Bacillus coahuilensis sp. nov., a moderately halophilic species from a desiccation lagoon in the Cuatro Ciénegas Valley in Coahuila, Mexico

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A moderately halophilic, Gram-positive and rod-shaped bacterium, strain m4-4^T, was isolated from a Chihuahuan desert lagoon in Cuatro Ciénegas, Coahuila, Mexico. Strain m4-4^T was found to grow optimally at 30–37 °C, pH 7.0–8.0 and 5 % NaCl and to tolerate from 0.5 % to 10 % NaCl. It was shown to be aerobic. The genomic DNA G + C content was about 37 mol%. Strain m4-4^T exhibited minimal or no growth on most sugars tested. Its major cellular fatty acids were C_{14:0}, C_{16:0} and C_{18:1}. Based on phylogenetic analysis of 16S rRNA and *recA* gene sequences, we observed that the closest relatives of the isolate are moderately halophilic *Bacillus* species, with 16S rRNA gene sequence similarity ranging from 96.6 to 97.4 % (*Bacillus marisflavi*, *Bacillus aquimaris* and *Bacillus vietnamensis*). Additionally, using genomic data it was determined that the type strain contains a total of nine rRNA operons with three slightly different sequences. On the basis of phenotypic and molecular properties, strain m4-4^T represents a novel species within the genus *Bacillus*, for which the name *Bacillus coahuilensis* sp. nov. is proposed, with the type strain m4-4^T (=NRRL B-41737^T =CECT 7197^T).

A number of halophilic and moderately halotolerant, Gram-positive, endospore-forming aquatic isolates in the genus *Bacillus* have been described. A large number of them have been isolated from marine environments (Siefert *et al.*, 2000; Yoon *et al.*, 2003, 2004; Noguchi *et al.*, 2004; Yoon & Oh, 2005; Lee *et al.*, 2006). However, little is known about species inhabiting non-marine, high salinity aquatic environments (Lim *et al.*, 2006; Souza *et al.*,

2006). In this study, the *Bacillus* strain m4-4^T was isolated in August 2003 from a desiccation lagoon in the Churince system, a hydrological system on the western side of the Cuatro Ciénegas Valley in Coahuila, Mexico (26° 50.830'N, 102° 09.335'W).

Strain m4-4^T was analysed using taxonomic and biochemical methods. Two markers were used for phylogenetic reconstruction (16S rRNA and *recA* gene sequences). Studies have shown that more robust results are obtained when additional markers such as housekeeping genes are used, especially in closely related isolates (Stackebrandt *et al.*, 2002; Zeigler, 2003). We determined the phylogenetic affiliation of the isolate m4-4^T by means of 16S rRNA gene phylogeny reconstruction and determined its taxonomic status as a representative of a novel species by using a polyphasic approach. The study also included genomic analysis to determine environmental genome size and diversity of ribosomal operons.

GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences: m4-4^T, EF014450, EF014451 and EF014452; and the *recA* gene sequences: m4-4^T, EF014455; *B. marisflavi* TF-11, EF014457; *B. vietnamensis* NRIC 0530, EF014458; *B. vietnamensis* NRIC 0531^T, EF014459; *B. vietnamensis* NRIC 0532, EF014460; *B. vietnamensis* NRIC 0533, EF014461.

Photomicrographs of *Bacillus coahuilensis* m4-4^T, a phylogenetic tree of the *recA* sequences of m4-4^T and other bacilli and a table showing the fatty acid composition of m4-4^T are available with the online version of this paper.

Strain m4-4^T was isolated from surface water samples that were taken and placed in sterile flasks. These were subjected to a shock temperature of 80 °C for 20 min by means of damp heat (Istock *et al.*, 2001). Subsequently, 1:100 and 1:1000 dilutions were made. Aliquots (100 µl) from each dilution, as well as from the original water samples, were placed in Petri dishes with marine agar 2216 medium (MA; Difco) and incubated at 37 °C for 2 days. Cultures were purified by subculturing on the same medium and maintained at –80 °C in 5 % MA and 15 % (w/v) glycerol.

