***Halomonas nitroreducens* sp. nov., a new nitrate and nitrite reducing species**

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

Supplementary figures are available on line in IJSEM

Abstract

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% to 20% w/v (optimum 5-7.5%), temperatures from 4ºC to 45º C (optimum 20-32º C) and within a pH range of 5 to 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 which our strain showed maximum 16S rRNA similarity values of 97.1% to 98.1%. Its G+C content is 65.3 mol %. DNA-DNA hybridization studies between strain 11S^T and *H. alimentaria* DSM 15356^T showed 54.2% relatedness and 47.2% relatedness between strain 11S^T and *H. organivorans* CECT 5995^T. Lower DNA-DNA hybridization percentages were obtained against other related *Halomonas* species. Its major fatty acids are $C_{12:0}$ 3-OH (5,56%), $C_{15:0}$ ISO 2OH/ $C_{16:1}$ w7c (22,30%), $C_{16:0}$ (27,80%) and $C_{18:1}$ w7c (29,92%). The proposed name for the new species is *Halomonas nitroreducens*, 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 microorganisms, *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 (see IJSEM web site). 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-Canovas *et al.,* 2004), respire with nitrate and nitrite and are therefore denitrifiers.

We have determined the taxonomic position of strain 11S^{T} and propose it as a new species of *Halomonas*, with the name *Halomonas nitroreducens.*

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 salt solution (Rodríguez-Valera *et al.*, 1981) at 32ºC.

The procedures followed for phenotypic characterisation have been described by Mata *et al.* (2002). We compared the new 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, HD20742, USA). Dendrogram showing clustering of strain 11S ^{T} and type strains of species of *Halomonas* is available on line (supplementary Fig. S1, http://ijs.sgmjournals.org). The closest phenotypically species was *H. alimentaria* (85% of similarity), but there were enough differences with 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 differentiating the new species 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 *Halomonadaceae* (Dobson & Franzmann, 1996) and the 4 signature nucleotides characteristic of the genus *Halomonas*.

The phylogenetic tree constructed using the neighbour-joining algorithm is shown in Fig. 1. The phylogenetic tree constructed using maximum-parsimony algorithm is available on line (supplementary Fig. S2, http://ijs.sgmjournals.org). 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.

The similarities between the 16S rRNA nucleotide sequence of the isolate and the other species of *Halomonas* were obtained by Megalign software (DNAstar package, Burland, 2000). The similarity values ranged from 88.3% with *H. indalinina* to 98.1% with *H .alimentaria. H. denitrificans* (97.9%), *H. organivorans* (97.9%) and *H. ventosae* (97.1%) are the other species 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% of relatedness between strain 11S^T and *H. alimentaria* DSM 15356^T and 47.2% of relatedness between strain 11S^T and *H. organivorans* CECT 5995^T. Lower DNA-DNA hybridization percentages than those were obtained against other related *Halomonas* species. All these results show that the new strain was not closely related to any of them.

The fatty acids of strain 11S^T were analysed at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) by high-resolution GLC. To this purpose, strain 11S^T was grown in MY medium (Moraine & Rogovin, 1966) with 7.5% w/v sea-salt solutions (Rodríguez-Valera *et al.*, 1981) at 32ºC. The results are given in the species description. The new strain 11S^T had a combination of fatty acids found in other species of *Halomonas* (Dobson & Franzmann, 1996), most importantly 18:1 w7c ; 16:0 ; 16:1 w7C/15:0 ISO 2OH ; 12:0 30H ; 12:0 ; 10:0 and 19:0 CYCLO w8c.

Figure 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 nitroreduc´ens L. sb. nitrum = nitrate + L. adj. from pres. part. of verb reduco = reduce, convert to a different condition – N.L. adj. nitroreducens = reducing nitrate.

Halomonas nitroreducens cells are Gram-negative rods, 1.5-2.2 x 0.4-0.5 μm, appearing either singly or in pairs. The cells are non-motile. It produces exopolysaccharide, accumulates PHA 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 moderately halophile, capable of growing in NaCl concentrations of 3% to 20% w/v, with an optimum between 5% and 7.5% w/v. It is not able to grow without NaCl and in NaCl concentrations lower than 3% w/v and higher than 20% w/v. The growth was tested

within the temperatures range of 4ºC to 45ºC (4, 15, 20, 25, 32, 37 and 45ºC), with positive results within whole range and with an optimum between 20ºC and 32ºC. The pH range was tested from 5 to 10 at intervals of 1 pH unit, growing between these edges (optimum between 7.0 and 9.0). It resists heating to 80ºC for 10 minutes. 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 oxidised. It does not produce acids from adonitol, D-fructose, D-galactose, D-glucose, myo-inositol, lactose, maltose, D-mannitol, D-mannose, D-melezitose, L-rhamnose, sucrose, D-salicin, D-sorbitol, sorbose nor D-trehalose. Indol, methyl red and Voges-Proskauer are negative. It reduces selenite, nitrate and nitrite. It hydrolyses urea, gelatin and tirosyne 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 D-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), cefalotine (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), doxacicline (30 μg), gentamicine (10μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), norfloxacine (10 μg), penicillin (10 μg), polymyxin B (300 UI), rifampycin (30 μg), sulphamide (250 μg) and

