NOTE

Alicyclobacillus herbarius sp. nov., a novel bacterium containing ω-cycloheptane fatty acids, isolated from herbal tea

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A thermo-acidophilic Gram-positive bacterium, strain CP-1^T, which grows aerobically at 35–65 °C (optimum 55–60 °C) and at pH 3·5–6·0 (optimum pH 4·5–5·0), was isolated from a herbal tea made from the dried flowers of hibiscus. Phylogenetic analysis of the 16S rRNA gene sequences showed that this bacterium was clearly distinguishable from previously described species of the genera *Alicyclobacillus* and *Sulfobacillus*. Strain CP-1^T had unique ω-cycloheptane fatty acids as the major membrane lipid component, a characteristic which is peculiar to *Alicyclobacillus cycloheptanicus*. However, phenotypic and chemotaxonomic characteristics of strain CP-1^T were different from those of the type strain of *A. cycloheptanicus*. DNA–DNA hybridization between the type strains of *Alicyclobacillus* species and *Sulfobacillus disulfidooxidans* was < 20 %, indicating that strain CP-1^T represents a distinct species. On the basis of these results, the name *Alicyclobacillus herbarius* is proposed for this organism. The type strain is strain CP-1^T (= DSM 13609^T = IAM 14883^T = NRIC 0477^T).

Keywords: *Alicyclobacillus herbarius* sp. nov., ω-cycloheptane fatty acids, thermophiles, acidophiles

The genus *Alicyclobacillus* comprises thermo-acidophilic, heterotrophic, endospore-forming bacteria. Recently, *Alicyclobacillus hesperidum* has been proposed as a new species of *Alicyclobacillus* (Albuquerque *et al*., 2000) along with three other species described previously: *Alicyclobacillus acidocaldarius*, *Alicyclobacillus acidoterrestris* and *Alicyclobacillus cycloheptanicus* (Wisotzkey *et al*., 1992). All these species possess ω-cyclohexane fatty acids (*A. acidocaldarius*, *A. acidoterrestris* and *A. hesperidum*) or ω-cycloheptane fatty acids (*A. cycloheptanicus*) as the major membrane lipid component. The ω-cyclohexane fatty acids have also been observed in *Curtobacterium pusillum* (Suzuki *et al*., 1981), *Propionibacterium cyclohexanicum* (Kusano *et al*., 1997) and the species of the genus *Sulfobacillus* (Dufresne *et al*., 1996), but of the bacteria examined only *A. cycloheptanicus* has been found to possess ω-cycloheptane fatty acids (Deinhard *et al*., 1987b). The genus *Sulfobacillus* embraces a group of acidophilic, endospore-forming bacteria similar to the genus *Alicyclobacillus*. However, the species of the genus *Sulfobacillus* are facultatively autotrophic bacteria, whereas the species of the genus *Alicyclobacillus* are heterotrophic bacteria (Darland & Brock, 1971; Golovacheva *et al*., 1979; Deinhard *et al*., 1987a, b; Wisotzkey *et al*., 1992; Tourova *et al*., 1994; Norris *et al*., 1996). This is one of the main characteristics which differentiates the genus *Alicyclobacillus* from the genus *Sulfobacillus* (Dufresne *et al*., 1996). In this paper, we describe the characteristics of a novel *Alicyclobacillus* species that possesses ω-cycloheptane fatty acids.

Dried hibiscus flowers were suspended in distilled water, large particles were allowed to settle and the supernatant was filtered through a membrane filter (ADVANTEC TOYO; mixed cellulose ester; pore size 0·45 μm, diameter 47 mm). The filter was placed on the surface of YSG medium solidified with agar and the

The DDBJ/GenBank/EMBL accession numbers for the 16S rRNA gene sequence of *A. herbarius* sp. nov. CP-1^T, *A. acidocaldarius* ATCC 27009^T, *A. acidoterrestris* ATCC 49025^T, *A. acidoterrestris* DSM 3923 and *A. cycloheptanicus* DSM 4006^T are AB042055, AB042056, AB042057, AB042058 and AB042059, respectively.

plate was incubated at 50 °C for 5 days. The YSG medium contained (l^{-1}) 2 g yeast extract (Difco), 1 g glucose and 2 g soluble starch (Difco), and the pH of the medium was adjusted to 3·7 with 1 M H_2SO_4. Many thermo-acidophilic spore-formers isolated from raw materials of various beverages have been identified as *A. acidocaldarius* or *A. acidoterrestris* based on partial 16S rDNA sequence analyses (Goto *et al.*, 2000). However, according to the above analyses, strain CP-1^T was found to be distinct from previously described species of the genus *Alicyclobacillus*.

