***Alicyclobacillus hesperidum* sp. nov. and a related genomic species from solfataric soils of São Miguel in the Azores**

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Several acidophilic, slightly thermophilic or thermophilic Gram-positive isolates were recovered from solfataric soil at Furnas on the Island of São Miguel in the Azores. Phylogenetic analysis of the 16S rRNA gene sequence showed that these organisms represented two novel species of the genus *Alicyclobacillus*. Strains FR-11^T and FR-1b had an optimum growth temperature of about 50 °C, whereas strains FR-3 and FR-6^T had an optimum growth temperature of about 60 °C. Biochemical, physiological and chemotaxonomic characteristics did not distinguish isolates FR-3 and FR-6^T from the type strain of *Alicyclobacillus acidocaldarius*; however, strains FR-11^T and FR-1b could be easily distinguished from the type strain of *Alicyclobacillus acidoterrestris* by the carbon source assimilation pattern and the fatty acid composition. On the basis of the phylogenetic analysis, physiological and biochemical characteristics, and fatty acid composition the name *Alicyclobacillus hesperidum* is proposed for the species represented by strains FR-11^T and FR-1b; a formal name for the new genomic species represented by strains FR-3 and FR-6^T is not proposed at this time.

Keywords: *Alicyclobacillus hesperidum* sp. nov., *Alicyclobacillus* genomic species, *Bacteria*, thermophiles

INTRODUCTION

The strictly aerobic, slightly thermophilic or thermophilic bacteria of the genus *Alicyclobacillus* were initially placed in the genus *Bacillus* with which they share the characteristic of endospore formation. However, phylogenetic analysis based on sequence comparisons of the 16S rRNA gene showed that species of the genus *Alicyclobacillus* belonged to a distinct line of descent within the low G+C Gram-positive lineage of the *Bacteria* that also included the closely related facultatively autotrophic species of the genus *Sulfolobus* (Wisotzkey *et al.*, 1992; Tourova *et al.*, 1994;

Durand, 1996). The three validly named species of the genus *Alicyclobacillus* are: *Alicyclobacillus acidocaldarius* (Darland & Brock, 1971), *Alicyclobacillus acidoterrestris* (Deinhard *et al.*, 1987a) and *Alicyclobacillus cycloheptanicus* (Deinhard *et al.*, 1987b). The species *A. acidoterrestris* and *A. cycloheptanicus* have optimum growth temperatures of 45–50 °C, whereas *A. acidocaldarius* has a higher optimum growth temperature of about 60–65 °C. All species have pH optima for growth of 3.5–4.0, a minimum pH for growth of about 2.5 and a maximum pH for growth of approximately 5.5. The most distinctive characteristic of these organisms is, in our view, the presence of ω -cyclohexyl or ω -cycloheptyl fatty acids; the species *A. acidoterrestris* and *A. acidocaldarius*, have large relative proportions of ω -cyclohexyl fatty acids (De Rosa *et al.*, 1971; Oshima & Ariga, 1975; Hippchen *et al.*, 1981), whereas ω -cycloheptyl fatty acids are the predominant fatty acids of *A. cycloheptanicus* (Poralla

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The EMBL accession numbers for the 16S rRNA gene sequences of strains FR-11^T, FR-1b, FR-3, FR-6^T and *Alicyclobacillus acidoterrestris* DSM 3922^T are AJ133633, AJ133632, AJ133634, AJ133635 and AJ133631, respectively.

& König, 1983; Allgaier *et al.*, 1985). With the exception of *Sulfobacillus* spp., which also possess ω -cyclohexyl fatty acids (Dufresne *et al.*, 1996), no other bacterium examined has ω -cyclic fatty acids, thus providing excellent phylogenetic markers for these acidophilic spore-forming bacteria. Strains of the genus *Alicyclobacillus* have been isolated from solfataric soils within geothermal areas and several non-geothermal soils (Darland & Brock, 1971; Deinhard *et al.*, 1987a, b). Most of the interest in these organisms revolves around the study of the role of ω -cyclic fatty acids and hopanoids on membrane function (Poralla *et al.*, 1980; Kannenberg *et al.*, 1984), but there is also considerable interest in the biotechnological application of thermostable enzymes derived from *A. acidocaldarius*-like strains (Schwermann *et al.*, 1994).

