Halopelagius inordinatus gen. nov., sp. nov., a new member of the family *Halobacteriaceae* isolated from a marine solar saltern

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Two extremely halophilic archaea, strains RO5-2^T and RO5-14, were isolated from Rudong marine solar saltern in Jiangsu, China. Cells of the two strains were pleomorphic, motile and stained Gram-negative. Colonies were red-pigmented. Strains RO5-2^T and RO5-14 were able to grow at 20–50 °C (optimum 37 °C), at 2.6–4.8 M NaCl (optimum 3.4–3.9 M NaCl), at 0.03–0.7 M MgCl₂ (optimum 0.5 M MgCl₂) and at pH 5.5–8.0 (optimum pH 6.5–7.0). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis was 12 % (w/v). The major polar lipids of the two strains were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and two major glycolipids chromatographically identical to sulfated mannosyl glucosyl diether (S-DGD-1) and mannosyl glucosyl diether (DGD-1). The 16S rRNA gene sequences of strains RO5-2^T and RO5-14 showed 93.4–93.8 % similarity to the closest cultivated relative, *Halosarcina pallida*. The DNA G + C content of strains RO5-2^T and RO5-14 was 61.0 mol% and 59.9 mol%, respectively. The DNA–DNA relatedness between strains RO5-2^T and RO5-14 was 86.0 %. The phenotypic, chemotaxonomic and phylogenetic properties suggest that strains RO5-2^T and RO5-14 represent a novel species in a new genus within the family *Halobacteriaceae*, for which the name *Halopelagius inordinatus* gen. nov., sp. nov. is proposed. The type strain is RO5-2^T (=CGMCC 1.7739^T =JCM 15773^T).

Extremely halophilic archaea that grow optimally in media containing 1.5–4.5 M NaCl are widely distributed in all kinds of hypersaline environments as well as in saline microniches in some non-hypersaline environments (Oren, 2006; Savage *et al.*, 2007, 2008). These micro-organisms are classified within the family *Halobacteriaceae*, the single family described within the order *Halobacteriales*. The identification of the genera and species belonging to the

family *Halobacteriaceae* is currently based on a polyphasic approach (Oren *et al.*, 1997). At the time of writing, the family *Halobacteriaceae* accommodated 27 recognized genera (Oren *et al.*, 2009), members of which inhabit both thalassohaline and athalassohaline environments.

Marine solar salterns, the typical thalassohaline hypersaline environments located in tropical and subtropical areas worldwide, are artificial shallow ponds for the production of common salt from seawater. There are more than 100 marine solar salterns built along the 18 000 km coastline of Eastern China. Some of these coastal salterns have been long-term targets for the study of halophilic archaeal resources (Cui *et al.*, 2007; Xu *et al.*, 2007). In this paper, we report on the isolation and characterization of two novel halophilic archaeal strains that represent a novel species in a novel genus of the family *Halobacteriaceae*.

Rudong solar saltern (32.2699° N 121.3999° E) is an artificial marine solar saltern located in Jiangsu Province, China. At the time of sampling, 28 March 2008, Rudong

Abbreviations: DGD-1, mannosyl glucosyl diether; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; S-DGD-1, sulfated mannosyl glucosyl diether.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains RO5-2^T and RO5-14 are EU887284 and FJ944013, respectively.

A scanning electron micrograph of strain RO5-2^T, a phase-contrast micrograph of strain RO5-14, thin-layer chromatograms and a maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences of strains RO5-2^T, RO5-14 and some other haloarchaea are available with the online version of this paper.

