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

Carmen M. González-Doménech, Fernando Martínez-Checa, Emilia Quesada and Victoria Béjar.

Microbial Exopolysaccharide Research Group, Department of Microbiology, Faculty of Pharmacy, Campus Universitario de Cartuja, University of Granada, 18071 Granada, Spain.

Running title: *Halomonas cerina* sp. nov.

Keywords: moderately halophilic bacteria; denitrification; exopolysaccharides; *Halomonas*; *Halomonas cerina.*

Subject category: Taxonomic note, New taxa, *Proteobacteria*

Author for correspondence: V. Béjar.
Tel: +34 958 243871;
Fax: +34 958 246235;
e-mail: vbejar@ugr.es

Abbreviation: EPS, exopolysaccharide.

The GenBank (EMBL/DDBJ) accession number for the 16S rDNA sequence of strains 15CR, SP4 and R53 are EF613111, EF613112 and EF613110, respectively.

Strain SP4^T (CECT 7282^T, LMG 24145^T) is the type strain.

Supplementary phylogenetic tree using the maximum parsimony algorithm is available at IJSEM online

Abstract

We describe here three bacterial strains isolated from different saline soils in Spain. They are moderately halophilic, exopolysaccharide-producing, Gram-negative, non-motile rods. They require NaCl and grow best with 7.5% - 10% w/v in the medium. They form wax-coloured colonies, are oxidase positive and show respiratory metabolism, using oxygen, nitrate and nitrite as terminal electron acceptors. They denitrify and do not produce acids from sugars. Their G+C content varies between 62.7 and 66.2. Phylogenetic analyses based on 16S rRNA gene sequences and sequence signatures of this gene show that all three isolates belong to the genus *Halomonas* in the γ-*Proteobacteria* class and form an independent genetic line. The most phylogenetically related species are *Halomonas alimentaria*, *Halomonas campaniensis, Halomonas gudaonensis* and *Halomonas ventosae*, with which our strains show 16S rRNA similarity values of between 96.3 and 95.2. The principal fatty acids of the new strains are 18:1 ω7c, 16:0, 16:1 ω7c and 19:0 cyclo ω8c. Their predominant respiratory lipoquinone is ubiquinone with nine isoprene units (Q-9). The name *Halomonas cerina* sp. nov. is proposed for these isolates. Strain SP4^T (CECT 7282^T, LMG 24145^T) is the type strain.

The *Halomonadaceae* family belongs to the class of γ-*Proteobacteria* 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 (Euzeby, 2007). Its members are Gram-negative, rod-shaped, non-sporulated, aerobic, chemo-organotrophs, with predominantly respiratory metabolisms, which use oxygen or, more rarely, nitrate as electron acceptors (cf. Mata *et al.*, 2002; Dobson and Franzman, 1996; Franzman *et al.*, 1988; Vreeland, 2005); a few species have fermentative metabolism. Colonies range from white to yellow in colour. Most *Halomonas* species are found in hypersaline habitats and tend to be moderate halophiles, although some members of the genus could be classified as being halotolerant. Their G+C content ranges widely, between 54% in *H. halocynthiae* (Romanenko *et al.* 2002) and 74.3% in *H. 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 and Tindall, 1990). The *Halomonas* species described by our group, *H. eurihalina* (Quesada *et al.* 1990), *H. maura* (Bouchotroch *et al.*, 2001), *H. almeriensis* (Martínez-Checa *et al.* 2005), *H. anticariensis* (Martínez-Cánovas *et al.* 2004a) and *H. ventosae* (Martínez-Cánovas *et al.* 2004b), produce exopolysaccharides (EPSs) with potential applications in biotechnology (Calvo *et al.*, 2002; Béjar *et al.*, 1998; Martínez-Checa *et al.*, 2002; Arias *et al.*, 2003; Quesada *et al.*, 2004). Our research indicates that besides the species cited other EPS-producing bacterial strains are to found in hypersaline habitats that cannot be assigned to any currently recognized *Halomonas* species.

We classify here three hitherto unassigned exopolysaccharide-producing *Halomonas* strains which are characterised 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, we propose a new species, *Halomonas cerina* sp. nov.

The new strains, 15CR, SP4 and R53, were isolated from hypersaline soils at Fuente de Piedra (Malaga), Santa Pola (Alicante) and Rambla Salada (Murcia) in the south of Spain. All 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 32ºC. For comparison we used strains from the culture collections listed in the figures and tables below.

