis	−	+[a]	−	+	−	ND
Use of carbohydrates:						
Glucose	+	−	−	+	ND	+
Lactose	−	−	−	+	+	−

[*] Data taken from following references:

[a], Grant & Larsen (1989);

[b], Stan-Lotter et al. (2002);

[c], Denner et al. (1994).