We studied the cell morphology and sporulation process for strain m4-4^T using phase-contrast microscopy. Cells were negatively stained with 1 % (w/v) malachite green and contrasted with 1 % (w/v) safranin. Characterization of strain m4-4^T included the study of cultural, physiological and biochemical parameters. Single carbon source assimilation tests were performed in MA (4 g l⁻¹), replacing the yeast extract and peptone with the main carbon source. Nitrate reduction was determined as described by Lányi (1987) in the presence and absence of 3 % (w/v) NaCl. Growth at different temperatures was measured on MA between 30 and 50 °C. Urease activity was determined as described previously by Cowan & Steel (1965).

For quantitative analysis of whole-cell fatty acids, strain m4-4^T was cultivated on MA for 2 days at 37 °C. The whole-cell fatty acid composition was determined by using a gas chromatograph (model 5890; Hewlett Packard) equipped with a capillary column HP-5MS (30 m × 0.25 mm i.d.; 0.25 µm film thickness) coupled to a mass spectrometer detector (model 5972; Hewlett Packard). Operating conditions were an injection temperature of

1500 °C for 3 min, increasing at the rate of 40 °C min⁻¹ to a final temperature of 3000 °C, which was maintained for 20 min. Helium was used as carrier gas with a constant flow of 1 ml min⁻¹. Fatty acid methyl esters were identified using the mass spectral library search (NIST MS Data Base) distributed by the National Institute of Standards and Technology (NIST).

A combination of Sanger (Nunally, 2005) and 454 Life Sciences sequencing methods (Margulies *et al.*, 2005) were used to sequence the m4-4^T genome, as described by Alcaraz *et al.* (2008). The genome sequencing found nine ribosomal operons; three of them had slight differences, giving sequences m4-4a, b and c (Fig. 1). The G+C content was obtained directly by genomic analysis.

The 16S rRNA gene was amplified using the 27F and 1492R primers under conditions described previously (Lane, 1991) in 100 µl final volume. The *recA* gene was chosen for sequencing and phylogenetic analysis. Oligonucleotide primers were designed using the *recA* genes of the complete genomes of *Bacillus* strains reported in GenBank. These primers extended from position 28 to 48 (5'-GATCG-TCARGCAGSCYTGWGAT-3') and from position 583 to 602 (5'-TTWCCRACCATAACSCCRAC-3'), yielding a 574 bp product. PCR mixtures (25 µl) were prepared with 1 U *Taq* polymerase (Roche), 2.5 mM MgCl₂, 1 mM dNTPs, 2 µM each *recA* primer and 1 µl DNA (25–100 ng µl⁻¹). The PCR program was one cycle of initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s and extension at 72 °C for 60 s, and a final extension cycle at 72 °C for 5 min. PCR products were purified using a gel extraction

Fig. 1. Unrooted phylogenetic tree using the neighbour-joining method and derived from the analysis of the 16S rRNA gene sequences of strain m4-4^T and other representative *Bacillus* strains. Numbers next to the branches represent bootstrap values expressed as percentages of 2500 replications; only values greater than 70 % are indicated. GenBank accession numbers of sequences are shown in parentheses. Bar, 0.01 substitutions per nucleotide position.

DNA kit (Qiagen). For the 16S rRNA gene, a fragment of approximately 1400 bp was sequenced with the primer set reported previously (Sacchi *et al.*, 2002). For the *recA* gene, a 450 bp segment was sequenced from strain m4-4^T, *B. marisflavi* TF-11^T and from four isolates of *B. vietnamensis* (NRIC 0530, 0531^T, 0532 and 0533). The sequencing reaction had a total volume of 15 µl consisting of 2 µl Big Dye Terminator sequencing buffer (Applied Biosystems), 1.6 µM primer and 5 µl purified amplified product. The amplification conditions were as follows: one cycle of 5 min at 95 °C, and 45 cycles of 10 s at 95 °C, 10 s at 50 °C and 4 min at 60 °C. Sequencing was done in a capillary sequencer (ABI-Avant 100). Sequences (GenBank accession numbers EF014450–EF014452, EF014455 and EF014457–EF014461) were manually edited with the BioEdit program (Hall, 1999). In the case of the 16S rRNA gene sequences, isolate identities were established by comparing the

sequences obtained with the Ribosomal Database Project and the NCBI databases. Phylogenetic reconstruction for the *recA* gene was done using seven complete genomes of *Bacillus* strains reported in the NCBI database. Sequences were aligned using the CLUSTAL_W program (Thompson *et al.*, 1994). Phylogenetic reconstruction for the 16S rRNA and *recA* genes was done using the neighbour-joining algorithm with Kimura two-parameter distances, as implemented in MEGA3 (Kumar *et al.*, 2004).

