Halomonas nitroreducens sp. nov., a novel nitrate- and nitrite-reducing species

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We have carried out a polyphasic taxonomic study of strain 11S^T, a halophilic, Gram-negative bacterium that is able to respire on nitrate and nitrite in anaerobiosis. Strain 11S^T was isolated from a solar saltern in Cahuil, a region next to Pichilemu (Chile). It grows at NaCl concentrations within the range of 3–20 % w/v (optimum 5–7.5 %), temperatures from 4 to 45 °C (optimum 20–32 °C) and within a pH range of 5–10 (optimum pH 7–9). Its 16S rRNA gene sequence indicates that it belongs to the genus *Halomonas* in the class *Gammaproteobacteria*. Its closest relatives are *Halomonas alimentaria*, *H. denitrificans*, *H. organivorans* and *H. ventosae*, with the type strains of which our strain showed maximum 16S rRNA gene sequence similarity values of 97.1–98.1 %. Its G+C content is 65.3 mol%. DNA–DNA hybridization studies showed 54.2 % relatedness between strain 11S^T and *H. alimentaria* DSM 15356^T and 47.2 % relatedness between strain 11S^T and *H. organivorans* CECT 5995^T. Lower DNA–DNA hybridization percentages were obtained against the type strains of other related *Halomonas* species. Its major fatty acids are $C_{12:0}$ 3-OH (5.56 %), iso-$C_{15:0}$ 2-OH/$C_{16:1}\omega7c$ (22.30 %), $C_{16:0}$ (27.80 %) and $C_{18:1}\omega7c$ (29.92 %). The proposed name for the novel species is *Halomonas nitroreducens* sp. nov., with strain 11S^T (=CECT 7281^T =LMG 24185^T) being the type strain.

Members of the family *Halomonadaceae* (Franzmann *et al.*, 1988; Dobson & Franzmann, 1996; Garrity *et al.*, 2005) form a monophyletic group within the order *Oceanospirillales* belonging to the *Gammaproteobacteria*. Currently, the family *Halomonadaceae* contains three genera of halophilic micro-organisms, *Halomonas* (Vreeland *et al.*, 1980; Dobson & Franzmann, 1996), *Chromohalobacter* (Ventosa *et al.*, 1989; Arahal *et al.*, 2001) and *Cobetia* (Arahal *et al.*, 2002), and two genera of non-halophilic bacteria, *Zymobacter* (Okamoto *et al.*, 1993) and *Carnimonas* (Garriga *et al.*, 1998).

Halomonas is the type genus of the *Halomonadaceae* and, at the time of writing, contains 46 validly published species names and four additional descriptions in press (http://ijs.sgmjournals.org/misc/pip.shtml). Characteristically, strains belonging to *Halomonas* have a respiratory type of metabolism with oxygen as the terminal acceptor. Nevertheless, the genus is very heterogeneous and includes diverse species in terms of their physiology, ecology and nutrition and, in fact, its type species, *Halomonas elongata*, and some others are able to respire anaerobically with nitrate. Eight further species, *Halomonas alimentaria* (Yoon *et al.*, 2002), *H. campisalis* (Mormile *et al.*, 1999), *H. denitrificans* (Kim *et al.*, 2007), *H. desiderata* (Berendes *et al.*, 1996), *H. gudaonensis* (Wang *et al.*, 2007a), *H. halodenitrificans* (Dobson & Franzmann, 1996), *H. shengliensis* (Wang *et al.*, 2007b) and *H. ventosae* (Martínez-Cánovas *et al.*, 2004), respire with nitrate and nitrite and are therefore denitrifiers. We have determined the taxonomic position of strain 11S^T and propose that it represents a novel species of *Halomonas*.

Strain 11S^T was found in a sample taken from a solar saltern in Cahuil, a region close to Pichilemu in Chile. It was maintained and routinely grown in MY medium (Moraine & Rogovin, 1966) with 7.5 % w/v sea-salts solution (Rodríguez-Valera *et al.*, 1981) at 32 °C.

The procedures followed for phenotypic characterization have been described by Mata *et al.* (2002). We compared the novel strain to 24 species of *Halomonas* by means of a numerical analysis based on data deriving from 107 phenotypic characteristics. Computer analysis was made with the TAXAN program (Information Resources Group, Maryland Biotechnology Institute, University of Maryland, College Park, USA). A dendrogram showing clustering of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 11S^T is EF613113.