Recently, several strains of aerobic acidophilic spore-formers were isolated from the solfataric soils at Furnas on the Island of São Miguel in the Azores. Here, the characteristics of these strains are described and an additional species of the genus *Alicyclobacillus* is proposed, *Alicyclobacillus hesperidum* sp. nov.

METHODS

Isolation and bacterial strains. Strains FR-3, FR-6^T, FR-1b and FR-11^T (T = type strain) were isolated from the Furnas area on the Island of São Miguel, the Azores. Soil was resuspended in water, large particles were allowed to sediment and the suspensions were filtered through membrane filters (Gelman type GN-6; pore size 0.45 μ m, diameter 47 mm). The filters were placed on the surface of *Bacillus acidocaldarius* medium (BAM), pH 4.0 solidified with agar (Deinhard *et al.*, 1987a) and the plates were wrapped in plastic bags during incubation. Strains FR-3 and FR-6^T were isolated from plates incubated at 60 °C, whereas strains FR-1b and FR-11^T were isolated from plates incubated at 45 °C. Cultures were purified by subculturing and were kept at -80 °C in BAM medium containing 15% glycerol. The type strains of *A. acidocaldarius* (DSM 446^T), *A. cycloheptanicus* (DSM 4006^T) and *A. acidoterrestris* (DSM 3922^T) were obtained from Deutsche Sammlung Von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Morphological, biochemical and tolerance characteristics. Cell morphology and motility were examined by phase-contrast microscopy during the exponential growth phase in BAM medium. Unless otherwise stated, all biochemical and tolerance tests were performed as described previously (Santos *et al.*, 1989; Manaia & da Costa, 1991) in BAM liquid medium or in BAM agar. The type strain of *Alicyclobacillus acidocaldarius* and strains FR-3 and FR-6^T were incubated at 60 °C whereas the type strain of *Alicyclobacillus acidoterrestris* and strains FR-1b and FR-11^T were incubated at 50 °C for up to 5 d. Catalase and oxidase activity was examined as described previously (Smibert & Krieg, 1981). The growth temperature range of the organisms was examined by measuring the turbidity (610 nm) of cultures incubated in 300 ml metal-capped Erlenmeyer flasks, containing 100 ml BAM medium, adjusted to pH 4.0, in a reciprocal water-bath shaker. The pH range for growth was examined at 60 or 50 °C in BAM medium with pH values adjusted by the addition of 1.0 M

H₂SO₄. All pH measurements were performed at room temperature.

Single-carbon source assimilation tests were performed in a minimal medium composed of BAM basal salts, to which filter-sterilized ammonium sulfate (0.5 g l⁻¹) and the carbon sources (2.0 g l⁻¹) were added. Yeast extract was added to carbon source assimilation tests of the type strain of *Alicyclobacillus acidoterrestris* (0.01 g l⁻¹) which required it for growth. Growth of the strains on single carbon sources was examined by measuring the turbidity of cultures incubated at 60 or 50 °C in 20 ml screw-capped tubes containing 10 ml medium after 5 d of growth. Positive and negative control cultures were grown in BAM medium and in minimal medium without carbon source, respectively.

Acidification of the medium was examined with API 50 CH test strips (bioMérieux) using BAM basal salts medium, pH 4.0, 0.5 g yeast extract l⁻¹, 3.0 g agar l⁻¹ and 0.03 g bromophenol blue l⁻¹. The micro-organisms were resuspended in BAM basal salts corresponding to tube No. 2 of the MacFarland series of standard opacities (Smibert & Krieg, 1981). These cell suspensions (approx. 5 ml) were added to 100 ml BAM medium, distributed in the API 50 CH test strip wells, as recommended by the manufacturer, and incubated at 60 °C for the type strain of *A. acidocaldarius*, strains FR-3 and FR-6^T, and at 50 °C for the type strain of *A. acidoterrestris*, FR-1b and FR-11^T for 5 d. The colour change of the medium from blue to yellow was regarded as acidification by metabolism of the carbon source.

