Bacillus isabeliae sp. nov., a halophilic bacterium isolated from a sea salt evaporation pond

Luciana Albuquerque,[1] Igor Tiago,[1] Marco Taborda,[1] M. Fernanda Nobre,[1] António Veríssimo[1] and Milton S. da Costa[2]

Correspondence
Milton S. da Costa
milton@ci.uc.pt

[1]Centro de Neurociências e Biologia Celular, Department of Zoology, University of Coimbra, 3004-517 Coimbra, Portugal

[2]Department of Biochemistry, University of Coimbra, 3001-401 Coimbra, Portugal

A low-G+C, Gram-positive isolate, designated strain CVS-8^T, was isolated from a sea salt evaporation pond on the Island of Sal in the Cape Verde Archipelago. This organism was found to be a catalase- and oxidase-positive, non-motile, spore-forming, aerobic, curved rod-shaped organism with an optimum growth temperature of about 35–37 °C and an optimum pH between 7.5 and 8.0. Optimal growth occurred in media containing 4–6 % (w/v) NaCl and no growth occurred in medium without NaCl. The cell-wall peptidoglycan was of the A1γ type with *meso*-diaminopimelic acid, the major respiratory quinone was MK-7, the major fatty acids were iso-15 : 0, 16 : 0, anteiso-15 : 0 and iso-16 : 0 and the major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unidentified aminoglycophospholipid. The G+C content of the DNA was 37.9 mol%. Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain CVS-8^T represented a novel species of the genus *Bacillus*, the highest levels of sequence similarity (mean pairwise similarity values of ~97.5 %) being found with respect to the type strains of *Bacillus shackletonii* and *Bacillus acidicola*. On the basis of the phylogenetic, physiological and biochemical data, strain CVS-8^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus isabeliae* sp. nov. is proposed. The type strain is CVS-8^T (=LMG 22838^T=CIP 108578^T).

Species of the genus *Bacillus* have been isolated from a large variety of aquatic and terrestrial environments, demonstrating their ubiquity. Among these, moderately halophilic *Bacillus* species have been isolated from salterns, estuarine water, salt lakes, salty foods, sea ice and seawater (Agnew *et al.*, 1995; Arahal *et al.*, 1999; Nielsen *et al.*, 1994; Ventosa *et al.*, 1989; Yoon *et al.*, 2004). During a survey of the bacterial diversity of a sea salt evaporation pond on the Island of Sal in the Cape Verde Archipelago, several halophilic, Gram-positive bacteria were isolated and characterized. One of the isolates, designated strain CVS-8^T, was found to be phylogenetically related to species of the genus *Bacillus*, sharing several physiological and biochemical characteristics with the strains belonging to the species *Bacillus acidicola* (Albert *et al.*, 2005) and *Bacillus shackletonii* (Logan *et al.*, 2004). Nevertheless, this novel organism had distinctly higher NaCl and pH requirements for optimal growth as well as a distinctive fatty acid profile. Here, we report the morphological, physiological, chemotaxonomic and phylogenetic characterization of strain CVS-8^T, which is proposed as representing a novel species of the genus *Bacillus*.

Strain CVS-8^T was isolated from a sea salt evaporation pond on the Island of Sal in the Cape Verde Archipelago (República de Cabo Verde), using solid R3A-V medium (Tiago *et al.*, 2006) at pH 7.0 and containing 2 % (w/v) NaCl. Soil samples (3 g) were resuspended in sterile blenders with sterile solution of 2 % (w/v) NaCl. Single drops of the homogenate were spread on R3A-V agar plates. These preparations were incubated at 37 °C for up 5 days. Despite repeated attempts to isolate additional strains, only one isolate was obtained. Cultures were purified by means of subculturing on the same medium and were maintained at −70 °C in Degryse medium 162 containing 3 % (w/v) NaCl and 15 % (w/v) glycerol (Degryse *et al.*, 1978). The organism was routinely cultured in Degryse medium 162 containing 5 % (w/v) NaCl at 37 °C for up 5 days, unless otherwise stated (Albuquerque *et al.*, 2005). The type strains of *Bacillus acidicola* (DSM 14745^T) and *B. shackletonii* (LMG 18435^T) were used for comparative purposes.

The temperature range for growth of strain CVS-8^T was tested in liquid medium in a reciprocal water-bath shaker

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CVS-8^T is AM503357.

