Halomonas avicenniae sp. nov., isolated from the salty leaves of the black mangrove *Avicennia germinans* in Puerto Rico

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A Gram-negative, short rod to oval-shaped bacterium (strain MW2a^T) was isolated from the surface of leaves of the black mangrove *Avicennia germinans* and subjected to a polyphasic taxonomic study. Strain MW2a^T was moderately halophilic, growing at NaCl concentrations in the range 0–25 % (w/v) with optimum growth at 5 % (w/v) NaCl. Growth occurred at 12–40 °C (optimum, 30–35 °C) and at pH 5.0–9.0 (optimum, pH 7.0–8.0). Strain MW2a^T was strictly aerobic. Phylogenetic analysis based on the 16S rRNA gene showed that the strain belongs to the genus *Halomonas*. The closest relative was *Halomonas marisflavi*, with 98.6 % 16S rRNA gene sequence similarity. The DNA G + C content of strain MW2a^T was 61.5 mol%, which is in the range of values for *Halomonas* species. DNA–DNA hybridization with *H. marisflavi* showed a relatedness of 42 % and lower values were obtained with respect to other related *Halomonas* species. The major fatty acids were C_{16:0}, C_{19:0} cyclo ω 8c, C_{18:1} ω 7c and C_{12:0} 3-OH. Overall, the phenotypic, genotypic and phylogenetic results presented in this study demonstrate that strain MW2a^T represents a novel species within the genus *Halomonas*. The name *Halomonas avicenniae* sp. nov. is proposed, with strain MW2a^T (= CECT 7193^T = CCM 7396^T) as the type strain.

The genus *Halomonas* belongs to the family *Halomonadaceae* within the *Gammaproteobacteria* and at the time of writing comprised 35 species that had been isolated mainly from saline or hypersaline environments (Arahal *et al.*, 2002; Bouchotroch *et al.*, 2001; Dobson & Franzmann, 1996; Garcia *et al.*, 2004; Lee *et al.*, 2005; Lim *et al.*, 2004; Martínez-Cánovas *et al.*, 2004; Martínez-Checa *et al.*, 2005; Mata *et al.*, 2002; Mormile *et al.*, 1999; Quillaguaman *et al.*, 2004; Romanenko *et al.*, 2002; Ventosa *et al.*, 1998; Vreeland *et al.*, 1980; Yoon *et al.*, 2001, 2002) or from unusual habitats such as dry mural paintings (Heyrman *et al.*, 2002) and deep-sea hydrothermal vents (Kaye *et al.*, 2004). Phylogenetic analysis using the 16S and 23S rRNA gene sequences and phenotypic studies demonstrated that this genus is very heterogeneous (Arahal *et al.*, 2002; Mata *et al.*, 2002). One distinct group is represented by a single species, *Halomonas marisflavi* (Yoon *et al.*, 2001). To date, there have been no reports of the occurrence of members of this genus being associated with mangroves that thrive on saline habitats. *Avicennia germinans* is a type of mangrove that has developed mechanisms to tolerate high salt concentrations

in unstable substrates, to obtain oxygen in almost anoxic sediments and to reproduce in harsh areas. One of these mechanisms consists of the secretion of salt crystals on the leaves when the concentration is in excess of that normally present in the vascular system (Lugo & Snedaker, 1975). In this study, we determined the taxonomic position of strain MW2a^T, which was isolated from the surface of leaves of the black mangrove *A. germinans* during a microbial diversity study. As a result of our studies, the name *Halomonas avicenniae* sp. nov. is proposed for this novel micro-organism.

Strain MW2a^T was isolated from the surface of leaves of *A. germinans*. For isolation, a total of 40 leaves were collected randomly from four trees growing near the solar salterns of Cabo Rojo, Puerto Rico. The leaves were placed in sterile bags (Whirl Pak) containing 1 % phosphate buffer at pH 7 with 15 % (w/v) NaCl and mixed. This procedure allowed suspension of the micro-organisms present in the salt crystals on the surface of the leaves. Ten millilitres of the suspension was then used to generate serial dilutions. The dilutions were poured into agar plates containing Seghal–Gibbons (SG) medium at 15 % NaCl (Seghal & Gibbons, 1960). The inoculated plates were incubated at 30 °C. After

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MW2a^T is DQ888315.

3 days of incubation, colonies were selected and purified by using the quadrant streak plate method. Pure cultures were transferred to SG medium for further analysis.

