Halomonas cerina sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium

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Three bacterial strains were isolated from different saline soils in Spain. The novel strains were moderately halophilic, exopolysaccharide-producing, Gram-negative, non-motile rods. The strains required NaCl and grew best with 7.5–10% (w/v) NaCl in the medium. They formed wax-coloured colonies, were oxidase-positive and showed respiratory metabolism, using oxygen, nitrate and nitrite as terminal electron acceptors. The novel strains were able to denitrify and did not produce acid from sugars. The DNA G + C contents varied between 62.7 and 66.2 mol%. Phylogenetic analyses based on 16S rRNA gene sequences and sequence signatures of this gene showed that all three novel isolates belonged to the genus *Halomonas* in the class *Gammaproteobacteria* and formed an independent phylogenetic line. The most phylogenetically related species were *Halomonas alimentaria*, *Halomonas campaniensis*, *Halomonas gudaonensis* and *Halomonas ventosae*, with which the novel strains showed 16S rRNA gene sequence similarity values of between 96.3 and 95.2%. The principal fatty acids of the novel strains were 16:0, 18:1 ω 7c, 16:1 ω 7c and 19:0 cyclo ω 8c. The predominant respiratory lipoquinone was ubiquinone with nine isoprene units (Q-9). The name *Halomonas cerina* sp. nov. is proposed for these isolates. The type strain is SP4^T (=CECT 7282^T=LMG 24145^T).

The family *Halomonadaceae* belongs to the class *Gammaproteobacteria* and includes three genera of halophilic bacteria: *Halomonas*, *Chromohalobacter* and *Cobetia*, plus two genera of non-halophilic bacteria, *Zymobacter* and *Carnimonas* (Garrity *et al.*, 2005). The genus *Halomonas* currently contains more than forty species (Euzéby, 2007). Its members are Gram-negative, rod-shaped, non-sporulating, aerobic, chemo-organotrophs with predominantly respiratory metabolisms, which use oxygen or, more rarely, nitrate as electron acceptors (Franzmann *et al.*, 1988; Dobson & Franzmann, 1996; Mata *et al.*, 2002; Vreeland, 2005); a few species have a fermentative metabolism. Colonies range from white to yellow in colour. Most species of the genus *Halomonas* are found in hypersaline habitats and tend to be moderate halophiles, although some members of the genus could be classified as being halotolerant. Their DNA G + C content ranges widely,

between 54 mol% for *Halomonas halocynthiae* (Romanenko *et al.*, 2002) and 74.3 mol% for *Halomonas ventosae* (Martínez-Cánovas *et al.*, 2004b). Their principal fatty acids are: 16:1 cis 9, 16:0, 17:0 cyclo, 18:1 and 19:0 cyclo 11-12 and the major isoprenoid quinone is ubiquinone 9 (Franzmann & Tindall, 1990). The *Halomonas* species described by our group, *Halomonas eurihalina* (Quesada *et al.*, 1990), *Halomonas maura* (Bouchotroch *et al.*, 2001), *Halomonas almeriensis* (Martínez-Checa *et al.*, 2005), *Halomonas anticariensis* (Martínez-Cánovas *et al.*, 2004a) and *H. ventosae* (Martínez-Cánovas *et al.*, 2004b), produce exopolysaccharides (EPSs) with potential applications in biotechnology (Arias *et al.*, 2003; Quesada *et al.*, 2004). Our research indicates that besides the species cited, other EPS-producing bacterial strains found in hypersaline habitats cannot be assigned to any currently recognized species of the genus *Halomonas*.

In this study, we classify three hitherto unassigned exopolysaccharide-producing *Halomonas* strains that are characterized by their capacity to denitrify. On the basis of their phenotypic features, comparative studies of their 16S rRNA gene sequences and DNA–DNA hybridization, followed by analyses of their fatty acid and isoprenoid quinone contents, they are proposed as representing a novel species of the genus *Halomonas*.

Abbreviations: EPS, exopolysaccharide; PHA, poly- β -hydroxyalkanoate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 15CR, SP4^T and R53 are EF613111, EF613112 and EF613110, respectively.

A dendrogram of phenotypic characteristics based on the simple-matching coefficient and UPGMA methods is available with the online version of this paper.

The novel strains, 15CR, SP4^T and R53, were isolated from hypersaline soils at Fuente de Piedra (Malaga), Santa Pola (Alicante) and Rambla Salada (Murcia), respectively, in the south of Spain. All of the strains were routinely grown in MY medium (Moraine & Rogovin, 1966) with 7.5 % (w/v) sea salt solutions (Rodríguez-Valera *et al.*, 1981) at 32 °C. The strains included in Fig. 1 and Supplementary Fig. S1 (available in IJSEM Online) were used for phylogenetic and phenotypic comparisons, respectively.