A maximum-parsimony phylogenetic dendrogram based on 16S rRNA gene sequences and a graph showing the effect of salt on growth of strain CVS-8^T are available as supplementary figures with the online version of this paper.

between 15 and 45 °C; the pH range for growth, from pH 5.5 to pH 10.5, was determined at 37 °C in the same medium buffered using 20 mM MES, TRIS and CAPSO. The NaCl range for growth was determined in liquid medium without additional NaCl and at NaCl concentrations of up to 16.0 % (w/v). Enzymic activities were determined using the API ZYM system (bioMérieux). Anaerobic growth was assessed on solidified Degryse medium with 5.0 % (w/v) NaCl and KNO_3 (1.0 g l^{-1}) in anaerobic chambers (GENbox anaer; bioMérieux). Tests to determine the assimilation of single carbon sources were performed in a defined medium composed of Degryse basal salts containing 5.0 % (w/v) NaCl, yeast extract (0.1 g l^{-1}), ammonium sulfate (0.5 g l^{-1}), single carbon sources (2.0 g l^{-1}) and 2 % (w/v) deionized water-washed agar (Oxoid). Plate cultures (48 h) were resuspended in the basal salts medium (turbidity equivalent to a McFarland no. 1 standard) and single drops of the suspension were placed on Petri dishes, each of which contained a single carbon source; the plates were examined visually for up to 5 days. Acid production from carbohydrates was determined with the API 50 CH system (bioMérieux) according to the manufacturer's instructions, using API 50 CHB/E medium (bioMérieux) containing 5.0 % (w/v) NaCl. Results were recorded after 24 h, 48 h and 5 days incubation at 37 °C.

Peptidoglycan analysis was performed by using the method of Schleifer & Kandler (1972) and Schleifer (1985); analysis of respiratory quinones was performed according Tindall (1989). Cultures for fatty acid analyses were grown on solidified Degryse medium 162 containing 5 % (w/v) NaCl buffered at pH 7.5, incubated in sealed plastic bags submerged in a water bath at 37 °C for 48 h. For comparison, fatty acid analyses were also performed on the type strains of *B. acidicola* and *B. shackletonii* grown on Degryse medium 162 buffered at pH 6.2 (*B. acidicola*) or pH 6.5 (*B. shackletonii*) with incubation in sealed plastic bags submerged in a water bath at 37 °C for 24 h. Salt was not added to the culture media used to grow *B. acidicola* and *B. shackletonii* because these organisms show optimum growth in media without added NaCl. Fatty acid methyl esters were extracted as described previously (Moreira *et al.*, 2000); their identification and quantification, as well as the numerical analysis of the fatty acid profiles, were performed by using the standard MIS Library Generation Software (Microbial ID). Polar lipid analyses were performed as described previously (Prado *et al.*, 1988).

The G+C content of the DNA was determined by HPLC as described by Mesbah *et al.* (1989). The 16S rRNA gene was amplified with a PCR and sequenced as described by Rainey *et al.* (1996). Phylogenetic analyses were performed using the ARB software package (Ludwig *et al.*, 2004). A phylogenetic tree was constructed using the neighbour-joining (Saitou & Nei, 1987) algorithm. Tree topologies were evaluated by performing a bootstrap analysis (Felsenstein, 1985) of 1000 resamplings of the dataset.

Comparative analyses of 1481 nucleotide positions of the 16S rRNA gene sequence of strain CVS-8^T with those of other lineages within the low-G+C, Gram-positive bacteria showed that the novel isolate belonged to the genus *Bacillus* (Fig. 1). The highest levels of pairwise 16S rRNA gene sequence similarity were found with respect to the type strains of *B. shackletonii* (97.5 %) and *B. acidicola* (97.3 %). Despite these similarity values, the phylogenetic tree, irrespective of the method used for construction,

