Alicyclobacillus ferrooxydans sp. nov., a ferrous-oxidizing bacterium from solfataric soil

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A novel mesophilic and acidophilic Gram-positive bacterium, designated strain TC-34^T, was isolated from solfataric soil. Strain TC-34^T grew aerobically at 17–40 °C and pH 2.0–6.0, and optimally at 28 °C and pH 3.0. Analysis based on 16S rRNA gene sequences showed that strain TC-34^T was phylogenetically related to members of the genus *Alicyclobacillus*, with the highest similarity (94.8%) to *Alicyclobacillus pomorum*. Strain TC-34^T showed a range of phenotypic characteristics that differentiated it from recognized *Alicyclobacillus* species, including growth temperature, assimilation of carbon sources and production of acids from a range of compounds. Strain TC-34^T was able to oxidize ferrous iron and its growth was correlated with the oxidation of Fe²⁺ in culture medium. ω -Alicyclic fatty acids were not detected. On the basis of these results, it was concluded that strain TC-34^T represents a novel species of the genus *Alicyclobacillus*, for which the name *Alicyclobacillus ferrooxydans* (type strain TC-34^T=JCM 15090^T=CGMCC 1.6357^T) is proposed.

The genus *Alicyclobacillus* was first proposed to accommodate three *Bacillus* species that contained ω -alicyclic fatty acids and were phylogenetically distant from other members of *Bacillus* (Wisotzkey *et al.*, 1992). Subsequently, the genus was emended by Goto *et al.* (2003) to include species that did not contain ω -alicyclic fatty acids, e.g. *Alicyclobacillus pomorum* (Goto *et al.*, 2003). Currently, the genus *Alicyclobacillus* contains 18 species with validly published names, two subspecies and two genomic species (Fig. 1 and <http://www.bacterio.cict.fr/a/alicyclobacillus.html>). Most members of the genus *Alicyclobacillus* are thermo-acidophilic, Gram-positive, spore-forming, heterotrophic organisms that often inhabit acidic geothermal environments such as geothermal water (Hiraishi *et al.*, 1997; Nicolaus *et al.*, 1998) and soil (Hippchen *et al.*, 1981; Tsuruoka *et al.*, 2003). Although the majority of *Alicyclobacillus* species mainly utilize organic compounds, *Alicyclobacillus tolerans* and *Alicyclobacillus disulfidooxidans* are able to oxidize ferrous iron, elemental sulfur and sulfides for growth (Kovalenko & Malakhova, 1983; Dufresne *et al.*, 1996). In this report, we describe a novel *Alicyclobacillus* strain, designated TC-34^T, which grows by means of oxidation of ferrous iron, pyrite, tetrasulfate and thiosulfate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TC-34^T is EU137838.

A table detailing the cellular fatty acid compositions of the type/reference strains of *Alicyclobacillus ferrooxydans* sp. nov. and related *Alicyclobacillus* species is available as supplementary material with the online version of this paper.

Strain TC-34^T was isolated from solfataric samples (temperature and pH ranges of 48–50 °C and 4.5–5.5, respectively) from Tengchong county (24° 38'–25° 52' N 98° 05'–98° 46' E), Yunnan Province, China, using modified Norris broth that contained a mineral salts medium (Norris *et al.*, 1996) supplemented with 14.0 g FeSO₄·7H₂O l⁻¹ and 0.2 g yeast extract l⁻¹. For the preparation of modified Norris solid medium, 14.0 g Gelrite Gellan Gum l⁻¹ (Sigma) was added to the modified broth. The samples collected were first enriched at 30 °C using modified broth. After three enrichments, the enriched culture was serially diluted (10-fold) in tubes containing 0.9 ml modified Norris broth. Aliquots (0.2 ml) of the diluted cultures were spread on plates to obtain individual colonies. Further purification of a single colony was carried out by repeated streaking on plates of modified Norris medium. The purity of strain TC-34^T was checked by means of 16S rRNA gene sequence analysis and the phenotypic homogeneity of the culture.

