

## *Halomonas organivorans* sp. nov., a moderate halophile able to degrade aromatic compounds

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A group of moderately halophilic bacteria able to degrade aromatic organic compounds contaminating hypersaline habitats in southern Spain have been isolated and characterized. The taxonomic position of these strains was determined using phenotypic, phylogenetic and genotypic methods. The G + C content of their DNA ranged from 61.0 to 62.9 mol%. DNA–DNA hybridization studies showed that they constitute a genospecies, having DNA–DNA hybridization values of 90–100%. Analysis of the complete 16S rRNA gene sequence revealed a high level of similarity with members of the genus *Halomonas*, sharing 98% 16S rRNA gene sequence similarity with the type strains of *Halomonas salina* and *Halomonas halophila*. However, phenotypic differences and the low level of DNA–DNA hybridization suggest the placement of these strains as a novel species within the genus *Halomonas*. The name *Halomonas organivorans* sp. nov. is proposed, with strain G-16.1<sup>T</sup> (=CECT 5995<sup>T</sup>=CCM 7142<sup>T</sup>) as the type strain. This novel species of *Halomonas* is characterized by its ability to use a wide range of organic compounds (benzoic acid, *p*-hydroxybenzoic acid, cinnamic acid, salicylic acid, phenylacetic acid, phenylpropionic acid, phenol, *p*-coumaric acid, ferulic acid and *p*-aminosalicylic acid), and it could be useful for the decontamination of polluted saline habitats.

The two main groups of micro-organisms adapted for life in hypersaline environments are extremely halophilic archaea and moderately halophilic bacteria. Moderately halophilic bacteria constitute a group able to grow in a wide range of saline environments. Several studies on their molecular adaptation to media with different salt concentrations have increased the scientific interest in these bacteria (Nieto & Vargas, 2002). Moreover, extremophiles in this group exhibit great potential for biotechnological exploitation: in fact, these bacteria have been used as a source of compatible solutes or hydrolytic enzymes (Mellado & Ventosa, 2003).

Saline and hypersaline environments are often contaminated with organic compounds as a result of industrial activities and urban water effluents. Although the use of different bacterial groups in bioremediation processes has been widely studied, the ability of native communities of halophilic bacteria to be used for the degradation of pollutants in saline habitats has not been studied in a systematic way, and only a few studies have reported the biotechnological potential of this group of extremophiles

in the decontamination of saline environments (Margesin & Schinner, 2001; Mellado & Ventosa, 2003). Thus, the identification of moderate halophiles that play an important role in the degradation of pollutant compounds is of great interest in the context of assessing acceleration of the environmental repair process for bioremediation in contaminated saline habitats.

Recently, we have focused on the characterization of the moderately halophilic bacterial community able to degrade different aromatic pollutants in saline habitats of southern Spain, the primary goal being the isolation of potential degraders able to grow at different salinities. In the course of this work, four novel bacterial strains, designated G-16.1<sup>T</sup>, G-2.5, G-17.1 and H-30.2, were isolated from saline soils in Isla Cristina (Huelva), Spain. These bacteria were isolated because of their ability to grow in phenol (strains G-16.1<sup>T</sup>, G-2.5, G-17.1) or *p*-coumaric acid (strain H-30.2) as the sole source of carbon and energy. For this purpose, an enrichment culture using sterile mineral medium M63 (Cohen & Rickenberg, 1956) supplemented with 10% NaCl was used. Phenol or *p*-coumaric acid was added to the mineral medium at a concentration of 0.05% (w/v). After three enrichment steps, the cultures obtained were plated on M63 + 10% NaCl medium supplemented with the organic compound and incubated at 37 °C. Colonies that differed in appearance from each other were picked and

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inoculated into the liquid selective medium previously described. Of the isolates, four strains showing a high level of degradative activity were selected for further studies.