The procedures followed for phenotypic characterisation are described in Mata *et al.* (2002), Quesada *et al*. (1983) and Ventosa *et al*. (1982). 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 distinguishing between the three strains are shown in Table 1. We compared the new strains to other species of *Halomonas* by 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 done with the TAXAN program (Information Resources Group, Maryland Biotechnology Institute, University of Maryland, College Park, HD20742, USA). The dendrogram thus obtained is shown in Figure 1, where it can be seen that at 88% similarity the three strains group into one phenon, which shares less than 78% similarity with any other species belonging to *Halomonas*.

Table 2 shows the main phenotypic differences between the type strains of *Halomonas cerina* and other phenotypically and phylogenetically related species of the genus.

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 and Hill (1979). The G+C content of reference DNA from *Escherichia coli* was taken to be 50.8 mol% (Owen & Pitcher, 1985). The values (mol%) were 62.7 for strain 15CR, 66.2 for strain R53 and for strain SP4.

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 (Molecular Evolutionary Genetics Analysis) version 3.1 (Kumar *et al*., 2004) after multiple alignments of data by CLUSTALX (Thomson *et al*., 1997). Distances and clustering with the neighbour-joining and maximum-parsimony methods were determined by using bootstrap values based on 1,000 replications.

We determined almost the entire16S rDNA sequences of the three strains: 15CR (1,436 bp), SP4(1,449 bp) and R53 (1,486 bp). The fragment analysed contained the 15 signature nucleotides defined for *Halomonadaceae* and the four defined for the *Halomonas* genus (Dobson and Franzmann, 1996). The phylogenetic tree constructed using the neighbour-joining algorithm appears in Figure 2. The three sequences share more than 99.4% similarity and are on the same separate phylogenetic branch. The most phylogenetically related species are *Halomonas alimentaria*, *Halomonas campaniensis, Halomonas gudaonensis* and *Halomonas ventosae*, with which our strains show 16S rRNA similarity values of between 96.3 and 95.2.

DNA-DNA hybridization was conducted following Lind & Ursing's methods (1986) with the modifications of Ziemke *et al.* (1998) and Bouchotroch *et al.* (2001). The comparative values for all three strains are higher than 80%, demonstrating that they 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. 3). The cells contain poly-β-hydroxyalkanoate (PHA) granules together with EPS clinging to the cell surface and in the medium surrounding the bacteria.

Fatty acids and quinones were identified by high-resolution GLC and HPLC respectively by the Identification Service of 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 *Halomonas*: predominantly 18:1ω7, 16:0, 16:1ω7c and 19:0 cyclo ω8c (Dobson & Franzmann, 1996). An analysis of the quinones shows that the three strains contain ubiquinone 9; strain R53 also contains a small quantity (6%) of ubiquinone 8.

On the basis of the data discussed and the full description provided below, we propose that a novel species of the genus *Halomonas*, called *Halomonas cerina*, be admitted to include the denitrifying EPS-producing strains 15CR, SP4 and R53.

Description of *Halomonas cerina* sp. nov.

Halomonas cerina sp. (ceri´na, L. adj., the colour of bees-wax, describing the colour of the mature colonies).

The strains described here are straight, Gram-negative rods, 1.9-2.8 x 0.7-0.9 μm, appearing either singly or in pairs. The cells are capsulated and non-motile. They accumulate PHA and do not form endospores. Cell colonies are wax coloured, circular, convex and mucoid. Their growth pattern is uniform in a liquid medium. They are moderate halophiles, capable of growing in mixed-sea-salt concentrations of 3% - 25% w/v, optimum growth occurring between 7.5% and 10%. They require NaCl and can grow within a range of 7.5% to 20% w/v, the optimum being 7.5% to10% w/v. They grow within a temperature range of 4ºC to 45ºC at pH values of between 5 and 10, the optimum values being 20ºC - 32ºC and pH 7-8. Catalase and oxidase are produced. They are chemo-organotrophic. Their metabolism is respiratory with oxygen, nitrate and nitrite as terminal electron acceptors. Respiration with fumarate is negative. Under aerobic conditions they reduce selenite and nitrate. They do not produce acids from sugar. Indol, methyl red, Voges-Proskauer, O/F and ONPG prove negative. They do not produce piocianin, fluorescein or pigment in tyrosine