Cell morphology and flagellation were examined by using transmission electron microscopy of cells in the exponential growth phase in modified Norris broth. Physiological and chemotaxonomical characterization of strain TC-34^T was carried out using BAM medium (Deinhard *et al.*, 1987a), modified by the addition of 2.0 g soluble starch l⁻¹ and a reduction in the concentrations of yeast extract and glucose to final values of 0.2 and 0.5 g l⁻¹, respectively. Gram-reactions were determined according to the method described by Gerhardt *et al.* (1994). Endospore formation was observed after malachite green staining of cells from a

Fig. 1. Phylogenetic tree constructed using the neighbour-joining method according to 16S rRNA gene sequence evolutionary distances among strain TC-34^T and other *Alicyclobacillus* species. *Bacillus subtilis* IAM 12118^T was used as the outgroup. GenBank accession numbers are given in parentheses. Numbers at nodes are bootstrap percentages (only values greater than 50 % are shown) based on 1000 replicates. Bar, evolutionary distance (K_{nuc}) of 0.02 substitutions per nucleotide position.

6 day-old culture in modified BAM broth. Unless otherwise stated, catalase and oxidase activities, the Voges-Proskauer reaction, carbon source utilization and other biochemical characterization tests were performed as described by Deinhard *et al.* (1987a, b), Albuquerque *et al.* (2000) and Goto *et al.* (2002) using modified BAM media. The strains were characterized biochemically using the API ZYM, API 20NE and API 50 CH systems (bioMérieux), with modified BAM basal salts medium. Cell concentrates were resuspended in 15 ml modified BAM medium, at pH 4.0 and with 0.03 g bromophenol blue l⁻¹ as indicator, and distributed in the API 50 CH test strip wells. The results for acid production were read after incubation at 30 °C for 48–96 h, according to the manufacturer's instructions. The temperature range for growth was examined with a TN3F temperature-gradient incubator (Advantec) and modified Norris broth. The pH range of 0.5–7.0 (in increments of 0.5 pH units, adjusted by the addition of 1 M H₂SO₄) for growth was examined using modified BAM medium. Cell growth was determined by measuring the increase in optical density at 460 nm (OD₄₆₀).

For analysis of the cellular fatty acids, strain TC-34^T was grown for 5 days at 30 °C on modified BAM agar. Cells were harvested from the plates, and the fatty acids were methylated and analysed by using the Sherlock Microbial Identification System according to the manufacturer's instructions (Microbial ID). Isoprenoid quinones were extracted from freeze-dried cells (200 mg) with chloroform/methanol (2:1, v/v), and then separated from other components by using thin-layer chromatography. Purified isoprenoid quinones were analysed by reversed-phase HPLC with a ZOBAX ODS C₁₈ column (Agilent Co.), with acetonitrile/isopropyl alcohol (2:1.2, v/v) as the mobile phase. The DNA base composition was determined by using the thermal denaturation method (Marmur & Doty, 1962) with DNA from *Escherichia coli* K-12 as a reference.

A nearly complete 16S rRNA gene of strain TC-34^T was amplified with the primers 27f (5'-AGAGTTTGATCTTG-GCTCAG-3') and 1492r (5'-TACGGCTACCTTGTTACG-ACTT-3') and sequenced. Alignments of 16S rRNA gene

sequences were performed using the program CLUSTAL_X, version 1.64b (Thompson *et al.*, 1994), and positions with insertions or deletions were excluded during calculations. A neighbour-joining phylogenetic tree was constructed using MEGA 3.1 (Saitou & Nei, 1987; Kumar *et al.*, 2004), based on evolutionary distances that were calculated with the Kimura two-parameter model (Kimura, 1980).

Cells of strain TC-34^T were Gram-positive, aerobic, endospore-forming, coccoids or rods. Flagella were not observed. Catalase and oxidase were positive. Colonies of strain TC-34^T on plates of modified Norris medium were brown at the centre and yellow–orange in the periphery. The colonies were circular and flat, with entire margins, with diameters of 0.3–0.5 mm after incubation for 5–6 days. Carbon compounds that supported growth and acid production are given in the species description.