solar saltern had been off-production for less than 1 year after more than 30 years flourishing, and plenty of salt granules could be found in the saline soil of the saltern. To isolate halophilic archaea, soil samples from the solar saltern were collected (using a sterile steel spoon) into sterile 50 ml plastic tubes with screw caps. The tubes were carried back to the laboratory at room temperature. The neutral oligotrophic haloarchaeal medium (NOM) used for the isolation procedure was modified according to the DBCM2 medium from the online Halohandbook (Dyall-Smith, 2008) and contained the following ingredients (l^{-1}): yeast extract (Oxoid), 0.05 g; fish peptone (Sinopharm Chemical Reagent Co.), 0.25 g; sodium pyruvate, 1.0 g; KCl, 5.4 g; K_2HPO_4 , 0.3 g; $CaCl_2$, 0.25 g; NH_4Cl , 0.25 g; $MgSO_4 \cdot 7H_2O$, 26.8 g; $MgCl_2 \cdot 6H_2O$, 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. One gram of soil was suspended in 9 ml of liquid NOM medium, serially diluted with the same medium and then plated onto NOM agar plates. The plates were incubated in the dark at 37 °C for at least 8 weeks in sealed plastic bags. The plate with 50 to 100 colonies was selected for picking the single colonies designated strains RO5-2^T and RO5-14. To ensure purity, a single colony of each strain was restreaked twice onto NOM plates. The strains were routinely grown aerobically at 37 °C in NOM-1 medium containing (l^{-1}): yeast extract, 0.2 g; fish peptone, 0.2 g; sodium pyruvate, 2.0 g; sodium lactate, 2.0 g; KCl, 5.4 g; K_2HPO_4 , 0.3 g; NH_4Cl , 0.5 g; $MgSO_4 \cdot 7H_2O$, 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 *Haloferax volcanii* CGMCC 1.2150^T, *Haloferax elongans* JCM 14791^T, *Halogeometricum borinquense* JCM 10706^T, *Halosarcina pallida* BZ256^T and *Haloplanus natans* JCM 14081^T 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 % (v/v). 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 % (v/v) ethanol solutions (10 min at each stage), critical-point dried, 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 sterile saline solutions containing NaCl 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 $NaNO_3$ 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} was tested in completely filled 10 ml stoppered tubes. Starch hydrolysis was determined on NOM agar plates supplemented with 2 g soluble starch l^{-1} 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 H_2S was tested by growing the isolates and reference strains in a tube with NOM liquid medium supplemented with 0.5 % (w/v) $Na_2S_2O_3$; a filter-paper strip impregnated with lead acetate was used for H_2S 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} . After 7–10 days of cultivation, the turbidity of the cultures was compared with those without added carbon sources. Sensitivity to antimicrobial agents was performed as described by Gutiérrez *et al.* (2008).

Cells of strains RO5-2^T and RO5-14 were motile and pleomorphic (rods and irregular cocci, see Supplementary Fig. S1 available in IJSEM Online) when grown in NOM-1 liquid medium. Cells stained Gram-negative and colonies were red-pigmented. Strains RO5-2^T and RO5-14 were able to grow at 20–50 °C (optimum 37 °C), at 2.6–4.8 M NaCl (optimum 3.4–3.9 M NaCl), at 0.03–0.7 M $MgCl_2$ (optimum 0.5 M $MgCl_2$) and at pH 5.5–8.0 (optimum pH 6.5–7.0). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis was 12 % (w/v). Both strains produced indole from tryptophan and did not hydrolyse gelatin, Tweens 20, 40, 60 and 80, starch and casein. They were able to grow in defined and complex media; D-glucose, D-mannose, D-galactose, D-fructose, maltose, sucrose, lactose, glycerol, D-sorbitol and acetate as carbon sources yielded the best growth. Acid was produced during growth on carbohydrates. The two strains differed in the utilization of single carbon sources for growth; strain RO5-2^T used succinate, L-malate and fumarate, but strain RO5-14 did not. More detailed results of phenotypic tests and nutritional features of the two strains are given in the genus and species descriptions.

Genomic DNA from the halophilic archaeal strains was prepared as described by Ng *et al.* (1995). The 16S rRNA genes were 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 (initial 5 min denaturing step at 95 °C; 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). The PCR products

were examined on a 1.0% (w/v) agarose gel and then cloned into the pEASY-T vector (TransGen Biotech) and transformed into *Escherichia coli* Mach1 cells. Eight transformants for each strain were randomly picked and sequenced at the Sino-GenoMax Company, Beijing, China, to determine whether the two strains possessed multiple distinct 16S rRNA gene sequences. Multiple sequence alignments were performed by using the CLUSTAL W program integrated in the MEGA4 software (Tamura *et al.*, 2007). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in MEGA4. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were calculated. 16S rRNA gene sequence similarity was calculated by comparison with sequences of related halophilic archaea from the online EzTaxon server (Chun *et al.*, 2007).