Polar lipid, lipoquinone and fatty acid composition. The cultures used for polar lipid analysis were grown in 1 l Erlenmeyer flasks containing 200 ml BAM medium at 60 or 50 °C in a reciprocal water-bath shaker until the exponential phase of growth. Harvesting of the cultures, extraction of the lipids and single dimensional TLC were performed as described previously (Prado *et al.*, 1988; Donato *et al.*, 1990).

Lipoquinones were extracted from freeze-dried cells and were purified by TLC as described previously (Tindall, 1989). The lipoquinones were separated with a Gilson HPLC by using a reverse-phase (RP18) Spherisorb S50DS2 column and methanol-heptane (10:2, v/v) as the mobile phase and were detected at 269 nm.

Cultures for fatty acid analysis were grown on BAM medium plates, in sealed plastic bags submerged in a water bath at 60 or 50 °C for 24 h. Fatty acid methyl esters were obtained from fresh wet biomass by saponification, methylation and extraction as described previously (Kuykendall *et al.*, 1988). The fatty acid methyl esters were separated using a Hewlett Packard model 5890 GC equipped with an FID fitted with a 5% phenylmethyl silicone capillary column (0.2 mm $\times$ 25 m; Hewlett Packard). The carrier gas was high-purity H₂; the column head pressure was 60 kPa; the septum purge was 5 ml min⁻¹; the column split ratio was 55:1; and the injection port temperature was 300 °C. The temperature of the oven was programmed at 170–270 °C at a rate of 5 °C min⁻¹. Identification and quantification of the fatty acid methyl esters, as well as the numerical analysis of the fatty acid profiles, were performed by using the standard MIS Library Generation Software (Microbial ID).

Determination of mean base composition of DNA and DNA–DNA reassociation studies. The G + C content of the overall genome was determined by HPLC as described by Mesbah *et al.* (1989). DNA for DNA–DNA reassociation

studies was extracted and purified by the procedure of Marmur (1961). The degree of DNA reassociation was determined spectrophotometrically from the initial renaturation rates, according to De Ley *et al.* (1970). The renaturation rates were measured in $1 \times \text{SSC}$ (0.15 M NaCl and 0.015 M trisodium citrate at pH 7.0), using a Uvikon 940 spectrophotometer (Kontron) equipped with a thermostat-controlled cuvette chamber. The optimal renaturation temperature used in each case was calculated from the G+C value (mol%) (De Ley *et al.*, 1970). Each hybridization experiment was executed at least twice.

16S rRNA gene sequence determination and phylogenetic analyses. The extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described previously (Rainey *et al.*, 1996). Sequence reaction products were purified by ethanol precipitation and electrophoresed with a model 373A DNA sequencer or a 310 Genetic Analyzer (Applied Biosystems). The ae2 editor (Maidak *et al.*, 1999) was used to align the 16S rRNA gene sequences obtained in this study with previously determined *Alicyclobacillus* sequences available from the public databases. The method of Jukes & Cantor (1969) was used to calculate evolutionary distances. Phylogenetic dendrograms were generated using various treeing algorithms contained in the PHYLIP package (Felsenstein, 1993).

The EMBL accession numbers of the 16S rRNA gene sequences determined in this study are as follows: strain FR-11^T, AJ133633; strain FR-1b, AJ133632; strain FR-3, AJ133634; strain FR-6^T, AJ133635; and *A. acidoterrestris* DSM 3922^T, AJ133631. The accession numbers and strain designations of the reference 16S rRNA gene sequences used in the phylogenetic analyses are as follows: *A. acidocaldarius* DSM 446^T, X60742; *A. cycloheptanicus* DSM 4006^T, X51928; *Bacillus tusciae* DSM 2912^T, Z26933; *Sulfobacillus disulfidooxidans* SD-11^T, U34974; and *Sulfobacillus thermo-sulfidooxidans* VKM B1269^T, Z21979.

