Halomonas ilicicola sp. nov., a moderately halophilic bacterium isolated from a saltern

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A Gram-negative, heterotrophic, aerobic, pale orange-pigmented, non-endospore-forming and motile bacterial strain, designated strain SP8^T, was isolated from a salty water sample from the solar salterns of Santa Pola, located on the Mediterranean coast of Spain. The strain grew optimally at 37 °C, pH 6.5 and in the presence of 10 % NaCl. A polyphasic taxonomic study was conducted in order to characterize the strain in detail. Phylogenetic analyses based on 16S rRNA gene sequence comparisons indicated that strain SP8^T clustered within the branch constituted by species of the genus *Halomonas*. The closest phylogenetic neighbours of strain SP8^T were *Halomonas muralis* LMG 20969^T (96.0 % sequence similarity), *Halomonas pantelleriensis* AAP^T (95.9 %) and *Halomonas campaniensis* 5AG^T (95.8 %). Phenotypic features, the fatty acid profile and the DNA G + C content of the novel strain further supported its placement in the genus *Halomonas*. On the basis of phenotypic, chemotaxonomic and phylogenetic distinctiveness, it is suggested that strain SP8^T represents a novel species for which the name *Halomonas ilicicola* sp. nov. is proposed. The type strain is SP8^T (=CECT 7331^T=CCM 7522^T=DSM 19980^T).

The family *Halomonadaceae* (Franzmann *et al.*, 1988) belongs to the class *Gammaproteobacteria* which, at the time of writing, comprises seven genera: *Halomonas* (Dobson & Franzmann, 1996), *Carnimonas* (Garriga *et al.*, 1998), *Chromohalobacter* (Ventosa *et al.*, 1989), *Cobetia* (Arahal *et al.*, 2002b), *Halotalea* (Ntougias *et al.*, 2007), *Modicisalibacter* (Ben Ali Gam *et al.*, 2007) and *Zymobacter* (Okamoto *et al.*, 1993). All members described in this family so far are able to grow at salinities between 5–10 % (w/v) except *Zymobacter palmae* (Okamoto *et al.*, 1993). The genus *Halomonas* was originally proposed with the sole species *Halomonas elongata* to accommodate a group of Gram-negative, rod-shaped and extremely halotolerant strains (Vreeland *et al.*, 1980). Taxonomically, the genus *Halomonas* is very heterogeneous and, at the time of writing, includes 49 species (<http://www.bacterio.cict.fr/>). It was established by Arahal *et al.* (2002a) that this genus is not monophyletic on the basis of 16S and 23S rRNA gene sequence analyses. These authors distinguished two phylogenetic groups comprising

different species with similar sequence similarities. In addition, several species did not clearly fall into either of the two groups mentioned and did not form a group themselves. All members of the family *Halomonadaceae* are aerobes, although some species of the genus *Halomonas* have been shown to grow anaerobically with nitrate, nitrite or fumarate as an electron acceptor and with glucose on solid media in sealed jars (Mata *et al.*, 2002). In this paper, the features of a new strain isolated from a saltern are described and it is shown to represent a novel species of the genus *Halomonas*.

The Bras del Port solar salterns are located on the eastern coast of Spain close to the city of Santa Pola (Alicante) (38° 11' 29.22" N 0° 35' 39.70" W). Strain SP8^T was isolated during the characterization of the microbial diversity in samples taken from a pond containing saturated brine collected during the salt washing process to remove particles and also to replace magnesium brine clinging to the salt crystals. This strain was selected for further study on the basis of its distinctive morphological features compared with the other isolates. The isolation was performed at 37 °C using the dilution plating technique and a modification of medium no. 29 from the CECT (<http://www.cect.org>), containing (% w/v): yeast extract (0.3), peptone (0.5), NaCl (15.0) and agar (2.0) and adjusted to pH 7.4. Subculturing was performed on 10 % MH medium that had the following composition (% w/v):

Abbreviation: SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SP8^T is EU218533.

