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***Halobacillus mangrovi* sp. nov., a moderately halophilic bacterium isolated from the
black mangrove *Avicennia germinans***

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Footnote: The GenBank accession number for the 16S rRNA gene sequence of strain
MS10^T is DQ888316.

24 **Summary**

25 A moderately halophilic spore-forming short rod was isolated from the surface of leaves
26 of the black mangrove *Avicennia germinans* during a microbial diversity study. This
27 Gram-positive strain was designated MS10^T and was subjected to a polyphasic taxonomic
28 study. Strain MS10^T is moderately halophilic growing at NaCl concentrations in the
29 range of 5-20 % (w/v) with an optimum of 10% (w/v) NaCl. Growth occurs at
30 temperatures from 10 to 50 °C (optimal 33-35°C) and pH range from 6.0 to 9.0 (optimal
31 7.0). Phylogenetic analysis based on the 16S rRNA gene sequence comparison revealed
32 that it is within the *Halobacillus* branch, and the most closely relative is *Halobacillus*
33 *dabanensis* JCM 12772^T having a 99.2% 16S rRNA gene sequence similarity. Its DNA
34 G+C content is 45.7 mol%, and the cell wall peptidoglycan is of the L-Orn-D-Asp type,
35 which are characteristics for *Halobacillus* species. DNA-DNA hybridization with
36 *Halobacillus dabanensis* JCM 12772^T showed a 29% relatedness and percentages below
37 70% were also obtained with respect to other related *Halobacillus* species. Its major fatty
38 acids are iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{14:0} and iso-C_{15:0}. Overall, the phenotypic,
39 genotypic and phylogenetic results presented in this study demonstrate that strain MS10^T
40 should be placed as a new species within the genus *Halobacillus*. The name *Halobacillus*
41 *mangrovi* sp. nov. is proposed, with strain MS10^T (=CECT 7206^T = CCM 7397^T) as the
42 type strain.

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45 The genus *Halobacillus* was first described by Spring *et al.* (1996) in order to
46 accommodate the species *Sporosarcina halophila* together with two new species
47 designated as *Halobacillus litoralis* and *Halobacillus trueperi*. Currently this genus
48 comprises nine validly published species names, *Halobacillus halophilus* (type species),
49 *Halobacillus litoralis*, *Halobacillus trueperi* (Spring *et al.*, 1996), *Halobacillus salinus*
50 (Yoon *et al.*, 2003), *Halobacillus karajensis* (Amoozegar *et al.*, 2003), *Halobacillus*
51 *locisalis* (Yoon *et al.*, 2004), *Halobacillus aidingensis*, *Halobacillus dabanensis* (Liu *et*
52 *al.*, 2005) and *Halobacillus yeomjeoni* (Yoon *et al.*, 2005). Besides the ability to produce
53 ellipsoidal or spherical endospores at central or subterminal position, one of the main
54 features of the genus *Halobacillus* is the cell-wall peptidoglycan type based on L-Orn-D-
55 Asp, which is different from the meso-diaminopimelic acid or L-lysine type cell-wall
56 peptidoglycan present on related genera (Shida *et al.*, 1997; Yoon *et al.*, 2003). So far,
57 there are no reports about the occurrence of members of this genus associated to
58 mangroves that thrive on saline habitats.

59 *Avicennia germinans* (black mangrove) is a type of mangrove that has developed
60 mechanisms to tolerate high salt concentrations in unstable substrates, to obtain oxygen
61 in almost anoxic sediments, and to reproduce in harsh areas. One of the mechanisms
62 consists in secreting salt crystals on the leaves when the concentration is in excess of that
63 normally present in the vascular system (Lugo & Snedaker, 1975). In this study, we have
64 determined the taxonomic position of strain MS10^T who was isolated from the surface of
65 leaves of the black mangrove *A. germinans* during a microbial diversity study. On the
66 basis of the results presented below, the name *Halobacillus mangrovi* sp. nov. is
67 proposed for this novel microorganism.

Strain MS10^T was isolated from the surface of the black mangrove leaves. For isolation, leaves from *Avicennia germinans* trees growing near the solar salterns of Cabo Rojo, Puerto Rico were obtained and printed against agar plates containing Seghal-Gibbons medium (15% NaCl, 2% MgSO₄ · 7H₂O, 0.2% KCl, 0.3% trisodium citrate, 0.002% FeCl₂ ; 0.75% casaminoacids, 1% yeast extract [w/v], pH 7.5-7.8) (Seghal & Gibbons, 1960) (SG). This procedure allowed the transfer of the microorganisms present in the salt crystals and the surface of the leaves to the growth medium. Plates were incubated at 30°C. After 3 days of incubation, the leaves were removed aseptically and colonies were then selected and purified by the quadrant streak plate method. Pure cultures were transferred into SG medium for further biochemical and molecular analysis.