medium. They do not hydrolyse starch, casein, lecithin or aesculin. They produce urease, phosphatase and DNase but not phenylalanine deaminase. Gluconate is oxidised. They do not produce H_2S from L-cysteine. They grow on MacConkey agar but not on cetrimide agar. Blood is not lysed. The following compounds are acceptable as sole carbon and energy sources: acetate, citrate, fumarate, D-gluconate, succinate, D-glucose, D-maltose and D-mannose, whilst aesculin, galactose, formate, malonate and sorbitol are unacceptable. L-alanine, L-isoleucine, L-cysteine, L-lysine, L-methionine and L-valine cannot be used as sole sources of carbon, nitrogen and energy. They are susceptible to amoxicillin+clavulanic acid (25μg), ampicillin (10μg), aztreonam (30μg), cephalothin (30μg), cefoxitin (30μg), ceftazidine (30μg), doxicicline (30 UI), gentamycin (10μg), nalidixic acid (30 μg), norfloxacin (10μg), nitrofurantoin (300 μg), polymyxin B (300 UI), rifampycin (30 μg), sulphamide (250 μg), and trimetroprim-sulphametoxazol (1.25 μg -23.75 μg). They are resistant to tobramycine (10 μg). Their principal fatty acids are 18:1 ω7c, 16:0 and 16:1 ω7c. The predominant respiratory lipoquinone is ubiquinone with nine isoprene units (Q-9). The G+C range is between 62.7 and 66.2

The type strain is strain SP4T Strain SP4^T (CECT 7282^T, LMG 24145^T) is the type strain. The description of the type strain is the same as that of the species. Additionally: strain SP4T can grow in a medium containing only 5% w/v NaCl. It hydrolyses tyrosine and Tween 80 but not gelatine. It does not survive for 10 min at 80ºC. Salicine, ethanol and serine are acceptable as sole carbon and energy sources. It does not consume arabinose, cellobiose, fructose, lactose, starch, trehalose, lactate, adonitol, glycerol, inositol, manitol or hystidine. Its principal fatty acids are (%): 16:0 (33.9); 16:1 ω7c (18.72); 18:1 ω7c (16.08); 19:0 cyclo ω8c (14.46) and 12:0 3OH (6.22). The predominant respiratory lipoquinone is ubiquinone with nine isoprene units (Q-9). Its DNA G+C content is 66.2 mol% (Tm method).

Acknowledgements

This research was supported by grants from the *Dirección General de Investigación Científica y Técnica* (BOS2003-0498; CGL2005-05947) and from the *Plan Andaluz de Investigación,* Spain. Thanks go to Mª José Martínez-

Cánovas, who isolated the strains and to Concepción Hernandez and David Porcel from the Centro de Instrumentación Científica (Universidad de Granada) for their expertise in microscope studies. We also thank our colleague Dr. J. Trout for revising our English text and for his help with the species name.

References

Arias, S., Del Moral, A. Ferrer, M. R., Quesada, E. & Béjar, V. (2003). Mauran, an exopolysaccharide produced by the halophilic bacterium *Halomonas maura*, with a novel composition and interesting properties for biotechnology. *Extremophiles* **7**, 319-326.

Béjar, V., Llamas, I., Calvo, C. & Quesada, E. (1998). Characterization of exopolysaccharides produced by 19 halophilic strains of the species *Halomonas eurihalina*. *J Biotechnol* **61**, 135-141.

Bouchotroch, S., Quesada, E, Del Moral, A., Llamas, I. & Béjar, V. (2001). *Halomonas maura* sp. nov., a novel moderately halophilic, exopolysaccharide-producing bacterium. *Int J Syst Evol Microbiol* **51**, 1625-1632.

Calvo C., Martinez-Checa F., Toledo F. L., Porcel J. & Quesada E. (2002). Characteristics of bioemulsifiers synthesised in crude oil media by *Halomonas eurihalina* and their effectiveness in the isolation of bacteria able to grow in the presence of hydrocarbons. *Appl Microbiol Biotechnol* **60**, 347-51.