Additional phylogenetic trees based on neighbour-joining and maximum-likelihood analysis of the 16S rRNA gene sequences of strain SP8^T and closely related species are available with the online version of this paper.

NaCl (8.1), MgCl₂ (0.7), MgSO₄ (0.96), CaCl₂ (0.036), KCl (0.2), NaHCO₃ (0.006), NaBr (0.0026), proteose peptone (Difco) (0.5), yeast extract (Difco) (1.0), glucose (0.1) and agar (1.5) (Ventosa *et al.*, 1982).

The procedures followed for phenotypic characterization have been described previously by Ventosa *et al.* (1982), Mata *et al.* (2002) and Arahall *et al.* (2007). Cellular morphology was examined by phase-contrast microscopy (model Axiolab; Zeiss) and scanning electron microscopy (SEM) (model DSM950; Zeiss) after growth on 10 % MH medium at 37 °C. SEM imaging was performed with cells grown on inclined glass coverslips embedded into culture medium in a Petri dish. Cells were fixed using 5 % formaldehyde in 0.2 M cacodylate buffer, pH 7.2 for 1 h, followed by two washings with the same buffer. Cells were dehydrated by washing four times with increasing gradients of ethyl alcohol ranging from 25 % to 100 % and twice with acetone for 10 min. The samples were stored overnight in anhydrous acetone. Critical point-drying was then performed using liquid carbon dioxide rinse cycles to remove traces of ethanol and acetone. Samples were heated to 30–40 °C to remove any remaining moisture. Samples were then sputter-coated with gold–palladium. The Gram reaction was performed as described by Gerhardt *et al.* (1994).

Cells were short rod-shaped with rounded ends; no endospores were observed (Fig. 1). Strain SP8^T was able to grow on MH medium supplemented with 2 to 17.5 % (w/v) total salts and in MH medium supplemented with 2 to 17.5 % NaCl (w/v). Optimal growth was found on MH medium with 10 % (w/v) NaCl. Thus this micro-organism can be considered as moderately halophilic (Ventosa *et al.*, 1998). Other morphological, physiological and biochemical characteristics of the novel strain are shown in Table 1 or are given in the species description.

For the determination of the DNA G + C content of strain SP8^T, the extraction and purification of genomic DNA

was performed according to Marmur (1961). The G + C content was determined using the thermal denaturation method (Marmur & Doty, 1962; Owen & Hill, 1979). The phylogenetic position of strain SP8^T was determined by 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified using oligonucleotide primers complementary to highly conserved regions of bacterial 16S rRNA genes. The forward primer and the reverse primer were 27F and 1492R (Reysenbach *et al.*, 2000). PCR products were purified using an UltraClean PCR Clean-up kit (Mo Bio), according to the manufacturer's instructions. Purified PCR products were sequenced using the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit and an Applied Biosystems 3130 Genetic Analyzer, using the protocols of the manufacturer (Applied Biosystems). Sequence assembly was performed using the CAP3 program (Huang & Madan, 1999). The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Subsequent sequence analysis was conducted using the ARB program package (Ludwig *et al.*, 2004). Following the recommendations of Ludwig *et al.* (1998), alternative treeing methods (maximum-parsimony, neighbour-joining and maximum-likelihood) were used. The 16S rRNA gene sequence of strain SP8^T was a continuous stretch of 1423 bp and was used for an initial BLAST search against the GenBank, EMBL and DDBJ databases. The 16S rRNA gene sequence of strain SP8^T and sequences of closely related strains were aligned and the phylogenetic trees showed that strain SP8^T clustered together with species of the genus *Halomonas*. The position of strain SP8^T in a tree constructed using maximum-parsimony is shown in Fig. 2. Similar topologies were obtained when other treeing methods were used (see Supplementary Fig. S1 in IJSEM Online). The sequence similarity matrix indicated that the closest relatives of strain SP8^T were *Halomonas muralis* LMG 20969^T (96.0 % sequence similarity), *Halomonas pantelleriensis* AAP^T (95.9 %) and *Halomonas campaniensis* 5AG^T (95.8 %). Sequence similarities to all other species of the genus *Halomonas* included in the phylogenetic analyses were equal to or below 95.5 %. The DNA G + C content of strain SP8^T was 66.9 mol%, similar to the values reported for the closely related species of *Halomonas* (Table 1).

