Haloplanus vescus sp. nov., an extremely halophilic archaeon from a marine solar saltern, and emended description of the genus Haloplanus

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An extremely halophilic archaeon, strain RO5-8T, was isolated from a disused marine solar saltern in China. The cells were pleomorphic and flat. In static liquid medium, cells floated to the surface. Strain RO5-8T was closely related to three strains of Haloplanus natans with similarities of 97.3–97.6 %. The DNA G+C content of strain RO5-8T was 62.1 mol%. The DNA–DNA hybridization value between strain RO5-8T and Haloplanus natans JCM 14081T was 51.6 %. It was concluded that strain RO5-8T represents a novel species of the genus Haloplanus, for which the name Haloplanus vescus sp. nov. is proposed. The type strain is RO5-8T (=CGMCC 1.8712T =JCM 16055T).

The genus Haloplanus was proposed by Bardavid et al. (2007) to accommodate three flat-shaped strains isolated from an experimental mesocosm filled with a mixture of water from the Dead Sea and the Red Sea, Israel. The extremely halophilic nature of these strains indicated that they may be derived from the Dead Sea (340 g total dissolved salts l−1) and not from the Red Sea (41 g total dissolved salts l−1). The most interesting characteristic is that cells of Haloplanus natans are pleomorphic, flat and in static liquid medium float to the surface, which reveals that these cells may contain gas vesicles. Several uncultured relatives (with 96–97 % similarities) to this group have also been detected in different hypersaline environments (He et al., 2008; Sørensen et al., 2005). To date, no really novel members related to H. natans have been isolated from other hypersaline environments such as salt lakes and marine solar salterns. During our surveys on halophilic archaeal diversity of marine solar salterns of Eastern China, an extremely halophilic archaeal strain related to H. natans was obtained. In this study, we characterize strain RO5-8T as a novel species of the genus Haloplanus.

Strain RO5-8T was isolated from sediment of Rudong solar saltern (32.2699° N 121.3999° E), which is a disused artificial marine solar saltern located in Jiangsu Province, China. The neutral oligotrophic haloarchaeal medium (NOM) used for the isolation procedure was modified from the DBCM2 medium described in the online Halohandbook (Dyall-Smith, 2008) and contained the following ingredients (l−1): yeast extract, 0.05 g; fish peptone, 0.25 g; sodium pyruvate, 1.0 g; KCl, 5.4 g; K2HPO4, 0.3 g; CaCl2, 0.25 g; NH4Cl, 0.25 g; MgSO4, 7H2O, 26.8 g; MgCl2, 6H2O, 23.0 g; NaCl, 184.0 g (pH adjusted to 7.0–7.2 with 1 M NaOH solution). The medium was solidified with 2.0 % agar (Difco). Strains were routinely grown aerobically at 37 °C in NOM-1 medium containing the following ingredients (l−1): yeast extract (Difco), 0.2 g; fish peptone, 0.2 g; sodium pyruvate, 2.0 g; sodium lactate, 2.0 g; KCl, 5.4 g; K2HPO4, 0.3 g;
NH₄Cl, 0.5 g; MgSO₄·7H₂O, 20.0 g; NaCl, 200.0 g (pH 7.0–7.2).

Phenotypic tests were performed according to the proposed minimal standards for the description of novel taxa in the order Halobacteriales (Oren et al., 1997). The type strains *Halofex volcanii* CGMCC 1.2150T, *Halofex elongans* JCM 14791T and *H. natans* JCM 14081T were selected as reference strains in positive and negative testing. Cell morphology and motility in exponentially growing liquid cultures were examined using a Leica microscope equipped with phase-contrast optics (model DM LB2). For scanning electron microscopy examination, 0.5 ml samples were fixed overnight at 4°C by adding glutaraldehyde to a final concentration of 5.0%. A 5 µl sample was smeared on a polylysine-coated coverslip and air-dried. The coverslip was then serially dehydrated in 40, 70, 90 and 100% ethanol solutions (10 min at each stage), vacuum dried at 55°C, mounted on a specimen stub, sputter-coated with gold and viewed in a JEOL JSM-7001F scanning electron microscope. Minimal salt concentration to prevent cell lysis was tested by suspending washed cells in serial sterile saline solutions containing NaCl ranging from 0 to 15% (w/v) and the stability of the cells was detected by light microscopic examination.