The morphology of the cells was examined by using the Nomarsky technique and scanning electron microscopy with cells at the exponential phase of growth under optimal conditions. Electron microscopy was performed as described previously (Díaz-Muñoz & Montalvo-Rodríguez, 2005). Gram stain was performed using both heat-fixed smears and smears fixed in 5 % acetic acid (Dussault, 1955). Macroscopic properties were determined using the classical characterization of colony appearance. Optimal conditions for growth were determined by growing strain MW2a^T in SG broth supplemented with 0, 5, 10, 15, 20, 25 and 30 % (w/v) NaCl at temperatures of 20, 25, 30, 35 and 40 °C. The pH range for growth was tested using SG medium containing 5 % NaCl and adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, with the addition of the appropriate buffering capacity to each medium as described previously (Montalvo-Rodríguez *et al.*, 2000). The cells were cultivated with constant agitation (150 r.p.m.) and growth was monitored by measuring the absorbance at 600 nm. Cells of strain MW2a^T are Gram-negative, non-spore-forming, motile short rods or ovals. At the exponential phase of growth in SG medium with 5 % NaCl at 30 °C, cells were 2–2.6 µm long and 1–2 µm wide. On SG medium with 5 % NaCl, colonies were orange, smooth, circular/slightly irregular and convex, with an entire margin. Strain MW2a^T grew at NaCl concentrations in the range 0–25 % (w/v) in SG medium. Growth did not occur at concentrations of NaCl greater than 25 % (w/v). The temperature range for growth was 12–40 °C and the pH range was 5.0–9.0. Optimal growth occurred in SG medium with 5 % (w/v) NaCl at 30–35 °C and pH 7.0–8.0. This combination of conditions produced the shortest generation time (1.42 h).

All of the biochemical tests were carried out with 5 % NaCl and at 30 °C, unless stated otherwise. Catalase activity was determined by addition of a 1 % (w/v) H₂O₂ solution to colonies on SG agar medium. Oxidase activity was tested using the Dry Slide (Difco) biochemical test (Montalvo-Rodríguez *et al.*, 1998). Hydrolysis of starch, Tween 80 and aesculin was determined as described by Cowan & Steel (1965), with the addition of basal salts [5 % NaCl, 2 % MgSO₄·7H₂O and 0.2 % KCl (w/v)] to the medium. Hydrolysis of gelatin and production of urease were determined according to Cowan & Steel (1965). Citrate utilization was determined on Simmons' citrate medium (Simmons, 1926) supplemented with basal salts. Acid production from carbohydrates was determined using a phenol red base supplemented with 0.7 % of the carbohydrate tested and basal salts. Motility was determined using SIM medium, supplemented with basal salts. Production of H₂S was determined using the API 20E system, as described previously (Yoon *et al.*, 2001). Growth under anaerobic conditions was determined by incubating strain MW2a^T in an anaerobic chamber in SG medium with

5 % NaCl. Tests for sugar fermentation and enzymes (qualitative) were carried out using API 20 NE and API ID 32E (bioMérieux), according to the manufacturer's instructions, with the inoculated fluid at 5 % NaCl and incubation at 30 °C. Nutritional features were determined using Koser medium (Koser, 1923), as modified by Ventosa *et al.* (1982), which contains the basal salts described above and (% w/v): KNO₃, 0.1; (NH₄)₂HPO₄, 0.1; and KH₂PO₄, 0.05. The filter-sterilized substrate was added to this medium at a final concentration of 0.1 % (w/v), with the exception of carbohydrates, which were used at a final concentration of 0.2 % (w/v). Strain MW2a^T was able to use L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose and glycerol as carbon and energy sources. Antibiotic susceptibility was determined according to the conventional Kirby–Bauer method (Bauer *et al.*, 1966). The phenotypic characteristics of strain MW2a^T are summarized and compared with those of the type strains of related *Halomonas* species in Table 1 and in the species description.

For the characterization and sequencing of the 16S rRNA gene, strain MW2a^T was grown in SG medium with 5 % NaCl and incubated at 30 °C. Genomic DNA was extracted from cells in lysis buffer followed by phenol/chloroform extraction and ethanol precipitation. The DNA was used as a template for subsequent PCR amplification. Reaction conditions and amplification protocols used were as described elsewhere (Hezayen *et al.*, 2002). The resulting amplicon was purified using a MinElute PCR purification kit, according to the manufacturer's instructions. Purified PCR products were sequenced at a DNA sequencing facility (Macrogen, Korea). Distance analysis of the resulting DNA sequence was performed using the PHYLIP program (version 3.63; Felsenstein, 1993). Multiple-sequence alignment was performed by using the CLUSTAL W program with 16S rRNA gene sequences of closely related organisms (as determined by BLAST analysis) (Maidak *et al.*, 1996). The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment. SEQBOOT was used to generate 100 bootstrapped datasets. Distance matrices were calculated with DNADIST. One hundred trees were inferred by using the neighbour-joining software. Any bias introduced by the order of sequence addition was minimized by randomizing the input order. The program CONSENSE was used to determine the most frequent branching order. The final tree was drawn using TREEVIEW (Page, 1996).