Fatty acids were analysed by GC at the Belgian Coordinated Collections of Micro-organisms, Laboratory of Microbiology of Gent (BCCM/LMG), Gent, Belgium (Kämpfer & Kroppenstedt, 1996; Miller, 1982). Cells were cultured on TSA medium supplemented with 10 % (w/v) NaCl for 48 h at 30 °C. Strain SP8^T had a fatty acid profile similar to those described for the type strains of the closely related *Halomonas* species (Table 2). The predominant fatty acids are C_{18:1ω7c} and C_{16:0}.

Based on these combined phenotypic and phylogenetic analyses, strain SP8^T is not closely affiliated with any recognized species. This organism is closely related to

Fig. 1. SEM image of cells of strain SP8^T. Cells were grown on 10 % MH medium at 37 °C for 3 days. Bar, 1 μm.

Fig. 2. Phylogenetic tree, based on maximum-parsimony, of 16S rRNA gene sequences, showing the position of strain SP8^T and closely related species. Sequence accession numbers are shown in parentheses. Bootstrap values higher than 50 % are indicated at branch-points. *Zymobacter palmae* T109^T was used as outgroup. Bar, 1 % sequence divergence.

species of the genus *Halomonas* and we propose to include it as a novel species of this genus. The name *Halomonas ilicicola* sp. nov. is proposed for the novel species. Some features that enable *Halomonas ilicicola* sp. nov. to be differentiated from related species of the genus *Halomonas* are shown in Table 1.

Description of *Halomonas ilicicola* sp. nov.

Halomonas ilicicola (i.li.ci'co.la. L. n. *ilici* the Roman name of Elche, the city close to the solar salterns where the type strain was isolated; L. suff. *-cola* inhabitant, dweller; N.L. n. *ilicicola* inhabitant of Ilici).

Table 1. Characteristics that differentiate strain SP8^T and some related species of the genus *Halomonas*

Taxa: 1, SP8^T; 2, *H. muralis* (7 isolates); 3, *H. pantelleriensis* DSM 9661^T; 4, *H. elongata* ATCC 33173^T. Data are from Arahall *et al.* (2002a); Heyrman *et al.* (2002); Martínez-Cánovas *et al.* (2004) and this study. +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4
Morphology	Short rods	Rods	Rods	Rods
Pigmentation	Pale orange	Colourless	Cream–pink	Colourless
Salt range (%)	2–17.5	0–15	1.2–15	3.5–20
Temperature range (°C)	25–42	10–35	10–44	15–45
pH range	6.0–9.0	5–10	7.5–11	5–9
Hydrolysis of gelatin	+	–	–	+
H ₂ S production	–	ND	+	–
Urease	–	–	+	+
Oxidase	–	+	+	–
Growth on:				
D-Fructose	+	–	+	+
D-Mannose	–	+ / –	+	+
DNA G + C content (mol%)	66.9	62.4	65	60.5
16S rRNA gene sequence similarity to strain SP8 ^T (%)	100	96.0	95.9	93.8

Table 2. Cellular fatty acid content (%) of strain SP8^T, *H. muralis* and *H. elongata*

Taxa: 1, strain SP8^T; 2, *H. muralis* LMG 20969^T; 3, *H. elongata* ATCC 33173^T. Values <0.5 % are not shown; –, not detected.