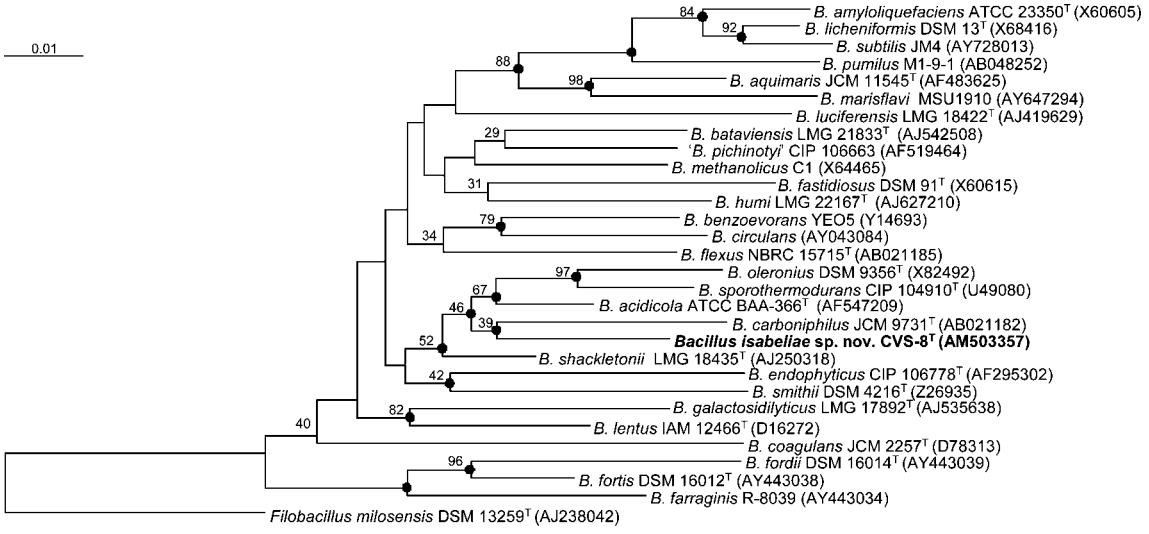

Fig. 1. Neighbour-joining phylogenetic dendrogram based on a comparison of the 16S rRNA gene sequences of strain CVS-8^T and the closest phylogenetic relatives. Numbers on the tree indicate bootstrap support (%), derived from 1000 replications. Bar, 1 inferred nucleotide substitutions per 100 nucleotides. Filled circles indicate branching topologies that are also present in the tree obtained using the maximum-parsimony method (see Supplementary Fig. S1 in IJSEM Online).

showed a peculiar topology whereby strain CVS-8^T formed a branch with the type strain of *Bacillus carboniphilus* (Fujita *et al.*, 1996). However, the 16S rRNA gene sequence similarity between strain CVS-8^T and that of the type strain of *B. carboniphilus* was only 96.4 %. Furthermore, the topology for the branch with *B. carboniphilus* and CVS-8^T showed poor bootstrap support (Fig. 1). Regrettably, the type strain of *B. carboniphilus* is not freely available for study, as discussed by the Subcommittee on the taxonomy of the Genus *Bacillus* and related organisms (Logan, 2005), and the description of the species (Fujita *et al.*, 1996) is based on a small number of characteristics, making it difficult to compare the phenotypic characteristics of this organism with those of strain CVS-8^T. Therefore we considered it appropriate to compare the phenotypic characteristics of strain CVS-8^T with the type strains of *B. shackletonii* and *B. acidicola*. The G+C content of the DNA of strain CVS-8^T was 37.9 mol%.

Strain CVS-8^T formed orange-pigmented colonies, the cells stained Gram-positive and were curved rods (Fig. 2). Endospores (positioned subterminally in swollen sporangia) were rarely observed, but their existence was confirmed in cells from a few colonies that formed after the incubation of cultures at 60 and 70 °C for 20 and 10 min, respectively. The isolate had an optimum growth temperature between 35 and 37 °C and did not grow at 15 or 45 °C. The optimum pH of the novel organism was between 7.5 and 8.0. Optimum growth occurred at NaCl concentrations between 4 and 6 % (w/v) and no growth occurred in medium without NaCl or in medium containing 16 % (w/v) NaCl (see Supplementary Fig. S2, available with the online version of this paper). On the other hand, the type strains of *B. acidicola* and *B. shackletonii* grew in medium without salt, showed a narrow range of salt tolerance and had a lower optimum pH for growth (Table 1).

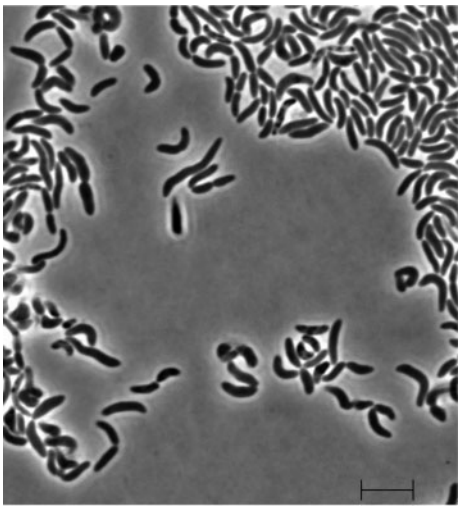

Fig. 2. Phase-contrast micrograph of cells of strain CVS-8^T cultivated for 24 h. Bar, 5 μm.

