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

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A moderately halophilic, spore-forming, Gram-positive, short-rod-shaped bacterium, designated strain MS10^T, was isolated from the surface of leaves of the black mangrove *Avicennia germinans* and was subjected to a polyphasic taxonomic study. Strain MS10^T was able to grow at NaCl concentrations in the range 5–20 % (w/v) with optimum growth at 10 % (w/v) NaCl. Growth occurred at temperatures of 10–50 °C (optimal growth at 33–35 °C) and pH 6.0–9.0 (optimal growth at pH 7.0). Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain MS10^T fell within the branch encompassing members of the genus *Halobacillus* and was most closely related to *Halobacillus dabanensis* JCM 12772^T (99.2 % 16S rRNA gene sequence similarity). The DNA G+C content of strain MS10^T was 45.7 mol%, the major respiratory isoprenoid quinone was MK-7 and the cell-wall peptidoglycan was of the L-Orn–D-Asp type, characteristics consistent with its affiliation to the genus *Halobacillus*. Strain MS10^T showed a level of DNA–DNA hybridization with *H. dabanensis* JCM 12772^T of 29 % and levels below 70 % were also obtained with respect to other recognized members of the genus *Halobacillus*. The major fatty acids of strain MS10^T were iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{14:0} and iso-C_{15:0}. Overall, the phenotypic, genotypic and phylogenetic results presented in this study demonstrate that strain MS10^T represents a novel species of the genus *Halobacillus*, for which the name *Halobacillus mangrovi* sp. nov. is proposed. The type strain is MS10^T (=CECT 7206^T=CCM 7397^T).

The genus *Halobacillus* was first described by Spring *et al.* (1996) in order to accommodate *Sporosarcina halophila* (Claus *et al.*, 1983) together with two novel species, namely *Halobacillus litoralis* and *Halobacillus trueperi*. At the time of writing, the genus comprises nine recognized species, *Halobacillus halophilus* (type species of the genus), *Halobacillus litoralis*, *Halobacillus trueperi* (Spring *et al.*, 1996), *Halobacillus salinus* (Yoon *et al.*, 2003), *Halobacillus karajensis* (Amoozegar *et al.*, 2003), *Halobacillus locisalis* (Yoon *et al.*, 2004), *Halobacillus aidingensis*, *Halobacillus dabanensis* (Liu *et al.*, 2005) and *Halobacillus yeomjeoni* (Yoon *et al.*, 2005). Besides the ability to produce ellipsoidal or spherical endospores at the central or subterminal position, one of the main features of the genus *Halobacillus* is the cell-wall peptidoglycan type based on L-Orn–D-Asp, in contrast to the *meso*-diaminopimelic

acid or L-lysine type cell-wall peptidoglycan found in related genera (Shida *et al.*, 1997; Yoon *et al.*, 2003). There have been no previous reports of members of the genus *Halobacillus* associated with mangroves that thrive in saline habitats.

Avicennia germinans (black mangrove) is a type of mangrove that has developed mechanisms to tolerate high salt concentrations in unstable substrates, to obtain oxygen from almost anoxic sediments and to reproduce under harsh environmental conditions. One of these mechanisms is the ability to secrete salt crystals on to the surface of its leaves when the salt concentration is in excess of that normally present in the vascular system (Lugo & Snedaker, 1975). In the present study, we determined the taxonomic position of bacterial strain MS10^T, which was isolated from the surface of leaves of the black mangrove during a microbial diversity study. On the basis of the results presented, we show that strain MS10^T represents a novel species of the genus *Halobacillus*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MS10^T is DQ888316.

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

Cell morphology was examined by use of the Nomarsky technique and scanning electron microscopy on cells at the exponential phase of growth under optimal conditions. Electron microscopy procedures were performed as described by Díaz-Muñoz & Montalvo-Rodríguez (2005). Gram staining was investigated by using both heat-fixed smears and smears fixed in 5 % acetic acid (Dussault, 1955). Macroscopic properties were documented by using the classical characterization of colony appearance. Optimal conditions for growth were determined by growing strain MS10^T in SG broth supplemented with 0, 5, 10, 15, 20, 25 and 30 % (w/v) NaCl and at temperatures of 10, 15, 20, 25, 30, 33, 35, 40 and 50 °C. The pH range for growth was tested in SG medium with 10 % NaCl adjusted to pH values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with the addition of the appropriate buffering ability to each medium as described by Montalvo-Rodríguez *et al.* (2000). Cells were cultivated in SG medium with constant agitation (150 r.p.m.) and growth was monitored by measuring absorbance at 600 nm. Growth curves used to test the conditions mentioned above were generated from duplicate experiments and the mean generation time was calculated.

