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

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Halomonas ventosae sp. nov. includes three moderately halophilic, exopolysaccharide-producing strains isolated from saline soils in Jaén (south-eastern Spain). These strains can grow anaerobically using either nitrate or nitrite as terminal electron acceptor and hydrolyse both tyrosine and phenylalanine. Their G+C content varies between 72-6 and 74-3 mol%. The affiliation of the isolates with the genus Halomonas was confirmed by 16S rRNA gene sequence comparison. DNA–DNA hybridization shows 70–82.7% relatedness among the three strains. Nevertheless, their relatedness is less than 43% compared to related reference strains. The proposed type strain for Halomonas ventosae is strain Al12T (=CECT 5797T = DSM 15911T). It grows best at 8% (w/v) sea salts and requires the presence of Na+. Its major fatty acids are 18:1 ω7c, 16:0, 16:1 ω7c, and 15:0 iso 2-OH. The predominant respiratory lipoquinone found in strain Al12T is ubiquinone with nine isoprene units (Q-9).

The Halomonadaceae include four genera of halophilic bacteria: Halomonas, Chromohalobacter, Alcanivorax and the recently described Cobetia, plus two genera of non-halophilic bacteria, Zymobacter and Carminonas (Arahall et al., 2002; Dobson & Franzmann, 1996; Garriga et al., 1999; Yakimov et al., 1998). The genus Halomonas was originally proposed to accommodate one species, Halomonas elongata (Vreeland et al., 1980). It now contains a large number of recently discovered species (Heyman et al., 2002; Romanenkov et al., 2002), as well as already known bacteria which were initially assigned to other genera, such as Deleya, Volcanella and Halovibrio (Mellado et al., 1998; Quesada et al., 1984, 1990; Valderrama et al., 1991; Ventosa et al., 1998), but have since been included in Halomonas.

Halomonas species are widely distributed throughout hypersaline environments. Some of them have been recognized for their potential use in biotechnology (Margesin & Schinner, 2001; Ventosa & Nieto, 1995). Halomonas eurihalina and Halomonas maura were isolated by our group from hypersaline soils and found to produce exopolysaccharides (EPSs) (Quesada et al., 1990; 1993; Bouchotroch et al., 2001), which have since been characterized and evaluated for potential applications in industry (Calvo et al., 1995, 1998; Béjar et al., 1998; Martínez-Checa et al., 2002; Arias et al., 2003; Quesada et al., 2004).

We classify here three unassigned EPS-producing strains, which are characterized in particular by their capacity to denitrify. On the basis of phenotypic features, DNA–DNA hybridization and comparison of 16S rRNA gene sequences, as well as an investigation for strain Al12T of polar lipids, isoprenoid quinones and salt requirements, we propose a novel species, Halomonas ventosae sp. nov.

The strains, referred to as Al12T, Al15 and Al16, were all isolated from a saline soil in Jaén, south-eastern Spain (Quesada et al., 2004). All the strains were routinely grown in MY medium (Quesada et al., 1993) at 32°C. For comparison, we used the strains from culture collections that appear in the figures and tables below.

Phenotypic studies (135 tests) and numerical analyses were made as described in previous publications (Mata et al., 2002; Quesada et al., 1983; Ventosa et al., 1982). The data were submitted to cluster analysis using the simple matching coefficient (SSM) (Sokal & Michener, 1958) and clustering was achieved by the unweighted-pair-group method of association (UPGMA) (Sneath & Sokal, 1973). Computer analysis was made with the TAXAN program (Information Resources Group, Maryland Biotechnology Institute). Test error was evaluated by examining 10 strains in duplicate (Sneath & Johnson, 1972).
DNA was purified using the technique 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). $T_m$ was determined by the graphic method described by Ferragut & Leclerc (1976) and the G+C content was calculated from this temperature using the equation of Owen & Hill (1979). The $T_m$ value of reference DNA from *Escherichia coli* NCTC 9001 was taken to be 74·6°C in 0·1× SSC (Owen & Pitcher, 1985).