The novel isolate was oxidase- and catalase-positive. Other enzyme activities are listed in the species description. Yeast extract was required for growth on single carbon sources. Strain CVS-8^T assimilated carbohydrates and organic acids. Of the amino acids tested, proline was the only one that was assimilated. Acid was produced from several carbohydrates. Nitrate was not reduced to nitrite and anaerobic growth in the presence of nitrate was not observed.

Like the great majority of endospore-forming bacteria, strain CVS-8^T possessed a cell-wall peptidoglycan structure of type A1γ with *meso*-diaminopimelic acid as the diagnostic diamino acid. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and an unknown aminoglycophospholipid were identified by using TLC. The major respiratory lipoquinone was MK-7. The major fatty acids of strain CVS-8^T were iso-15 : 0 (24.8 %), 16 : 0 (17.5 %), anteiso-15 : 0 (15.6 %) and iso-16 : 0 (11.6 %) (Table 2). However, strain CVS-8^T and the type strains of *B. acidicola* and *B. shackletonii* could be clearly distinguished from each other from the relative amounts of these fatty acids. Furthermore, strain CVS-8^T possessed

Table 1. Differentiating characteristics of strain CVS-8^T and the type strains of *B. acidicola* and *B. shackletonii*

Taxa: 1, strain CVS-8^T; 2, *B. acidicola* DSM 14745^T; 3, *B. shackletonii* LMG 18435^T. All data are from this study unless indicated otherwise. +, Positive; −, negative; (+), weakly positive.

Characteristic	1	2	3
Cell motility	−	+	+
Colony pigmentation	Orange	Cream	Cream
NaCl requirement for growth	+	−	−
NaCl range (%)	1–14	0–2	0–3
NaCl optimum (%)	4–6	0	0
Growth temperature (°C) range	20–40	20–42.5	20–52.5
Optimum temperature (°C)	35–37	35–37	42.5–50
pH range	6.5–8.5	3.5–7.0	4.5–8.5
Optimum pH	7.5–8.0	5.0–6.5	5.5–7.0
Oxidase	+	−	+
Starch hydrolysis	+	+	−
Acid production from:			
Glycerol	−	+	−
D-Ribose	(+)	+	(+)
D-Xylose	(+)	+	−
D-Galactose	−	+	(+)
D-Glucose	−	+	+
D-Fructose	(+)	+	(+)
D-Mannose	−	+	(+)
L-Sorbose	(+)	−	−
D-Mannitol	−	+	(+)
Maltose	−	+	(+)
D-Lactose	−	+	(+)
Sucrose	(+)	+	−
DNA G+C content (mol%)	37.9	43.2*	35.4–36.8†

*Data from Albert *et al.* (2005).
†Data from Logan *et al.* (2004).

Table 2. Fatty acid contents (%) of strain CVS-8^T and the type strains of *B. acidicola* and *B. shackletonii*

Strains: 1, CVS-8^T; 2, *B. acidicola* DSM 14745^T; 3, *B. shackletonii* LMG 18435^T. Values from this study are given as means ±SD (three replicates). –, Not detected; tr, trace (<0.5 %).

Fatty acid	1	2	3
iso-13 : 0	1.0 ± 0.1	tr	–
iso 14 : 0	6.9 ± 0.3	0.6 ± 0	0.5 ± 0
14 : 0	4.2 ± 0	1.2 ± 0.1	0.5 ± 0
iso-15 : 0	24.8 ± 0.1	58.4 ± 0.2	29.6 ± 0.1
anteiso-15 : 0	15.6 ± 0.4	19.4 ± 0.1	22.2 ± 0.4
15 : 0	3.1 ± 0.2	–	tr
16 : 1ω7*c* alcohol	1.8 ± 0.1	–	–
iso-16 : 0	11.6 ± 0	1.2 ± 0	6.4 ± 0.3
16 : 1ω11*c*	2.0 ± 0	–	–
Summed feature 3*	2.7 ± 0	–	–
16 : 0	17.5 ± 0.2	2.6 ± 0.1	1.9 ± 0.1
iso-17 : 0	2.1 ± 0.1	6.3 ± 0	3.1 ± 0.1
anteiso-17 : 0	4.1 ± 0.1	10.0 ± 0.1	33.7 ± 0.6

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained one or more of the following fatty acids: 16 : 1ω7*c*/16 : 1ω6*c*/iso-15 : 0 2-OH.

straight-chain 15 : 0 as well as 16 : 1ω7*c* alcohol, 16 : 1ω11*c* and summed feature 3 (16 : 1ω7*c*/16 : 1ω6*c*/iso-15:0 2-OH), which were not detected in the type strains of *B. acidicola* and *B. shackletonii*.