Cells of strain MS10^T were Gram-positive, spore-forming, short rods. Cells were 1.0–2.0 µm long and 0.5–1.0 µm wide at the exponential phase of growth in SG medium with 10 % NaCl at 30 °C. Cells were motile. On SG medium with 10 % NaCl, colonies were cream-brown, entire, circular, convex and elevated. Strain MS10^T grew at NaCl concentrations in the range 5–20 % (w/v) in SG medium. It was not able to grow at NaCl concentrations above 20 % (w/v). Growth was observed at temperatures of 10–50 °C and at pH values of 6.0–9.0. Optimal growth conditions in SG medium were at 10 % (w/v) NaCl, 33–35 °C and pH 7.0. This combination of conditions produced the shortest generation time (0.52 h).

All biochemical tests were carried out at 10 % NaCl and 30 °C unless stated otherwise. Catalase activity was determined by adding a 1 % (w/v) H₂O₂ solution to colonies on SG agar medium. The oxidase test was performed by 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 Simmons' citrate medium (SIM; Simmons, 1926) supplemented with basal salts. Acid production from carbohydrates was determined by using phenol red base supplemented with 0.7 % of the carbohydrate and basal salts. Motility was determined by 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 enzyme activities (qualitative) were carried out by using the API ID32E system (bioMérieux) inoculated according to the manufacturer's instructions, with the inoculated fluid at 10 % NaCl and incubation at 35 °C. Nutritional characteristics were determined by using Koser medium (Koser, 1923) as modified by Ventosa *et al.* (1982), which contains the basal salts described above, together with (w/v) 0.1 % KNO₃, 0.1 % (NH₄)₂HPO₄ and 0.05 % KH₂PO₄. 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 with those of the type strains of related *Halobacillus* species in Table 1 and also in the species description below.

For 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 by Hezayen *et al.* (2002). The resulting amplicon was purified by using the MinElute PCR purification kit according to the manufacturer's instructions (Qiagen). Purified PCR products were sent to a DNA sequencing facility (Macrogen, Republic of Korea). Distance analysis of the resulting DNA sequences was performed by 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 identified by BLAST analysis) (Maidak *et al.*, 1996). 16S rRNA gene sequence similarity values were calculated by pairwise comparison of the sequences within the alignment. The program 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 package. Any bias introduced by the order of sequence addition was minimized by randomizing the input order. The CONSENSE

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

Taxa: 1, strain MS10^T; 2, *H. dabanensis* (data from Liu *et al.*, 2005); 3, *H. aidingensis* (Liu *et al.*, 2005); 4, *H. karajensis* (Amoozegar *et al.*, 2003); 5, *H. halophilus* (Spring *et al.*, 1996); 6, *H. litoralis* (Spring *et al.*, 1996). +, Positive; –, negative.

Characteristic	1	2	3	4	5	6
Cell morphology	Rods	Rods	Clubs	Rods	Cocci or ovals	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	+	+	+	+	+	–
DNA G + C content (mol%)	45.7	41.4	42.2	41.3	40.1–40.9	42.0

program was used to determine the most frequent branching order. The final tree was drawn by using TreeView (Page, 1996).

In silico analysis by using the BLAST program (National Center for Biotechnology) of the almost complete 16S rRNA gene sequence (1460 bp) of strain MS10^T revealed that this strain was related to members of the genus *Halobacillus* and showed highest sequence similarity to *H. dabanensis* JCM 12772^T (99.2%). Strain MS10^T showed levels of 16S rRNA gene sequence similarity of 97.9, 98.6, 98.5, 98.2, 98.7 and 98.8% to *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, respectively. Phylogenetic analysis according to the neighbour-joining algorithm revealed that strain MS10^T belonged to the cluster encompassing members of the genus *Halobacillus* (Fig. 1). Similar results were obtained for strain MS10^T when the maximum-parsimony and maximum-likelihood algorithms were used.