To study the ability of the isolates to degrade a variety of aromatic compounds, substrates were added to the saline minimal medium M63 + 10% NaCl at different concentrations (0.05% for phenol and *p*-coumaric acid and 1–5 mM for the other substrates). This medium was inoculated with strains G-16.1<sup>T</sup>, G-2.5, G-17.1 and H-30.2, and the utilization of these compounds was tested by monitoring the increase in turbidity. This group of isolates utilized, in addition to phenol and *p*-coumaric acid, a diverse range of compounds as sole carbon and energy sources (Table 1), indicating that they constitute promising potential degraders in bioremediation processes. The aim of this work was to study these four isolates in detail by using a polyphasic taxonomic approach.

A preliminary taxonomic study based on the determination and comparison of partial sequences of 16S rRNA genes placed these bacterial isolates in the genus *Halomonas*. This genus – currently one of the largest among the moderate halophiles – was originally proposed by Vreeland *et al.* (1980) with a single species, *Halomonas elongata*. Currently, the genus comprises more than 20 species, including bacterial isolates from different terrestrial and aquatic environments with, in most cases, high salt concentrations. This genus has been considered as a model system among the moderate halophiles and has been used extensively for studies concerning both osmoregulatory mechanisms and physiological adaptations (Ventosa *et al.*, 1998; Nieto *et al.*, 2000). On the other hand, the genus *Halomonas* is a taxon comprising strains that are metabolically versatile: some *Halomonas* species have been described as denitrifying halophilic bacteria useful in the reduction of nitrate during the treatment of saline waste (Berendes *et al.*, 1996; Mormile *et al.*, 1999; Romano *et al.*, 1996; Dobson & Franzmann, 1996). In addition, there are also a few reports on *Halomonas* strains able to degrade aromatic pollutants (Maltseva *et al.*, 1996; Muñoz *et al.*, 2001).

**Table 1.** Ability of strains of *H. organivorans* sp. nov. to use a variety of aromatic substrates as sole carbon and energy sources

All strains were able to grow on phenylacetic acid, phenol, *p*-coumaric acid and ferulic acid. Symbols: +, growth; –, no growth.

Aromatic compound	G-16.1 <sup>T</sup>	G-2.5	G-17.1	H-30.2
Benzoic acid	+	–	+	+
<i>p</i> -Hydroxybenzoic acid	+	–	+	+
Cinnamic acid	+	+	–	+
Salicylic acid	+	+	+	–
Phenylpropionic acid	+	+	+	–
<i>p</i> -Aminosalicylic acid	+	–	+	–

The isolates were cultured on a complex medium (SW-10) containing 10% salts supplemented with 0.5% (w/v) yeast extract (Ventosa *et al.*, 1982). The strains were routinely grown at 200 r.p.m. in an orbital shaker (New Brunswick Scientific) at 37 °C. When necessary, solid medium was prepared by adding 20 g Bacto-agar l<sup>-1</sup> (Difco).

The methods used for phenotypic characterization have been described previously in detail (Ventosa *et al.*, 1982; Quesada *et al.*, 1984; García *et al.*, 1987). Isolates G-16.1<sup>T</sup>, G-2.5, G-17.1 and H-30.2 were Gram-negative rods that were oxidase-negative, catalase-positive and strictly aerobic. On SW-10 medium incubated at 37 °C, the strains produced circular colonies, each 2–3 mm in diameter. Colonies were flat/slightly convex with irregular edges.

The salt requirement of these strains was determined in complex medium SW at different salt concentrations. The isolates grew in a wide range of salinities (from 1.5 to 30%, w/v, salt), producing optimal growth in media containing 7.5–10% (w/v) salt. They did not grow in the absence of NaCl. These results indicated that the four isolates were strictly halophilic and could be assigned to the group of moderately halophilic bacteria (Kushner & Kamekura, 1988). Some other physiological characteristics of the strains are shown in Table 2 and in the species description.