Despite similar characteristics and close phylogenetic relatedness, strain CVS-8^T can be clearly distinguished from the type strains of *B. acidicola* and *B. shackletonii*, i.e. from the NaCl requirement for growth, the pH range, the fatty acid composition and other phenotypic traits. On the basis of these findings, strain CVS-8^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus isabeliae* sp. nov. is proposed.

Description of *Bacillus isabeliae* sp. nov.

Bacillus isabeliae (i.sa.be.li′ae. N.L. gen. fem. n. *isabeliae* of Isabel, in honour of Portuguese microbiologist Isabel Spencer-Martins).

Cells are curved rods that are 0.5–1.0 μm in width and 2.8–5.7 μm in length. Gram stain is positive. Cells are non-motile and form rarely observed endospores in subterminal swollen sporangia. Colonies on Degryse 162 medium containing 5 % (w/v) NaCl are orange. Strictly aerobic and heterotrophic. Oxidase- and catalase-positive. Optimum growth temperature is approximately 35–37 °C; growth does not occur at 15 or 45 °C. Optimum pH is between 7.5 and 8.0; growth does not occur at pH 6.0 or pH 9.0. Optimum growth occurs at NaCl concentrations between 4 and 6 % (w/v); no growth occurs in medium without NaCl or in medium containing 16 % (w/v) NaCl. Cell-wall peptidoglycan is of the A1γ type with *meso*-diaminopimelic acid as the diagnostic diamino acid. The major respiratory quinone is MK-7. Predominant fatty acids are iso-15 : 0, 16 : 0, anteiso-15 : 0 and iso-16 : 0; smaller amounts of iso-13 : 0, iso-14 : 0, 14 : 0, 15 : 0, 16 : 1ω7*c* alcohol, 16 : 1ω11*c*, summed feature 3, iso-17 : 0 and anteiso-17 : 0 are also present. The major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and an unknown aminoglycophospholipid. Yeast extract or growth factors are required for growth. Nitrate is not reduced. Aesculin, arbutin, starch and xylan are hydrolysed, but gelatin, casein, elastin, hippurate and Tweens 20 to 80 are not hydrolysed. Positive for DNase, esterase lipase (C8), leucine arylamidase, valine arylamidase and α-chymotrypsin. Negative for alkaline phosphatase, esterase (C4), lipase (C14), cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucosidase, β-galactosidase, α-galactosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Glucose, fructose, xylose, sucrose, maltose, trehalose, cellobiose, succinate, α-ketoglutarate, lactate, malate, pyruvate, citrate, acetate and proline are assimilated. Mannose, galactose, sorbose, D-arabinose, L-arabinose, ribose, lactose, melezitose, melibiose, L-rhamnose, raffinose, fucose, ribitol, xylitol, sorbitol, erythritol, arabitol, mannitol, *myo*-inositol, glycerol, aspartate, glutamate, alanine, asparagine, cysteine, phenylalanine, glycine, histidine, isoleucine, lysine, methionine, glutamine, arginine, serine, threonine, valine and ornithine are not assimilated. Acid is produced from ribose, xylose, fructose, sorbose, cellobiose, sucrose, trehalose, melezitose, glycogen, gentiobiose, turanose, tagatose and potassium 5-ketogluconate. Acid is not produced from glycerol, erythritol, arabinose, L-xylose, ribitol, methyl β-D-xylopyranoside, galactose, glucose, mannose, rhamnose, galactitol, inositol, mannitol, sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, maltose, lactose, melibiose, inulin, raffinose, starch, xylitol, lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate or 2-ketogluconate. The DNA G+C content is 37.9 mol% (HPLC method).

The type strain, strain CVS-8^T (=LMG 22838^T=CIP 108578^T), was isolated from a sea salt evaporation pond on the Island of Sal in the Cape Verde Archipelago.

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