A UPGMA dendrogram based on phenotypic characteristics and a 16S rRNA gene sequence-based maximum-parsimony tree are available as supplementary material with the online version of this paper.

strain 11S^T and the type strains of species of *Halomonas* is available as Supplementary Fig. S1 in IJSEM Online. The closest species phenotypically was *H. alimentaria* (85 % similarity to the type strain), but there were sufficient differences from it, mainly in NaCl and sea salt ranges of growth and in nutritional tests. The characteristics of strain 11S^T are given in the species description. Phenotypic features that differentiate the novel strain from other related species of *Halomonas* are included in Table 1.

The DNA of strain 11S^T was extracted by the method of Marmur (1961). The G+C DNA content was estimated from the midpoint value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979). The G+C content of reference DNA from *Escherichia coli* NCTC 9001^T was taken to be 50.9 mol% (Owen & Pitcher, 1985). The result was 65.3 mol% for strain 11S^T.

Phylogenetic analyses based on the 16S rRNA gene sequence were made as described in Bouchotroch *et al.* (2001). We determined the almost-complete 16S rRNA gene sequence (1441 bp) of strain 11S^T. The fragment analysed contained the 15 signature nucleotides defined for the *Halomonadaceae* (Dobson & Franzmann, 1996) and the four signature nucleotides characteristic of the genus *Halomonas*.

Table 1. Characteristics that distinguish *H. nitroreducens* sp. nov. 11S^T from other related species of *Halomonas*

Strains/species: 1, 11S^T; 2, *H. alimentaria* (data from Yoon *et al.*, 2002); 3, *H. denitrificans* (Kim *et al.*, 2007 and this study); 4, *H. halodenitrificans* (Mata *et al.*, 2002); 5, *H. maura* (Mata *et al.*, 2002); 6, *H. organivorans* (García *et al.*, 2004 and this study); 7, *H. ventosae* (Martínez-Cánovas *et al.*, 2004). ND, No data available; +, positive; −, negative; EPS, exopolysaccharide.

Characteristic	1	2	3	4	5	6	7
EPS	+	−	−	−	+	+	+
Motility	−	−	+	−	−	+	+
Oxidase	+	+	+	+	+	−	+
Sea-salt range (% w/v)	3–20*	1–23*	2–20*	3–20	1–20	1.5–30	1–15
Sea-salt optimum (% w/v)	5–7.5*	1–13*	8–10*	5–9	9	7.5–10	8
Acid from D-glucose	−	−	−	−	−	ND	−
Hydrolysis of:							
Aesculin	−	−	−	−	−	+	−
Gelatin	+	−	−	−	−	−	−
DNA	+	+	−	−	−	−	−
Production of H_2S	+	−	−	+	+	+	+
Respiration on nitrate	+	+	+	+	+	−	+
Respiration on nitrite	+	+	+	+	−	−	+
Gas production from nitrate	+	+	+	+	−	−	+
Growth on:†							
D-Fructose	+	−	+	+	+	+	+
D-Glucose	+	+	−	+	+	+	+
Lactose	+	+	ND	−	−	−	+
Maltose	+	+	−	−	+	−	+
D-Mannose	+	+	−	+	+	+	−
D-Salicin	−	+	−	−	−	ND	−
Trehalose	+	+	ND	+	−	+	+
Malonate	−	+	+	+	+	+	+
Propionate	+	+	+	+	+	−	+
Succinate	+	−	ND	+	+	+	+
Adonitol	−	−	ND	−	−	−	+
myo-Inositol	−	−	−	+	+	+	+
D-Mannitol	+	+	−	+	+	+	+
Sorbitol	+	+	−	+	+	+	+
Growth on:‡							
L-Alanine	+	+	+	+	+	+	−
L-Histidine	+	+	−	−	+	+	−
L-Serine	+	+	+	+	+	+	−
DNA G+C content (mol%)	65.3	63.0	53.8	64–66	64.1	61.0	74.3

*Tested using NaCl.
†When supplied as the sole source of carbon and energy.
‡When supplied as the sole source of carbon, nitrogen and energy.

The phylogenetic tree constructed using the neighbour-joining algorithm is shown in Fig. 1 and the phylogenetic tree constructed using maximum-parsimony algorithm is available as Supplementary Fig. S2. The trees obtained by the two methods show that 11S^T formed a sufficiently separate lineage in the genus *Halomonas*. Evolutionary distances, including a correction factor for reverse mutations (Jukes & Cantor, 1969), were calculated for sequence pairs by using a 'mask' (Lane, 1991) for non-homologous or uncertain nucleotide positions.