Nutritional features of the isolates were determined using GN MicroPlates (Biolog). Strains were grown on isolate medium (Biolog) at 37 °C for 24 h. Suspensions of the strains were made using prewarmed sterile saline medium (3% NaCl), within the density range specified by the manufacturer. Immediately after suspension of the cells in the saline solution, the suspensions were transferred into sterile multichannel pipetter reservoirs (Biolog). Biolog GN MicroPlates were inoculated with 125 µl cell suspension per well by means of an eight-channel repeating pipetter (Biolog). The inoculated Biolog GN MicroPlates were incubated at 37 °C for 24 h and the results were read with a MicroPlate Reader, using Microlog 3.59 computer software to perform automated reading. The results of the nutritional tests are shown in the description of the species. The four isolates show a greater nutritional versatility than other related *Halomonas* species.

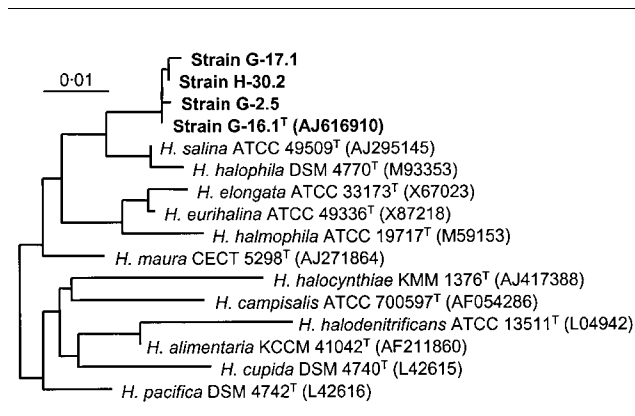
Chromosomal DNA from the four strains was isolated and purified according to the methods described by Wilson (1987) and Marmur (1961). The 16S rRNA genes of the four isolates were amplified by a PCR using two universal primers, as described previously (Mellado *et al.*, 1995), and almost-complete nucleotide sequences (approx. 1450 bp) were determined. The ARB software package (Ludwig & Strunk, 1996) was used for 16S rRNA gene sequence analysis.

16S rRNA gene phylogenetic analysis performed on the basis of the neighbour-joining method (Saitou & Nei, 1987) clearly showed the position of this group of strains within the family *Halomonadaceae*, in the genus *Halomonas*.

**Table 2.** Differential phenotypic characteristics of *H. organivorans* sp. nov. and other related species of the genus *Halomonas*Data are from Arahall *et al.* (2002), Mata *et al.* (2002), Vreeland *et al.* (1980) and this study. +, Positive; -, negative; ND, not determined.

Characteristic	<i>H. organivorans</i> sp. nov.	<i>H. salina</i>	<i>H. halophila</i>	<i>H. elongata</i>
Morphology	Rods	Short rods	Short rods	Rods
Cell length (µm)	2.0–3.0	2.0–2.5	1.5–2.0	ND
Cell width (µm)	1.0–1.2	0.7–0.8	0.5–0.7	ND
Motility	+	–	+	+
Pigmentation	Cream	Yellow-cream	Cream	Cream
Oxidase	–	+	+	–
Salt growth range (%)	1.5–30	2.5–20	2–30	0–20
Hydrolysis of aesculin	+	–	+	+
H <sub>2</sub> S production	+	+	+	–
Growth on:				
Tween 80	+	–	–	+
Glycerol	+	–	+	+
D-Arabitol	+	–	–	+
α-D-Lactose	–	+	–	+
D-Glucuronic acid	+	–	–	+
L-Aspartic acid	+	–	–	+
L-Histidine	+	–	–	+
L-Leucine	+	–	+	+
Inosine	+	–	+	+
Uridine	+	–	+	–
DNA G+C content (mol%)	61.0–62.9	60.4–64.2	66.7	60.5

Maximum-parsimony- and maximum-likelihood-based trees using the full dataset or a selection of sequences were also obtained which showed the same phylogenetic position of the group of isolates, i.e. in the genus *Halomonas*, grouped together and forming a cluster separate from the other species (Fig. 1). Base-frequency filters were applied in the sequence comparison analysis and the effects on the results were evaluated.