In silico analysis of strain MW2a^T using the BLAST program (National Center for Biotechnology) with an almost complete 16S rRNA gene sequence (1433 bp) revealed that this strain was related to the genus *Halomonas*, with the closest sequence similarity to *H. marisflavi* KCCM 80003^T (98.6 %), a species that represents one of the various groups of this genus. 16S rRNA gene sequence similarities between strain MW2a^T and *Halomonas elongata* ATCC 33173^T, *Halomonas salina* DSM 5928^T and *Halomonas halodurans* DSM 5160^T were 92.8, 92.8 and 91.7 %, respectively. Strain

Table 1. Phenotypic characteristics that distinguish strain MW2a^T from the type strains of other species of the genus *Halomonas*

Strains: 1, MW2a^T; 2, *H. marisflavi* KCCM 80003^T; 3, *H. elongata* ATCC 33173^T; 4, *H. salina* ATCC 49509^T; 5, *H. halodurans* DSM 5160^T; 6, *Halomonas halmophila* DSM 5349^T; 7, *Halomonas marina* DSM 4741^T; 8, *Halomonas eurihalina* DSM 5720^T. Data from Arahall *et al.* (2001, 2002), Baumann *et al.* (1983), Franzmann *et al.* (1988), Hebert & Vreeland (1987), Mata *et al.* (2002), Mellado *et al.* (1995), Yoon *et al.* (2001) and this study. +, Positive; –, negative; +/–, weak reaction; ND, no data.

Characteristic	1	2	3	4	5	6	7	8
Cell morphology	Rods or ovals	Rods or ovals	Short rod	Short rod	Rod	Short rod	Rod	Rod
Pigmentation	Orange	Yellow	White	Cream	None	Cream	Cream	Cream
β-Galactosidase	+	–	+	+	–	–	+	+
Motility	+	+	+	–	+	+	+	–
Exopolysaccharide production	–	–	–	–	–	–	+	+
Oxidase	–	–	–	+	+	+	–	–
Facultative anaerobe	–	+	+	–	–	–	–	–
Acid production from:								
D-Glucose	+	+	+	–	+	+	+	–
Sucrose	+	+	+	–	+	+	–	–
Maltose	+/–	+	+	–	–	+	–	–
Mannitol	+	+/–	+	–	+	+	+	–
Xylose	+	+	–	ND	–	–	–	+
D-Fructose	+	+	–	–	+	+	+	–
Lactose	–	+	+	–	–	+	–	–
L-Arabinose	+	+	+	–	–	+	–	–
Nitrate reduction	–	–	+	+	–	–	–	+
Simmons' citrate	+	+	+	+	+	–	–	+
Hydrolysis of:								
Gelatin	+	+	–	–	–	–	–	+
Urea	–	–	+	+	+	–	+	+
Tween 80	–	–	–	–	+	–	+	+
Aesculin	+	+	–	–	+	–	–	+
DNA G+C content (mol%)	61.5	59.0	60.5	64.2	63.2	63.0	62.8	65.7

MW2a^T also showed low sequence similarity to *Chromohalobacter salexigens* DSM 3043^T (93.7 %). Phylogenetic analysis using the neighbour-joining algorithm revealed that strain MW2a^T was closely related to *H. marisflavi*, forming a branch in this cluster with a bootstrap value of 100 % (Fig. 1). The phylogenetic position of strain MW2a^T was also confirmed in a tree generated using the maximum-parsimony algorithm.

Fatty acids were analysed by using GC at the Belgian Co-ordinated Collections of Microorganisms, Laboratory of Microbiology of Gent (BCCM/LMG), Gent, Belgium (Kämpfer & Kroppenstedt, 1996; Miller, 1982). Cells were cultured on SW10 medium [8.1 % NaCl, 0.7 % MgCl₂, 0.96 % MgSO₄, 0.036 % CaCl₂, 0.2 % KCl, 0.006 % NaHCO₃, 0.0026 % NaBr, 0.5 % yeast extract, w/v (Difco)] (Ventosa *et al.*, 1982) for 24 h at pH 7.0 and 30 °C. The predominant fatty acids of strain MW2a^T were C_{16:0}, C_{19:0} cyclo ω8c, C_{18:1}ω7c and C_{12:0} 3-OH. This composition is very similar to those described for other *Halomonas* species (Table 2).

The G+C content of genomic DNA was determined from the mid-point value (*T*_m) of the thermal denaturation

profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979), as described in detail by Ventosa *et al.* (1999). The DNA G+C content of strain MW2a^T was 61.5 mol%, which is in the range for species belonging to the genus *Halomonas* (Arahall & Ventosa, 2005; Franzmann *et al.*, 1988).