Similarities between the 16S rRNA gene nucleotide sequence of the isolate and the other species of *Halomonas* were obtained by MEGALIGN software (DNASTAR package; Burland, 2000). The similarity to the type strains ranged from 88.3 % with *Halomonas indalinina* to 98.1 % with *H. alimentaria*. The type strains of *H. denitrificans* (97.9 %), *H. organivorans* (97.9 %) and *H. ventosae* (97.1 %) are the others with the highest values of similarity.

DNA–DNA hybridization was conducted following the methods of Lind & Ursing (1986) with the modifications of Ziemke *et al.* (1998) and Bouchotroch *et al.* (2001). The results of DNA–DNA hybridization were 54.2 % relatedness between strain 11S^T and *H. alimentaria* DSM 15356^T and 47.2 % relatedness between strain 11S^T and *H. organivorans* CECT 5995^T. Lower DNA–DNA hybridization percentages were obtained against the type strains of other related *Halomonas* species. All these results show that the novel strain was not closely related to any of them.

The fatty acids of strain 11S^T were analysed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH by high-resolution GLC. For this purpose, strain 11S^T was grown in MY medium (Moraine & Rogovin, 1966) with 7.5 % w/v sea-salts solution (Rodríguez-Valera *et al.*, 1981) at 32 °C. The results are given in the species description. Strain 11S^T had a combination of fatty acids found in other species of *Halomonas* (Dobson & Franzmann, 1996), most importantly 18 : 1ω7*c*, 16 : 0, 16 : 1ω7*c*/iso-15 : 0 2-OH, 12 : 0 3-OH, 12 : 0, 10 : 0 and 19 : 0 cyclo ω8*c*.

Fig. 2 shows the cell size and morphology of strain 11S^T. The transmission electron micrograph was made as described in Bouchotroch *et al.* (2001).

On the basis of the data discussed and the full description provided below, we propose a novel species that respires with nitrate, *Halomonas nitroreducens*, to include strain 11S^T.

Description of *Halomonas nitroreducens* sp. nov.

Halomonas nitroreducens (ni.tro.re.du′cens. Gr. n. *nitron* potassium nitrate, saltpetre; L. adj. from pres. part. of verb *reduco* reduce, convert to a different condition; N.L. adj. *nitroreducens* reducing nitrate).

Cells are Gram-negative rods, 1.5–2.2 × 0.4–0.5 μm, appearing either singly or in pairs. The cells are non-motile. It produces exopolysaccharide, accumulates polyhydroxyalkanoate and does not form endospores. Cell colonies are circular, convex, creamy white in colour and quite mucoid. Its growth pattern is uniform in a liquid medium. It is a moderate halophile, capable of growing in NaCl concentrations of 3–20 % w/v, with optimum growth at 5–7.5 % w/v. It is not able to grow without NaCl or in NaCl concentrations lower than 3 % w/v or higher than 20 % w/v. Growth was tested within the temperature range of 4–45 °C (4, 15, 20, 25, 32, 37 and 45 °C), with positive results within the whole range and with optimum growth between 20 and 32 °C. The pH range for growth was tested