Strain m4-4^T was subjected to morphological and physiological tests that showed significant differences with respect to other closely related *Bacillus* species (Table 1). Strain m4-4^T grew on only three carbon sources (starch, glycerol and trehalose). Cells were rod-shaped, approximately 0.5–0.7 µm in diameter and 1.5–3 µm in length after 2 days of cultivation at 37 °C (Supplementary Fig. S1, available in

Table 1. Differential characteristics of strain m4-4^T and closely related strains

Strains: 1, m4-4^T; 2, *B. marisflavi* JCM 11544^T; 3, *B. aquimaris* JCM 11545^T; 4, *B. vietnamensis* NRIC 0531^T. The four strains were positive for utilization of starch, glycerol, L-glutamine, citrate, trehalose and fumarate. All strains were negative for nitrate reduction, H₂S and urease.

Characteristic	1	2	3	4
Spore position*	C	s or C	C	C
Colony colour†	LY	PY	PO	O
Utilization of:				
D-Mannose	–	+	–	–
Raffinose	–	+	–	–
Sucrose	–	+	+	+
D-Glucose	–	+	+	+
Lactose	–	+	+	+
Arabinose	–	+	+	+
Dulcitol	–	+	+	+
Fructose	–	+	+	+
Adonitol	–	+	+	+
D-Sorbitol	–	+	+	+
Salicin	–	+	+	+
D-Mannitol	–	+	+	+
D-Xylose	–	+	+	+
L-Rhamnose	–	+	+	+
Optimal temperature for growth (°C)	30–37	30–37	30–37	30–40
Growth at 45 °C	–	+	–	+
Optimal pH for growth	7.0–8.0	6.0–8.0	6.0–7.0	6.0–8.0
Growth at:				
pH 4.5	–	+	–	–
pH 9.0	+	+	–	+
NaCl for growth (% w/v):				
Optimum	5	5	9	5
Maximum	10	10	15	15
Major cellular fatty acids (% of total)‡	C _{14:0} (29.4 %), C _{16:0} (22.3 %), C _{18:1} (15.2 %)	ai-C _{15:0} (27.7 %), i-C _{15:0} (22.9 %), i-C _{14:0} (9.1 %)	i-C _{15:0} (46.9 %), ai-C _{15:0} (22.2 %), i-C _{14:0} (6.5 %)	ai-C _{15:0} (48.3 %), i-C _{15:0} (16.2 %), ai-C _{17:0} (13.6 %)
DNA G + C content (mol%)	37	49	38	44

*C, Central; s, subterminal.

†LY, Light yellow; O, orange; PO, pale orange; PY, pale yellow.

‡i, iso; ai, anteiso.

IJSEM Online). The G + C content of 37 mol% for strain m4-4^T is significantly different from that for *B. marisflavi* (49 mol%) and *B. vietnamensis* (43–44 mol%), but not from that for *B. aquimaris* (38 mol%).

The major cellular fatty acids of strain m4-4^T were C_{14:0} (29.4 %), C_{16:0} (22.3 %), C_{18:1} (15.2 %) and C_{17:0} (7.9 %). Fatty acids occurring in minor amounts were C_{12:0} (1.3 %), anteiso-C_{17:0} (4.7 %) and anteiso-C_{15:0} (4.8 %) (Supplementary Table S1). Fatty acids profile comparisons between strain m4-4^T and other species of the genus *Bacillus* reveal significant differences (Table 1).