The morphology of cells was examined by Nomarsky technique and scanning electron microscopy (SEM) at logarithmic phase of growth under optimal conditions. Electron microscopy procedures were performed as previously described (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 documented using the classical characterization of colony appearance. Optimal conditions for growth were determined by growing MS10^T in SG broth supplemented with 0, 5, 10, 15, 20, 25 and 30% (w/v) NaCl at temperatures of 10, 15, 20, 25, 30, 33, 35, 40 and 50°C, respectively. The pH range for the isolate was tested in SG medium with 10% NaCl adjusted to the following pH values: 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 previously described (Montalvo-Rodríguez *et al.*, 2000). Cells were cultivated in SG medium with constant

agitation (150 rpm) and growth was monitored by measuring absorbance at 600 nm. Growth curves testing the conditions mentioned above were performed in duplicate and the average generation time was calculated. Strain MS10^T is a Gram-positive, spore-forming short rod. Cells are 1.0-2.0 µm long and 0.5-1.0 µm wide at logarithmic phase of growth in SG medium with 10% NaCl at 30°C. Cells are motile. On SG medium with 10% NaCl colonies are cream-brown, entire, circular, convex and elevated. Strain MS10^T grows at NaCl concentrations in the range of 5-20% (w/v) in SG medium. It does not grow above 20% (w/v) of NaCl. Growth is observed at temperatures from 10-50°C and pH range from 6.0-9.0. The optimal growth conditions occur in SG medium at 10% (w/v) NaCl at 33-35°C and a pH of 7.0. This combination of conditions produced the shortest generation time (0.52 hours).

All biochemical tests were carried out at 10% NaCl and 30°C, unless it is stated otherwise. Catalase activity was determined by adding a 1% (w/v) H₂O₂ solution to colonies on SG agar medium. Oxidase test was performed using the Dry Slide (Difco) biochemical test (Montalvo-Rodríguez *et al*; 1998). Hydrolysis of starch, Tween 80, and aesculin were determined as described by Cowan & Steel (1965) with the addition of Basal Salts (10% 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 Simmon's Citrate medium (Simmons, 1926) supplemented with Basal Salts. Acid production from carbohydrates was determined using phenol red base supplemented with 0.7% of the carbohydrate and Basal Salts. Motility was determined using SIM medium, supplemented with Basal Salts.

Growth under anaerobic conditions was determined by incubating strain MS10^T in an anaerobic chamber in SG medium with 10% (w/v) NaCl. Tests for enzymes activities (qualitative) were carried out using API ID32E (bioMérieux) inoculated according to the manufacturer's instructions using the inoculated fluid at 10% of NaCl and incubated at 35°C. Nutritional features were determined using the Koser medium (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). Antibiotic susceptibility was determined according to the conventional Kirby-Bauer method (Bauer *et al.*, 1966). The phenotypic characteristics of strain MS10^T are summarized and compared to the type strains of related *Halobacillus* species in Table 1 and also in the species description.

For the characterization and sequencing of the 16S rRNA gene, strain MS10^T was grown in SG medium at 10% (w/v) NaCl and incubated at 30°C. Genomic DNA was extracted from cells in lysis buffer followed by phenol/chloroform extraction and ethanol precipitation. This DNA was used as template for subsequent PCR amplification. Reaction conditions and amplification protocols were performed as described elsewhere (Hezayen *et al.*, 2002). The resulting amplicon was purified using the MinElute PCR purification kit according to the manufacturer instructions (QIAGEN). Purified PCR products were sent to a DNA sequencing facility (Macrogen in Korea). Distance analysis of the resulting DNA sequences were performed using the PHYLIP program (version

3.63) (Felsenstein, 1993). A multiple-sequence alignment was made 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 data sets. Distance matrices were calculated with DNADIST. One hundred trees were inferred by using the NEIGHBOR software. Any bias introduced by the order of sequence addition was minimized by randomizing the input order. CONSENSE was used to determine the most frequent branching order. The final tree was drawn using TREEVIEW (Page, 1996).

In silico analysis using the BLAST program (National Center for Biotechnology) of strain MS10^T using an almost complete 16S rRNA gene sequence (1460 bp) revealed that this strain was related to *Halobacillus*, having the closest sequence similarity to *Halobacillus dabanensis* JCM 12772^T (99.2%). Additional 16S rRNA gene sequence similarities between MS10^T and *H. aidingensis* JCM 12771^T, *H. yeomjeoni* DSM 17110^T, *H. karajensis* DSM 14948^T, *H. locisalis* DSM 16468^T, *H. trueperi* CCM 4593^T, and *H. litoralis* DSM 10405^T were 97.9, 98.6, 98.5, 98.2, 98.7, and 98.8 %, respectively.