Cell morphology was examined by phase-contrast microscopy. Motility was observed using a microscope and prick test with *Bacillus acidocaldarius* medium (BAM medium) containing 0·05% of gellan gum. All biochemical tests were basically performed as described previously (Darland & Brock, 1971; Deinhard *et al.*, 1987a, b; Albuquerque *et al.*, 2000). These tests were repeated at least four times. Strain CP-1^T and *A. acidocaldarius* ATCC 27009^T were incubated at 55 °C, and *A. acidoterrestris* ATCC 49025^T and *A. cycloheptanicus* DSM 4006^T were incubated at 50 °C. The growth temperature range was examined by measuring the turbidity at 578 nm. The pH range for growth was examined in BAM medium with different pH values adjusted by addition of 1 M H_2SO_4. Acidification was examined with API 50 CH test strips (bioMérieux) in BAM basal salts medium. In the case where the API 50 CH test was unclear, the degree of acidification was judged by measuring the pH decrease ($> 0·4$) of the culture using BAM basal salts medium without indicator, to which carbon source (2 g l^{-1}) was added. Acidification was observed everyday for 7 days and the reading recorded was always the one with the strongest acidification.

The cultures used for quinone analysis were grown in 500 ml Erlenmeyer flasks containing 100 ml BAM medium at 50 or 55 °C in a reciprocal shaker for 4 days. Quinones were extracted from freeze-dried cells as described previously (Sano *et al.*, 1996) and were analysed by Alliance HPLC system equipped with 996 photodiode array detector (Waters). Mightysil RP-18 column (4·6 × 250 mm; Kanto Chemical) was used for separation and methanol:2-propanol (3:1) was used as the mobile phase. UV spectra of the peaks were used for identification of quinones.

The cultures for fatty acid analysis were grown in 500 ml Erlenmeyer flasks containing 100 ml semisynthetic basal medium (Hippchen *et al.*, 1981) at 50 or 55 °C in a reciprocal shaker for 4 days. Fatty acid methyl esters were obtained from fresh wet biomass by saponification, methylation and extraction as described previously (Bligh *et al.*, 1959; Metcalfe *et al.*, 1996; Kusano *et al.*, 1997). The fatty acid methyl esters were analysed using a Hewlett Packard HP5890 GC equipped with JEOL DX-303 mass spectrometer. A DB-1 fused silica capillary column (0·25 mm × 30 m; J & W) was used for separation. The temperature of the injection port was 250 °C, and that of the oven was programmed at 150–250 °C at a rate of 5 °C min^{-1}. Identification of the fatty acid methyl esters was performed by comparing mass spectra and retention time with fatty acid methyl ester standards (Supelco). The peak areas on the total ion chromatogram were used for quantification of the fatty acid methyl esters.

Preparation of genomic DNA and 16S rDNA sequencing were carried out as described previously (Goto *et al.*, 2000). The DNA G+C content was determined by HPLC as described previously (Tamaoka *et al.*, 1984). Levels of DNA relatedness were determined fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microplates. Multiple sequence alignment, calculation of nucleotide substitution rates (K_{nuc} values), construction of a neighbour-joining phylogenetic tree (Kimura, 1980; Saitou & Nei, 1987), and bootstrap analysis with 1000 replicates for evaluation of phylogenetic tree topology were carried out with the CLUSTAL W version 1.7 program (Thompson *et al.*, 1994). Alignment gaps and unidentified base positions were not taken into account for the calculations.

Cells of strain CP-1^T were Gram-positive, spore-forming, motile rods with rounded ends. Spores were oval and subterminal with swollen sporangia. Optimal growth occurred aerobically at 55–60 °C (range 35–65 °C) and pH 4·5–5·0 (range 3·5–6·0). Strain CP-1^T could grow heterotrophically in an inorganic medium containing glucose or starch, without any growth factors. Generation time in BAM medium at 55 °C was about 2·5 h. Strain CP-1^T had nitrate reductase and catalase was weakly detected. Voges–Proskauer test was negative. Starch and gelatin were not hydrolysed. Strain CP-1^T and reference strains produced acid from L-arabinose, ribose, D-glucose, D-fructose, D-mannose and mannitol, and none of the strains produced acid from adonitol, methyl β-xyloside, *N*-acetyl-glucosamine, dulcitol, inositol, inulin, L-fucose, D-arabitol, L-arabitol, gluconate and 2-keto-gluconate. In some cases, our results for acidification were in contradiction with those of previous reports (Deinhard *et al.*, 1987a, b; Albuquerque *et al.*, 2000). This may be because acidification can not always be clearly determined since liberation of ammonium from the amino acids which are constituents of the yeast extract masks the results (Berkeley *et al.*, 1984; Deinhard *et al.*, 1987a, b). Differential phenotypic characteristics of strain CP-1^T and *Alicyclobacillus* reference strains are shown in Table 1.

