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Halomonas fontilapidosi sp. nov., a moderately halophilic, denitrifying bacterium

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We have made a polyphasic taxonomic study of strain 5CR^T, isolated from Fuente de Piedra, Málaga, southern Spain. The strain is a moderately halophilic, Gram-negative rod, oxidase-positive and motile by a single polar flagellum. It does not produce acids from sugars and shows respiratory metabolism, using oxygen, nitrate and nitrite as terminal electron acceptors. It requires NaCl and grows best with 5–7.5 % w/v at temperatures of between 32 and 45 °C within a pH range of 6–8. Its 16S rRNA gene sequence indicates that strain 5CR^T belongs to the genus *Halomonas* in the class *Gammaproteobacteria*. Its closest relatives are *Halomonas alimentaria*, *H. nitroreducens*, *H. shengliensis* and *H. ventosae*, with the type strains of which our strain showed 16S rRNA gene sequence similarity values of 96.7–97.8 %. DNA–DNA hybridization studies between strain 5CR^T and *H. ventosae* CECT 5797^T, the phylogenetically nearest type strain, showed 40 % relatedness. Its G+C content is 65.7 mol%. Its major fatty acids are $C_{18:1}\omega7c$ (31.36 %), $C_{16:0}$ (25.55 %), $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH (23.23 %), $C_{19:0}$ cyclo $\omega8c$ (8.14 %), $C_{12:0}$ 3-OH (5.76 %) and $C_{10:0}$ (2.22 %) and the predominant respiratory lipoquinone is ubiquinone with nine isoprene units (Q-9). The proposed name for the novel species is *Halomonas fontilapidosi* sp. nov., strain 5CR^T (=CECT 7341^T =LMG 24455^T) being the type strain.

The family *Halomonadaceae* of the class *Gammaproteobacteria* comprises seven genera, *Carnimonas*, *Chromohalobacter*, *Cobetia*, *Halomonas*, *Halotalea*, *Modicisalibacter* and *Zymobacter* (Euzéby, 2008). *Chromohalobacter*, *Cobetia*, *Halomonas* and *Modicisalibacter* are composed of halophilic bacteria whereas *Carnimonas*, *Halotalea* and *Zymobacter* contain non-halophilic bacteria (Ben Ali Gam *et al.*, 2007; Garrity *et al.*, 2005; Ntougias *et al.*, 2007). The genus *Halomonas* currently contains more than 50 species (Euzéby, 2008). Its members are Gram-negative, rod-shaped, non-sporulated, aerobic chemo-organotrophs, with predominantly respiratory metabolism, using oxygen, nitrate or nitrite as electron acceptors. Few *Halomonas* species produce acids from sugars (see Mata *et al.*, 2002; Dobson & Franzmann, 1996; Franzmann *et al.*, 1988; Vreeland, 2005; Arahal *et al.*, 2007). They are widely distributed throughout hypersaline environments. Some of them are recognized for their potential use in biotechnology (Margesin & Schinner, 2001; Ventosa & Nieto, 1995), producing exopolysaccharides (Arias *et al.*, 2003; Mata *et al.*, 2006; Martínez-Checa *et al.*, 2002), denitrifying (Peyton *et al.*, 2001; Yoshie *et al.*, 2006) or degrading aromatic compounds (García *et al.*, 2004).

We have already described some novel halophilic taxa isolated from Fuente de Piedra (Málaga, Spain): *Idiomarina fontislapidosi* (Martínez-Cánovas *et al.*, 2004c), *Halomonas anticariensis* (Martínez-Cánovas *et al.*, 2004b) and strain 15CR of *Halomonas cerina* (González-Domenech *et al.*, 2008a). Furthering our research into bacteria in this area, we subjected strain 5CR^T to a polyphasic taxonomic investigation and as a result propose it as the type strain of a novel species.

Strain 5CR^T was isolated from a soil sample collected from Fuente de Piedra, an endorreic, saline wetland in the province of Málaga in southern Spain. The strain was isolated, maintained and routinely grown in MY medium (Moraine & Rogovin, 1966) supplemented with 7.5 % w/v sea-salt solution (Rodríguez-Valera *et al.*, 1981) at 32 °C. The procedures followed for phenotypic characterization are described in Mata *et al.* (2002). Growth at different salt concentrations was determined in MY medium with the following sea-salt concentrations: 0, 0.5, 1, 3, 5, 7.5, 10, 15, 20, 25 and 30 % w/v. pH tolerance between 5 and 10 was determined in MY medium containing 7.5 % w/v sea salts.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 5CR^T is EU541349.