RESULTS

Isolation, and biochemical and physiological characteristics of the strains

The organisms were isolated from acidic soils within the small solfataric area at Furnas on the Island of São Miguel in the Azores. Enrichments performed at 45 or 60 °C on BAM agar led to the isolation of several organisms, of which strains FR-1b, FR-11^T, FR-3 and FR-6^T were characterized. Strains FR-1b and FR-11^T had optimum growth temperatures (about 50 °C) that were very similar to that of the type strain of *A. acidoterrestris*. The optimum and maximum growth temperatures of strains FR-3 and FR-6^T were about 2–5 °C lower than that of the type strain of *A. acidocaldarius*, which was very noticeable at supra-optimum growth temperatures. The type strain of *A. acidocaldarius* grows at 70 °C, whereas the Azorean strains did not (Fig. 1). The pH range for growth of the isolates and the two type strains was similar (Fig. 2).

All the organisms were cytochrome oxidase-negative and catalase was not detected or was weak. Unlike the type strain of *A. acidoterrestris*, strains FR-1b, FR-11^T, FR-3 and FR-6^T did not require yeast extract or co-factors for growth. All organisms hydrolysed

Fig. 1. Effect of temperature on the growth rates of the type strain of *A. acidocaldarius* (●), strain FR-6^T (○), the type strain of *A. acidoterrestris* (■) and strain FR-11^T (□).

Fig. 2. Effect of pH on the growth rates of the type strain of *A. acidocaldarius* (●), strain FR-6^T (○), the type strain of *A. acidoterrestris* (■) and strain FR-11^T (□).

gelatin, hippurate, arbutin and aesculin. *A. acidoterrestris* did not hydrolyse xylan and hide powder azure. Strains FR-3, FR-6^T and *A. acidocaldarius* had practically identical single carbon source assimilation patterns, being generally unable to grow on most of the polyols, except glycerol and mannitol. Strains FR-1b and FR-11^T had a radically different single carbon source assimilation pattern from *A. acidoterrestris*; several sugars, amino acids and organic acids, in

addition to most polyols, were not used for growth. All strains produced acid from virtually the same sugars and sugars derivatives used as carbon sources (Table 1).

Polar lipid, lipoquinone and fatty acid composition

All strains had virtually identical polar lipid patterns on single dimensional TLC, being composed of two phospholipids, one aminoglycolipid, one glycolipid and a sulfonolipid. The major respiratory quinone was menaquinone 7. The major fatty acid of all strains was ω -cyclohexyl 17:0 (Table 2). The type strain of *A. acidocaldarius* and the high temperature strains FR-3 and FR-6^T had very similar fatty acid compositions that made them difficult to distinguish from each other. The type strain of *A. acidoterrestris* had very large relative proportions of ω -cyclohexyl 17:0 that reached about 70% of the total fatty acids; acyl components, other than ω -cyclohexyl 19:0, which reached about 25% of the total fatty acids, were vestigial or were not detected in this strain. Strains FR-1b and FR-11^T, on the other hand, had lower relative proportions of ω -cyclohexyl fatty acids and higher proportions of branched-chain fatty acids than *A. acidoterrestris*. Anteiso fatty acids reached about 20% of the total and iso fatty acids reached about 18% of the total fatty acids in strain FR-1b and FR-11^T.

16S rRNA gene sequence based phylogenetic analysis and DNA–DNA reassociation studies

Almost complete 16S rRNA gene sequences of 1482–1485 nucleotides in length were determined for the four new isolates and *A. acidoterrestris* DSM 3922^T. Phylogenetic analyses based on a data set comprising 1420 unambiguous nucleotides between positions 36 and 1510 [*Escherichia coli* positions (Brosius *et al.*, 1978)] showed that the new isolates clustered with the previously described species of the genus *Alicyclobacillus* (Fig. 3). Two of the four strains, strains FR-6^T and FR-3, shared 99.3% 16S rRNA gene sequence similarity and clustered with *A. acidocaldarius* (97.8–98.0% similarity). Strains FR-11^T and FR-1b had identical 16S rRNA gene sequences and clustered with *A. acidoterrestris* at 97.7% similarity.