16S rRNA gene sequence similarity between strain m4-4^T and type strains of other phylogenetically closely related *Bacillus* species (*B. marisflavi*, *B. aquimaris* and *B. vietnamensis*) ranged from 96.6 to 97.4 %. Values obtained in this study meet widely accepted criteria for delineating species in current bacteriology (Stackebrandt & Goebel, 1994). A 16S rRNA gene-sequence-based neighbour-joining phylogeny analysis revealed that the three different ribosomal operons of strain m4-4^T formed a tight and highly supported clade (100 % bootstrap support) nested within a deeper cluster that comprises *B. aquimaris*, *B. marisflavi*, *B. vietnamensis* and *Bacillus seohaeanensis* at a bootstrap confidence level of 87 % (Fig. 1). In addition, a *recA*-based neighbour-joining tree also grouped strain m4-4^T as a strongly supported monophyletic lineage (Supplementary Fig. S2), which is distinct from the clade comprising *B. marisflavi* and *B. vietnamensis*.

Our results show that strain m4-4^T can grow in medium containing NaCl in the range 0.5 to 10 % (w/v). From these data we concluded that this *Bacillus* strain is moderately halophilic (Ventosa *et al.*, 1998).

In this study we described a *Bacillus* isolate using biochemical and genomic data as well as phylogenetic reconstructions involving 16S rRNA and *recA* gene sequences. This approach showed that m4-4^T is a member of a distinct group within the genus *Bacillus*. The strain displayed characteristics typical of *Bacillus* species, like spore production and low DNA G + C content (37 mol%). However, the fatty acid composition for strain m4-4^T is completely different from those of other closely related *Bacillus* species (Supplementary Table S1). Chains C₁₄, C₁₆, and C₁₈ are characteristic for this novel isolate. Phylogenetic analysis using 16S rRNA gene sequences showed that the novel isolate formed a distinct clade compared with the closely related type strains of *B. marisflavi* (JCM 11544^T), *B. aquimaris* (JCM 11545^T), *B. vietnamensis* (NRIC 0531^T) and *B. seohaeanensis* (DSM 16464^T). Phylogenetic reconstruction using *recA* gene sequences also showed that the novel isolate formed a distinct group compared with *B. marisflavi* JCM 11544^T and *B. vietnamensis* strains NRIC 0531^T, 0530, 0532, and 0533. We suggest, on the basis of the data described above, that strain m4-4^T should be placed within the genus *Bacillus* as a representative of a novel species, for which the name *Bacillus coahuilensis* sp. nov. is proposed.

Description of *Bacillus coahuilensis* sp. nov.

Bacillus coahuilensis (co.a.hui.len'sis. N.L. masc. adj. *coahuilensis* in reference to Coahuila, the state in Mexico where the type strain was collected).

Vegetative cells are rod-shaped, occurring in large chains (Supplementary Fig. S1a), approximately 0.5–0.7 µm in diameter by 1.5–3 µm in length. Central ellipsoidal endospores are observed in swollen sporangia and are 1.0 µm wide and 1.5–1.7 µm long (Supplementary Fig. S1b, c). Colonies on MA are light yellow and 2–5 mm in diameter after 2 days growth at 37 °C; they are low, convex, circular and slightly irregular. Optimal growth temperature is 30–37 °C and the maximum growth temperature is 45 °C. Minimum pH for growth lies between 5.0 and 5.5, the optimum pH for growth is between 7 and 8 and the maximum pH for growth is 9. Acid is produced from glycerol, but not from D-glucose or lactose. Citrate and fumarate can be utilized. Nitrate reduction was not present. H₂S and urease are not produced. Does not utilize sucrose, lactose, arabinose, dulcitol, fructose, adonitol, D-sorbitol, salicin, D-mannitol, D-xylose, L-rhamnose and L-glutamine as sole carbon and energy sources. DNA G + C content of the type strain is 37 mol%. Halotolerant, growing in NaCl salt concentration from 0.5 to 10 %. The major fatty acids are C_{14:0}, C_{16:0} and C_{18:1}. Additionally, based on genome analysis, strain m4-4^T showed nine ribosomal operons with three different sequences (Fig. 1).

The type strain, m4-4^T (=NRRL B-41737^T =CECT 7197^T), was isolated from a desiccation lagoon in the Cuatro Ciénegas Valley in Coahuila, Mexico.