Dobson, S. J. & Franzmann, P. D. (1996). Unification of the genera *Deleya* (Bauman *et al.,* 1993), *Halomonas* (Vreeland *et al.,* 1980), and *Halovibrio* (Fendrich, 1988) and the species *Paracoccus halodenitrificans* (Robinson and Gibbons, 1952) into a single genus, *Halomonas*, and placement of the genus *Zymobacter* in the family *Halomonadaceae*. *Int J Syst Bacteriol* **46**, 550-558.

Euzeby, J. P. (2007). List of Prokaryotic Names with Standing in Nomenclature. http://www.bacterio.cict.fr/

Franzmann, P. D., Wehmeyer, U. & Stackebrandt, E. (1988). *Halomonadaceae* fam. nov., a new family of the class *Proteobacteria* to accommodate the genera *Halomonas* and *Deleya*. *Syst Appl Microbiol* **11**, 16-19.

Franzmann, P. D. & Tindall, B. J. (1990). A chemotaxonomic study of members of the family *Halomonadaceae*. *Syst Appl Microbiol* **13**, 142-147.

Garrity, G.M., Bell, J.A. & Liburn, T. (2005). Family IV. *Halomomadaceae* Franzmann, Wehmeyer and Stackebrandt 1989, 205 ^{VP} emend. Dobson and Franzmann 1996, 558. *In*: Bergey's Manual of Systematic Bacteriology, 2nd ed. , vol. 2. Edited by D.J. Brenner, N.R. Krieg, J.T. Staley & G.M. Garrity. New York: Springer.

Kumar, S., Tamura, K., & Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* **5**,150-163.

Lind, E. & Ursing, J. (1986). Clinical strains of *Enterobacter agglomerans* (Synonyms, *Erwinia herbicola, Erwinia milletiae*) identified by DNA-DNA hybridization. *Acta Pathol Microbiol Immunol Scand. Sect* B **94**: 205-213.

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109-118.

Martínez-Checa, F., Toledo, F. L., Vílchez, R., Quesada, E. & Calvo, C. (2002). Yield production, chemical composition and functional properties of emulsifier H28 synthesized by *Halomonas eurihalina* strain H-28 in media containing various hydrocarbons. *Appl Microbiol Biotechnol* **58**, 358-363.

Moraine, R. A. & Rogovin, P. (1966). Kinetics of polysaccharide B-1459 fermentation. *Biotechnol Bioengin* **8**, 511-524.

Martínez-Cánovas, M. J., Béjar, V., Martínez-Checa, F. & Quesada, E. (2004a). *Halomonas anticariensis* sp. nov., from Fuente de Piedra, a saline-wetland wild-fowl reserve in Málaga, southern Spain. *Int J Syst Evol Microbiol* 54, 1329 - 1332.

Martinez-Canovas, M. J., Quesada E., Llamas I. & Bejar V. (2004b). *Halomonas ventosae* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. *Int J Syst Evol Microbiol* **54**, 733-7.

Martinez-Checa, F., Béjar V., Martinez-Cánovas M. J., Llamas I. & Quesada E. (2005). *Halomonas almeriensis* sp. nov., a moderately halophilic, exopolysaccharide-producing bacterium from Cabo de Gata, Almería, south-east Spain. *Int J Syst Evol Microbiol* **55**, 2007-2011.

Mata, J. A., Martínez-Cánovas, M. J., Quesada, E. & Béjar, V. (2002). A detailed phenotypic characterization of the type strains of *Halomonas* species. *Syst Appl Microbiol* **25,** 360-375.

Owen, R. J. & Hill, L. R. (1979). The estimation of base compositions, base pairing and genome size of bacterial deoxyribonucleic acids. In *Identifications Methods for Microbiologists*, pp. 277-296, 2nd ed. Edited by F.A. Skinner & D.W. Lovelock. London: Academic Press.

Owen R. J. & Pitcher, D. (1985). Current methods for estimating DNA composition and levels of DNA-DNA hybridization. In *Chemical Methods in Bacterial Systematics*, pp.67-93. Edited by M. Goodfellow & D.E. Minnikin. London: Academic Press

Quesada, E., Ventosa, A., Rodríguez-Valera, F., Megías, L. & Ramos-Cormenzana, A. (1983). Numerical taxonomy of moderately halophilic Gram-negative bacteria from hypersaline soils. *J Gen Microbiol* **129**, 2649-2657.