The distinct species status of strain FR-6^T was demonstrated by a DNA–DNA reassociation value of 53.3% with the type strain of *A. acidocaldarius*. Strain FR-11^T had a DNA–DNA reassociation value of 60.6% with the type strain of *A. acidoterrestris*.

DISCUSSION

Acidophilic strains of slightly thermophilic or thermophilic spore-forming bacteria appear to be common in solfataric soils. Some of the thermophilic organisms

that have growth temperature ranges similar to those of the type strain of *A. acidocaldarius*, however, may be difficult to distinguish from the type strain of the species. In this study, the only thermo-acidophilic spore-forming strains isolated were, in fact, very similar to *A. acidocaldarius*. However, the 2.0–2.2% 16S rRNA gene sequence difference, and the low DNA–DNA reassociation values between strains FR-3 and FR-6^T and the type strain of *A. acidocaldarius* clearly show that these strains represent a new genomic species. The only noticeable difference between the two species was the slightly lower optimum and maximum growth temperatures. The biochemical, physiological and chemotaxonomic characteristics of the new species represented by strains FR-3 and FR-6^T were indistinguishable from those of the type strain of *A. acidocaldarius*. Yet, it is considered that this feature is not sufficient to clearly distinguish strains FR-3 and FR-6^T from *A. acidocaldarius*, particularly since only the type strain of the latter species could be examined and a typical phenotype cannot be ascribed for this species. Therefore, the absence of distinct phenotypic characteristics points against proposing a formal name for strains FR-3 and FR-6^T at this time. Nevertheless, these strains show that genomic species, other than *A. acidocaldarius*, are found in solfataric soils that might be mistaken for the type species of this genus because of similar phenotypic characteristics.

The carbon source assimilation pattern and the fatty acid composition clearly distinguishes strains FR-1b and FR-11^T from the type strain of *A. acidoterrestris*. Moreover, DNA–DNA reassociation values between *A. acidoterrestris* and strain FR-11^T and the phylogenetic analysis show that these strains are distinct, but related to *A. acidoterrestris* rather than to *A. cycloheptanicus*. The 16S rRNA gene sequence similarity values also support the novel species status of these strains. Based on the distinct biochemical and chemotaxonomic characteristics, and the 16S rRNA gene sequence comparisons, the name *Alicyclobacillus hesperidum* is proposed for strains FR-11^T and FR-1b.

Description of *Alicyclobacillus hesperidum* sp. nov.

Alicyclobacillus hesperidum (hes.pe.ri'dum. L. fem. plur. N. *hesperidum* of the Hesperides, mythological figures whom the Greeks believed to have lived at the Western edge of the Earth in a miraculous garden, which we interpret as the Azores).

Rod-shaped cells that are 2.1–3.9 μ m long and 0.5–0.7 μ m wide. Gram stain is positive. Cells are non-motile and terminal spores are formed; sporangia are not swollen. Colonies on BAM medium are 1–2 mm in diameter after 72 h of growth and are not pigmented. Growth occurs above 35 °C and below 60 °C; the optimum growth temperature for strains FR-11^T and FR-1b is 50–53 °C. The optimum pH is 3.5–4.0; growth does not occur at pH 2.0 or pH 6.0. Strains are

Table 1. Phenotypic characteristics that distinguish the type strain of *A. acidocaldarius* from strains FR-3 and FR-6^T after growth at 60 °C and the type strain of *A. acidoterrestris* from strains FR-1b and FR-11^T after growth at 50 °C