trimetroprim-sulphametoxazol (1.25-23.75 µg). It is resistant to tobramycin (10 µg) and vancomycin (30 µg). Principal fatty acids are (%): 18:1 w7c (29.92); 16:0 (27.80), 16:1 w7C/15:0 ISO 2OH (22.30); 12:0 30H (5.56), 12:0 (2.10), 10:0 (2.08) and 19:0 CYCLO w8c (7.02). Its DNA G+C content 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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Table 1. Characteristics that distinguish *H. nitroreducens* sp. nov. (11S ^{T}) from other related type strains of *Halomonas*

1, 11 S ^{T}*;* **2,** *H. alimentaria* (Yoon *et al.,* 2002); **3**, *H. denitrificans* (Kim *et al.,* 2007)**; 4,** *H. halodenitrificans* (Mata *et al.,* 2002); **5,** *H. Maura* (Mata *et al.,* 2002); **6,** *H. organivorans* (García *et al.*, 2004); **7,** *H. ventosae* (Martínez-Cánovas *et al.*, 2004). ND: Not determined; +: Positive; -: Negative; EPS: Exopolysaccharide

Characteristic	1	2	3	4	5	6	7
EPS	+	-	ND	-	+	ND	+
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	+	+	ND	+	+	-	+
Respiration on nitrite	+	+	ND	+	-	-	+
Gas production from nitrate	+	+	ND	+	-	-	+
Growth on †							
D-fructose	+	-	+	+	+	+	+
D-glucose	+	+	-	+	+	+	+
lactose	+	+	ND	-	-	-	+
maltose	+	+	-	-	+	-	+
D-mannose	+	+	-	+	+	+	-
D-salicin	-	+	-	-	-	ND	-
D-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

* NaCl

† When supplied as the sole source of carbon and energy

‡ When supplied as the sole source of carbon, nitrogen and energy

Fig. S1. Dendrogram based on 107 phenotypic characteristics. The simple matching (SSM) coefficient and unweighted-pairgroup method with average (UPGMA) clustering were used.