The procedures followed for phenotypic characterization were as described in Ventosa *et al.* (1982), Quesada *et al.* (1983) and Mata *et al.* (2002). Anaerobic nitrate and nitrite reduction were tested according to Stanier *et al.* (1966). Characteristics common to all three strains are given in the species description. Phenotypic features that varied between the three strains are shown in Table 1. We compared the novel strains to other species of the genus *Halomonas* by a numerical analysis based on 107 phenotypic data. The data were submitted to cluster analysis using the simple matching coefficient (S_{SM}) (Sokal & Michener, 1958) and clustering was achieved by the unweighted pair group method of association (UPGMA) (Sneath & Sokal, 1973). Computer analysis was performed with the TAXAN program (Information Resources Group). The dendrogram obtained is shown in Supplementary Fig. S1, where it can be seen that at 88 % similarity, the three strains group into one phenon, which shared less than 78 % similarity with any other species belonging to the genus *Halomonas*.

Table 2 shows the main phenotypic differences between the novel species and other related species of the genus *Halomonas*.

The DNA G + C 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 50.8 mol% (Owen & Pitcher, 1985). The values obtained were 62.7 mol%, 66.2 mol% and 66.3 mol% for strains 15CR, R53 and SP4^T, respectively.

Phylogenetic analyses based on the 16S rRNA gene sequence 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 MEGA software version 3.1 (Kumar *et al.*, 2004) after multiple alignments of 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 sequences of the three novel strains: 15CR (1436 bp), SP4^T (1449 bp) and R53 (1486 bp). The fragments 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 is shown in Fig. 1. The maximum-parsimony algorithm gave a similar result (data not shown). The three sequences shared more than 99.4 % similarity and were on the same separate phylogenetic branch. The most

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of the novel group of three isolates compared with other species of the genus *Halomonas*. The tree was obtained using the neighbour-joining algorithm. GenBank/EMBL/DDJB accession numbers are given in parentheses. Numbers at nodes are bootstrap percentages from 1000 replications. Bar, 1 % sequence divergence.

Table 1. Characteristics that distinguish between strains 15CR, R53 and SP4^T

Characteristic	15CR	SP4 ^T	R53
Size (µm)	2.8 × 0.7	1.9 × 0.7	2.2 × 0.9
Growth on 5 % (w/v) NaCl	+	+	–
Resistance to 80 °C for 10 min	–	–	+
Tween 80 hydrolysis	+	+	–
Gelatinase	–	–	+
Tyrosine hydrolysis	+	+	–
Growth on:			
Adonitol	–	–	+
Arabinose	–	–	+
Cellobiose	+	–	–
Ethanol	+	+	–
Fructose	+	–	–
Glycerol	+	–	–
Histidine	+	–	–
Inositol	+	–	–
Lactate	+	–	+
Lactose	–	–	+
Mannitol	–	–	+
Salicin	–	+	–
Serine	+	+	–
Starch	+	–	+
Trehalose	+	–	–
Principal fatty acids (%):*			
10:0	2.30	2.60	2.35
12:0	3.07	3.19	3.26
12:0 3-OH	5.43	6.22	5.98
16:1ω7c	20.81	18.72	14.19
16:0	30.91	33.93	24.01
18:1ω7c	23.76	16.08	42.23
18:0	3.06	1.55	3.79
19:0 cyclo ω8c	9.40	14.46	4.18
Lipoquinones	Ubiquinone 9	Ubiquinone 9	Ubiquinone 9, 94 %; Ubiquinone 8, 6 %
DNA G + C (mol%)	62.7	66.2	66.2

*Only fatty acids with a percentage higher than 2 % are shown.

phylogenetically related species were *Halomonas alimentaria*, *Halomonas campaniensis*, *Halomonas gudaonensis* and *H. ventosae*, with which the novel strains showed 16S rRNA gene sequence similarity values of between 96.3 and 95.2 %.

DNA–DNA hybridization was conducted following Lind & Ursing's methods (Lind & Ursing, 1986) with the modifications of Ziemke *et al.* (1998) and Bouchotroch *et al.* (2001). The comparative values for all three strains were higher than 80 % (15CR with SP4^T: 89.16 %; 15CR with R53: 80.50 %; R53 with SP4^T: 87.37 %; the reciprocal values were very similar). These data demonstrate that the three novel strains belong to the same species.

Transmission electron micrographs obtained using the methods of Bouchotroch *et al.* (2001) show the cell morphology of the three strains (Fig. 2). The cells contained poly-β-hydroxyalkanoate (PHA) granules.