Strains: 1, *A. acidocaldarius*; 2, FR-3; 3, FR-6^T; 4, *A. acidoterrestris*; 5, FR-1b; 6, FR-11^T. w, Weakly positive result; —, negative result; +, positive result. All strains: degraded arbutin, aesculin, gelatin, hippurate, Tween 40, Tween 60 and Tween 80; assimilated D-glucose, D-fructose, D-galactose, D-mannose, maltose, sucrose, lactose, D-cellobiose, D-trehalose, D-xylose, L-arabinose, glycerol and D-mannitol; produced acid from glycerol, L-arabinose, D-galactose, D-glucose, D-fructose, D-mannose, mannitol, cellobiose, maltose, lactose, sucrose and trehalose. None of the strains: had cytochrome oxidase activity; degraded elastin and fibrin; produced nitrite; assimilated L-sorbose, L-fucose, L-serine and L-asparagine; produced acid from D-arabinose, L-xylose, D-xylose, adonitol, methyl β -xyloside, L-sorbose, dulcitol, methyl α -D-mannoside, methyl α -D-glucoside, *N*-acetyl-glucosamine, amygdalin, arbutin, aesculin, salicin, inulin, melezitose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate and 5-keto-gluconate.

Characteristic	1	2	3	4	5	6
Presence of:						
Catalase	w	w	—	w	w	—
DNase	+	+	+	+	—	—
Degradation of:						
Hide powder azure	+	+	+	—	+	+
Starch	+	—	+	—	+	+
Xylan	+	+	+	—	+	+
Tween 20	+	+	+	—	+	+
Growth in:						
3% NaCl	—	—	—	+	+	+
4% NaCl	—	—	—	+	—	—
Assimilation of:						
L-Rhamnose	+	+	+	+	—	—
D-Melibiose	+	+	+	—	—	—
D-Raffinose	+	+	+	—	+	+
D-Ribose	+	+	+	+	—	—
D-Arabinose	—	—	—	—	+	+
<i>i</i> -Erythritol	—	—	—	+	—	—
Xylitol	—	—	—	+	—	—
D-Arabitol	—	—	—	+	—	—
D-Sorbitol	—	—	—	+	—	—
Ribitol	—	—	—	+	—	—
<i>myo</i> -Inositol	—	+	—	+	—	—
Pyruvate	+	+	+	+	—	—
Citrate	+	+	+	+	—	—
Malate	+	+	+	—	+	—
Succinate	+	+	+	+	—	—
L-Glutamate	+	+	+	+	—	—
L-Proline	+	+	+	+	—	—
L-Glutamine	+	+	+	+	—	—
Arginine	+	—	+	+	—	—
Acid production from:						
Erythritol	—	—	—	+	—	—
Ribose	+	+	—	+	—	+
Rhamnose	—	+	—	+	—	—
Inositol	—	+	—	+	—	—
Sorbitol	—	—	—	+	—	—
Melibiose	+	+	—	w	w	—
D-Raffinose	+	w	+	—	—	—
Starch	—	—	—	—	w	w
Glycogen	—	—	—	—	+	+
Xylitol	—	—	—	+	—	—
β -Gentiobiose	—	—	—	—	—	w
D-Turanose	—	+	+	—	+	+

Table 2. Mean fatty acid composition of the type strain of *Alicyclobacillus acidocaldarius* and strains FR-3 and FR-6^T after growth in medium BAM at 60 °C and of the type strain of *Alicyclobacillus acidoterrestris* and strains FR-1b and FR-11^T after growth in medium BAM at 50 °C

Strains: 1, *Alicyclobacillus acidocaldarius*; 2, FR-3; 3, FR-6^T; 4, *Alicyclobacillus acidoterrestris*; 5, FR-1b; 6, FR-11^T.

