Halomonas almeriensis sp. nov., a moderately halophilic, exopolysaccharide-producing bacterium from Cabo de Gata, Almería, south-east Spain

Fernando Martínez-Checa, Victoria Béjar, M. José Martínez-Cánovas, Inmaculada Llamas and Emilia Quesada

Correspondence
Emilia Quesada
equesada@ugr.es

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

Halomonas almeriensis sp. nov. is a Gram-negative non-motile rod that was isolated from a saltern in the Cabo de Gata-Níjar wildlife reserve in Almería, south-east Spain. It is moderately halophilic, capable of growth at concentrations of 5–25 % w/v sea-salt mixture, the optimum being 7.5 % w/v. It is chemo-organotrophic and strictly aerobic, produces catalase but not oxidase, does not produce acid from any sugar and does not synthesize hydrolytic enzymes. The most notable difference between this micro-organism and other *Halomonas* species is that it is very fastidious in its use of a carbon source. It forms mucoid colonies due to the production of an exopolysaccharide. Its G + C content is 63.5 mol%. A comparison of 16S rRNA gene sequences confirmed its relationship to *Halomonas* species. The most closely related species is *Halomonas halmophila* with 95.8 % similarity between their 16S rRNA gene sequences. DNA–DNA hybridization with *H. halmophila* is 10.1 %. Its major fatty acids are 18 : 1 ω 7c, 16 : 0, 16 : 1 ω 7c/15 : 0 iso 2-OH, 12 : 0 3-OH, 12 : 0, 11-methyl 18 : 1 ω 7c and 10 : 0. The proposed name is *Halomonas almeriensis* sp. nov., with strain M8^T (= CECT 7050^T = LMG 22904^T) as the type strain.

The genus *Halomonas*, belonging to the family *Halomonadaceae* within the class ‘*Gammaproteobacteria*’, contains to date 32 species of moderately halophilic bacteria, most of which have been isolated from hypersaline habitats (Dobson & Franzmann, 1996; Mata *et al.*, 2002; Ventosa *et al.*, 1998; Vreeland *et al.*, 1980). Taxonomically *Halomonas* is a heterogeneous bacterial group. On the basis of 16S and 23S rRNA gene sequences, Arahal *et al.* (2002) have established three clearly distinguishable phylogenetic groups, in addition to which another three groups can also be identified by phenotypic studies, according to their capacity to produce acids from glucose and their use of a variety of compounds as sole sources of carbon and energy (Mata *et al.*, 2002). Some of the *Halomonas* species, including *Halomonas eurihalina*, *Halomonas maura*, *Halomonas*

ventosae and *Halomonas anticariensis*, which were isolated and characterized by our research group (Quesada *et al.*, 1990; Bouchotroch *et al.*, 2001; Martínez-Cánovas *et al.*, 2004a, b), produce extracellular polysaccharides with potential biotechnological applications (Calvo *et al.*, 2002; Béjar *et al.*, 1998; Martínez-Checa *et al.*, 2002; Arias *et al.*, 2003; Quesada *et al.*, 2004).

We describe here a novel exopolysaccharide-producing species belonging to the genus *Halomonas*, with the proposed name *Halomonas almeriensis*.

Strain M8^T was isolated from a water sample taken from a saltern in the Cabo de Gata-Níjar wildlife reserve in the province of Almería in south-east Spain during a wide range of samplings made by our research group in 18 hypersaline habitats in Spain and Morocco (Martínez-Cánovas *et al.*, 2004c). It was routinely kept and grown at 32 °C in MY medium (Moraine & Rogovin, 1966) with 7.5 % w/v marine salts (Rodríguez-Valera *et al.*, 1981).

Phenotypic characterization, on the basis of 112 tests, was done as described by Mata *et al.* (2002). We compared the novel strain with *Halomonas* species using the software TAXAN (Information Resources Group, Maryland Biotechnology Institute, University of Maryland, College Park,

Published online ahead of print on 13 May 2005 as DOI 10.1099/ij.s.0.63676-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M8^T is AY858696.