The 16S rRNA gene was amplified by PCR using standard protocols (Saiki et al., 1988). The forward primer, 16F27 (5’-AGAGTTTGATCMTGGCTCAG-3’), annealed at position 8–27, and the reverse primer, 16R1488 (5’-CGGT-TACCTTGTTAGGACTTCACC-3’), annealed at position 1511–1488 (E. coli numbering according to Brosius et al., 1978). Both primers were obtained from Pharmacia. PCR products were purified using Qiagen PCR clean-up kit (Qiagen). Direct sequence determination of PCR-amplified DNAs was carried out with the ABI PRISM 377 sequencer (Perkin-Elmer) and an ABI PRISM dye-terminator cycle-sequencing ready-reaction kit (Perkin-Elmer). DNA was purified using the technique of Marmur (1961). Phylogenetic analysis was carried out using MEGA (Molecular Evolutionary Genetics Analysis) version 2.1 (Kumar et al., 2001), 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.

DNA–DNA hybridization was conducted following the methods of Lind & Ursing (1986) with the modifications of Ziemke et al. (1998) and Bouchotroch et al. (2001).

The fatty-acid analysis of strain Al12T was carried out by Microbial Identification Systems (Williston, VT, USA), using the MID/Hewlett Packard Microbial Identification System (MIS), which employs high-resolution GC to obtain the fatty-acid profile. Isoprenoid quinones were identified by HPLC by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH).

Transmission and scanning electron micrographs were obtained by the methods of Bouchotroch et al. (2001).

Salt requirements and optimum concentrations for strain Al12T were determined in MY medium according to the methods described by Bouchotroch et al. (2001). The salt concentrations assayed ranged from 1 to 15% (w/v) and were prepared from a mixture of sea salts (Rodriguez-Valera et al., 1981). We also tested to see whether strain Al12T needed NaCl alone or whether it required other magnesium and/or potassium salts.

The three strains described here were strictly halophilic, being unable to grow in the absence of sea salts. They were characterized by their capacity to produce EPS. Phenotypic characteristics common to all three strains are given in the species description; differential phenotypic features among the three strains and the dendrogram obtained from numerical analysis are available as Supplementary Table A and Fig. A, respectively, in IJSEM Online. The estimated error in the phenotypic study was no more than 3% in any of the tests, which would not affect the cluster analysis significantly. No test was found to be so non-reproducible (test variance $\geq 0·2$) as to be excluded from the analysis. The test responsible for most error was gluconate oxidation. At an 85% similarity level, the novel strains grouped into a phenon sharing the highest similarity (about 80%) with the species *Halomonas halodenitrificans* and *Halomonas maura*. The novel EPS-producing strains were characterized by their denitrifying capability; they were able to reduce nitrate and nitrite under anaerobic conditions; this capacity is uncommon among halophilic bacteria and may prove very useful for biotechnological applications (Mormile et al., 1999; Peyton et al., 2001). They also hydrolyse tyrosine and produce the enzyme phenylalanine deaminase; the latter capacity has only been observed hitherto in *Halomonas desiderata* (Mata et al., 2002). They differ from *H. halodenitrificans*, which is non-motile and does not produce EPS or phenylalanine deaminase.

The G+C content of the three novel strains was 74·3 mol% for strain Al12T, 74·2 for strain Al15 and 72·6 for strain Al15. These G+C values are very high and considerably above the typical G+C for *Halomonas* species of 52–68 mol% (Franzmann et al., 1988). Nevertheless, the phenotypic data point to the conclusion that this cluster does indeed belong to *Halomonas* and this is supported by the chemotaxonomic, genotypic and phylogenetic analyses.

The results of DNA–DNA hybridization are shown in Supplementary Table B. In this respect, none of the three strains was closely related to the type strains of related species, showing less than 43% hybridization with them. These reference strains were chosen because of their phenotypic or phylogenetic relationship to the new isolates. Additionally, some of them produce EPS (*H. eurihalina* and *H. maura*) or can grow anaerobically with nitrate as terminal electron acceptor (*H. halodenitrificans*, *Halomonas campisalis*, *H. desiderata* and *Marinobacter hydrocarbonoclasticus*). Nevertheless, our results show that the new strains were not closely related to any of them.

We determined almost complete 16S rDNA sequences for strains Al12T (1395 bp), Al15 (1398 bp) and Al16 (1396 bp), corresponding to positions 46 and 1445 of the *E. coli* 16S rRNA gene. The fragment analysed contained the 15 signature nucleotides defined for the *Halomonadaceae*, as described by Dobson & Franzmann (1996). The three sequences share 99·7–100% similarity among themselves and are on the same phylogenetic branch. The phylogenetic tree constructed using the neighbour-joining method is

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shown in Supplementary Fig. B. The maximum-parsimony algorithm gave a similar result (data not shown).