Strain TC-34^T grew at 17–40 °C and pH 2.0–6.0, and optimally at 28 °C and pH 3.0. The following compounds were tested as energy sources: 5.0 g elemental sulfur l⁻¹, 10 mmol K₂S₄O₆ l⁻¹, 5 mmol Na₂S₂O₃ l⁻¹ and 5.0 g FeS₂ l⁻¹. Strain TC-34^T oxidized ferrous iron, K₂S₄O₆ and pyrite as electron donors for growth, but not elemental sulfur. Fig. 2 clearly shows that the biomass production of strain TC-34^T was proportional to the oxidation of Fe²⁺. The graph in Fig. 2 also shows that oxidation of ferrous iron by strain TC-34^T was affected by temperature. Maximum oxidation of ferrous iron occurred at 28–30 °C, the same range as that for the optimal growth temperature. Oxidation of ferrous iron by other *Alicyclobacillus* species such as *A. tolerans*, *A. disulfidooxidans* (Karavaiko *et al.*, 2005) and *A. pohliae* (Imperio *et al.*, 2008) has been reported previously. This property of members of the genus *Alicyclobacillus* is considered to be related to habitat and is ecologically important.

Fig. 2. Growth of and Fe²⁺ oxidation by strain TC-34^T at different temperatures. Data shown are means of three parallel experiments. Error bars indicate SD. ■, Fe²⁺ oxidation rate; ●, OD₄₆₀. The experiments were conducted using modified Norris medium (Norris *et al.*, 1996).

The predominant cellular fatty acids of strain TC-34^T were anteiso-C_{15:0} (37.2 %), anteiso-C_{17:0} (25.3 %), iso-C_{16:0} (22.2 %) and iso-C_{15:0} (9.8 %). ω -Alicyclic fatty acids were not detected. This property was similar to *A. pomorum* (Goto *et al.*, 2003), but was different from the majority of *Alicyclobacillus* species. Detailed information on the cellular fatty acid composition is provided in the species description and a comparison of the fatty acid profiles of various *Alicyclobacillus* species is provided in Supplementary Table S1 (available in IJSEM Online). The major respiratory quinone of strain TC-34^T was menaquinone 7 (MK-7). A trace amount of MK-6 was also detected. The G+C content of the DNA of strain TC-34^T was 48.6 mol%, which was close to the range for other *Alicyclobacillus* species (48.7–62.7 mol%) (Karavaiko *et al.*, 2005).

The 16S rRNA gene sequence analysis showed that strain TC-34^T was phylogenetically related to members of the genus *Alicyclobacillus* (90.8–94.8 % similarity), with the highest similarity (94.8 %) with *A. pomorum*. The neighbour-joining tree (Fig. 1) showed that strain TC-34^T clustered with *Alicyclobacillus* species.

Based on the above phenotypic and phylogenetic studies, it is clear that strain TC-34^T represents a novel species of the genus *Alicyclobacillus*. Strain TC-34^T showed a range of phenotypic characteristics that were different from those of recognized *Alicyclobacillus* species (Table 1). The most distinctive features were that strain TC-34^T is mesotrophic, oxidizes ferrous iron and does not contain ω -alicyclic fatty acids. Other differences include the ability to assimilate various carbon sources and to oxidize inorganic ferrous iron or sulfuric compounds. The major cellular fatty acids are also significantly different from those of other *Alicyclobacillus* species.

Based on the results presented here, it is concluded that strain TC-34^T represents a novel species of the genus *Alicyclobacillus*, for which the name *Alicyclobacillus ferrooxydans* sp. nov. is proposed.

Description of *Alicyclobacillus ferrooxydans* sp. nov.

Alicyclobacillus ferrooxydans (fer.ro.ox'y.dans. L. n. *ferrum* iron; Gr. adj. *oxys* sharp, acid; N.L. v. *oxydare* to make acid, to oxidize; N.L. part. adj. *ferrooxydans* iron-oxidizing).