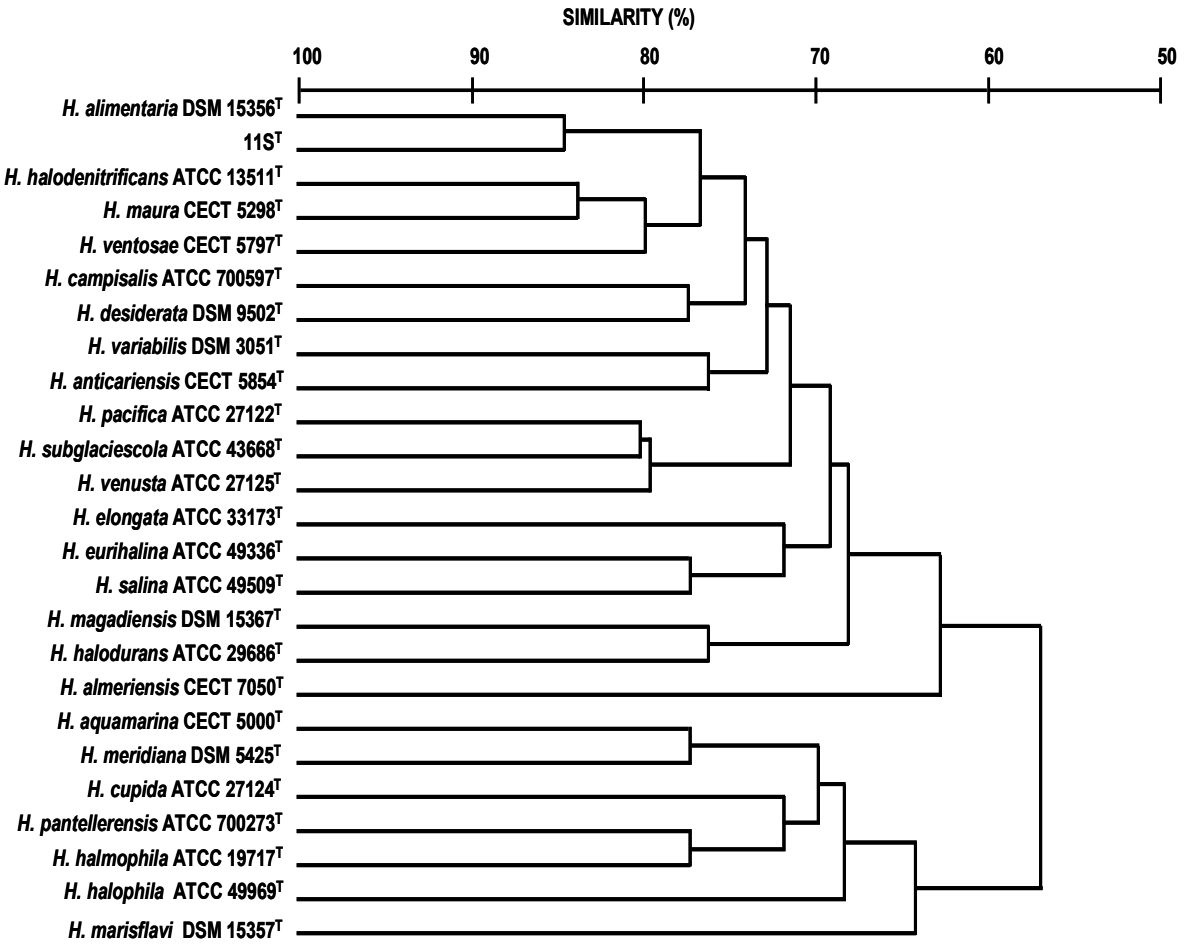

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of the novel isolate with respect to other members of the family *Halomonadaceae*. The tree was obtained using the neighbour-joining algorithm. GenBank/EMBL/DDBJ accession numbers are given in parenthesis. Bar, 1% sequence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