Fatty acids were analysed by 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 by GC at the Belgian Co-ordinated Collections of Micro-organisms, Laboratory of Microbiology (BCCM/LMG), Gent, Belgium (Miller, 1982; Kämpfer & Kroppenstedt, 1996). Cell-wall composition analysis was carried out by the Identification Service of the DSMZ (Braunschweig, Germany) by using the methods described by Schleifer & Kandler (1972). The analysis of respiratory quinones was carried out by the Identification Service of the DSMZ (Braunschweig, Germany). 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 was of L-Orn–D-Asp type and the main isoprenoid quinone was menaquinone 7 (MK-7).

The G + C content of the genomic DNA of strain MS10^T was determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) by 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 was 45.7 mol%. This value is close to those reported for recognized species of the genus *Halobacillus* (Spring *et al.*, 1996; Amoozegar *et al.*, 2003; Yoon *et al.*, 2003, 2004, 2005; Liu *et al.*, 2005).

DNA–DNA hybridization studies were performed according to the competition procedure of the membrane method (Johnson, 1994), as described in detail by Mormile *et al.* (1999). The hybridization temperature was 48.7 °C, which was within the limit of validity for the filter method (De Ley & Tijtgaat, 1970) and the percentage hybridization was calculated according to Johnson (1994). The experiments were carried out in triplicate. The level of DNA–DNA hybridization between strain MS10^T and *H. dabanensis* JCM 12772^T was 29%. Strain MS10^T showed levels of DNA–DNA hybridization to *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 and *H. salinus* JMC 11546^T of 44, 32, 49, 36, 51, 44 and 36%, respectively. These values are sufficiently low to classify strain MS10^T as representing a genotypically distinct species within the genus *Halobacillus* (Wayne *et al.*, 1987).

Notable phenotypic differences were found between strain MS10^T and *H. dabanensis* JCM 12772^T, its closest phylogenetic relative. Strain MS10^T was unable to produce

MS10^T can be considered to represent a species distinct from *H. halophilus*.

Description of *Halobacillus mangrovi* sp. nov.

Gram-positive, spore-forming, short rods, 1–2 µm long and 0.5–1 µm wide. Cells are motile. Colonies are cream-brown, entire, circular, convex and elevated. Moderately halophilic, growing at NaCl concentrations in the range of 5–20 % (w/v) with optimum growth at 10 % (w/v) NaCl. Growth occurs at temperatures of 10–50 °C (optimal 33–35 °C) and pH 6.0–9.0 (optimal pH 7.0). Strictly aerobic. Catalase and oxidase are produced. Negative for aesculin, indole, H₂S production, methyl red, Voges–Proskauer reaction, urease and Tween 80. Gelatin, casein and starch are hydrolysed. Nitrate is not reduced. No acid production from glucose, sucrose, maltose, mannitol, xylose, fructose, mannose, D-melibiose, L-rhamnose, D-sorbitol, D-galactose, inositol, sorbitol, adonitol, L-arabitol, D-arabitol, L-arabinose, palatinose, trehalose, cellobiose, galacturonic acid, potassium 5-ketogluconate or sodium pyruvate. Starch, D-glucose, D-sorbitol and succinate are used as carbon and energy sources. Negative for arginine dihydrolase, ornithine decarboxylase and lipase (C14). Positive for β -galactosidase, α -glucosidase, α -galactosidase and α -mannosidase. Negative for lysine decarboxylase, β -glucosidase,

Taxa: 1, strain MS10^T; 2, *H. dabanensis*; 3, *H. aidingensis*; 4, *H. litoralis*; 5, *H. trueperi*; 6, *H. salinus*; 7, *H. locisalis*; 8, *H. halophilus*. 1–3, Data from the present study; 4–8, 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.

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β -glucuronidase, *N*-acetyl- β -glucosaminidase and L-aspartic acid arylamidase. Resistant to (μ g per disc) polymyxin (300 U), neomycin (30), streptomycin (10) and sulfamethoxazole/trimethoprim (23.75/1.25). Shows intermediate resistance to (μ g per disc) erythromycin (15), nalidixic acid (30) and tetracycline (30). Sensitive to (μ g per disc) kanamycin (30), rifampicin (5), bacitracin (10 U), vancomycin (30), novobiocin (30), cephalothin (30), penicillin (10 U) and chloramphenicol (30). The cell-wall peptidoglycan is of L-Orn-D-Asp type. The major respiratory quinone is MK-7. The major fatty acids are iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{14:0} and iso-C_{15:0}. The DNA G+C content is 45.7 mol%.

The type strain, MS10^T (=CECT 7206^T=CCM 7397^T), was isolated from the leaf surface of *Avicennia germinans* (black mangrove).

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