Cells are Gram-positive, strictly aerobic, non-motile, endospore-forming coccoids or rods with rounded ends (0.4–0.6 × 1.0–1.5 µm). Colonies on plates of modified Norris broth are brown in the centre, with the peripheries being yellow–orange, with diameters of 0.3–0.5 mm. Colonies on modified BAM agar are non-pigmented, pinpoint and circular, with entire margins, with diameters of 0.3–0.5 mm. Growth is stimulated by yeast extract. Growth occurs in the presence of 0–3 % NaCl, but not in 4 % NaCl. Oxidase, catalase and indole production are positive, but nitrate reduction, methyl red and Voges–Proskauer tests are negative. Hydrogen sulfide (H₂S) is not

Table 1. Phenotypic characteristics that differentiate strain TC-34^T from other *Alicyclobacillus* species

Strains: 1, strain TC-34^T (*A. ferrooxydans* sp. nov.); 2, *A. pomorum* 3A^T; 3, *A. contaminans* 3-A191^T; 4, *A. tolerans* K1^T (Karavaiko *et al.*, 2005); 5, *A. hesperidum* DSM 12489^T; 6, *A. macrosporangiidus* DSM 17980^T; 7, *A. acidoterrestris* DSM 3922^T; 8, *A. sacchari* DSM 17974^T; 9, *A. cycloheptanicus* DSM 4006^T; 10, *A. acidiphilus* TA-67^T; 11, *A. fastidiosus* DSM 17978^T; 12, *A. kakegawensis* DSM 17979^T; 13, *A. shizuokensis* DSM 17981^T; 14, *A. herbarius* DSM 13609; 15, *A. disulfidooxidans* DSM 12064^T (Karavaiko *et al.*, 2005); 16, *A. vulcanalis* CsHg2^T (Simbahan *et al.*, 2004); 17, *A. sendaiensis* JCM 11817^T (Tsuruoka *et al.*, 2003); 18, *A. pohliae* MP4^T (Imperio *et al.*, 2008); 19, *A. acidocaldarius* subsp. *acidocaldarius* 101-1A^T. If not indicated otherwise, data are from this study. For all tests, *A. hesperidum* DSM 12489^T was run in parallel with strain TC-34^T. When the results for *A. hesperidum* DSM 12489^T from this study were different from those reported by Albuquerque *et al.* (2000) and Goto *et al.* (2007), the results from this study are given and the previous results are given in parentheses. +, Positive; −, negative; ND, not determined; w, weakly positive; v, variable.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Temperature range for growth (°C)	17–40	30–60	35–60	20–55	35–60	35–60	35–55	30–55	40–53	20–50	20–55	40–60	35–60	35–65	4–40	35–65	40–65	42–60	45–70
pH range for growth	2.0–6.0	3.0–6.0	3.0–6.0	1.5–5.0	2.0–6.0	3.0–6.5	2.2–5.8	2.0–6.5	3.0–5.5	2.5–5.5	2.0–5.5	3.0–6.5	3.0–6.5	3.0–6.5	0.5–6.0	2.0–6.0	2.5–6.5	4.5–7.5	2.0–6.0
DNA G + C content (mol%)	48.6	53.1	60.3	48.7 ± 0.6	53.3	62.5	52.2	56.6	55.6	54.1	53.9	61.5 ± 0.2	60.5	56.2	53.0	62.0	62.3	55.1	60.3
Oxidase	+	+	−	w	−	−	−	−	+	−	−	−	−	−	−	ND	−	−	−
Catalase	+	+	−	w	− (+)	w	+	−	+	+	+	w	+	+	−	−	−	−	+
Hydrolysis of:																			
Gelatin	−	+	+	−	+	−	+	+	−	−	+	−	−	−	−	ND	ND	ND	+
Starch	+	+	−	+	+	−	−	+	−	−	−	−	−	−	+	+	ND	ND	+
Nitrate reduced to nitrite	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	ND	+	ND	−
Growth at 5% NaCl	−	−	−	ND	−	+	+	−	+	−	−	−	+	+	ND	−	ND	−	−
Acid production from:																			
Glycerol	−	+	+	w	+	+	+	+	−	−	−	−	−	+	+	ND	+	−	+
D-Arabinose	−	−	−	w	−	+	−	−	+	+	+	+	−	+	+	ND	+	+	−
L-Arabinose	+	−	+	w	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+
D-Xylose	−	−	+	+	+	+	−	+	+	+	+	+	+	+	+	ND	ND	+	+
L-Xylose	−	−	−	+	−	+	−	−	+	−	−	+	+	−	+	ND	ND	+	−
Methyl β-D-xyloside	+	−	−	+	−	−	−	+	−	−	+	−	−	−	ND	−	ND	−	−
D-Galactose	−	−	+	+	+	+	+	+	−	+	+	+	+	+	−	ND	+	+	+
L-Sorbose	−	+	v	+	−	+	−	−	+	+	−	+	−	−	+	ND	ND	+	−
L-Rhamnose	−	−	v	−	−	+	+	+	+	−	+	+	−	+	−	−	−	+	+
D-Mannitol	−	+	+	+	+	−	+	+	+	−	+	+	+	+	+	+	ND	−	+
D-Sorbitol	+	−	−	−	−	+	+	−	+	+	−	+	−	−	−	−	−	−	−
Methyl α-D-mannoside	+	−	−	+	−	+	−	−	−	−	−	+	−	+	ND	ND	ND	−	−
Amygdalin	−	+	−	+	−	−	−	−	−	−	−	+	−	+	+	ND	−	−	−
Arbutin	−	−	+	−	+	−	−	+	−	+	−	+	+	+	−	w	+	w	+
Aesculin	+	+	+	+	+	+	−	−	+	+	−	+	+	+	+	ND	−	+	+
Salicin	−	+	v	−	−	+	−	+	−	+	−	+	+	+	−	−	+	w	+
Cellobiose	−	−	+	−	+	−	+	+	−	+	−	+	+	+	+	ND	+	+	+
Maltose	−	+	+	w	+	+	+	+	−	+	−	+	+	+	+	ND	+	+	+