Phylogenetic analysis using the neighbour joining algorithm revealed that strain MS10^T belonged to the *Halobacillus* cluster (Fig. 1). Similar results were obtained for strain MS10^T when the maximum-parsimony and maximum-likelihood algorithms were used.

Fatty acids were analysed using the MIDI system (Microbial Identification System). Cells were cultured on Marine Agar supplemented with 8.1% NaCl for 24-48 h at 35°C and the cellular fatty acids were analysed using GC at the Belgian Co-ordinated Collections of Microorganisms, Laboratory of Microbiology of Gent (BCCM/LMG), Gent, Belgium (Miller, 1982; Kämpfer & Kroppenstedt, 1996). Cell wall composition analysis was carried out by the Identification Service of DSMZ Braunschweig (Germany) by using the methods described by Schleifer and Kandler (1972). The predominant fatty acids of strain MS10^T were iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{14:0} and iso-C_{15:0} (Table 2). The cell wall peptidoglycan is of the L-Orn-D-Asp type.

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 previously described in detail by Ventosa et al. (1999). The DNA G+C content of strain MS10^T is 45.7 mol %. This value is closely related to those reported for other *Halobacillus* species (Spring et al., 1996; Yoon et al., 2003, 2004, 2005; Amoozegar et al., 2003; Liu et al., 2005).

DNA-DNA hybridization studies were performed by the competition procedure of the membrane method (Johnson, 1994), described in detail by Mormile et al. (1999). The hybridization temperature was 48.7°C, which is within the limit of validity for the filter method (De Ley & Titjgat, 1970) and the percentage of hybridization was calculated according to Johnson (1994). The experiments were carried out in triplicate. The percentage of DNA-DNA hybridization between strain MS10^T and *H. dabanensis* JCM 12772^T was 29%. Additional percentages of DNA-DNA hybridization between strain

MS10^T and *H. locisalis* DSM 16468^T, *H. trueperi* CCM 4593^T, *H. aidingensis* JCM 12771^T, *H. litoralis* DSM 10405^T, *H. karajensis* DSM 14948^T, *H. yeomjeoni* DSM 17110^T, *H. salinus* JMC 11546^T were 44, 32, 49, 36, 51, 44, and 36% respectively. These levels of DNA-DNA hybridization are low enough to classify strain MS10^T as a genotypically distinct species within the genus *Halobacillus* (Wayne *et al.*, 1987).

There are notable phenotypic differences between strain MS10^T and *H. dabanensis* JCM 12772^T, its closest phylogenetical relative. Strain MS10^T is unable to produce acid from the carbohydrates tested in contrast to *H. dabanensis* which has this property. Strain MS10^T has the ability to hydrolyze gelatin, a property that is absent in *H. dabanensis*. The biochemical properties of strain MS10^T are very similar to *H. halophilus*, however there are differences in cell shape and colony pigmentation (Table 1). Also, differences in fatty acid composition together with 16S rRNA gene sequence similarities suggest that they can be considered as two different species.

Overall, the phenotypic, genotypic and phylogenetic results presented in this study demonstrate that strain MS10^T should be placed as a new species within the genus *Halobacillus*. The name *Halobacillus mangrovi* sp. nov. is proposed for this novel species.

Description of *Halobacillus mangrovi* sp. nov.

Halobacillus mangrovi (man.gro'vi. N.L. n. mangrovum, mangrove; N.L. gen. n. mangrovi, of a mangrove).