Fatty acid	1	2	3
C _{10:0}	2.3	2.7	2.4
C _{10:0} 3-OH	3.8	–	3.1
C _{12:0}	2.6	4.1	2.8
C _{12:0} 3-OH	8.2	8.8	7.7
Summed feature 2*	1.1	–	0.9
Summed feature 3*	8.9	18.8	5.8
C _{16:0}	20.0	17.3	20.7
C _{16:0} 3-OH	0.9	–	0.8
C _{17:0} cyclo	0.7	–	0.7
C _{18:1} ω7c	46.3	43.6	47.3
C _{19:0} cyclo ω8c	3.4	2.0	6.1
C _{19:0} 10 methyl	1.0	–	0.7

*Summed features are groups of fatty acids which were not resolved chromatographically; summed feature 2 consists of C_{16:1} iso 1 and/or iso-C_{14:0} 3-OH; summed feature 3 consists of C_{16:1} ω7c and/or iso-C_{15:0} 2-OH.

Cells are short rods, 0.5–0.6 × 0.8–1.1 μm, occur singly or in pairs, are motile, do not form endospores and stain Gram-negative. Colonies are circular, smooth, convex, pale orange, mucoid and 1.0–1.2 mm in diameter after 3 days incubation on MH medium at 37 °C. In liquid medium, grows producing a uniform turbidity. Exopolysaccharide is not produced. Poly-β-hydroxyalkanoates are not accumulated. Moderately halophilic, capable of growing in concentrations of between 2 and 17.5 % (w/v) salts. No growth occurs in the absence of salt. Optimal growth at 10 % (w/v) salts or NaCl. The temperature range for growth is 25–42 °C with optimal growth at 37 °C. No growth detected at 15, 20 or 45 °C. Growth occurs at pH 6.0–9.0 and is optimal at pH 6.5. Chemo-organotrophic. Metabolism is respiratory, with oxygen, nitrate or nitrite as terminal electron acceptor. Cells do not grow anaerobically in the presence of fumarate. Catalase is produced but not oxidase. D-Glucose is not fermented. Acid is produced from D-galactose, D-glucose, D-fucose, D-sorbitol, turanose and potassium 5-ketogluconate, but not from D-adonitol, L-arabinose, D-fructose, *myo*-inositol, D-lactose, maltose, D-mannose, melezitose, L-rhamnose, glycerol, D-mannitol, L-sorbose, trehalose or D-xylose. Gelatin and aesculin are hydrolysed, but not starch, casein, Tween 80, DNA or tyrosine. Nitrate and nitrite are reduced (with gas formation). Grows on MacConkey and cetrimide agars supplemented with 10 % NaCl. Methyl red test is positive. Indole and H₂S production are negative. The Voges–Proskauer test and tests for activities of urease and lysine-, ornithine- and arginine decarboxylases, ONPG and phenylalanine deaminase are negative. The following compounds can be used as sole carbon and energy sources:

acetate, citrate, D-fructose, fumarate, D-galactose, gluconate, D-glucose, D-mannitol and propionate, but not aesculin, L-arabinose, cellobiose, ethanol, formate, lactose, D-mannitol, D-mannose, malonate, maltose, *myo*-inositol, D-ribose, starch, succinate, trehalose or D-xylose. L-Alanine can be used as sole carbon, nitrogen and energy sources, but not L-cysteine, L-histidine, L-lysine, L-methionine, L-serine or L-valine. Cells are sensitive to (μg per disc) trimethoprim/sulfamethoxazole (1.25/23.75). Intermediate resistance to (μg per disc): erythromycin (15), nalidixic acid (30) and rifampicin (5). Resistant (μg per disc) to penicillin (10 U), vancomycin (30), chloramphenicol (30), streptomycin (10), bacitracin (10), cephalothin (30), novobiocin (30) neomycin (30), kanamycin (30) and tetracycline (30). The predominant fatty acids are C_{18:1} ω7c and C_{16:0} (Table 2).

The type strain, SP8^T (=CECT 7331^T=CCM 7522^T=DSM 19980^T), was isolated from a salty water sample from the solar salterns of Santa Pola (Alicante), located on the Mediterranean coast of Spain. The DNA G + C content of the type strain is 66.9 mol% (*T_m* method).

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