Fig. 1 shows the cell morphology of strain Al12<sup>T</sup>. Thin sections reveal a typical Gram-negative cell envelope profile; the cells also contain poly-β-hydroxyalkanoate (PHA) granules. EPS appears associated with the cell surface and also in the medium surrounding the bacterium.

Strain Al12<sup>T</sup> is euryhaline and can grow in media containing between 1 and 15 % (w/v) sea salts, although it grew best under our experimental conditions in a concentration of 8 % (w/v), within which it required the presence of 1·38 M Na<sup>+</sup>. It did not require magnesium or potassium. Under optimum conditions, its growth rate was 1·47 h<sup>−1</sup>.

The results of chemotaxonomic analysis are given in the species description.

Table 1 shows the features that distinguish Halomonas ventosae sp. nov. from other phenotypically or phylogenetically related taxa.

Accordingly, on the basis of differences in phenotypic characteristics, phylogenetic inference and genetic distinctiveness, strains Al12<sup>T</sup>, Al15 and Al16 should be placed as a novel species of the genus Halomonas, for which the name Halomonas ventosae sp. nov. is proposed.

**Description of Halomonas ventosae sp. nov.**

Halomonas ventosae (ven.to.sa’e, N.L. sb. gen. ventosae of Ventosa, named in honour of Professor A. Ventosa, a renowned expert on halophilic bacteria).

Cells are capsulated, motile, Gram-negative rods, 1·2–1·4 × 0·7–0·8 μm, occurring either singly or in pairs. They accumulate PHA. On MY solid medium the bacteria grow in circular, convex, cream-coloured, mucoid colonies. In liquid medium their growth pattern is uniform. Moderately halophilic, capable of growing in salt concentrations (mixture of sea salts) of 3–15 % (w/v), whereas no growth occurs in the absence of salt. Grow within the temperature range 15–50 °C and at pH values between 6 and 10. Chemoorganotrophic. Metabolism is respiratory, with oxygen, nitrate or nitrite as terminal electron acceptor. Cells do not grow anaeroberically in the presence of fumarate. Catalase and oxidase are produced. They do not produce acids from sugars. Indol, methyl-red and Voges–Proskauer are negative. Selenite is reduced, phenylalanine deaminase is produced and tyrosine and TWEEN 20 are hydrolysed, but not TWEEN 80, starch, urea, gelatin, DNA, lecithin or casein. There is no growth on MacConkey agar and cetrime agar. Blood is not haemolysed. The following can be used as sole carbon and energy sources: acetate, citrate, D-galactose, D-glucuronate, D-glucose, lactate, malonate, maltose, propionate, D-sorbitol and succinate, but not L-arabinose, D-cellobiose, ethanol and formate. The following are not used as sole carbon, nitrogen and energy sources: L-alanine, L-cysteine, L-histidine, isoleucine, L-lysine, L-methionine and L-valine. Cells are susceptible to amoxycillin (25 μg), ampicillin (10 μg), carbenicillin (100 μg), cefamandole (30 μg), cefotaxime (30 μg), cefoxitin (30 μg), chloramphenicol (30 μg), kanamycin (30 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), polymyxin B (300 IU), rifampicin (30 μg), sulfonamide (250 μg) and trimethoprim/sulfamethoxazole (1·25–2·375 μg). The three strains were isolated from a saline soil in Jaén, south-eastern Spain. The DNA G + C content is 72·6–74·3 mol% (T<sub>m</sub> method).

The type strain is strain Al12<sup>T</sup> (= CECT 5797<sup>T</sup> = DSM 15911<sup>T</sup>). The description of the type strain is the same as that of the species. Additionally, this strain is able to grow in a medium containing 1 % (w/v) mixture of sea salts, although it grows best in 8 % (w/v). It requires sodium but not magnesium or potassium; the optimum NaCl concentration is 1·38 M. It produces H<sub>2</sub>S from L-cysteine. Phosphatase and aesculin are not hydrolysed. Glucuronate is oxidized. The following can be used as sole carbon and energy sources: adonitol, D-fructose, fumarate, DL-glycerol, myo-inositol, lactose, D-mannitol and D-trehalose, but not D-mannose, D-salicin, L-serine and starch. Al12<sup>T</sup> is resistant to tobramycin (10 μg). The major fatty acids (up to 83 %) are 18:1 ω7c, 16:0, 16:1 ω7c and 15:0 iso 2-OH, and it
has a ubiquinone with nine isoprene units. Its DNA G+C content is 74·3 mol% (Tm method).

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References