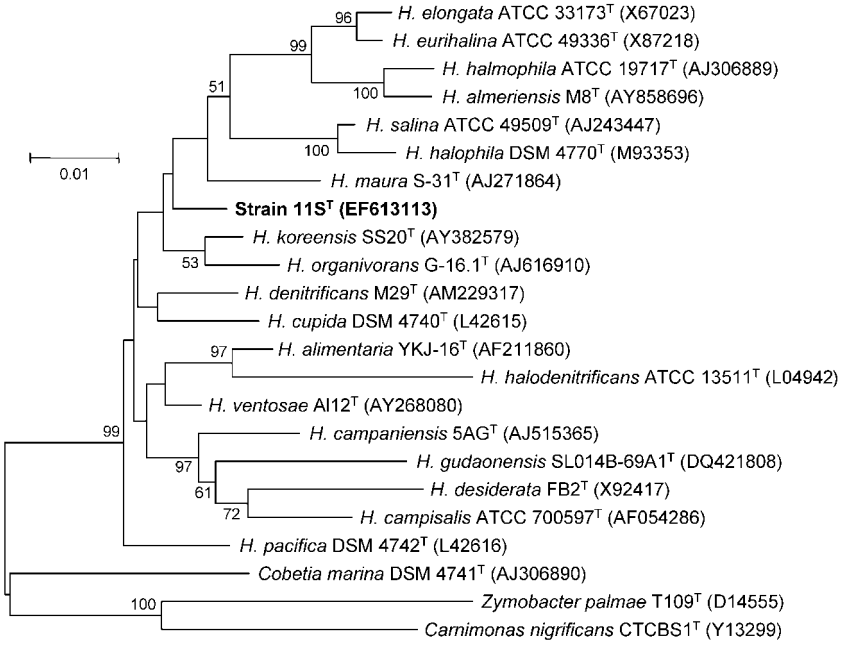

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of the novel isolate with respect to other members of the family *Halomonadaceae*. The tree was obtained using the neighbour-joining algorithm. GenBank accession numbers are given in parentheses. Bar, 1 % sequence divergence. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points.

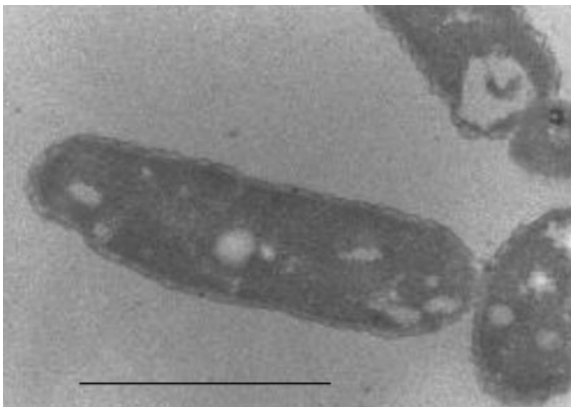

Fig. 2. Transmission electron micrograph of cells of strain 11S^T stained with ruthenium red. Bar, 1 μm.

from pH 5 to 10 at intervals of 1 pH unit, growing between these limits (optimum growth between pH 7.0 and 9.0). It resists heating to 80 °C for 10 min. It is chemo-organotrophic. Its metabolism is of the respiratory type with oxygen, nitrate and nitrite as terminal electron acceptors. It produces gas from nitrate and nitrite in anaerobiosis. It is catalase- and oxidase-positive. It produces H_2S from L-cysteine. Gluconate is not oxidized. It does not produce acids from adonitol, D-fructose, D-galactose, D-glucose, *myo*-inositol, lactose, maltose, D-mannitol, D-mannose, melezitose, L-rhamnose, sucrose, D-salicin, D-sorbitol, sorbose or trehalose. Indole, methyl red and Voges–Proskauer tests are negative. It reduces selenite, nitrate and nitrite. It hydrolyses urea, gelatin and tyrosine but not aesculin, starch, Tween 20, Tween 80, lecithin, casein or blood. DNase is produced but not phosphatase. It does not produce phenylalanine deaminase or pigment from tyrosine. It grows on MacConkey agar and cetrimide agar. ONPG is negative. The following compounds are acceptable as sole carbon and energy sources: acetate, citrate, ethanol, D-fructose, lactose, fumarate, D-galactose, DL-glycerol, gluconate, D-glucose, lactate, maltose, D-mannitol, D-mannose, propionate, sorbitol, starch, succinate and trehalose. The following compounds are not acceptable as sole carbon and energy sources: adonitol, aesculin, L-arabinose, *myo*-inositol, malonate and D-salicin. L-Alanine, L-lysine, L-histidine and L-serine are used as sole sources of carbon, nitrogen and energy. L-Cysteine, DL-isoleucine, L-methionine and L-valine are not used as sole sources of carbon, nitrogen and energy. It is susceptible to amoxicillin (25 μg), ampicillin (10 μg), aztreonam (30 μg), cefalotin (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), doxycycline (30 μg), gentamicin (10 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), penicillin (10 μg), polymyxin B (300 IU), rifampicin (30 μg), sulfamide (250 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg). It is resistant to tobramycin (10 μg) and vancomycin (30 μg). Principal fatty acids are (%): 18 : 1ω7*c* (29.92), 16 : 0 (27.80), 16 : 1ω7*c*/iso-15 : 0 2-OH (22.30), 12 : 0 3-OH (5.56), 12 : 0 (2.10), 10 : 0 (2.08) and 19 : 0 cyclo ω8*c* (7.02). The DNA G+C content of the type strain is 65.37 mol% (T_m method).

The type strain is strain 11S^T (=CECT 7281^T =LMG 24185^T). It was isolated from a solar saltern in Cahuil, a region close to Pichilemu in Chile.

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