Sixteen complete 16S rRNA gene sequences (1472 nt each) of strains RO5-2^T and RO5-14 were obtained. Sequence comparisons indicated that both strains have one kind of 16S rRNA gene sequence; they are 99.3% similar to each other. Both strains showed low levels of 16S rRNA gene sequence similarity to other members of the family Halobacteriaceae. The most closely related recognized species were representatives of the genera *Halosarcina* (*Halosarcina pallida*, 93.4–93.8% similarity), *Haloferax* (*Haloferax volcanii*, 93.2–93.6% similarity), *Halogeometricum* (*Halogeometricum borinquense*, 93.1–93.5% similarity), *Haloquadratum* (*Haloquadratum walsbyi*, 90.4–90.7% similarity) and *Haloplanus* (*Haloplanus natans*, 90.1% similarity). Phylogenetic analysis using the neighbour-joining algorithm revealed that the two strains distantly clustered with the *Haloferax*, *Haloquadratum*, *Halogeometricum* and *Halosarcina* clades (Fig. 1). The phylogenetic position was also confirmed in a tree generated using the maximum-parsimony algorithm (see Supplementary Fig. S2). The single clade comprising strains RO5-2^T and RO5-14, separated from the genera *Haloferax* and *Haloquadratum*, suggests that the two strains represent a novel phylogenetic taxon.

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. To detect phospholipids, the plate 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 & Kostetsky, 1968). To detect 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% α -naphthol in methanol/water (1:1, v/v). The plate was then sprayed with sulfuric acid/ethanol (1:2, v/v) followed by heating at 150 °C for 3 min to detect phospholipids and glycolipids. Strains RO5-2^T and RO5-14 contained phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and two glycolipids chromatographically identical to sulfated mannosyl glucosyl diether (S-DGD-1) and mannosyl glucosyl diether (DGD-1) (Supplementary Fig. S3). The absence of phosphatidylglycerol sulfate (PGS) within this group helps to differentiate the two strains from the member of the genus *Haloplanus*, which contains PGS (Bardavid *et al.*, 2007). The glycolipid profile sets strains RO5-2^T and RO5-14 apart from *Halogeometricum borinquense*, which possesses a novel non-sulfate-containing glycolipid (Montalvo-Rodríguez *et al.*, 1998), and the sole member of the genus *Haloquadratum*, which only contains S-DGD-1 (Burns *et al.*, 2007).

DNA G + C content of the novel strains 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 strains RO5-2^T and RO5-14 was 61.0 and 59.9 mol%, respectively. These values are lower than those of *Halosarcina pallida* (65.4 mol%; Savage *et al.*, 2008) and *Haloplanus natans* (66.2 mol%; Bardavid *et al.*, 2007), but higher than the values reported for *Haloquadratum walsbyi*

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain RO5-2^T, strain RO5-14 and other close relatives within the family Halobacteriaceae. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 75% bootstrap support. Bar, 0.02 substitutions per nucleotide position.

(46.9 mol%) (Burns *et al.*, 2007) and *Halogeometricum borinquense* (59.1 mol%; Montalvo-Rodríguez *et al.*, 1998). The DNA–DNA relatedness between strains RO5-2^T and RO5-14 was 86.0%. The data show that the two strains should be classified in the same species, since the generally accepted threshold value to separate two species is 70% (Stackebrandt & Goebel, 1994).

In view of the morphological and physiological properties of the new strains, the low levels of 16S rRNA gene sequence similarity with other genera within the family *Halobacteriaceae* and the distinctive components of the polar lipid profiles, we suggest that strains RO5-2^T and RO5-14 represent a novel species of a new genus within the family *Halobacteriaceae*, for which the name *Halopelagius inordinatus* gen. nov., sp. nov. is proposed. Characteristics that distinguish strains RO5-2^T and RO5-14 from other genera within the family *Halobacteriaceae* are shown in Table 1.

Description of *Halopelagius* gen. nov.

Halopelagius (Ha.lo.pe.la'gi.us. Gr. masc. n. *hals halos* salt; L. masc. adj. *pelagius* pertaining to the sea; N.L. masc. n. *Halopelagius* salt organism from the sea).

Cells are pleomorphic under optimal growth conditions and stain Gram-negative. Aerobic heterotrophs. Cells lyse in distilled water. Oxidase and catalase tests are positive. Temperatures between 20 and 50 °C support growth. Sugars are metabolized, in some cases with formation of acids. Cells contain PG and PGP-Me, but PGS is absent.

Two main glycolipids are present and chromatographically identical to S-DGD-1 and DGD-1. The genomic DNA G+C content is 59.9–61.0 mol%. The type species is *Halopelagius inordinatus*. Recommended three-letter abbreviation: *Hpl*.

Description of *Halopelagius inordinatus* sp. nov.

Halopelagius inordinatus (i.nor.di.na'tus. L. masc. adj. *inordinatus* not arranged, irregular).