**Fig. 1.** Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of the novel group of four isolates with respect to other *Halomonas* species. The tree was obtained using the maximum-likelihood method. GenBank/EMBL/DDBJ accession numbers are given in parentheses. Bar, 1% sequence divergence.

The 16S rRNA gene sequence of strain G-16.1<sup>T</sup> was very similar to those of strains G-2.5, G-17.1 and H-30.2, with sequence similarities of 99.8, 99.7 and 99.9%, respectively. All four strains are on the same phylogenetic branch and their 16S rRNA gene sequences contain the four signature nucleotides that define the genus *Halomonas* as well as the 15 signature nucleotides characteristic of the family *Halomonadaceae*, as described by Dobson & Franzmann (1996).

The similarities between the 16S rRNA gene nucleotide sequences of the isolates and those of the other 25 currently accepted *Halomonas* species were between 93 and 98%. The highest similarity values between the sequence of strain G-16.1<sup>T</sup> and those of the type strains of species of the genus were for *Halomonas salina* (98.0%) and *Halomonas halophila* (98.0%). These two species have 99.0% 16S rRNA gene sequence similarity but are considered different species on the basis of other phenotypic and genotypic data (Valderrama *et al.*, 1991).

Recently, Arahall *et al.* (2002) performed a phylogenetic analysis of the genus *Halomonas* based on 23S and 16S rRNA gene sequence comparisons: two clear phylogenetic groups were observed. Group 1 comprises the type species of *Halomonas*, *H. elongata* and four other species, *Halomonas eurihalina*, *Halomonas halmophila*, *H. halophila* and *H. salina*. Since the publication of this work, a number of novel species belonging to the genus *Halomonas* have been described, and our phylogenetic analysis based on the

sequence of 16S rRNA genes reveals that *Halomonas maura* could also be included in group 1. The phylogenetic analyses performed also show that strains G-16.1<sup>T</sup>, G-2.5, G-17.1 and H-30.2 cluster with species belonging to the above-mentioned *Halomonas* group 1 (Fig. 1) and show clearly that they are members of the *Halomonas* 'sensu stricto group'.

The G+C content of genomic DNA was determined by the method of Marmur & Doty (1962) using the equation of Owen & Hill (1979): the values for our isolates ranged between 61.0 and 62.9 mol%. These values fall within the range described for species of the genus *Halomonas* (52–68 mol%; Franzmann *et al.*, 1988). However, the G+C content is lower than those of the most phylogenetically related species (64.2 mol% for *H. salina*, 66.7 mol% for *H. halophila*).

To define the taxonomic position of the novel isolates able to degrade different organic pollutants, DNA–DNA hybridization studies were carried out according to the competition procedure of Johnson (1994), described in detail in Mormile *et al.* (1999). The hybridization temperature was 57 °C, which is within the limit of validity for the filter method (De Ley & Tijtgaat, 1970), and the percentage of hybridization was calculated according to Johnson (1994). The values obtained in the DNA–DNA hybridization studies between strain G-16.1<sup>T</sup> and the type strains of the *Halomonas* species with which it formed a phylogenetically coherent cluster are shown in Table 3. The DNA–DNA hybridization between strain G-16.1<sup>T</sup> and isolates G-2.5, G-17.1 and H-30.2 was equal to, or greater than, 90 %, confirming the phylogenetic data and the phenotypic characteristics shared by this group of strains. On the other hand, DNA–DNA hybridization between strain G-16.1<sup>T</sup> and closely related species of the genus *Halomonas* was significantly below 70 %, showing relatedness values of

49 % with *H. salina* and 11 % with *H. halophila*, indicating that this strain is genotypically distinct from the type strains of these species (Wayne *et al.*, 1987).

