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NOTE

Alicyclobacillus acidiphilus sp. nov., a novel thermo-acidophilic, ω-alicyclic fatty acid-containing bacterium isolated from acidic beverages

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A novel thermo-acidophilic bacterium was isolated from an acidic beverage that had the odour of guaiacol. The cells are aerobic, Gram-positive, spore-forming rods. The organism, strain TA-67^T, grows at temperatures from 20 to 55 °C (optimum, 50 °C) and at pH values from 2·5 to 5·5 (optimum, pH 3·0). It possesses ω-cyclohexane fatty acid as a major cellular fatty acid. The G+C content of the DNA is 54·1 mol%. Phylogenetic analysis of the 16S rRNA gene sequences indicated that strain TA-67^T constituted a distinct lineage in the *Alicyclobacillus* cluster, with *Alicyclobacillus acidoterrestris* as the closest neighbour (96·6% homology). Phenotypically, it is similar to, but can be distinguished from, ω-cyclohexane fatty acid-possessing alicyclobacilli (*A. acidoterrestris*, *Alicyclobacillus acidocaldarius*, *Alicyclobacillus hesperidum* and '*Alicyclobacillus mali*') by the morphology of spores and sporangia, by the growth response to different temperatures, and by the profiles for acid production from carbon sources. It is the alicyclobacillus that produces guaiacol, a causative substance for an 'off' flavour of orange juice. On the basis of the phenotypic and phylogenetic evidence, it is concluded that strain TA-67^T represents a new species of the genus *Alicyclobacillus*, for which the name *Alicyclobacillus acidiphilus* is proposed. The type strain is TA-67^T (= DSM 14558^T = IAM 14935^T = NRIC 6496^T).

Keywords: *Alicyclobacillus acidiphilus* sp. nov., ω-cyclohexane fatty acid, thermo-acidophile

The genus *Alicyclobacillus* is characterized by endospore-formation, a thermo-acidophilic nature, and the possession of ω-alicyclic fatty acid as a major cellular fatty acid (Hippchen *et al*., 1981; Poralla & Konig, 1983). Those organisms had been assigned to the genus *Bacillus* for two decades. In 1992, on the basis of phylogenetic distinctness revealed by comparative 16S rRNA gene sequence analysis and the possession of ω-alicyclic fatty acid, three thermo-acidophilic species, *Bacillus acidocaldarius* (Darland & Brock, 1971; Rosa *et al*., 1971), *Bacillus acidoterrestris* (Deinhard *et al*., 1987a), *Bacillus cycloheptanicus* (Deinhard *et al*., 1987b), were reclassified in a new genus, i.e. *Alicyclobacillus* (Wisotzkey *et al*., 1992). Recently, *Alicyclobacillus hesperidum* (Albuquerque *et al*., 2000), '*Alicyclobacillus mali*' and '*Alicyclobacillus acidocaldarius* subsp. *rittmannii*' (Nicolaus *et al*., 1998), which possess ω-cyclohexane fatty acid, and *Alicyclobacillus herbarius* (Goto *et al*., 2002), which possesses ω-cycloheptane fatty acid, have been reported. In the preceding studies, strains of the genus *Alicyclobacillus* had been isolated from geothermal sources, and, subsequently, also from non-geothermal soils. The isolation of thermo-acidophiles from spoiled apple juice was reported by Cerny *et al*. (1984); these were later recognized as *Alicyclobacillus acidoterrestris*. Since then, the occurrence of this species, which is the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Alicyclobacillus acidiphilus* sp. nov. TA-67^T is AB076660.

causative organism of an 'off' flavour, in spoiled fruit juices, has been reported in the US and Japan (Jensen, 1999; Pettipher *et al*., 1997; Splittstoesser *et al*., 1994; Yamazaki *et al*., 1996). In the hot summer of 1994, an outbreak of spoilage of fruit-juice products caused by thermo-acidophiles occurred in Europe. A microbiological survey of orange trees and garden soil conducted in Brazil, a raw-material-exporting country, showed that the primary niche of these micro-organisms was possibly soil (Eguchi *et al*., 1999). Alicyclobacilli are now generally considered to inhabit soil, and to contaminate, and occasionally grow in, fruit juices. Recently, they have been focused on as target organisms in quality control in the production of acidic beverages.