The Gram stain was performed by following the method outlined by Dussault (1955). Most miscellaneous biochemical tests and nutritional tests were performed as described and cited by Bardavid et al. (2007). Briefly, growth and gas formation with nitrate as electron acceptor were tested in 10 ml stoppered tubes, completely filled with liquid growth medium to which 5 g NaNO₃ l⁻¹ had been added, and containing an inverted Durham tube. The formation of nitrite was monitored colorimetrically. Anaerobic growth in the presence of L-arginine and 5 g DMSO l⁻¹ were tested in completely filled 10 ml stoppered tubes. Starch hydrolysis was determined on NOM agar plates supplemented with 2 g soluble starch per litre and detected by flooding the plates with Lugol’s iodine solution. Gelatin hydrolysis was performed by growing colonies on NOM agar plates amended with 0.5% (w/v) gelatin and flooding the plates with Frazier reagent after growth was established. Esterase activity was detected as outlined by Gutiérrez & González (1972). Tests for catalase and oxidase activities were performed as described by Gonzalez et al. (1978). Production of H₂S was tested by growing the isolates and reference strains in a tube with NOM liquid medium supplemented with 0.5% (w/v) sodium thiosulfate; a filter-paper strip impregnated with lead acetate was used for H₂S detection (Cui et al., 2007). To test for growth on single carbon sources, fish peptone and sodium pyruvate were omitted from the NOM medium and the compound to be tested was added at a concentration of 5 g l⁻¹. Sensitivity to antimicrobial agents was performed as described by Gutiérrez et al. (2008).

Cells of strain RO5-8T are motile, pleomorphic, flat and contain gas vesicles (see Supplementary Fig. S1 available in IJSEM Online). Cells stained Gram-negative and colonies were pink-pigmented. Strain RO5-8T was able to grow at 30–50°C (optimum 40°C), at 2.6–4.3 M NaCl (optimum 3.1 M NaCl), at 0.03–0.5 M MgCl₂ (optimum 0.03 M MgCl₂) and at pH 5.5–7.5 (optimum pH 6.0–6.5). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis was 12% (w/v). Strain RO5-8T produced indole from tryptophan, but did not hydrolyse gelatin, Tween 20, 40, 60 and 80, starch or casein. H₂S was not produced from sodium thiosulfate. The following substrates were utilized as single carbon and energy sources for growth: D-glucose, D-mannose, maltose, lactose, D-mannitol, D-sorbitol, acetate, pyruvate and DL-lactate. Acid was not produced from D-glucose, D-mannose, maltose or lactose. More detailed results of phenotypic tests and nutritional features of strain RO5-8T are given in the species description.

Genomic DNA from halophilic archaeal strains was prepared as described by Ng et al. (1995). The 16S rRNA gene was amplified via PCR by using primers 0018F and 1518R (Cui et al., 2009). PCR was performed in a thermal cycler (MJ Research PTC-150) for 30 cycles (5 min denaturing step at 95°C in the first cycle; 1 min denaturing at 95°C, 1 min annealing at 60°C and 1.5 min elongation at 72°C; final extension step at 72°C for 10 min). PCR products were examined on a 1.0% (w/v) agarose gel and cloned into the pEASY-T vector (TransGen Biotech) and transformed into cells of *Escherichia coli* Mach1. Ten transformants were randomly picked and sequenced at the Sino-GenoMax Company (Beijing, China), to determine whether the strain possessed multiple distinct 16S rRNA gene sequences. Multiple sequence alignments were performed by using the CLUSTAL W program integrated in the MEGA 4 software (Tamura et al., 2007). Phylogenetic trees were reconstructed by using the neighbour-joining algorithm (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms in MEGA 4. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replications) are shown next to the branches. 16S rRNA gene sequence similarity was calculated by comparison with those of related halophilic archaea from the online EzTaxon server (Chun et al., 2007).

Ten complete 16S rRNA gene sequences (1472 nt each) of strain RO5-8T were obtained. Sequence comparisons indicated that strain RO5-8T has one kind of 16S rRNA gene sequence. It was closely related to three strains of *H. natans*, the single species of the genus *Haloplanus*, with similarities of 97.3–97.6%. Phylogenetic analysis using the neighbour-joining algorithm revealed that strain RO5-8T clustered with *H. natans*, forming a branch in the cluster with a bootstrap value of 100% (Fig. 1). The phylogenetic position was also confirmed in a tree generated using the maximum-parsimony algorithm (see Supplementary Fig. S2).

The 16S rRNA gene-based phylogenetic analysis results supported the placement of strain RO5-8T in the genus *Haloplanus*. 

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Polar lipids were extracted by using a chloroform/methanol system and analysed by using one- and two-dimensional TLC, as described previously (Kates, 1986). Merck silica gel 60 F254 aluminium-backed thin-layer plates were used in TLC analysis. For detecting phospholipids, the plate dotted with sample was subjected to two-dimensional development, with the first solvent of chloroform/methanol/water (65 : 25 : 4, by vol.) followed by the second solvent of chloroform/methanol/acetic acid/water (85 : 12 : 15 : 4, by vol.), then sprayed with the phosphate stain reagent (Vaskovsky & Kostetsk, 1968). For detecting glycolipids, the plate was subjected to single development in the solvent chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, by vol.) and detected by spraying with 0.5 % \(\alpha\)-naphthol in methanol/water (1 : 1, v/v), and then sprayed with sulfuric acid/ethanol (1 : 2, v/v) followed by heating at 150 °C for 3 min to detect phospholipids and glycolipids. Strain RO5-8\(^T\) contained PG (phosphatidylglycerol), PGP-Me (phosphatidylglycerol phosphate methyl ester), PGS (phosphatidylglycerol sulfate) and one major glycolipid chromatographically identical to the sulfated mannosyl glucosyl diether S-DGD-1, in a pattern chromatographically identical to the polar lipid profiles of \(H.\) natans JCM 14081\(^T\) (see Supplementary Fig. S3). The polar lipid composition supports classification of strain RO5-8\(^T\) in the genus \(Haloplanus\).