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References

- Alcaraz, L. D., Olmedo, G., Bonilla, G., Cerritos, R., Hernández, G., Cruz, A., Ramírez, E., Putonti, C., Jiménez, B. & other authors (2008). The genome of *Bacillus coahuilensis* reveals adaptations essential for survival in the relic of an ancient marine environment. *Proc Natl Acad Sci U S A* (in press).
- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. Cambridge: Cambridge University Press.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41, 95–98.
- Istock, C. A., Ferguson, N., Istock, N. L. & Duncan, K. E. (2001). Geographical diversity of genomic lineages in *Bacillus subtilis* (Ehrenberg) Cohn *sensu lato*. *Org Divers Evol* 1, 179–191.

- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5, 150–163.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Lányi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* 19, 1–67.
- Lee, J.-C., Lim, J.-M., Park, D.-J., Jeon, C. O., Li, W.-J. & Kim, C.-J. (2006). *Bacillus seohaeanensis* sp. nov., a halotolerant bacterium that contains L-lysine in its cell wall. *Int J Syst Evol Microbiol* 56, 1893–1898.
- Lim, J. M., Jeon, C. O., Lee, S. M., Lee, J. C., Xu, L. H., Jiang, C. L. & Kim, C. J. (2006). *Bacillus salarius* sp. nov., a halophilic, spore-forming bacterium isolated from a salt lake in China. *Int J Syst Evol Microbiol* 56, 373–377.
- Margulies, M., Elgholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bembien, L. A., Berka, J., Braverman, M. S., Chen, Y. J. & other authors (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437, 376–380.
- Noguchi, H., Uchino, M., Shida, O., Takano, K., Nakamura, L. K. & Komagata, K. (2004). *Bacillus vietnamensis* sp. nov., a moderately halotolerant, aerobic, endospore-forming bacterium isolated from Vietnamese fish sauce. *Int J Syst Evol Microbiol* 54, 2117–2120.
- Nunally, B. K. (2005). *Analytical Techniques in DNA Sequencing*. Boca Raton, FL: Taylor & Francis CRC Press.
- Sacchi, C. T., Whitney, A. M., Mayer, L. W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R. S. & Popovic, T. (2002). Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis*. *Emerg Infect Dis* 8, 1117–1123.
- Siefert, J. L., Larios-Sanz, M., Nakamura, L. K., Slepecky, R. A., Paul, J. A., Moore, E. R., Fox, G. E. & Jurtshuk, P., Jr (2000). Phylogeny of marine bacillus isolates from the Gulf of Mexico. *Curr Microbiol* 41, 84–88.
- Souza, V., Espinosa-Asuar, L., Escalante, A. E., Eguiarte, L., Farmer, J., Forney, L., Lloret, L., Rodriguez-Martinez, J. M., Soberon, X. & other authors (2006). An endangered oasis of aquatic microbial diversity in the Chihuahuan desert. *Proc Natl Acad Sci U S A* 103, 6565–6570.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* 44, 846–849.
- Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, C. J. M., Nesme, X., Rosselló-Mora, R., Swings, J. & other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* 52, 1043–1047.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22, 4673–4680.
- Ventosa, A., Nieto, J. & Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* 62, 504–544.
- Yoon, J.-H. & Oh, T.-K. (2005). *Bacillus litoralis* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* 55, 1945–1948.
- Yoon, J. H., Kim, I. G., Kang, K. H., Oh, T. K. & Park, Y. H. (2003). *Bacillus marisflavi* sp. nov. and *Bacillus aquimaris* sp. nov., isolated from sea water of a tidal flat of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* 53, 1297–1303.
- Yoon, J.-H., Kim, I.-J., Kang, K. H., Oh, T.-K. & Park, Y.-H. (2004). *Bacillus hwajinpoensis* sp. nov. and an unnamed *Bacillus* genomospecies, novel members of *Bacillus* rRNA group 6 isolated from sea water of the East Sea and the Yellow Sea in Korea. *Int J Syst Evol Microbiol* 54, 803–808.
- Zeigler, D. R. (2003). Gene sequences useful for predicting relatedness of whole genomes in bacteria. *Int J Syst Evol Microbiol* 53, 1893–1900.