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
D-Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β -Gentiobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Turanose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Lyxose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Tagatose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5-Ketogluconate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

produced. Starch and casein are hydrolysed, but gelatin, Tween 80, aesculin and DNase are not. Phosphatase, esterase and lipase activities are positive. L-Lysine decarboxylases, L-ornithine decarboxylases and L-phenylalanine deaminase are absent. Utilizes the following carbon sources: L-arabinose, cellobiose, erythritol, L-fucose, D-mannose, maltose, D-fructose, D-sorbitol, D-galactose and D-glucose, but not glycerol, aesculin, melezitose, D-mannitol, raffinose, inositol, D-lactose, L-phenylalanine, L-cysteine, peptone or xylitol. Acid is produced from L-arabinose, methyl β -D-xyloside, methyl α -D-mannoside, trehalose, sorbitol, D-glucose, turanose, D-lyxose, D-tagatose, 5-ketogluconate and aesculin, but not from glycerol, erythritol, D-arabinose, D-xylose, L-xylose, D-galactose, L-sorbose, L-rhamnose, inositol, D-mannitol, methyl α -D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, maltose, D-lactose, melibiose, sucrose, inulin, melezitose, raffinose, glycogen, xylitol, β -gentiobiose, D-fucose, L-fucose or D-arabitol. Uses Fe^{2+} , $\text{K}_2\text{S}_4\text{O}_6$ and pyrite for energy. Cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0} and iso-C_{15:0}. Major isoprenoid quinone is menaquinone 7 (MK-7). The DNA G+C content of the type strain is 48.6 mol%.

The type strain, TC-34^T (=JCM 15090^T=CGMCC 1.6357^T), was isolated from solfataric soil of Tengchong county of Yunnan province, China.

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