The DNA G + C content of strain RO5-8\(^T\) was determined by the HPLC method (Mesbah et al., 1989). DNA–DNA hybridization analyses were performed according to the thermal denaturation and renaturation method of De Ley et al. (1970) as modified by Huß et al. (1983). The DNA G + C content of strain RO5-8\(^T\) was 62.1 mol%, lower than those of strains representing \(H.\) natans (66.1–66.4 mol%) (Bardavid et al., 2007). The DNA relatedness between strain RO5-8\(^T\) and \(H.\) natans JCM 14081\(^T\) was 51.6 %, which indicated that strain RO5-8\(^T\) represents a distinct genospecies of the genus \(Haloplanus\), since the generally accepted threshold value to separate two species is less than 70 % (Stackebrandt & Goebel, 1994).

This polyphasic taxonomic study provides evidence that strain RO5-8\(^T\) represents a novel species of the genus \(Haloplanus\), for which the name \(Haloplanus vescus\) sp. nov. is proposed. Characteristics that distinguish strain RO5-8\(^T\) from \(H.\) natans JCM 14081\(^T\) are shown in Table 1.

### Emended description of the genus Haloplanus

Bardavid et al. 2007

Cells are pleomorphic, flat and contain gas vesicles. In static liquid culture, cells float to the surface. Gram-negative. Strictly aerobic. Extremely halophilic. Cells lyse in distilled water. Oxidase- and catalase-positive. Cells contain PG, PGP-Me, PGS and one major glycolipid that is chromatographically identical to S-DGD-1. The genomic DNA G + C content is 62.1–66.4 mol% (as determined by HPLC). The type species is \(Haloplanus natans\).

### Description of Haloplanus vescus sp. nov.

**Haloplanus vescus** (ves′cus. L. masc. adj. vescus thin and weak).

Cells are pleomorphic, flat (1.0–2.0 \(\mu\)m) under optimal growth conditions, contain gas vesicles and stain Gram-negative. Colonies on agar plates containing 3.1 M NaCl are pink, elevated and round. Chemo-organotrophic and aerobic. Growth occurs at 30–50 °C (optimum 40 °C), at 2.6–4.3 M NaCl (optimum 3.1 M NaCl), at 0.03–0.5 M \(\text{MgCl}_2\) (optimum 0.03 M \(\text{MgCl}_2\)) and at pH 5.5–7.5 (optimum pH 6.0–6.5). Cells lyse in distilled water;

### Table 1. Characteristics that distinguish strain RO5-8\(^T\) from Haloplanus natans JCM 14081\(^T\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<tr>
<td>Cell size ((\mu)m)</td>
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<td>2.5–8</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>6.0–6.5</td>
<td>7.0</td>
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<tr>
<td>pH range</td>
<td>5.5–7.5</td>
<td>6.5–8.5</td>
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<tr>
<td>(\text{H}_2\text{S}) formation</td>
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<td>+</td>
</tr>
<tr>
<td>Sensitivity to rifampicin</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Utilization of D-ribose</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Utilization of maltose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>62.1</td>
<td>66.2</td>
</tr>
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</table>
minimal NaCl concentration to prevent cell lysis is 12% (w/v). Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite is observed and nitrite is reduced. H₂S is not produced from sodium thiosulfate. Positive for indole formation. Tweens 20, 40, 60 and 80, casein, starch and gelatin are not hydrolysed. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, maltose, lactose, D-mannitol, D-sorbitol, acetate, pyruvate and DL-lactate. D-Galactose, D-fructose, L-sorbose, D-ribose, D-xyllose, sucrose, starch, glycerol, succinate, L-malate, fumarate, citrate, glycin, L-alanine, L-arginine, L-aspartate, L-glutamate, L-lysine and L-ornithine are not utilized as carbon sources. Sensitive to the following antibiotics (μg per disc, unless otherwise indicated): bacitracin (0.04 IU per disc), novobiocin (30), rifampicin (5), anisomycin (20) and aphidicolin (20). Resistant to the following antibiotics: erythromycin (15), neomycin (30), chloramphenicol (30), ampicillin (10), penicillin G (10 IU per disc), norfloxacin (10), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid (30). The major polar lipids are PG, PG-P, and PG-S. One major glycolipid chromatographically identical to S-DGD-I. The DNA G+C content of the type strain is 62.1% (as determined by HPLC).

The type strain is RO5-8T (=CGMCC 1.8712T =JCM 16055T) and was isolated from Rudong marine solar saltern in Jiangsu province, China.

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