The major respiratory quinone of strain CP-1^T was menaquinone 7 (MK7) which reached 74% of the total, MK3 was also present at 24%, and MK8 was vestigial (2%). The major fatty acids of strain CP-1^T were ω-cycloheptane fatty acids, and the relative proportions of ω-cycloheptane fatty acids (ω-cycloheptylundecanoic acid, ω-cycloheptyltridecanoic acid and ω-cycloheptyl-α-hydroxyundecanoic acid) reached 73% of the total fatty acids (Table 2). The remainder was a mixture of straight- and branched-

Table 1. Differential characteristics of strain Cp-1^T and other species of the genus *Alicyclobacillus*

Strains: 1, Cp-1^T; 2, *A. cycloheptanicus* DSM 4006^T; 3, *A. acidocaldarius* ATCC 27009^T; 4, *A. acidoterrestris* DSM 3923; 5, *A. hesperidum* DSM 12489^T. +, Positive result; w, weakly positive result; −, negative result.

Characteristic	1	2	3	4	5*
Catalase	+	+	+	+	w
Oxidase	−	+	−	−	−
Starch hydrolysis	−	−	+	−	+
Gelatin hydrolysis	−	−	−	−	+
Nitrate reduced to nitrite	+	−	−	−	−
Growth in 5% NaCl	+	+	−	+	−
Acid production from:					
Glycerol	+	−	+	+	+
Erythritol	−	−	−	+	−
D-Arabinose	+	+	−	−	−
D-Xylose	+	+	+	+	−
L-Xylose	−	+	−	−	−
D-Galactose	+	−	+	+	+
L-Sorbose	−	+	−	−	−
Rhamnose	+	+	+	+	−
Sorbitol	−	+	−	+	−
Methyl α-D-mannoside	+	−	−	−	−
Methyl α-D-glucoside	+	−	+	+	−
Amygdalin	+	−	−	−	−
Arbutin	+	−	+	+	−
Aesculin	+	+	+	+	−
Salicin	+	−	+	+	−
Cellobiose	+	−	+	+	+
Maltose	+	−	+	+	+
Lactose	+	−	+	+	+
Melibiose	+	−	+	−	−
Sucrose	+	−	+	+	+
Trehalose	+	−	+	−	+
Melezitose	+	−	−	−	−
D-Raffinose	+	−	+	−	−
Starch	−	−	+	−	w
Glycogen	−	−	+	−	+
Xylitol	−	−	−	+	−
β-Gentiobiose	+	−	−	+	w
D-Turanose	+	−	−	−	+
D-Lyxose	−	+	−	−	−
D-Tagatose	−	+	−	−	−
D-Fucose	+	−	−	−	−
5-Keto-gluconate	+	+	−	−	−

* Data from Albuquerque *et al.* (2000).

chain fatty acids. The type strain of *A. cycloheptanicus* had predominantly ω-cycloheptane fatty acids and principally the same fatty acid composition as strain CP-1^T, while the type strains of *A. acidocaldarius* and *A. acidoterrestris* had large proportions of ω-cyclohexane fatty acids.

The G+C content of strain CP-1^T was 56·2 mol% which falls in the range for the genera *Alicyclobacillus* (53–60%) and *Sulfobacillus* (53–57%). Almost complete 16S rDNA sequences comprising 1514–1529 nucleotides of strain CP-1^T and four strains of the genus *Alicyclobacillus* were determined. Phylogenetic analyses based on the 16S rDNA sequences comprising 1389 nucleotides between positions 29 and 1476 [*Escherichia coli* positions (Brosius *et al.*, 1978)] showed that strain CP-1^T was located in the phylogenetic cluster composed of the species of the genera *Alicyclobacillus* and *Sulfobacillus* (Fig. 1). The sequence similarity values of strain CP-1^T to the species of the genus *Alicyclobacillus* and *Sulfobacillus disulfidooxidans* were 91·3–92·6%, whereas the similarity values of strain CP-1^T to *Sulfobacillus thermosulfidooxidans* and *Sulfobacillus acidophilus* were 84·7 and 82·1%, respectively. The phylogenetic analyses showed that strain CP-1^T was more closely related to the *Alicyclobacillus* species and *S. disulfidooxidans* than *S. thermosulfidooxidans* and *S. acidophilus*. The DNA–DNA reassociation values for strain CP-1^T and related strains, *A. acidocaldarius* ATCC 27009^T, *A. acidoterrestris* ATCC 49025^T, *A. cycloheptanicus* DSM 4006^T and *S. disulfidooxidans* DSM 12064^T were 10–16%. All of these values were well below the 70% cut-off point recommended by Wayne *et al.* (1987) for the recognition of genomic species.

Table 2. Cellular fatty acid composition of strain Cp-1^T and *Alicyclobacillus* strains

Strains: 1, Cp-1^T; 2, *A. cycloheptanicus* DSM 4006^T; 3, *A. acidocaldarius* ATCC 27009^T; 4, *A. acidoterrestris* DSM 3923; 5, *A. hesperidum* DSM 12489^T. TR, Trace.