A dendrogram based on the simple-matching coefficient and UPGMA method, a neighbour-joining tree showing the phylogenetic relationships between *H. almeriensis* and other *Halomonas* species and taxa of Gram-negative halophilic bacteria, and a transmission electron micrograph showing the morphology of strain M8^T are available as supplementary material in IJSEM Online.

Table 1. Characteristics that distinguish *H. almeriensis* from other phylogenetically related type strains of the genus *Halomonas*

1, *H. almeriensis* CECT 7050^T; 2, *H. halmophila* ATCC 19717^T; 3, *Halomonas elongata* CECT 4279^T; 4, *H. eurihalina* ATCC 49336^T. Data from this study and from Dobson *et al.* (1990), Mata *et al.* (2002) and Vreeland *et al.* (1980).

Characteristic	1	2	3	4
Morphology	Short rod	Rod	Rod	Short rod
Exopolysaccharide	+	–	–	+
Motility	–	+	+	–
Oxidase	–	+	–	–
Sea-salt range (% w/v)	5–25	3–25	0–20	0·5–25
Sea-salt optimum (% w/v)	7·5	7·5	3–8	7·5
Temperature range (°C)	15–37	15–45	4–45	4–45
Acid from:				
Adonitol	–	+	–	–
L-Arabinose	–	+	+	–
D-Fructose	–	+	–	–
D-Galactose	–	+	–	–
D-Glucose	–	+	+	–
myo-Inositol	–	+	–	–
Lactose	–	+	+	–
Maltose	–	+	+	–
D-Mannitol	–	+	+	–
D-Mannose	–	+	–	–
D-Melezitose	–	+	–	–
D-Rhamnose	–	+	–	–
Sucrose	–	+	+	–
D-Salicin	–	+	+	–
D-Sorbitol	–	–	+	–
Sorbose	–	+	–	–
D-Trehalose	–	+	+	–
Hydrolysis of:				
Aesculin	–	–	–	+
Gelatin	–	–	–	+
Tween 20	–	+	–	+
Tween 80	–	–	–	+
Tyrosine	–	–	–	+
ONPG	–	–	+	+
Urea	–	–	+	+
Pigment from tyrosine	–	–	–	+
H ₂ S production	–	+	+	+
Nitrate reduction	–	–	+	+
Respiration on nitrate	–	–	+	–
Gluconate oxidation	+	–	+	+
Growth on:				
Agar cetrimide	–	–	+	+
Aesculin	–	+	–	–
L-Arabinose	–	–	+	+
D-Cellobiose	–	+	+	+
D-Fructose	–	+	+	+
D-Galactose	–	+	+	+
D-Glucose	–	+	+	+
Lactose	–	+	+	+
Maltose	–	+	–	+

Table 1. cont.

Characteristic	1	2	3	4
D-Mannose	–	+	+	+
D-Melezitose	–	+	+	+
D-Salicin	–	–	–	+
D-Trehalose	–	+	+	+
Acetate	–	+	+	+
Citrate	–	+	+	+
Formate	–	+	–	–
Fumarate	–	–	+	+
Gluconate	+	–	+	+
Malonate	–	–	+	+
Propionate	–	–	+	+
Succinate	–	–	+	+
Adonitol	–	–	+	+
Ethanol	–	–	+	–
Glycerol	–	+	–	–
myo-Inositol	–	–	+	+
D-Mannitol	–	–	+	+
Sorbitol	–	+	+	+
L-Histidine	–	+	+	+
DL-Isoleucine	–	–	+	+
L-Lysine	–	–	+	+
L-Valine	–	+	+	+
Susceptibility to:				
Amoxicillin (25 µg)	+	+	–	–
Ampicillin (10 µg)	+	+	–	–
Carbenicillin (100 µg)	+	+	–	–
Erythromycin (15 µg)	+	–	+	+
Nalidixic acid (30 µg)	–	+	+	+
Polymyxin B (300 IU)	–	+	–	+
Streptomycin (10 µg)	+	+	+	–
Sulphamide (250 µg)	–	+	+	+
G + C content (mol%)	63·5	63·0	60·5	59·1–65·7