Fatty acid	Fatty acid composition (%) in strains:					
	1	2	3	4	5	6
14:0 anteiso	—	—	—	1.0	—	—
15:0 iso	2.2	0.5	1.7	—	2.6	5.4
15:0 anteiso	—	—	0.8	—	3.0	6.6
16:0 iso	1.1	—	1.4	—	0.8	0.9
16:0	0.7	0.7	0.9	1.1	2.3	2.1
17:0 iso	10.8	2.6	8.3	—	3.1	4.9
17:0 anteiso	3.0	2.3	8.4	0.6	7.2	10.3
ω -Cyclohexyl C17:0	48.9	53.1	51.8	71.6	66.2	56.8
ω -Cyclohexyl C19:0	33.3	40.9	26.7	25.8	14.8	13.3

cytochrome oxidase-negative and catalase is weak or negative. Yeast extract or growth factors are not required for growth. The major fatty acids are ω -cyclohexyl C17:0 and ω -cyclohexyl C19:0; branched-chain fatty acids are also present in large proportions. Strains do not reduce nitrate to nitrite. Gelatin, hide powder and starch are hydrolysed, but elastin and fibrin are not. Strains FR-11^T and FR-1b utilize many hexoses and disaccharides, but pentoses and polyols, with the exception of mannitol and glycerol, are not utilized as single carbon sources. L-Serine, L-asparagine, L-glutamate, L-glutamine, L-proline and L-arginine are not utilized as single carbon sources. Acid is produced from virtually the same sugars that are utilized as single carbon sources. The DNA of strain FR-11^T has a G+C content of 53.3 mol%. This bacterium was isolated from solfataric soils in the Furnas area of the Island of São Miguel, the Azores. Strain FR-11^T has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany as strain DSM 12489^T. Strain FR-1b (= DSM 12766) is an additional strain of this species.

Description of *Alicyclobacillus* genomic species 1

Alicyclobacillus species 1 strains form rod-shaped cells that are 2.1–4.2 μ m long and 0.5–0.8 μ m wide. Gram stain is positive. The cells are non-motile and terminal spores are formed. Sporangia are not swollen. Colonies on BAM medium are 1–2 mm in diameter after 72 h of growth and are not pigmented. Growth occurs above 40 °C and below 70 °C; the optimum growth temperature for strains FR-3 and FR-6^T is 60–63 °C. The

Fig. 3. 16S rRNA gene sequence based neighbour-joining phylogenetic tree showing the position of the new isolates within the cluster comprising the *Alicyclobacillus* species and their closest relatives. The position of the root was determined by the inclusion of *Bacillus subtilis* as an outgroup sequence. Scale bar, 5 inferred nucleotide substitutions per 100 nucleotides.

optimum pH is 3.5–4.0; growth does not occur at pH 2.0 or pH 6.0. Strains are cytochrome oxidase-negative and catalase is weak or negative. Yeast extract or growth factors are not required for growth. The major fatty acids are ω -cyclohexyl C17:0 and ω -cyclohexyl C19:0; branched-chain fatty acids are also present. Strains do not reduce nitrate to nitrite. Gelatin and hide powder are hydrolysed, but elastin and fibrin are not. Strain FR-3 does not hydrolyse starch. Strains FR-3 and FR-6^T utilize many hexoses and disaccharides, but pentoses and polyols, with the exception of mannitol and glycerol, are not utilized as single carbon sources. Several amino acids are utilized as single carbon sources. Acid is produced from virtually the same sugars that are utilized as single carbon sources. The DNA of strain FR-6^T has a G+C content of 60.3 mol%. This bacterium was isolated from solfataric soils in the Furnas area of the Island of São Miguel, the Azores. Strain FR-6^T has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany as strain DSM 11984^T. Strain FR-3 (= DSM 11983) is an additional strain of this species.

ACKNOWLEDGEMENTS

This work was supported in part by the European Community Biotech Programme (Extremophiles as Cell Factories) Contract No. BIO4-CT96-0488 and PRAXIS XXI Program (PRAXIS/PCNA/BIO/46/76), Portugal. L.A. was supported by a PRAXIS XXI scholarship (PRAXIS XXI/BM/12769/97). We also thank J. Burghardt (DSM, Germany) for the DNA–DNA reassociation values.

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