A dendrogram based on phenotypic characteristics, a transmission electron micrograph of a cell of strain 5CR^T and a 16S rRNA gene sequence-based maximum-parsimony tree are available as supplementary material with the online version of this paper.

The temperature range was tested in a similar medium by incubating the bacterium at temperatures of 4, 15, 20, 25, 32, 37, 45 and 50 °C at pH 7. Denitrifying ability was tested by conducting a respiratory assay according to the method of Callies & Mannheim (1978) with modifications by Stanier *et al.* (1966). This assay consists of growing the bacterium in a Weinberg tube under anaerobic conditions, with nitrate or nitrite as sole electron acceptor. We used phenol red as pH indicator in the medium to avoid any possibility of a false-positive result caused by a fermentative metabolism. We compared the novel strain with the type strains of 31 species of *Halomonas* by means of a numerical analysis based on data deriving from 104 phenotypic characteristics. Computer analysis was done with the NTSYSpc program, version 2.0.1.5 (Applied Biostatistics). A dendrogram showing the clustering of strain 5CR^T and type strains of other species of *Halomonas* is available as Supplementary Fig. S1 (available in IJSEM Online). The closest species phenotypically were *Halomonas cerina* and *Halomonas desiderata* (73.25 % similarity with both species).

The characteristics of strain 5CR^T are given in the species description. Phenotypic features that distinguish the novel species from other phenotypically and phylogenetically related species of *Halomonas* are included in Table 1. Strain 5CR^T and *Halomonas* species included in Table 1 shared the following features: Gram-negative rods, positive for oxidase and catalase but negative for haemolysis and the hydrolysis of starch, aesculin and casein. Their metabolism is respiratory. They are all able to respire on nitrate and nitrite anaerobically as well as aerobically. They produce acids from sugars.

The fatty acids and quinones of strain 5CR^T were analysed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) by high-resolution GLC and HPLC, respectively. To this end, strain 5CR^T was grown in MY medium (Moraine & Rogovin, 1966) with 7.5 % w/v sea-salt solution (Rodríguez-Valera *et al.*, 1981) at 32 °C. The strain contained a combination of fatty acids found in species of *Halomonas* (Dobson & Franzmann, 1996), predominantly $C_{18:1}\omega7c$ (31.36 %), $C_{16:0}$ (25.55 %), $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH (23.23 %), $C_{19:0}$ cyclo $\omega8c$ (8.14 %), $C_{12:0}$ 3-OH (5.76 %) and $C_{10:0}$ (2.22 %). An analysis of the quinones showed that the strain contained ubiquinone with nine isoprene units (Q-9) as the predominant respiratory lipoquinone (92 %); it also contained a small quantity (8 %) of ubiquinone 8.

Transmission electron micrographs obtained using the methods of Bouchotroch *et al.* (2001) revealed the strain's size and cell morphology and its single flagellum (Supplementary Fig. S2).

The DNA of strain 5CR^T was extracted by the method of Marmur (1961). The G+C content of the DNA 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 G+C content of the novel strain was 65.7 mol%. This result is similar to others from denitrifying bacteria but somewhat lower than the G+C content of *Halomonas ventosae* (74.3 mol%).

Phylogenetic analyses based on the 16S rRNA gene were made as described in Bouchotroch *et al.* (2001). The sequences were compared to reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center of Biotechnology Information database using the BLAST search. Phylogenetic analyses were carried out using the software MEGA version 4 (Tamura *et al.*, 2007) after multiple alignments of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances and clustering with the neighbour-joining and maximum-parsimony methods were determined by using bootstrap values based on 1000 replications.