USA) based on numerical analysis. The dendrogram obtained by the simple-matching coefficient (*S*_{SM}) (Sokal & Michener, 1958) and UPGMA method (Sneath & Sokal, 1973) (see Supplementary Fig. S1 in IJSEM Online) shows that strain M8^T was related to the non-acid-producing group of *Halomonas* species (Mata *et al.*, 2002), although it shares less than 63 % similarity with them. This low similarity can be put down to the fact that strain M8^T is extremely fastidious nutritionally. The main phenotypic differences between *H. almeriensis* (M8^T) and its nearest phylogenetically related strains of the genus *Halomonas* are shown in Table 1.

The G + C content of the DNA of strain M8^T was estimated from the midpoint value (*T*_m) of the DNA thermal denaturation profile, as described by Marmur & Doty (1962) and Ferragut & Leclerc (1976). The G + C content of the DNA of the novel strain was 63·5 mol%, within the range proposed for *Halomonas* species of 52–68 mol% (Franzmann *et al.*, 1988).

A partial fragment of the 16S rRNA gene was amplified by PCR using the protocol of Saiki *et al.* (1988). The forward primer, 16F27 (5'-AGAGTTTGATCATGGCT-CAG-3'), annealed at positions 8–27 and the reverse primer, 16R1488 (5'-CGGTTACCTTGTTAGGACTTCA-CC-3') (both from Pharmacia), annealed at the complement of positions 1511–1488 (*Escherichia coli* numbering according to Brosius *et al.*, 1978). To complete the sequence we designed an internal primer, 5'-GAGGATGATCAGCC-ACACTG-3', which annealed at positions 401–421. The PCR product was purified using the GFX PCR DNA and Gel Band purification kit (Amersham Biosciences). Direct sequence determinations of PCR-amplified DNAs were made with an ABI PRISM dye-terminator, cycle-sequencing, ready-reaction kit (Perkin-Elmer) and an ABI PRISM 377 sequencer (Perkin-Elmer) according to the manufacturer's instructions. The sequence obtained (1459 bp) was compared with 16S rRNA reference gene sequences retrieved from the GenBank and EMBL databases by BLAST search. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 (Kumar *et al.*, 2004) after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances and clustering were determined using the neighbour-joining and maximum-parsimony algorithms, and a bootstrap analysis (1000 replications) was made to determine the stability of the clusters. The neighbour-joining tree is available as Supplementary Fig. S2 in IJSEM Online. A similar result (not shown) was obtained using the maximum-parsimony algorithm. The taxa included in the tree shown in Fig. 1 represent only the nearest neighbours. Our analyses confirmed that the novel strain belongs to the genus *Halomonas*, is located within group 1 of *Halomonas* species described by Arahall *et al.* (2002) and shares 95.8% 16S rRNA gene sequence similarity with *Halomonas halmophila* (Dobson *et al.*, 1990). The

16S rRNA gene fragment analysed contains the 15 signature nucleotides defined for the family *Halomonadaceae* (Dobson & Franzmann, 1996).

DNA–DNA hybridization was carried out according to the method of Lind & Ursing (1986) with the modifications introduced by Ziemke *et al.* (1998) and Bouchotroch *et al.* (2001). The result shows the low hybridization (10.1%) with *H. halmophila*, which was chosen on the basis of our phylogenetic study as being the most closely related *Halomonas* species.

The fatty acids were analysed at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) by high-resolution GLC using a moist pellet of the cells obtained from a culture in MY medium supplemented with 7.5% w/v sea-salt mixture. Strain M8^T shows a combination of fatty acids found in other species of *Halomonas* (Dobson & Franzmann, 1996) (see species description), although it also contains a relatively high proportion of 10:0 (2.11%), 12:0 (1.22%) and 11-methyl 18:1 ω 7c (2.75%).