We isolated a new thermo-acidophilic, ω-cyclohexane fatty acid-possessing, spore-forming bacterium, strain TA-67^T, from an 'off'-flavoured acidic beverage as an organism capable of producing guaiacol. Phylogenetic analysis based on 16S rRNA gene sequences and characterization of phenotypic features revealed that the organism should constitute a new species in the genus *Alicyclobacillus*, for which the name *Alicyclobacillus acidiphilus* is proposed.

Strain TA-67^T was isolated from an 'off'-flavoured, acidic beverage. Diluted samples were spread on agar plates of YSG medium containing the following (l^{-1}): 2 g yeast extract (Difco), 2 g soluble starch (Merck), 1 g D-glucose and 15 g agar, pH 3·7, adjusted with 1 M H_2SO_4; the plates were incubated at 45 °C for 3 days. *A. acidocaldarius* ATCC 27009^T and *A. acidoterrestris* DSM 3923, obtained from the American Type Culture Collection (ATCC; Manassas, VA) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany), respectively, were used as reference strains. Strain TA-67^T and the reference strains were cultivated in *B. acidocaldarius* medium (BAM medium) at 45 °C unless otherwise stated (Deinhard *et al*., 1987a). For solid medium, 1·5% of agar was added. For cellular fatty acid analysis, strains were cultivated in a semi-synthetic basal medium for 3 days (Cerny *et al*., 1984).

For young cells, morphology was examined by phase-contrast microscopy. Motility was observed microscopically for young cells and by the prick test with BAM medium containing 0·05% gellan gum. The other biochemical tests were carried out according to *Bergey's Manual of Systematic Bacteriology* (Claus & Berkeley, 1986) and Deinhard *et al*. (1987a) with BAM medium as the basal medium. Acid formation from carbon compounds was determined using the method described by Deinhard *et al*. (1987a) with the API 50 CH kit (bioMérieux). When acidification was ambiguous, strains were cultivated in BAM basal salts medium with 0·2% carbon compounds added and the pH indicator omitted. After cultivation, a decrease in the pH values of cultured broths were measured with a pH meter. Carbon compounds that gave pH values lower than that of the control (i.e. culture without carbon compounds) by 0·4 or more were scored as positive. Growth responses to temperature and pH were examined by measuring the turbidity (at 578 nm) of cultures. The pH range and the optimum pH for growth were determined at optimum temperature for each strain. For guaiacol measurement, culture supernatants were filtered through Sep-Pak Plus tC18 (Waters) and a dehydration column. GC/MS was performed on a 5973 mass spectrometer (Hewlett Packard) in conjuction with a 6890 gas chromatograph (Hewlett Packard) equipped with an HP-5MS capillary column (30 m × 0·25 mm; Hewlett Packard).

Quinones were extracted from freeze-dried cells (200 mg) with a chloroform/methanol mixture (2:1) and examined by HPLC with a Crest Pack (C18T-5; Japan Spectroscopic Co.) according to the method described by Tamaoka *et al*. (1983). Cell lipids were prepared from about 1 g wet cells, according to the Bligh–Dyer method (Bligh & Dyer, 1959). Cells were broken using a Waring blender with glass beads (80–100 mesh), and the lipids were extracted with a chloroform/methanol mixture (2:1). The extracted lipids were hydrolysed at 60 °C for 5 min with methanol containing 7% boron trifluoride (Metcalfe *et al*., 1996). GC/MS was performed on a 5971 mass spectrometer (Hewlett Packard) in conjuction with a 5890 gas chromatograph (Hewlett Packard) equipped with a DB-23 capillary column (0·25 mm × 30 m; J&B Scientific). The column temperature was increased by 3 °C min^{-1} from 130 to 210 °C. Identification of the methyl esters was performed by comparing mass spectra and retention times with fatty acid methyl ester standards (Larodan).