Cells are motile, pleomorphic when grown in NOM-1 liquid medium and stain Gram-negative. Colonies on agar plates containing 3.9 M NaCl are red, elevated and round. Chemo-organotrophic and aerobic. Growth occurs at 20–50 °C (optimum 37 °C), at 2.6–4.8 M NaCl (optimum 3.4–3.9 M NaCl), at 0.03–0.7 M MgCl₂ (optimum 0.5 M MgCl₂) and at pH 5.5–8.0 (optimum pH 6.5–7.0). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 12% (w/v). Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine and DMSO. Nitrate reduction to nitrite is not observed. Nitrite is reduced. H₂S is produced from Na₂S₂O₃. Positive for indole formation. Tweens 20, 40, 60 and 80 are not hydrolysed. Negative for caseinase, amylase and gelatinase. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, D-fructose, maltose, sucrose, lactose, glycerol, D-sorbitol, acetate, pyruvate, DL-lactate, succinate, L-malate, fumarate, L-alanine, L-glutamate and L-ornithine. L-Sorbose, D-ribose, D-xylose, starch, D-mannitol, citrate,

Table 1. Differential characteristics between strain RO5-2^T, strain RO5-14 and closely related genera within the order *Halobacteriales*

Taxa: 1, Strains RO5-2^T and RO5-14; 2, *Haloferax* (Allen *et al.*, 2008); 3, *Haloquadratum* (Burns *et al.*, 2007); 4, *Halogeometricum* (Montalvo-Rodríguez *et al.*, 1998); 5, *Halosarcina* (Savage *et al.*, 2008); 6, *Haloplanus* (Bardavid *et al.*, 2007). +, Positive; –, negative; UG, unidentified glycolipid. All data was confirmed in the present study.

Characteristic	1	2	3	4	5	6
Cell shape	Pleomorphic	Pleomorphic	Squares	Pleomorphic	Cocci	Pleomorphic
Colony colour	Red	Red to pink	Red to pink	Pink	Pink	Pink
Optimum NaCl (M)	3.4–3.9	1.7–4.3	3.1	3.1–4.3	3.1	3.0
Optimum temperature (°C)	37	32–53	45	40	30	40
Optimum pH	6.5–7.0	7.0–7.5	7.0	7.0	6.5	7.0
Anaerobic growth with nitrate	–	+/–	–	+	–	–
Catalase activity	+	+	–	+	+	+
Oxidase activity	+	+/–	–	+	+	+
Indole formation	+	+/–	–	+	+	+
Starch hydrolysis	–	+/–	–	–	–	–
Gelatin liquefaction	–	+/–	–	+	–	–
Glucose used	+	+	–	+	+	+
Glycerol used	+	+	–	+	+	–
DNA G+C content (mol%)	59.9–61.0	59.5–65.3	46.9	59.1	65.4	66.2
Presence of PGS	–	–	–	–	–	+
Types of glycolipids present	S-DGD-1, DGD-1	S-DGD-1, DGD-1	S-DGD-1	UG	S-DGD-1, DGD-1	S-DGD-1

glycine, L-arginine, L-aspartate and L-lysine are not utilized as carbon sources. Sensitive to the following antibiotics (μg per disc, unless otherwise indicated): rifampicin (5), novobiocin (30), bacitracin (0.04 IU per disc), 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, PGP-Me, S-DGD-1 and DGD-1. The DNA G + C content of the type strain is 61.0 % (as determined by HPLC).

The type strain is RO5-2^T (=CGMCC 1.7739^T =JCM 15773^T), and was isolated from the Rudong solar saltern in Jiangsu province, China.