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206 Gram-positive spore-forming short rod, 1-2 μm long and 0.5-1 μm wide. Cells are motile.
207 Colonies are cream-brown, entire, circular, convex and elevated. Moderately halophilic
208 growing at NaCl concentrations in the range of 5-20% (w/v) with an optimum of 10%
209 (w/v) NaCl. Growth occurs at temperatures from 10-50°C (optimal 33-35 °C) and pH
210 range from 6.0-9.0 (optimal 7.0). Strictly aerobic. Catalase and oxidase are produced.
211 Negative results for aesculin, indole, H₂S production, methyl red, Voges-Proskauer,
212 urease and Tween 80. Gelatin, casein and starch were hydrolyzed. Nitrate was not
213 reduced. No acid production from glucose, sucrose, maltose, mannitol, xylose, fructose,
214 mannose, D-melibiose, L-rhamnose, D-sorbitol, D-galactose, inositol, sorbitol, adonitol,
215 L-arabitol, D-arabitol, L-arabinose, palatinose, trehalose, cellobiose, galacturonic acid,
216 potassium 5-ketogluconate, and sodium pyruvate. Starch, D-glucose, D-sorbitol and
217 succinate are used as carbon and energy sources. Negative for arginine dihydrolase,
218 ornithine decarboxylase and lipase (C14). β -galactosidase, α -glucosidase, α -galactosidase
219 and α -mannosidase activities are present. Negative for lysine decarboxilase, β -
220 glucosidase and β -glucuronidase, N-acetyl- β -glucosaminidase and L-aspartic acid
221 arylamidase. Resistant (μg per disc) to polymixin (300 units), neomycin (30),
222 streptomycin (10), sulfamethoxazole/trimethoprim (23.75/1.25). Intermediate resistance
223 to (μg per disc): erythromycin (15), nalidixic acid (30) and tetracycline (30). Sensitive
224 (μg per disc) to kanamycin (30), rifampicin (5), bacitracin (10 units), vancomycin (30),
225 novobiocin (30), cephalothin (30), penicillin (10 units), and chloranphenicol (30). The
226 cell wall peptidoglycan is of the L-Orn-D-Asp type. The major fatty acids are iso-C_{16:0},
227 anteiso-C_{15:0}, iso-C_{14:0} and iso-C_{15:0}. The DNA G + C content was 45.7 mol%. Isolated

from the leaf surface of *Avicennia germinans* (black mangrove). The type strain is MS10^T which has been deposited at the CECT 7206^T and the CCM 7397^T.

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Table 1. Characteristics that distinguish strain MS10^T from other type strains of related species of the genus *Halobacillus*.

Symbols: +, Positive; -, negative.

Characteristic	Strain MS10 ^T	<i>Halobacillus dabanensis</i> ¹	<i>Halobacillus aidingensis</i> ¹	<i>Halobacillus karajensis</i> ²	<i>Halobacillus halophilus</i> ³	<i>Halobacillus litoralis</i> ³
Cell morphology	rods	rods	clubs	rods	cocci or oval	rods
Motility	+	+	+	-	+	+
Pigmentation	cream	orange	orange	white	Orange	orange
Acid production from:						
D-Fructose	-	+	+	+	-	+
D-Glucose	-	+	+	+	-	+
D-Mannitol	-	+	+	+	-	+
Maltose	-	+	+	+	-	+
Sucrose	-	+	+	-	-	+
D-Xylose	-	+	-	-	-	+
Hydrolysis of:						
Aesculin	-	-	-	+	-	-
Casein	+	+	+	+	+	-
Gelatin	+	-	+	+	+	+
Starch	+	+	+	+	+	-
G+C content (mol %)	45.7	41.4	42.2	41.3	40.1-40.9	42.0

¹Data from Liu *et al.* (2005), ²Data from Amoozegar *et al.* (2003), ³Data from Spring *et al.* (1996).

Table 2. Cellular fatty acid composition (%) of strain MS10^T and closely related *Hallobacillus* species.

Species: 1, Strain MS10^T (data from this study); 2, *H. dabanensis* (data from this study); 3, *H. aidingensis* (data from this study), 4, *H. litoralis* (data from Yoon *et al.*, 2003); 5, *H. trueperi* (data from Yoon *et al.*, 2003); 6, *H. salinus* (data from Yoon *et al.*, 2003); 7, *H. localis* (data from Yoon *et al.*, 2003); 8, *H. halophilus* (data from Yoon *et al.*, 2003). All strains were grown on marine agar supplemented with 8.1 % NaCl (final NaCl concentration of 10%) according to Yoon *et al.* (2003). Values are percentages of total fatty acids. ND, not detected.

Fatty acid	1	2	3	4	5	6	7	8
C _{15:0}	ND	ND	ND	1.1	ND	1.6	1.1	1.5
C _{16:0}	1.5	2.3	1.4	0.6	0.9	1.0	0.9	0.9
anteiso-C _{13:0}	ND	ND	ND	0.4	ND	ND	0.5	0.4
anteiso- C _{15:0}	22.8	49.9	21.7	57.0	25.3	31.7	42.0	47.3
anteiso- C _{17:0}	4.5	19.3	6.5	8.2	6.5	6.2	13.0	11.9
iso- C _{14:0}	17.1	1.9	19.8	6.4	21.7	9.4	11.2	12.2
iso- C _{15:0}	14.8	7.5	10.8	15.8	7.7	26.3	8.4	7.5
iso- C _{16:0}	32.2	14.2	31.7	5.4	31.5	15.7	15.9	15.2
iso- C _{17:0}	4.4	4.4	5.2	1.5	2.1	4.2	1.4	1.2
C _{16:1} ω7c alcohol	2.6	0.5	3.0	2.0	3.8	4.0	5.7	1.8
Iso-C _{17:1} ω10c	ND	ND	ND	0.2	ND	ND	ND	ND