Quesada, E, Valderrama, M.J., Béjar, V., Ventosa, A., Gutiérrez, M.C., Ruíz-Berraquero, F. & Ramos-Cormenzana, A. (1990). *Volcaniella eurihalina* gen nov., sp. nov., a moderately halophilic nonmotile gram-negative rod. *Int J Syst Evol Microbiol* **40**, 261-267.

Quesada, E., Béjar, V., Del Moral, A., Ferrer, M. R., Calvo, C., Llamas, I., Martínez-Checa, F., Arias, S., Ruiz-García, C., Martínez-Cánovas J. & Páez R. (2004). Moderately halophilic, exopolysaccharide-producing bacteria. In *Halophilic Microorganisms* pp. 297-314. Edited by Ventosa, A. Heilderberg: Springer-Verlag.

Rodríguez-Valera, F., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1981). Characteristics of the heterotropic bacterial populations in hypersaline environments of different salt concentrations. *Microbiol Ecol* **7**, 235-243.

Romano, I., Giordano, A., Lama, L., Nicolaus, B. & Gambacorta, A. (2005). *Halomonas campaniensis* sp. nov., a haloalkaliphilic bacterium isolated from a mineral pool of Campania Region, Italy. *Syst Appl Microbiol* **28**, 610-618.

Romanenko, L. A., Schumann, P., Rohde, M., Mikhailov, V. V. & Stackebrandt, E. (2002) *Halomonas halocynthiae* sp. nov., isolated from the marine ascidian *Halocynthia aurantium*. *Int J Syst Evol Microbiol* **52,** 1767-1772

Sneath, P. H. A. & Sokal, R. R. (1973). *Numerical taxonomy. The Principles and Practice of Numerical Classification*. San Francisco: Freeman, Williams & Wilkins Co.

Sokal, R. R. & Michener, C. D. (1958). A statistical method for evaluating systematic relationships. *Univ Kansas Sci Bull* **38**, 1409-1438.

Stanier, R.Y., Palleroni, N. J. & Duodoroff, M. (1966). The aerobic pseudomonads: A taxonomic study. *J Gen Microbiol* **43,** 159-272.

Thompson, J. D., Gibson, T. J., Plewniak, K., Jeanmougin, F. & Higgins, D. G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignments aided by quality analysis tools. *Nucleic Acids Res* **24**, 4876-4882

Ventosa, A., Quesada, E., Rodríguez-Valera, F., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. *J Gen Microbiol* **128**, 1959-1968

Ventosa, A., Nieto, J.J. & Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* **62**, 504-544.

Vreeland, R. H. (2005). Genus *Halomonas*. In *Bergey's Manual of Systematic Bacteriology* 2nd ed., vol. 2 The *Proteobacteria*, pp. 316-319. Edited by G. Garrity, D. J. Brenner, N. R. Krieg & J. T. Staley. New York, NY: Springer.

Yoon, J. H., Lee, K. C., Kho, Y. H., Kang, K. H., Kim, C. J. & Park, Y. H. (2002). *Halomonas alimentaria* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood *Int J Syst Evol Microbiol,* **52**, 123 - 130.

Wang, Y., Cai, H., Yu, S., Wang, Z., Liu, J. & Wu, X. (2007). *Halomonas gudaonensis* sp. nov., isolated from a saline soil contaminated by crude oil. *Int J Syst Evol Microbiol* **57**, 911-915.

Ziemke, F., Manfred, G.H., Lalucat, J. & Roselló-Mora, R. (1998). Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179-186.

Table 1. Distinguishing characteristics between *Halomonas azotoformans* strains.

Characteristics	Strains 15CR	Strain SP4^T	Strain R53
Size (µ)	2.8 x 0.7	1.9 x 0.7	2.2 x 0.9
Growth on 5% w/v NaCl	+	+	-
Resistance to 80ºC for 10 min	-	-	+
Tween 80 hydrolysis	+	+	-
Gelatinase	-	-	+
Tyrosine hydrolysis	+	+	-
Growth on:			
Arabinose	-	-	+
Cellobiose	+	-	-
Fructose	+	-	-
Lactose	-	-	+
Salicine	-	+	-
Starch	+	-	+
Trehalose	+	-	-
Lactate	+	-	+
Adonitol	-	-	+
Ethanol	+	+	-
Glycerol	+	-	-
Inositol	+	-	-
Manitol	-	-	+
Hystidine	+	-	-
Serine	+	+	-
Principal fatty acids			
10:0	2.30	2.60	2.35
12:0	3.07	3.19	3.26
12:0 3OH	5.43	6.22	5.98
16:1 ω7c	20.81	18.72	14.19
16:0	30.91	33.93	24.01
18:1 ω7c	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%
%G+C	62.7	66.2	66.2