We determined almost the entire 16S rRNA gene sequence of strain 5CR^T (1411 bp). The fragment analysed contained the 15 signature nucleotides defined for the family *Halomonadaceae* and the four defined for the genus *Halomonas* (Dobson & Franzmann, 1996). The phylogenetic tree constructed using the neighbour-joining algorithm appears in Fig. 1; the phylogenetic tree constructed using the maximum-parsimony algorithm is available as Supplementary Fig. S3. The most phylogenetically related species are all denitrifying species: *Halomonas alimentaria*, *H. nitroreducens*, *H. shengliensis* and *H. ventosae*, with the type strains of which our strain shows 16S rRNA gene sequence similarity values of 97.0, 97.5, 96.7 and 97.8 %, respectively. The trees obtained by the two methods show that 5CR^T formed a separate lineage in the genus *Halomonas* and warrants placement within a novel species. 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.

DNA–DNA hybridization was undertaken by two methods, the method of Lind & Ursing (1986), with the modifications of Ziemke *et al.* (1998) and Bouchotroch *et al.* (2001), and the spectroscopic DNA–DNA hybridization technique performed by the DSMZ. The latter method consists of isolating and purifying DNA by chromatography on hydroxyapatite as described by Cashion *et al.* (1977), followed by DNA–DNA hybridization as described by De Ley *et al.* (1970) and modified by Huß *et al.* (1983). The results of DNA–DNA hybridization revealed approximately 40 % relatedness between strain 5CR^T and *H. ventosae* CECT 5797^T, the closest phylogenetic relative, with both methods (40.3 and 40.8 % respectively).

The differences in G+C content, the similarity of its 16S rRNA gene sequence, the DNA–DNA hybridization values and the phenotypic and chemotaxonomic data show that the novel strain was not related closely enough to belong to any *Halomonas* species described to date and thus, on the

Table 1. Distinguishing characteristics of *Halomonas* species phenotypically and phylogenetically related to strain 5CR^T

Reference type strains: 1, *H. alimentaria* DSM 15356^T (data from this study and from Yoon *et al.*, 2002); 2, *H. cerina* CECT 7282^T (González-Domenech *et al.*, 2008a); 3, *H. desiderata* DSM 9502^T (Berendes *et al.*, 1996; Mata *et al.*, 2002); 4, *H. nitroreducens* CECT 7281^T (González-Domenech *et al.*, 2008b); 5, *H. shengliensis* LMG 23897^T (Wang *et al.*, 2007); 6, *H. ventosae* CECT 5797^T (Martínez-Cánovas *et al.*, 2004a). ++, Strongly positive; +, positive; D, delayed; −, negative; ND, no data available. 2

Characteristic	5CR^T	1	2	3	4	5	6
Morphology*	SR	C/SR	SR	R	R	SR	R
Cell width (μm)	0.96	0.8–1.2	0.7–0.9	0.4–0.6	0.4–0.5	0.6–0.8	0.7–0.8
Cell length (μm)	1.73	1.3–1.9	1.9–2.8	1.0–2.6	1.5–2.2	1.0–1.6	1.2–1.4
Pigmentation†	CRB	CRY‡	WX	CR	CRW	CRB	CR
Exopolysaccharide production	−	−	+	−	++	−	++
Motility	+	−	−	+	−	+	+
Flagellation	Polar	Absent	Absent	Peritrichous	Absent	Lateral	Lateral
Sea-salt concentration for growth (% w/v)							
Range	3–25	0–30	3–25	0–20	0.5–30	0.5–30	1–15
Optimum	7.5–10	1–13	7.5–10	1–5	3–5	5–15	8
pH for growth							
Range	5–9	5–10	5–10	7–11	5–10	5–10	6–10
Optimum	6–8	6.5–7.5	7–8	9.5	7–9	8.5	7–8
Temperature for growth (°C)							
Range	15–45	4–45	4–45	10–45	4–45	4–45	15–50
Optimum	32–45	30	20–32	37–42	20–32	30	32
Strictly halophilic	Yes	No	Yes	No	Yes	Yes	Yes
Hydrolysis of:							
Gelatin	−	−	−	−	+	−	−
Tween 20	+	+	+	+	−	+	+
Tween 80	−	−	+	+	−	−	−
DNA	+	+	+	+	+	−	−
Tyrosine	+	−	+	+	+	−	+
Tyrosine pigment	+	−	−	−	−	−	−
H_2S generation	+	+	−	+	+	+	+
Lecithinase	+	−	−	−	−	−	−
Phosphatase	−	−	+	+	−	+	−
Urease	−	+	+	+	+	+	−
Gluconate oxidation	+	+	+	+	−	−	+
Growth on:							
MacConkey agar	−	+	+	+	+	+	−
Cetrimide agar	−	−	−	+	+	+	−
Growth on:§							
Aesculin	−	−	−	−	−	−	+
L-Arabinose	+	+	−	+	−	−	−
Cellobiose	−	+	−	−	−	+	−
D-Fructose	+	−	−	+	+	−	+
D-Galactose	+	+	−	−	+	+	+
D-Glucose	+	+	+	−	+	+	+
Maltose	+	+	+	+	+	−	+
D-Mannose	+	+	+	+	+	+	−
D-Salicin	−	−	+	−	−	+	−
Starch	+	+	−	+	+	+	−
Trehalose	−	+	−	+	+	+	+
Acetate	−	+	+	+	+	+	+
Citrate	+	+	+	−	+	−	+
Formate	−	−	−	+	−	−	−
Fumarate	−	+	+	+	+	+	+
Malonate	−	+	−	+	−	+	+
Propionate	−	+	−	−	+	+	+
Adonitol	+	−	−	−	−	+	+