411 Legend to the Figure:

412

413 **Figure 1.** Neighbour-joining distance tree using the 16Sr RNA sequences of strain

414 MS10^T and closely related species. Bar represents 1 substitution per 100 nucleotides.

415 Bootstrap values higher than 40% are shown. *Gracilibacillus dipsosauri* DSM 11125^T

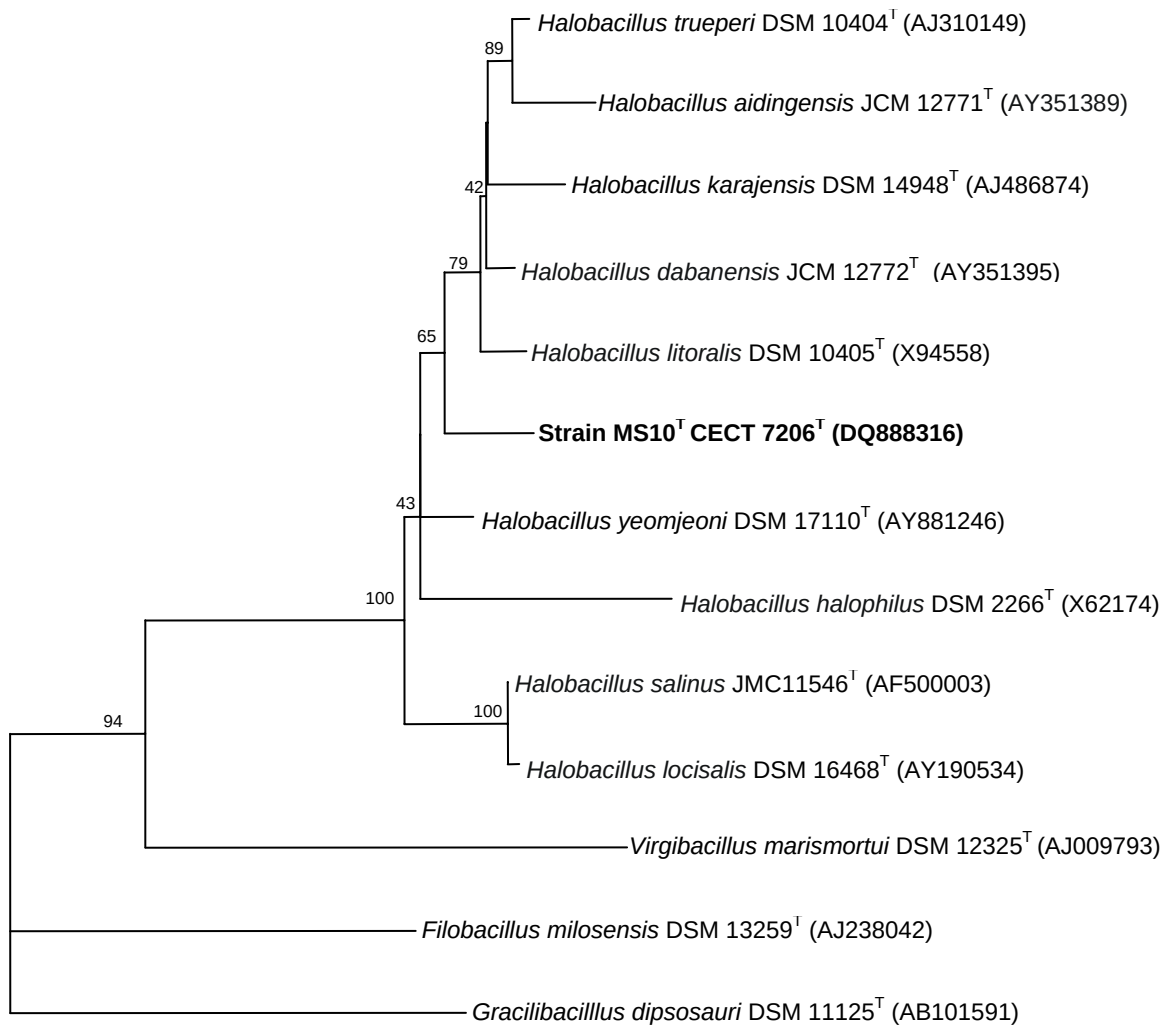
416 was used as the outgroup.

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