Particularly remarkable is the extreme nutritional versatility presented by these novel isolates in comparison with their closest relatives. This metabolic difference constitutes a key differentiating property of this novel group of moderate halophiles. Other differential features are shown in Table 2. Overall, the phenotypic, genotypic and phylogenetic results suggest that isolates G-16.1<sup>T</sup>, G-2.5, G-17.1 and H-30.2 should be assigned to a novel species within the genus *Halomonas*. On the basis of the ability of these isolates to degrade different aromatic organic compounds, the name *Halomonas organivorans* sp. nov. is proposed.

### Description of *Halomonas organivorans* sp. nov.

*Halomonas organivorans* (N.L. n. *organum* organic compound; L. v. *vorare* to eat, to consume; N.L. part. adj. *organivorans* devouring organic compounds).

Cells are motile, Gram-negative rods, 2.0–3.0 × 1.0–1.2 µm. Colonies are cream, flat/slightly convex, opaque and raised with slightly irregular edges on SW-10 medium. Moderately halophilic, growing in a wide range (1.5–30 %, w/v) of salt concentrations, with optimum growth at 7.5–10 % (w/v) salts. No growth in the absence of NaCl. No other salt requirements have been determined. Growth occurs at 15–45 °C (optimal temperature 37 °C) and at pH 6.0–10.0 (optimal pH 7.0). Strictly aerobic. Catalase is produced, aesculin is hydrolysed. Negative results in oxidase, indole, methyl red and Voges–Proskauer tests. Gelatin, casein, starch and DNA are not hydrolysed. As determined with the Biolog GN panel, the following compounds are utilized: glycogen, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, D-arabitol, i-erythritol, D-fructose, D-galactose, α-D-glucose, *m*-inositol, D-mannitol, D-mannose, D-psicose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, methylpyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-glucuronic acid, D-gluconic acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, D-saccharic acid, succinic acid, bromosuccinic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-proline, L-serine, L-threonine, urocanic acid, inosine, uridine, putrescine, 2-aminoethanol and glycerol. The following compounds are not utilized: α-cyclodextrin, dextrin, *N*-acetyl-D-galactosamine, adonitol, L-arabinose, cellobiose, L-fucose, gentiobiose, α-D-lactose, lactulose, maltose, D-melibiose, methyl β-D-glucoside, D-raffinose, turanose, xylitol, formic acid, D-glucosaminic acid, α-hydroxybutyric acid, γ-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, propionic acid, quinic acid, sebacic acid, succinamic acid, glucuronamide, alaninamide, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-ornithine, L-phenylalanine, L-pyroglutamic

**Table 3.** DNA G+C content and levels of DNA–DNA hybridization between the novel isolates and some phylogenetically related species of the genus *Halomonas*

Data are from this study and from Valderrama *et al.* (1991) unless indicated.

Source of unlabelled DNA	G+C content (mol%)	Relatedness to <sup>3</sup> H-labelled DNA from G-16.1 <sup>T</sup> (%)
G-16.1 <sup>T</sup>	61.0	100
G-2.5	61.5	90
G-17.1	62.5	93
H-30.2	62.9	90
<i>H. salina</i> ATCC 49509 <sup>T</sup>	64.2*	49
<i>H. halophila</i> CCM 3662 <sup>T</sup>	66.7*	11
<i>H. elongata</i> ATCC 33173 <sup>T</sup>	60.5*	14

\*Data from Arahal *et al.* (2002).

acid, D-serine, DL-carnitine,  $\gamma$ -aminobutyric acid, thymidine, phenylethylamine, 2,3-butanediol, DL- $\alpha$ -glycerol phosphate, D-glucose 1-phosphate and D-glucose 6-phosphate. The DNA G+C content ranges between 61.0 and 62.9 mol%.

Isolated from saline soils. The type strain is G-16.1<sup>T</sup> (=CECT 5995<sup>T</sup> =CCM 7142<sup>T</sup>). The DNA G+C content of the type strain is 61.0 mol%. One important property of this strain is its ability to utilize various organic compounds (Table 1) considered as pollutants as sole sources of carbon and energy.

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