A transmission electron micrograph showing the morphology and cell size of strain M8^T and the presence of an extracellular polymer that is released into the external medium is available as Supplementary Fig. S3 in IJSEM Online. The TEM method used is fully described by Bouchotroch *et al.* (2001).

On the basis of phylogeny, DNA–DNA hybridization, fatty acid composition and phenotypic differences between the novel and previously described species within the genus *Halomonas*, we consider that strain M8^T represents a novel species, for which we propose the name *Halomonas almeriensis* sp. nov.

Fig. 1. Phylogenetic relationships between *Halomonas almeriensis* and other related *Halomonas* species. The tree was constructed using the neighbour-joining algorithm based on 16S rRNA gene sequences. Only bootstrap values greater than 50% are shown (1000 replications). Bar, 1% estimated sequence divergence.

Description of *Halomonas almeriensis* sp. nov.

Halomonas almeriensis (al.me.ri.en'sis. N.L. fem. adj. *almeriensis* denizen of the province of Almería in south-east Spain, where the strain was isolated).

Cells are Gram-negative, non-motile rods, $2\text{--}2.5 \times 0.75 \mu\text{m}$, occurring singly or in pairs. They accumulate poly- β -hydroxyalkanoates and produce exopolysaccharide. Colonies are round, convex, creamy-white and mucoid. Their growth pattern is uniform in a liquid medium. It is moderately halophilic, capable of growth in salt concentrations (mixture of sea salts) of 5–25% w/v. It grows at 15–37 °C and pH 6–10. It is chemo-organotrophic. Its metabolism is respiratory with oxygen as the terminal electron acceptor. The cells do not grow anaerobically in the presence of nitrate, nitrite or fumarate. Catalase is produced but not oxidase. It does not produce acids from sugars. Indole, methyl red and Voges–Proskauer are negative. It does not hydrolyse starch, aesculin, gelatin, casein, Tween 20, Tween 80, DNA or tyrosine. It produces phosphatase and grows on MacConkey agar, but does not produce phenylalanine deaminase, urease, ONPG or lecitinase. Gluconate is oxidized. It does not produce pigment from tyrosine, H_2S from L-cysteine, grow on cetrimide agar or lyse blood. D-Gluconate is acceptable as a sole carbon energy source, whereas aesculin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, D-melezitose, salicin, starch, D-trehalose, D-xylose, acetate, citrate, formate, fumarate, malonate, propionate, succinate, adonitol, ethanol, glycerol, *myo*-inositol, D-mannitol and sorbitol are not. L-Alanine and L-serine are used as sole sources of carbon, nitrogen and energy, whereas L-histidine, DL-isoleucine, L-lysine, L-methionine and L-valine are not. It is susceptible to amoxicillin (25 μg), ampicillin (10 μg), carbenicillin (100 μg), cefotaxime (30 μg), cefoxitin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg), nitrofurantoin (300 μg), rifampicin (30 μg), streptomycin (10 μg), tobramycin (10 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg). It is resistant to nalidixic acid (30 μg), polymyxin B (300 IU) and sulphamide (250 μg). Principal fatty acids (greater than 1%) are 18:1 ω 7c (50.66%); 11-methyl 18:1 ω 7c (2.75%); 16:0 (21.08%); 16:1 ω 7c/15:0 iso 2-OH (14.16%); 12:0 3-OH (5.64%); 12:0 (1.22%) and 10:0 (2.11%). The DNA G+C content of the type strain is 63.5 mol% (T_m method).

The type strain, M8^T (=CECT 7050^T=LMG 22904^T), was isolated from a hypersaline water sample taken from a saltern at Cabo de Gata (Almería, south-east Spain).

Acknowledgements

This research was supported by grants from the Dirección General de Investigación Científica y Técnica (BOS2003-00498) and from the Plan Andaluz de Investigación, Spain. Thanks go to our colleague Dr J. Trout for revising our English text.