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References

- Allen, M. A., Goh, F., Leuko, S., Echigo, A., Mizuki, T., Usami, R., Kamekura, M., Neilan, B. A. & Burns, B. P. (2008). *Haloferax elongans* sp. nov. and *Haloferax mucosum* sp. nov., isolated from microbial mats from Hamelin Pool, Shark Bay, Australia. *Int J Syst Evol Microbiol* **58**, 798–802.
- Bardavid, R. E., Mana, L. & Oren, A. (2007). *Haloplanus natans* gen. nov., sp. nov., an extremely halophilic, gas-vacuolate archaeon isolated from Dead Sea–Red Sea water mixtures in experimental outdoor ponds. *Int J Syst Evol Microbiol* **57**, 780–783.
- Burns, D. G., Janssen, P. H., Itoh, T., Kamekura, M., Li, Z., Jensen, G., Rodriguez-Valera, F., Bolhuis, H. & Dyall-Smith, M. L. (2007). *Haloadaptatus walsbyi* gen. nov., sp. nov., the square haloarchaeon of Walsby, isolated from saltern crystallizers in Australia and Spain. *Int J Syst Evol Microbiol* **57**, 387–392.
- Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y.-W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.
- Cui, H.-L., Lin, Z.-Y., Dong, Y., Zhou, P.-J. & Liu, S.-J. (2007). *Halorubrum litoreum* sp. nov., an extremely halophilic archaeon from a solar saltern. *Int J Syst Evol Microbiol* **57**, 2204–2206.
- Cui, H.-L., Zhou, P.-J., Oren, A. & Liu, S.-J. (2009). Intraspecific polymorphism of 16S rRNA genes in two halophilic archaeal genera, *Haloarcula* and *Halomicrobium*. *Extremophiles* **13**, 31–37.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Dussault, H. P. (1955). An improved technique for staining red halophilic bacteria. *J Bacteriol* **70**, 484–485.
- Dyall-Smith, M. L. (2008). The Halo handbook: protocols for haloarchaeal genetics. <http://www.haloarchaea.com/resources/halo handbook>
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Gonzalez, C., Gutierrez, C. & Ramirez, C. (1978). *Halobacterium vallismortis* sp. nov. An amyolytic and carbohydrate-metabolizing, extremely halophilic bacterium. *Can J Microbiol* **24**, 710–715.
- Gutiérrez, C. & González, C. (1972). Method for simultaneous detection of proteinase and esterase activities in extremely halophilic bacteria. *Appl Microbiol* **24**, 516–517.
- Gutiérrez, M. C., Castillo, A. M., Kamekura, M. & Ventosa, A. (2008). *Haloterrigena salina* sp. nov., an extremely halophilic archaeon isolated from a salt lake. *Int J Syst Evol Microbiol* **58**, 2880–2884.
- HuB, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Kates, M. (1986). In *Techniques of Lipidology*, 2nd revised edn, pp. 106–107, 187–188 and 251–254. Amsterdam: Elsevier.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Montalvo-Rodríguez, R., Vreeland, R. H., Oren, A., Kessel, M., Betancourt, C. & López-Garriga, J. (1998). *Halogeometricum borinquense* gen. nov., sp. nov., a novel halophilic archaeon from Puerto Rico. *Int J Syst Bacteriol* **48**, 1305–1312.
- Ng, W.-L., Yang, C.-F., Halladay, J. T., Arora, A. & DasSarma, S. (1995). Protocol 25. Isolation of genomic and plasmid DNAs from *Halobacterium halobium*. In *Archaea: a Laboratory Manual: Halophiles*, pp. 179–180. Edited by S. DasSarma & E. M. Fleischmann. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Oren, A. (2006). The order *Halobacteriales*. In *The Prokaryotes: a Handbook on the Biology of Bacteria*, 3rd edn, vol. 3, pp. 113–164. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.
- Oren, A., Ventosa, A. & Grant, W. D. (1997). Proposed minimal standards for description of new taxa in the order *Halobacteriales*. *Int J Syst Bacteriol* **47**, 233–238.
- Oren, A., Arahall, D. R. & Ventosa, A. (2009). Emended descriptions of genera of the family *Halobacteriaceae*. *Int J Syst Evol Microbiol* **59**, 637–642.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Savage, K. N., Krumholz, L. R., Oren, A. & Elshahed, M. S. (2007). *Haladaptatus paucihalophilus* gen. nov., sp. nov., a halophilic archaeon isolated from a low-salt, sulfide-rich spring. *Int J Syst Evol Microbiol* **57**, 19–24.
- Savage, K. N., Krumholz, L. R., Oren, A. & Elshahed, M. S. (2008). *Halosarcina pallida* gen. nov., sp. nov., a halophilic archaeon from a low-salt, sulfide-rich spring. *Int J Syst Evol Microbiol* **58**, 856–860.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Vaskovsky, V. E. & Kostetsky, E. Y. (1968). Modified spray for the detection of phospholipids on thin-layer chromatograms. *J Lipid Res* **9**, 396.
- Xu, X.-W., Wu, Y.-H., Wang, C.-S., Oren, A., Zhou, P.-J. & Wu, M. (2007). *Haloferax larsenii* sp. nov., an extremely halophilic archaeon from a solar saltern. *Int J Syst Evol Microbiol* **57**, 717–720.