Fatty acid	Composition (%)				
	1	2	3	4	5*
14:0	0·4	0·8			
15:0 iso	0·4	0·5	1·4	TR	5·4
15:0 anteiso	TR	TR		TR	6·6
15:0	TR	0·6			
16:0 iso	3·8	1·0	1·4		0·9
16:0	5·1	2·4		2·5	2·1
17:0 iso	5·6	2·7	1·9	1·2	4·9
17:0 anteiso	3·0	1·3	2·3	4·2	10·3
17:0	0·5				
18:0 iso	3·2				
18:0	2·4	1·3			
ω-Cyclohexane C17:0			78·0	67·4	56·8
ω-Cyclohexane C19:0			16·0	24·6	13·3
ω-Cycloheptane C18:0	67·1	86·8			
ω-Cycloheptane (2-OH) C18:0	3·5	2·3			
ω-Cycloheptane C20:0	4·5	TR			

* Data from Albuquerque *et al.* (2000).

With regard to nutrition, *Alicyclobacillus* is hetero-

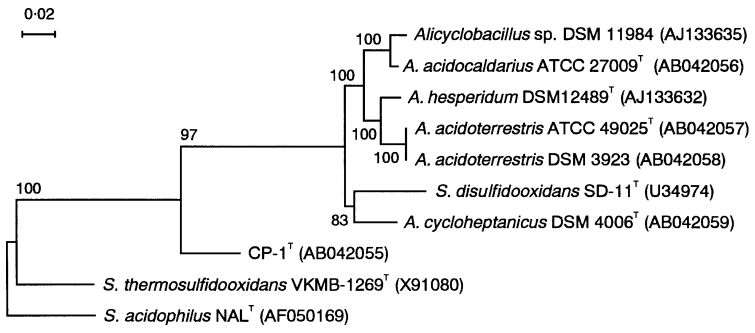

Fig. 1. Phylogenetic tree, based on neighbour-joining (Saitou & Nei, 1987), derived from an alignment comprising 16S rDNA sequences from *Alicyclobacillus* species and *Sulfobacillus* species. *S. acidophilus* NAL^T served as outgroup. The dataset was resampled 1000 times by using the bootstrap option and the percentage values are given at the nodes. Scale bar, number of substitutions per nucleotide position.

trophic, yielding energy by the oxidation of organic carbon compounds; whereas *Sulfobacillus* is mixotrophic, yielding energy by the oxidation of either organic carbon compounds or reduced sulfur (and iron) compounds, and this defines these two genera as being distinct from each other. All species of the genus *Alicyclobacillus* produce acid from various carbohydrates, while on the other hand no acid formation from carbohydrates is observed in any species of the genus *Sulfobacillus* (Darland & Brock, 1971; Golovacheva *et al.*, 1979; Deinhard *et al.*, 1987a, b; Wisotzkey *et al.*, 1992; Tourova *et al.*, 1994; Dufresne *et al.*, 1996; Norris *et al.*, 1996). Judging from these characteristics, we consider that it is appropriate to classify strain CP-1^T as a member of the genus *Alicyclobacillus*. On the basis of all obtained data, we conclude that strain CP-1^T is a new species of the genus *Alicyclobacillus*, for which the name *Alicyclobacillus herbarius* is proposed.

Description of *Alicyclobacillus herbarius* sp. nov.

Alicyclobacillus herbarius (her.ba′ri.us. L. fem. adj. *herbarius* herb, from which the organism was isolated).

Gram-positive, strictly aerobic, motile, spore-forming straight rods with rounded ends. Endospores are oval and subterminal with swollen sporangia. Colonies on BAM agar are circular and 2–3 mm in diameter after 72 h and are not pigmented. Temperature range for growth is 35–65 °C; the optimum growth temperature is 55–60 °C. The pH optimum is 4·5–5·0; growth does not occur at pH 3·0 or pH 6·5. Growth factors are not required, but growth is further increased by adding yeast extract to the inorganic medium. Oxidase-negative and catalase-positive. Nitrate is reduced to nitrite. Voges–Proskauer test and indole production are negative. Gelatin and starch are not hydrolysed. Acid formation is from the following carbon sources: glycerol, D-arabinose, L-arabinose, ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, rhamnose, mannitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arubutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, D-raffinose, β-gentiobiose, D-turanose, D-fucose, 5-keto-gluconate. Aesculin is hydrolysed. The major fatty acid is ω-cycloheptane C18:0. The main quinone is menaquinone 7. The G+C content is 56·2 mol%. Type strain is strain CP-1^T (= DSM 13609^T = IAM 14883^T = NRIC 0477^T).

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