Table 1. cont.

Characteristic	5CR^T	1	2	3	4	5	6
Ethanol	−	+	+	−	+	+	−
Glycerol	−	+	−	+	+	+	+
myo-Inositol	−	−	−	−	−	+	+
D-Mannitol	−	+	−	−	+	−	+
Sorbitol	+	−	−	−	+	+	+
L-Alanine	−	+	−	−	+	+	−
L-Histidine	−	+	−	−	+	−	−
DL-Isoleucine	+	+	−	−	−	+	−
L-Lysine	−	D	−	−	+	−	−
L-Serine	+	D	+	+	+	+	−
L-Valine	−	+	−	−	−	−	−
Susceptibility to:							
Polymyxin B (300 μg)	−	+	+	+	+	+	+
Norfloxacine (10 μg)	+	+	+	ND	+	+	ND
Tobramycin (10 μg)	+	−	−	+	−	−	−
DNA G+C content (mol%)	65.7	63.0	66.2	66.0	65.37	66.6	74.3

*C, Coccus; R, rod; SR, short rod.

†CR, Cream; CRB, cream–brown; CRW, cream–white; CRY, cream–yellow; WX, wax-coloured.

‡On marine agar.

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

basis of the data discussed and the full description provided below, we propose a novel species, *Halomonas fontilapidosi* sp. nov., to include strain 5CR^T.

Description of *Halomonas fontilapidosi* sp. nov.

Halomonas fontilapidosi (fon.ti.la.pi.do′si. L. n. *fons -tis* spring; L. adj. *lapidosus* stony; N.L. gen. n. *fontilapidosi* of the stony spring, i.e. from Fuente de Piedra, the site from which the type strain was isolated).

Cells are Gram-negative rods, 0.96 × 1.73 μm (means of *x* measurements). The cells are motile by a single polar flagellum and contain poly-*β*-alkanoate granules. It does not produce exopolysaccharide or form endospores. Cell colonies are circular, convex and creamy-brown in colour. Its growth pattern is uniform in a liquid medium. It is moderately halophilic and incapable of growing without NaCl; it grows at sea-salt concentrations of between 3 and 25 % w/v (optimum 7.5–10 % w/v) and NaCl concentrations of between 3 and 20 % w/v (optimum 5–7.5 % w/v). It grows within a temperature range of 15–45 °C and at pH values of between 5 and 9, the optimum values being 32–45 °C and pH 6–8. It resists heating to 80 °C for 10 min. It is chemo-organotrophic and catalase- and oxidase-positive. 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. Respiration with fumarate is negative. It produces H_2S from L-cysteine, DNase and phenylalanine deaminase but not phosphatase. Gluconate is 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, O/F, ONPG and Voges–Proskauer tests are negative. Reduction of selenite, nitrate and nitrite is positive. It hydrolyses tyrosine and produces pigment from it. It hydrolyses Tween 20 and lecithin, but not aesculin, starch, Tween 80, urea, gelatin, casein or blood. It does not grow on either MacConkey agar or cetrimide agar. The following compounds are acceptable as sole carbon and energy sources: adonitol, L-arabinose, citrate, D-fructose, lactose, D-galactose, gluconate, D-glucose, lactate, maltose, D-mannose, sorbitol, starch and succinate. The following compounds are not acceptable as sole carbon and energy sources: acetate, aesculin, cellobiose, ethanol, formate, fumarate, DL-glycerol, D-mannitol, *myo*-inositol, malonate, propionate, D-salicin and trehalose. DL-Isoleucine and L-serine are used as sole sources of carbon, nitrogen and energy. L-Alanine, L-cysteine, L-lysine, L-histidine, L-methionine and L-valine are not used as sole sources of carbon, nitrogen and energy. It is susceptible to amoxicillin (25 μg), cefalothin (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), doxycycline (30 μg), gentamicin (10 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), norfloxacine (10 μg), rifampicin (30 μg), sulfamide (250 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), tobramycin (10 μg) and vancomycin (30 μg). It is resistant to polymyxin B (300 IU). Principal fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH. The predominant respiratory lipoquinone is ubiquinone with nine isoprene units (Q-9). The DNA G+C content of the type strain is 65.7 mol% (T_m method).