Table 2. Distinguishing characteristics of *Halomonas* species related to *Halomonas cerina*

Data from Mata *et al.* (2002), Yoon et al. (2001), Martínez-Cánovas *et al.* (2004b), Romano *et al.* (2005), Wang *et al.* (2007), Ventosa *et al.* (1998) and from this work. Species: 1, *H. cerina*; 2, *H. alimentaria*; 3, *H. campaniensis*; 4, *H. campisalis*; 5, *H. desiderata*; 6, *H. gudaonensis*; 7, *H. ventosae*

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[a]	1-15
Sea-salt optimum (% w/v)	5-10	1-13	10	5	1-5	10-15[a]	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 (ºC)	4-45	4-45	10-43	4-50	10-45	10-42	15-50
Optimum temperature	20-32	30	37	30	37-42	30	32
Strictly halophilic	+	+	-	+	-	+	+
Acid from:							
D-glucose	-	-	ND	-	-	ND	-
Hydrolysis of:							
aesculin	-	-	ND	-	-	ND	-
casein	-	-	-	-	-	ND	-
tween 20	+	-	ND	+	+	ND	+
tween 80	+	-	+	-	+	-	-
DNA	+	+	ND	+	+	ND	-
tyrosine	+	-	+	-	+	ND	+
H_2S	-	-	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	+
MacConkey growth	+	ND	ND	-	+	ND	-
Cetrimide agar growth	-	ND	ND	-	+	ND	-
Haemolysis	-	-	ND	-	-	ND	-
Growth on [b]							
aesculin	-	-	ND	-	-	ND	+
L-arabinose	-	+	-	-	+	-	-
D-cellobiose	-	+	+	+	-	-	-
D-fructose	-	-	+	+	+	-	+
D-galactose	-	ND	-	-	-	+	+
D-mannose	+	+	+	-	+	+	-
D-melezitose	-	ND	ND	-	+	ND	ND
D-salicin	+	+	ND	-	-	ND	-
starch	-	+	ND	+	+	ND	-
D-trehalose	-	+	ND	+	+	+	+
citrate	+	+	ND	+	-	ND	+
formate	-	-	ND	-	+	ND	-
fumarate	+	+	ND	+	+	ND	+
gluconate	+	+	ND	+	+	+	+
malonate	-	+	ND	-	+	ND	+
propionate	-	+	ND	+	-	ND	+
succinate	+	-	ND	+	+	ND	+
adonitol	-	-	ND	-	-	ND	+
ethanol	+	+	ND	-	-	+	-
glycerol	-	+	+	+	+	+	+
myo-inositol	-	-	ND	+	-	ND	+
D-mannitol	-	+	ND	-	-	+	+
sorbitol	-	+	ND	-	-	ND	+
L-alanine	-	+	ND	+	-	+	-
L-cysteine	-	-	ND	-	-	ND	-
L-histidine	-	+	ND	-	-	ND	-
DL-isoleucine	-	D	ND	-	-	+	-
L-lysine	-	+	ND	+	-	-	-
L-methionine	-	ND	ND	-	-	ND	-
L-serine	+	+	ND	+	+	ND	-
L-valine	-	+	ND	-	-	ND	-
G +C content (%)	66.2	63	63.7	66	66	64	74.3

[a] NaCl
[b] When supplied as the sole source of carbon and energy or as sole source of carbon, nitrogen and energy

All species are Gram-negative rods, catalase and oxidase positive, reduce nitrate to nitrite and grow with acetate, maltose or glucose as sole carbon and energy source. They do not hydrolyse starch or gelatine.

Figure legends

Fig. 1. Dendrogram based on 107 phenotypic data. The simple-matching (SSM) coefficient and UPGMA were used.

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of the novel group of three isolates compared to other *Halomonas* species. The tree was obtained using the neighbour-joining algorithm. GenBank/EMBL/DDBJ accession numbers are given in parenthesis. Bar, 1% sequence divergence.

Fig. 3. Transmission electron micrograph of strains 15CR (A), R53 (B) and SP4 (C) stained with ruthenium red. Bars 1 μm