References

- Arahal, D. R., Ludwig, W., Schleifer, K. H. & Ventosa, A. (2002). Phylogeny of the family *Halomonadaceae* based on 23S and 16S rDNA sequence analyses. *Int J Syst Evol Microbiol* **52**, 241–249.
- 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.
- Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci U S A* **75**, 4801–4805.
- Calvo, C., Martínez-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**, 347–351.
- Dobson, S. J. & Franzmann, P. D. (1996). Unification of the genera *Deleya* (Baumann *et al.* 1983), *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.
- Dobson, S. J., James, S. R., Franzmann, P. D. & McMeekin, T. A. (1990). Emended description of *Halomonas halmophila* (NCMB 1971^T). *Int J Syst Bacteriol* **40**, 462–463.
- Ferragut, C. & Leclerc, H. (1976). Étude comparative des méthodes de détermination du T_m de l'ADN bactérien. *Ann Microbiol* **127A**, 223–235 (in French).
- 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.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **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-Cánovas, M. J., Quesada, E., Llamas, I. & Béjar, V. (2004a). *Halomonas ventosae* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. *Int J Syst Evol Microbiol* **54**, 733–737.
- Martínez-Cánovas, M. J., Béjar, V., Martínez-Checa, F. & Quesada, E. (2004b). *Halomonas anticariensis* sp. nov., from Fuente de Piedra, a saline-wetland wildfowl reserve in Málaga, southern Spain. *Int J Syst Evol Microbiol* **54**, 1329–1332.
- Martínez-Cánovas, M. J., Quesada, E., Martínez-Checa, F. & Béjar, V. (2004c). A taxonomic study to establish the relationship between exopolysaccharide-producing bacterial strains living in diverse hypersaline habitats. *Curr Microbiol* **48**, 348–353.

- Martínez-Checa, F., Toledo, F. L., Vilchez, 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.
- Mata, J. A., Martínez-Cánovas, J., Quesada, E. & Béjar, V. (2002).** A detailed phenotypic characterisation of the type strains of *Halomonas* species. *Syst Appl Microbiol* **25**, 360–375.
- Moraine, R. A. & Rogovin, P. (1966).** Kinetics of polysaccharide B-1459 fermentation. *Biotechnol Bioeng* **8**, 511–524.
- Quesada, E., Valderrama, M. J., Bejar, V., Ventosa, A., Gutierrez, M. C., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1990).** *Volcaniella eurihalina* gen. nov., sp. nov., a moderately halophilic nonmotile Gram-negative rod. *Int J Syst Bacteriol* **40**, 261–267.
- Quesada, E., Béjar, V., Ferrer, M. R. & 8 other authors (2004).** Moderately halophilic, exopolysaccharide-producing bacteria. In *Halophilic Microorganisms*, pp. 297–314. Edited by A. Ventosa. Heidelberg: Springer.
- Rodríguez-Valera, F., Rúa-Berraquero, F. & Ramos-Cormenzana, A. (1981).** Characteristics of the heterotrophic bacterial populations in hypersaline environments of different salt concentrations. *Microb Ecol* **7**, 235–243.
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988).** Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.
- Sneath, P. H. A. & Sokal, R. R. (1973).** *Numerical Taxonomy. The Principles and Practice of Numerical Classification*. San Francisco: Freeman, Williams & Wilkins.
- Sokal, R. R. & Michener, C. D. (1958).** A statistical method for evaluating systematic relationships. *Univ Kansas Sci Bull* **38**, 1409–1438.
- Thompson, J. D., Gibson, T. J., Plewniak, K., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Ventosa, A., Nieto, J. J. & Oren, A. (1998).** Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* **62**, 504–544.
- Vreeland, R. H., Litchfield, C. D., Martin, E. L. & Elliot, E. (1980).** *Halomonas elongata*, a new genus and species of extremely salt-tolerant bacteria. *Int J Syst Bacteriol* **30**, 485–495.
- Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.