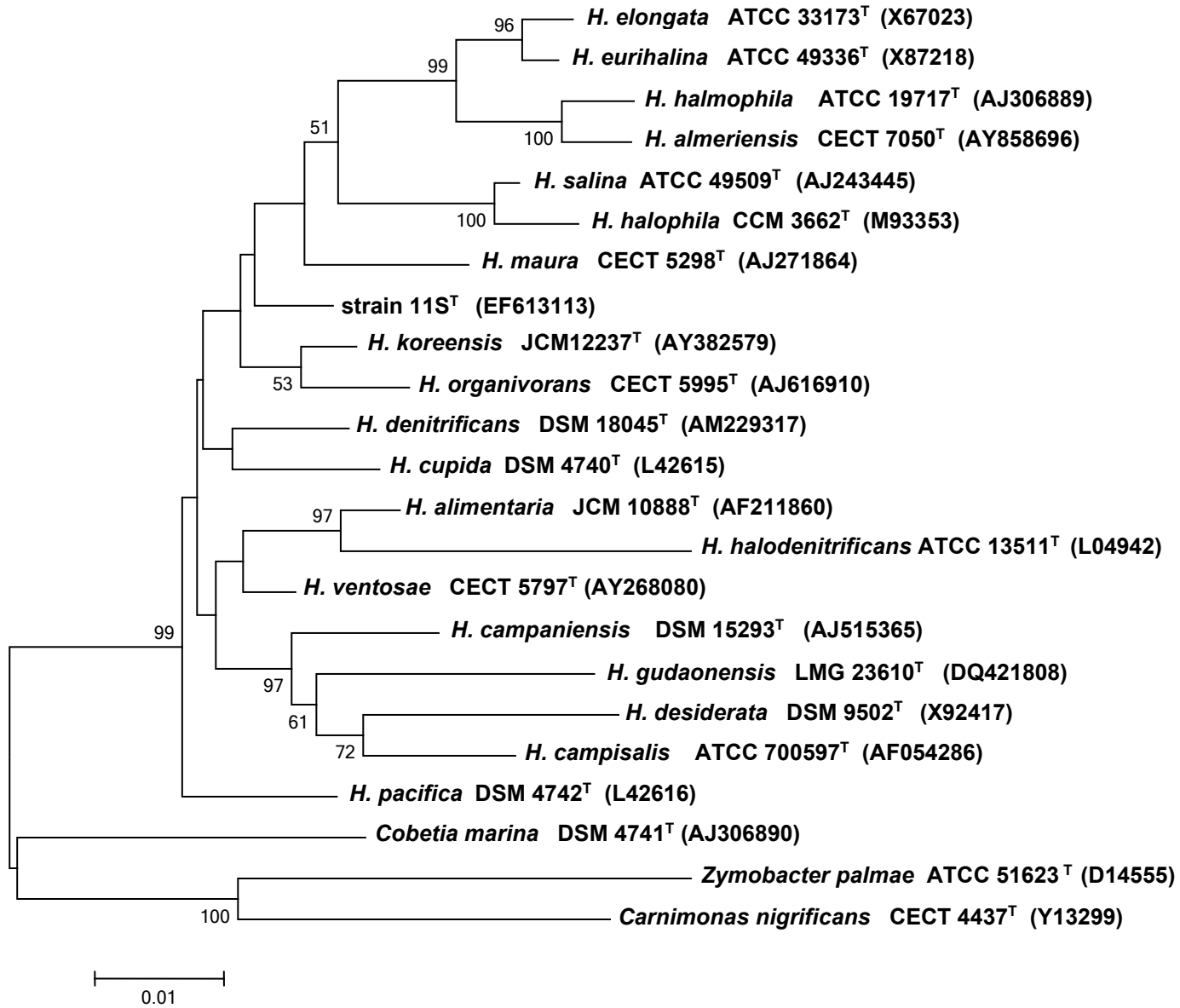

Fig. S2. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of the novel isolate with respect to other members of the family *Halomonadaceae.* The tree was obtained using the maximum parsimony algorithm. GenBank/EMBL/DDBJ accession numbers are given in parentheses. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points.

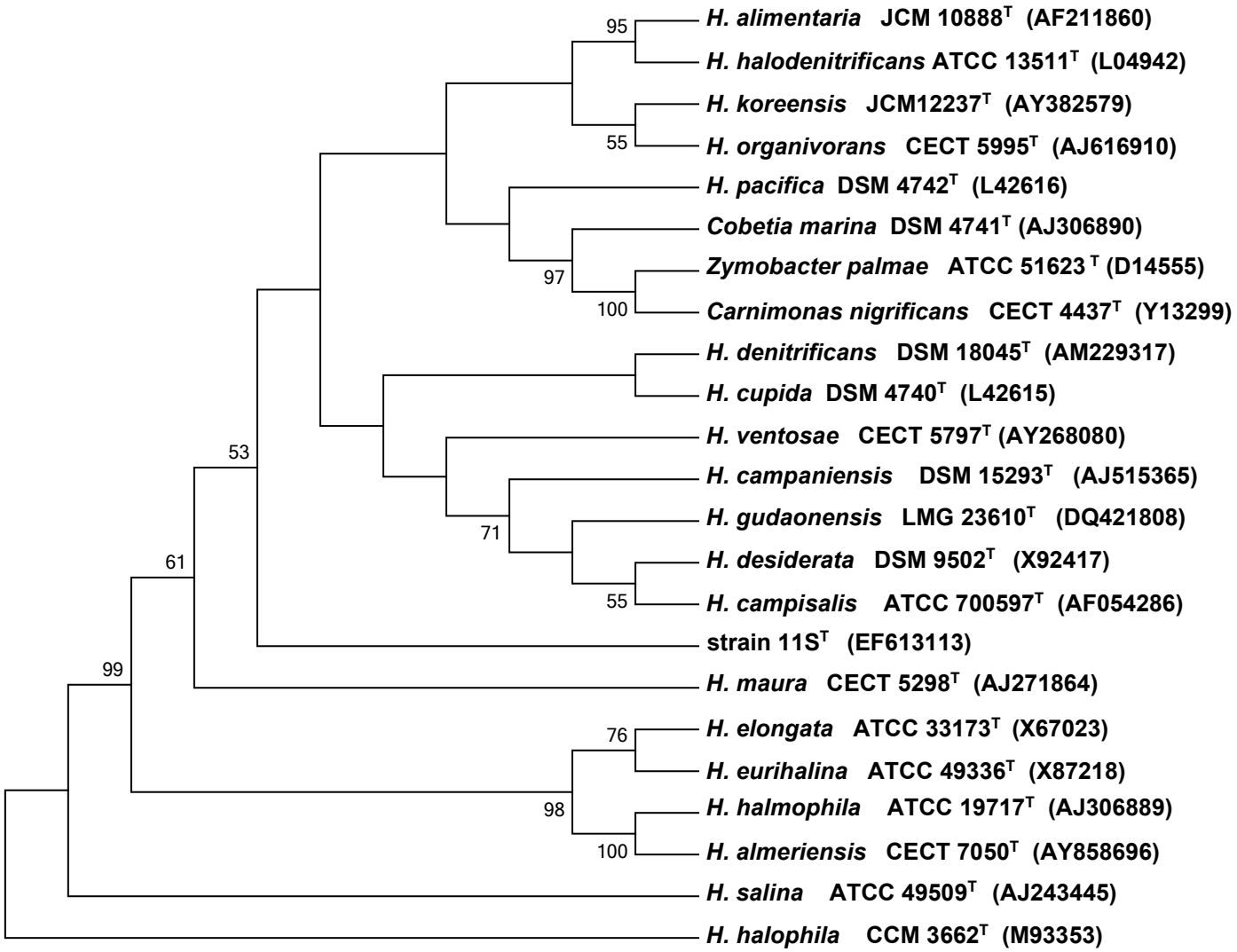

Fig. 2. Transmission electron micrograph of strain 11S^T stained with ruthenium red. (Bar 1 μm).