For DNA preparation, about 1 g wet cells grown aerobically for 3 days was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8·0). Then, lysozyme (final concentration 20 mg ml^{-1}), achromopeptidase (20 mg ml^{-1}; Sigma), RNase A (50 μg ml^{-1}) and NaCl (10 mM) were added and the mixture incubated at 37 °C for 1 h. Total lysis was achieved by adding 10% SDS (final concentration 1%). Proteins were digested by incubation with Proteinase K (Boehringer Mannheim) at 65 °C for 2 h. The residual proteins were removed by either phenol or hexadecyltrimethylammonium bromide treatment, depending on the strain. For DNA–DNA hybridization experiments, DNAs were further purified by equilibrium centrifugation in CsCl-ethidium bromide gradients. Hybridization was carried out by using the photobiotin-microplate method described by Ezaki *et al*. (1989). For measurement of the G+C contents (mol%), the purified DNAs were hydrolyzed into nucleosides and analyzed by HPLC using a C18T-5 Crest Pack. The 16S rRNA genes were amplified by the PCR technique in combination with the 16S rRNA gene-specific primer pair. The PCR products were purified by agarose-gel electrophoresis. PCR-amplified 16S rDNA fragments were sequenced by using the reagents in a Dye Terminator FS Ready Reaction kit (Perkin-Elmer) and they were analyzed on an ABI 373A DNA sequencer (Perkin-

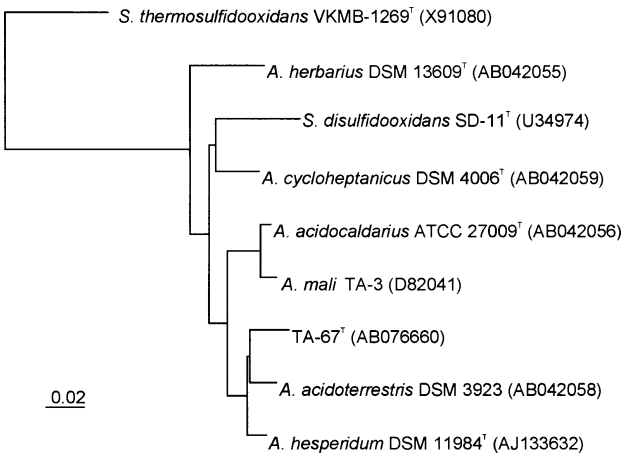

Fig. 1. Phylogenetic positions of the isolate based on analysis of 16S rRNA sequences. Scale bar, 2 inferred nuclotide substitutions per 100 nucleotides.

Elmer). Seven internal primers were designed at 3′ positions 248, 407, 525, 823, 985, 1240 and 1387 (*Escherichia coli* sequence numbering). The primary sequences of the 16S rRNA gene were generated by alignment and combining of the partial sequences by DNASIS software (Hitachi Software Engineering). The sequence of strain TA-67^T was aligned with published sequences obtained from the DDBJ, EMBL and GenBank databases (see Fig. 1). Bases that were uncertain, or gaps generated by the comparative alignment, were not taken into account for calculations. A total of 1387 nucleotides were used for analysis. A phylogenetic tree, calculated from a matrix of K_{nuc} values, was constructed using CLUSTAL W software, version 1.7 (Thompson *et al.*, 1994).

Differential characteristics for strain TA-67^T and other alicyclobacilli are listed in Table 1. Strain TA-67^T was a Gram-positive, motile, rod-shaped bacterium 0·9–1·1 μm in length and 4·8–6·3 μm in width. After 3 days incubation at 45 °C, the colonies on the surface of BAM agar plates were round, smooth, creamy white and opaque, and were 1·1–3·8 mm in diameter. Spores were ellipsoidal to oval and terminal to subterminal, and sporangia were swollen. The range of temperatures and the optimum were similar to those of *A. acidoterrestris*, *A. hesperidium* and '*A. mali*' and were lower than those of *A. acidocaldarius*. The pH range and optimum for growth were similar to those of the four reported species. It did not require growth factors, growing on glucose/NH_3-salts-based medium, as did other alicyclobacilli except *A. cycloheptanicus*. The isolate produced acid from various kinds of sugars. Sugar alcohols (except sorbitol and xylitol) were not used for acid production.