DNA–DNA hybridization studies were performed by using the competition procedure of the membrane method (Johnson, 1994), as described in detail by Mormile *et al.* (1999). The hybridization temperature used was 57.3 °C, which is within the limit of validity for the filter method (De Ley & Tjtgat, 1970) and the percentage DNA–DNA relatedness was calculated according to Johnson (1994). The experiments were carried out in triplicate. The DNA–DNA relatedness between strain MW2a^T and *H. marisflavi* KCCM 80003^T was 42 %, whereas the relatedness with *H. elongata* ATCC 33173^T and *H. salina* DSM 5928^T was 23 and 13 %, respectively. These levels of DNA–DNA relatedness are low enough to classify strain MW2a^T as representing a genotypically distinct species within the genus *Halomonas* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Overall, the phenotypic, phylogenetic, chemotaxonomic and genotypic results presented in this study demonstrate

Fig. 1. Neighbour-joining distance tree based on the 16S rRNA gene sequences of strain MW2a^T and closely related species. Bar, 1 substitution per 100 nucleotides. Bootstrap values greater than 40 % are shown. *Zymobacter palmae* DSM 10491^T was used as the outgroup.

that strain MW2a^T represents a novel species within the genus *Halomonas*, for which the name *Halomonas avicenniae* sp. nov. is proposed.

Description of *Halomonas avicenniae* sp. nov.

Halomonas avicenniae [a.vi.cen.ni'ae. N.L. n. *Avicennia* scientific name of a botanical genus which encompasses *Avicennia germinans* (the black mangrove); N.L. gen. n. *avicenniae* of *Avicennia*, isolated from leaves of *Avicennia germinans*].

Cells are Gram-negative, motile, non-spore-forming short rods or oval-shaped, 2–2.6 µm long and 1–2 µm wide. Colonies are orange, smooth, circular/slightly irregular and convex, with an entire margin. Moderately halophilic, growing at NaCl concentrations in the range 0–25 % (w/v) with optimum growth at 5 % (w/v) NaCl. Growth occurs at 12–40 °C (optimum, 30–35 °C) and pH 5.0–9.0 (optimum, pH 7.0–8.0). Strictly aerobic. Catalase-positive and gelatin and aesculin are hydrolysed. Production of indole and H₂S, hydrolysis of starch, DNA and Tween 80, and urease and

oxidase are negative. Nitrate is not reduced. Acid is produced from D-glucose, sucrose, xylose, D-fructose and L-arabinose and weakly produced from maltose. Negative for arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase. β-Galactosidase, β-glucosidase and α-glucosidase activities are present. Negative for β-glucuronidase, N-acetyl-β-glucosaminidase, α-galactosidase, α-maltosidase and L-aspartic acid arylamidase. L-Arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose and glycerol are used as carbon and energy sources. Shows intermediate resistance to erythromycin (15 µg). Resistant to penicillin (10 units), bacitracin (10 units), cephalothin (30 µg), tetracycline (30 µg), rifampicin (5 µg), streptomycin (10 µg), neomycin (30 µg), kanamycin (30 µg), vancomycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg) and chloramphenicol (30 µg). Major fatty acids are C_{16:0}, C_{19:0} cyclo ω8c, C_{18:1} ω7c and C_{12:0} 3-OH. The DNA G + C content of the type strain is 61.5 mol%.

The type strain is MW2a^T (=CECT 7193^T=CCM 7396^T), which was isolated from the leaf surface of *Avicennia germinans* (black mangrove).

Table 2. Cellular fatty acid compositions (%) of strain MW2a^T, the closely related species *H. marisflavi* and the type species of the genus, *H. elongata*

The strains were grown on SW10 medium for 24 h at pH 7.0 and 30 °C. –, Not detected.

Fatty acid	Strain MW2a ^T	<i>H. marisflavi</i> KCCM 80003 ^T	<i>H. elongata</i> ATCC 33173 ^T
C _{10:0}	1.0	2.4	4.7
C _{10:0} 3-OH	–	–	3.6
C _{12:0}	0.8	–	4.7
C _{12:0} 2-OH	3.2	3.2	–
C _{12:0} 3-OH	11.3	11.0	15.3
C _{14:0}	0.4	0.5	–
C _{16:1} ω7c*	2.6	3.5	4.5
C _{16:0}	37.4	33.4	25.6
C _{16:0} 3-OH	–	–	1.0
C _{17:0} cyclo	3.2	2.6	1.6
C _{17:0}	–	–	0.5
C _{18:1} ω7c	16.6	25.7	25.6
C _{18:0}	1.2	2.1	0.6
C _{19:0} cyclo ω8c	22.3	15.7	10.6
C _{19:0} 10-methyl	–	–	0.5

*Included in summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH).

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