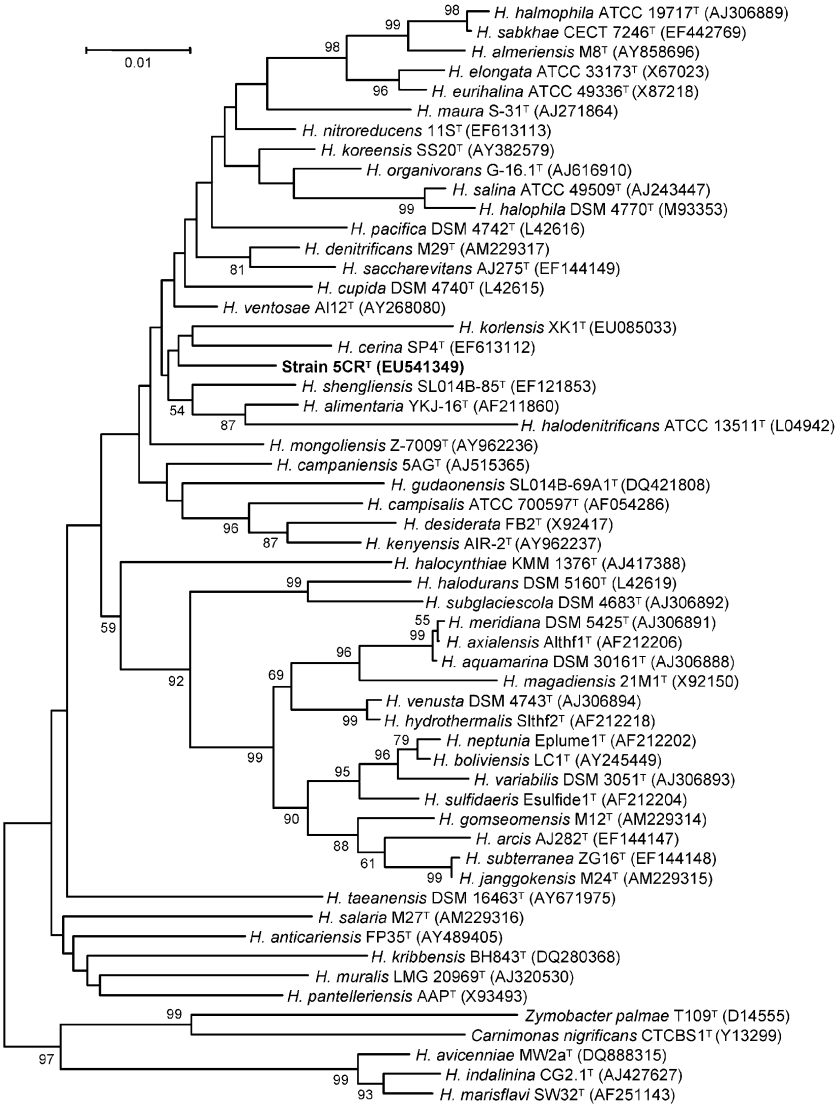

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of the novel isolate with respect to other members of the family *Halomonadaceae*. The tree was obtained using the neighbour-joining algorithm. GenBank/EMBL/DDBJ accession numbers are given in parentheses. Bar, 1 % sequence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

The type strain is strain 5CR^T (=CECT 7341^T =LMG 24455^T), isolated from saline soil at Fuente de Piedra (Málaga, Spain).

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