When orange juice was inoculated with the isolate and incubated at 40–50 °C, a minute amount of guaiacol was produced (data not shown). This is the substance that causes the 'off' flavour of fruit juices, and has been reported to be produced characteristically by *A. acidoterrestris* among alicyclobacilli (Yamazaki *et al.*, 1996).

Table 1. Differential characteristics of strain TA-67^T and other species of the genus *Alicyclobacillus*

Strains: 1, TA-67^T; 2, *A. acidocaldarius* ATCC 27009^T; 3, *A. acidoterrestris* DSM 3923; 4, *A. hesperidum* DSM 12489^T; 5, '*A. mali*' TA-3. +, Positive result; W, weakly positive result; −, negative result; ND, no data available. Acid is produced by all strains from the following: L-arabinose, arubtin, cellobiose, aesculin, D-fructose, galactose, D-glucose, maltose, D-mannose, sucrose, treharose, D-xylose. No acid is produced by any of the strains from the following: *N*-acetylglucosamine, adonitol, amygdalin, L-arabitol, dulcitol, D-fucose, L-fucose, D-lyxose, D-tagatose, gluconate, inulin, 2-ketoglucanate, 5-ketoglucanate, methyl β-xyloside, L-xylose.

Characteristic	1	2	3	4*	5
Catalase	+	+	+	−	W
Voges–Proskauer test	W	−	−	ND	−
Starch hydrolysis	−	+	−	+	+
Growth in 5% NaCl	−	−	+	−	−
Growth with lysozyme present	+	−	−	ND	−
Acid production from:					
D-Arabinose	+	−	−	+	−
Erythritol	−	−	+	−	−
β-Gentiobiose	+	+	+	W	−
Glycogen	−	+	−	+	+
Inositol	−	−	−	−	+
Lactose	+	+	+	+	−
Mannitol	−	+	+	+	+
Melezitose	+	−	+	−	−
Melibiose	−	+	−	−	−
Methyl α-D-glucoside	+	−	+	−	−
Methyl α-D-mannoside	−	−	+	−	−
D-Raffinose	+	+	−	−	−
Rhamnose	−	+	+	−	−
Ribose	+	+	+	+	−
Salicin	+	+	+	−	+
Sorbitol	+	−	+	−	−
L-Sorbose	+	−	−	−	−
Starch	−	+	−	−	+
D-Turanose	+	−	+	+	−
Xylitol	+	−	+	−	−

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

Strain TA-67^T had only MK-7 as a respiratory quinone system, whereas the other four species possessed MK-3 as a minor component besides MK-7 as a major one (Collins & Landworthy, 1983; Hiraishi *et al.*, 1997). The predominance of MK-7 is characteristic of the species of *Alicyclobacillus* and 'classical' *Bacillus*. Whole-cell fatty acid compositions are shown in Table 2. The possession of ω-cyclohexane fatty acid as the major acid distinguishes the isolate from *A. cycloheptanicus* and *A. herbarius*, which possess ω-cycloheptane fatty acid as the major acid. ω-Alicyclic acid is unique

Table 2. Cellular fatty acid composition of strain TA-67^T and *Alicyclobacillus* species

Strains: 1, TA-67^T; 2, *A. acidocaldarius* ATCC 27009^T; 3, *A. acidoterrestris* DSM 3923; 4, *A. hesperidum* DSM 12489^T; 5, '*A. mali*' TA-3. TR, Trace.

Fatty acid	1	2	3	4*	5
15:0 iso	–	1·4	TR	5·4	1·1
15:0 anteiso	0·3	–	TR	6·6	1·3
16:0 iso	–	1·4	–	0·9	1·4
16:0	1·5	–	2·5	2·1	1·7
17:0 iso	0·6	1·9	1·2	4·9	2·9
17:0 anteiso	2·6	2·3	4·1	10·3	5·8
ω-Cyclohexane C17:0	82·6	78·0	65·8	56·8	61·2
ω-Cyclohexane C19:0	12·3	16·0	24·0	13·3	23·5

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

as a cellular fatty acid among organisms and is characteristic of alicyclobacilli and sulfobacilli at the taxonomic level of genus; other micro-organisms reported as having these acids are *Curtobacterium pussilum* (Suzuki *et al.*, 1981) and *Propionibacterium cyclohexanicum* (Kusano *et al.*, 1997). Sulfobacilli are thermo-acidophilic and have been reported to possess ω-cyclohexane fatty acid as a major lipid component, but they differ in nutrition from alicyclobacilli as they are facultatively chemolithotrophic (Dufresne *et al.*, 1996; Golovacheva & Karavailo, 1978; Norris *et al.*, 1996).