Fatty acids and quinones were identified by high-resolution GLC and HPLC, respectively, by the Identification Service of the DSMZ, Braunschweig, Germany. The results are given in Table 1 and in the species description. The three strains showed a combination of fatty acids found in species of the genus *Halomonas*: predominantly 16:0, 18:1ω7, 16:1ω7c and 19:0 cyclo ω8c (Dobson & Franzmann, 1996). An analysis of the quinones showed that all three strains contained ubiquinone 9; strain R53 also contained a small quantity (6 %) of ubiquinone 8.

On the basis of the phylogenetic evidence, DNA–DNA hybridization values, fatty acid profiles, quinones and differences in phenotypic characteristics, we suggest that the denitrifying EPS-producing strains 15CR, SP4^T and R53 represent a novel species of the genus *Halomonas*, for which the name *Halomonas cerina* sp. nov. is proposed.

Description of *Halomonas cerina* sp. nov.

Halomonas cerina (cer.i'na. L. fem. adj. *cerina* the colour of beeswax, describing the colour of the mature colonies).

Cells are straight, Gram-negative rods, 1.9–2.8 × 0.7–0.9 µm, appearing either singly or in pairs. Cells are capsulated and non-motile. Cells accumulate PHA and do not form endospores. Cell colonies are wax coloured, circular, convex and mucoid. The growth pattern is uniform in a liquid medium. Moderate halophile, capable of growing in mixed-sea salt concentrations of 3–25 % (w/v), with optimum growth occurring between 7.5 and 10 %. Requires NaCl and can grow within a range of 7.5–20 % (w/v), the optimum being 7.5–10 %. Grows within a temperature range of 4–45 °C at pH values of between 5 and 10, the optimum values being 20–32 °C and pH 7–8. Catalase and oxidase are produced. Chemo-organotrophic. Metabolism is respiratory with oxygen, nitrate and nitrite as terminal electron acceptors. Respiration with fumarate is negative. Under aerobic conditions, selenite and nitrate are reduced. Acids are not produced from adonitol, L-arabinose, D-fructose, D-galactose, D-glucose, glycerol, myo-inositol, lactose, maltose, D-mannitol, D-mannose, melezitose, L-rhamnose, D-salicin, D-sorbitol, sorbose, sucrose, trehalose or D-xylose. Indole, methyl red, Voges–Proskauer, O/F and ONPG tests give a negative result. Does not produce pyocyanin, fluorescein or pigment in tyrosine medium. Does not hydrolyse starch, casein, lecithin or aesculin. Produces urease, phosphatase and DNase but not phenylalanine deaminase. Gluconate is oxidized. Does not produce H₂S from L-cysteine. Grows on MacConkey agar but not on cetrimide agar. Blood is not lysed. The following compounds serve as sole carbon and energy sources: acetate, citrate, fumarate, D-gluconate, succinate, D-glucose, maltose and D-mannose, whilst aesculin,

Table 2. Characteristics that distinguish *Halomonas cerina* SP4^T sp. nov. from other related species of the genus *Halomonas*

Taxa: 1, *H. cerina* SP4^T sp. nov.; 2, *H. alimentaria*; 3, *H. campaniensis*; 4, *H. campisalis*; 5, *H. desiderata*; 6, *H. gudaonensis*; 7, *H. ventosae*. Data are from Ventosa *et al.* (1998), Mata *et al.* (2002), Yoon *et al.* (2002), Martínez-Cánovas *et al.* (2004b), Romano *et al.* (2005), Wang *et al.* (2007) and from this study. All species are Gram-negative rods, are catalase- and oxidase-positive, are able to reduce nitrate to nitrite and are able to grow with acetate, maltose or glucose as sole carbon and energy source. They do not hydrolyse starch or gelatin.