The G+C content of the DNA of TA-67^T was 54·1 mol%, which was close to that of *A. acidoterrestris* (51·6 to 53·3 mol%) but lower than that of *A. acidocaldarius* (61·2 to 62·2 mol%) by 7–8 mol%. The phylogenetic tree reconstructed from the analysis of 16S rRNA gene sequences comprising 1404 nucleotides between positions 36 and 1490 showed that strain TA-67^T occupies a position in the ω-cyclohexane fatty acid-possessing cluster composed of *A. acidocaldarius*, *A. acidoterrestris*, *A. hesperidium* and '*A. mali*' (Fig. 1). The level of similarity between TA-67^T and *A. acidoterrestris* DSM 3923, the closest neighbour, was 96·6%. The DNA–DNA reassociation value between them was 48%. This value is rather higher than that expected from the similarity value of the 16S rRNA gene sequences, but is well below the relatedness that has been accepted for conspecific strains (Stackebrandt & Goebel, 1994). Strain TA-67^T clearly constitutes a distinct genomic species.

The phenotypic, morphological, physiological, biochemical and chemotaxonomic properties, the shape of spores and sporangia, the growth responses to different temperatures, the profiles of acid formation from carbon compounds, the resistance to lysozyme, the molecular composition of the respiratory quinone system, and the cellular fatty acid composition clearly differentiate strain TA-67^T from ω-cyclohexane fatty acid-possessing alicyclobacilli, as well as from ω-cycloheptane fatty acid-possessing counterparts.

The isolate clearly constitutes a distinct genomic species, as it had a 3·4% difference in similarity value with 16S rRNA gene sequences from the closest species, *A. acidoterrestris*, and a low DNA–DNA reassociation value with it. On the basis of the evidence mentioned above, we conclude that strain TA-67^T represents a new species of the genus *Alicyclobacillus*, for which the name *Alicyclobacillus acidiphilus* is proposed.

Description of *Alicyclobacillus acidiphilus* sp. nov.

Alicyclobacillus acidiphilus (a.ci.di'phi.lus. L. n. *acidum* acid, G. adj. *philus* loving; N.L. adj. *acidiphilus* acid-loving).

Cells are aerobic, Gram-positive, motile, spore-forming rods with the dimensions 0·9–1·1 × 4·8–6·3 μm. Colonies on BAM agar after 3 days incubation are circular, creamy white, flat and 1·1–3·8 mm in diameter. Catalase-positive and oxidase-negative. Growth factors are not required. The Voges–Prokauer test is weakly positive. Does not hydrolyse gelatin or starch. Tyrosine and phenylalanine are not degraded. Does not reduce nitrate to nitrite and does not form indole. Does not form gas from glucose. Acids are formed from D-arabinose, L-arabinose, arubtin, cellobiose, aesculin, D-fructose, galactose, β-gentiobiose, D-glucose, lactose, maltose, D-mannose, methyl α-D-glucoside, melezitose, D-raffinose, ribose, sucrose, salicin, sorbitol, L-sorbose, treharose, D-turanose and D-xylose. Does not produce acid from *N*-acetylglucosamine, adonitol, amygdalin, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, gluconate, glycerol, glycogen, inositol, inulin, 2-ketogluconate, 5-ketogluconate, D-lyxose, mannitol, melibiose, methyl α-D-mannoside, β-methylxyloside, rhamnose, starch, D-tagatose or L-xylose. The G+C content of the DNA is 54·1 mol%. The major menaquinone is MK-7. The major cellular fatty acids are ω-cyclohexylundecanoic acid and ω-cyclohexyltridecanoic acid. The type strain is TA-67^T (= DSM 14558^T = IAM 14935^T = NRIC 6496^T).

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