Characteristic	1	2	3	4	5	6	7
Morphology	Short rod	Coccus, short rod	Rod	Rod	Rod	Rod	Rod
Pigmentation	Wax coloured	Cream-yellow	Cream-pink	White	Cream	Cream	Cream
PHA	+	ND	+	+	+	ND	+
EPS	+	—	—	—	—	ND	+
Motility	—	—	+	+	+	+	+
Sea salt range (% w/v)	3–25	1–23	0–16	0.5–15	0–20	1–20*	1–15
Sea salt optimum (% w/v)	5–10	1–13	10	5	1–5	10–15*	8
pH range	5–10	5–10	7–10	8–11	7–11	8–9	6–10
pH optimum	7–10	6.5–7.5	9	9.5	9.5	8	7–8
Temperature range (°C)	4–45	4–45	10–43	4–50	10–45	10–42	15–50
Optimum temperature (°C)	20–32	30	37	30	37–42	30	32
Strictly halophilic	+	+	—	+	—	+	+
Acids from D-glucose	—	—	ND	—	—	ND	—
Hydrolysis of:							
Aesculin	—	—	ND	—	—	ND	—
Casein	—	—	—	—	—	ND	—
DNA	+	+	ND	+	+	ND	—
Tween 20	+	—	ND	+	+	ND	+
Tween 80	+	—	+	—	+	—	—
Tyrosine	+	—	+	—	+	ND	+
H ₂ S	—	—	ND	—	+	ND	+
Respiration on nitrate	+	+	ND	+	+	+	+
Respiration on nitrite	+	+	ND	+	+	+	+
Gas from nitrate	+	+	ND	+	+	+	+
Phosphatase	+	ND	ND	—	+	ND	—
Urease	+	+	+	—	+	+	—
Phenylalanine deaminase	—	ND	—	—	+	ND	+
Gluconate oxidation	+	ND	ND	+	+	ND	+
Selenite reduction	+	ND	ND	+	+	ND	+
Growth on:							
MacConkey agar	+	ND	ND	—	+	ND	—
Cetrimide agar	—	ND	ND	—	+	ND	—
Haemolysis	—	—	ND	—	—	ND	—
Growth on:†							
Aesculin	—	—	ND	—	—	ND	+
Adonitol	—	—	ND	—	—	ND	+
L-Alanine	—	+	ND	+	—	+	—
L-Arabinose	—	+	—	—	+	—	—
Cellobiose	—	+	+	+	—	—	—
Citrate	+	+	ND	+	—	ND	+
L-Cysteine	—	—	ND	—	—	ND	—
Ethanol	+	+	ND	—	—	+	—
Formate	—	—	ND	—	+	ND	—
D-Fructose	—	—	+	+	+	—	+
Fumarate	+	+	ND	+	+	ND	+
D-Galactose	—	ND	—	—	—	+	+
Gluconate	+	+	ND	+	+	+	+
Glycerol	—	+	+	+	+	+	+
L-Histidine	—	+	ND	—	—	ND	—
DL-Isoleucine	—	ND	ND	—	—	+	—
myo-Inositol	—	—	ND	+	—	ND	+
L-Lysine	—	+	ND	+	—	—	—

Table 2. cont.

Characteristic	1	2	3	4	5	6	7
Malonate	—	+	ND	—	+	ND	+
D-Mannitol	—	+	ND	—	—	+	+
D-Mannose	+	+	+	—	+	+	—
Melezitose	—	ND	ND	—	+	ND	ND
L-Methionine	—	ND	ND	—	—	ND	—
Propionate	—	+	ND	+	—	ND	+
D-Salicin	+	+	ND	—	—	ND	—
L-Serine	+	+	ND	+	+	ND	—
Sorbitol	—	+	ND	—	—	ND	+
Starch	—	+	ND	+	+	ND	—
Succinate	+	—	ND	+	+	ND	+
Trehalose	—	+	ND	+	+	+	+
L-Valine	—	+	ND	—	—	ND	—
DNA G + C content (mol%)	66.2	63	63.7	66	66	64	74.3

*NaCl.

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

galactose, formate, malonate and sorbitol do not. L-Alanine, L-isoleucine, L-cysteine, L-lysine, L-methionine and L-valine cannot be used as sole sources of carbon, nitrogen and energy. Susceptible to (amounts per disc): amoxicillin + clavulanic acid (25 µg), ampicillin (10 µg), aztreonam (30 µg), cephalothin (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), doxycycline (30 U), gentamicin (10 µg), nalidixic acid (30 µg), norfloxacin (10 µg), nitrofurantoin (300 µg), polymyxin B (300 U), rifampicin (30 µg), sulphamide (250 µg) and trimetoprim + sulphamethoxazol (1.25–23.75 µg). Resistant to tobramycin (10 µg). Principal fatty acids are 16:0, 18:1 ω 7c and 16:1 ω 7c. The predominant respiratory lipoquinone is ubiquinone with nine isoprene units (Q-9). The DNA G + C content is between 62.7 and 66.2 mol%.

The type strain, SP4^T, grows in a medium containing only 5 % (w/v) NaCl. It hydrolyses tyrosine and Tween 80 but not gelatin. It does not survive for 10 min at 80 °C. Salicin, ethanol and serine serve as sole carbon and energy sources. It does not utilize arabinose, cellobiose, fructose, lactose, starch, trehalose, lactate, adonitol, glycerol, inositol, mannitol or histidine. The principal fatty acids of the type strain are: 16:0 (33.9 %); 16:1 ω 7c (18.72 %); 18:1 ω 7c (16.08 %); 19:0 cyclo ω 8c (14.46 %) and 12:0 3-OH (6.22 %). The predominant respiratory lipoquinone is ubiquinone with nine isoprene units (Q-9). The DNA G + C content of the type strain is 66.2 mol% (T_m method).

The type strain, SP4^T (=CECT 7282^T=LMG 24145^T), was isolated from a saline soil.

Fig. 2. Transmission electron micrographs of cells of strains 15CR (a), R53 (b) and SP4^T (c) stained with ruthenium red. Bars, 1 µm.

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