Alicyclobacillus aeris sp. nov., a novel ferrous- and sulfur-oxidizing bacterium isolated from a copper mine

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A novel mesophilic, acidophilic, endospore-forming bacterium, designated strain ZJ-6^T, was isolated from Zi-Jin copper mine in Inner Mongolia, China. Cells of strain ZJ-6^T were rod-shaped, stained Gram-positive or were Gram-variable, and grew aerobically at 25–35 °C (optimum, 30 °C) and pH 2.0–6.0 (optimum, pH 3.5). 16S rRNA gene sequence analysis showed that strain ZJ-6^T was related phylogenetically to members of the genus *Alicyclobacillus*, with 16S rRNA gene sequence similarities of 89.5–94.2 %. Cells contained MK-7 as the major quinone and the DNA G+C content was 51.2 mol%. Strain ZJ-6^T possessed a number of phenotypic characteristics that differentiated it from recognized *Alicyclobacillus* species, including its growth temperature, assimilation of various carbon sources, production of acids from a range of compounds, and the ability to grow chemoautotrophically using ferrous iron, elemental sulfur and tetrathionate as electron donors. The predominant cellular fatty acids of strain ZJ-6^T were anteiso-C_{15:0} (67.1 %), iso-C_{16:0} (7.7 %) and anteiso-C_{17:0} (7.4 %); ω -alicyclic fatty acids were not found. On the basis of these results, it is concluded that strain ZJ-6^T represents a novel species within the genus *Alicyclobacillus*, for which the name *Alicyclobacillus aeris* sp. nov. is proposed; the type strain is ZJ-6^T (=CGMCC 1.7072^T=NBRC 104953^T).

Members of the genus *Alicyclobacillus* stain Gram-positive or are Gram-variable and are strictly aerobic, thermophilic or moderately thermophilic, acidophilic, spore-forming rods. Many members of the genus formerly belonged to the genus *Bacillus*, but were reclassified as members of a new genus, *Alicyclobacillus* (Wisotzkey *et al.*, 1992), because of their distinct 16S rRNA gene sequences and the occurrence of unique ω -alicyclic fatty acids. However, the description of the genus was subsequently amended to include organisms that possess straight- and branched-chain fatty acids due to the discovery of some species that do not possess ω -alicyclic fatty acids, e.g. *Alicyclobacillus pomorum* (Goto *et al.*, 2003), *Alicyclobacillus macrosporangiidus* and *Alicyclobacillus contaminans* (Goto *et al.*, 2007), *Alicyclobacillus pohliae* (Imperio *et al.*, 2008) and *Alicyclobacillus ferrooxydans* (Jiang *et al.*, 2008). At the time of writing (June 2008), the genus *Alicyclobacillus* was composed of 21 species with validly published names, two

subspecies and two genomic species (Fig. 1), with *Alicyclobacillus acidocaldarius* as the type species (Wisotzkey *et al.*, 1992; Goto *et al.*, 2006). Although most members of the genus *Alicyclobacillus* are heterotrophic organisms that often inhabit fruit beverages, 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), *Alicyclobacillus tolerans*, *Alicyclobacillus disulfidooxidans* and *A. ferrooxydans* are able to oxidize ferrous iron, elemental sulfur and sulfides for growth (Kovalenko & Malakhova, 1983; Dufresne *et al.*, 1996; Jiang *et al.*, 2008). Recently, some *Alicyclobacillus*-like strains that were also capable of oxidizing ferrous iron and metal sulfides were isolated from metal- or metal sulfide-rich ores and from a bioleaching column (Johnson & Hallberg, 2007; Wakeman, *et al.*, 2008).

During the course of a microbiological consortium survey of solfataric mine drainage (temperature and pH ranges were 25–35 °C and 2.5–5.5, respectively) of Zi-Jin copper mine located in Inner Mongolia, China, an acidophilic bacterial strain, strain ZJ-6^T, was isolated and purified. Strain ZJ-6^T grew heterotrophically as well as chemolithotrophically. It oxidized ferrous iron, elemental sulfur and tetrathionate and obtained energy for growth. Results of a

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ZJ-6^T is FM179383.

Phylogenetic trees based on 16S rRNA gene sequences constructed using the minimum-evolution and maximum-parsimony methods and a table showing the cellular fatty acid compositions of strain ZJ-6^T and related strains are available as supplementary material with the online version of this paper.

Fig. 1. Phylogenetic tree constructed with the neighbour-joining method based on 16S rRNA gene sequence evolutionary distances among strain ZJ-6^T and other *Alicyclobacillus* species. *B. subtilis* IAM 12118^T was used as the outgroup. GenBank accession numbers are given in parentheses. Numbers at nodes represent confidence levels from 1000 replicate bootstrap samplings; only values greater than 50 % are shown. Bar, 0.02 substitutions per nucleotide position.

polyphasic taxonomic study of strain ZJ-6^T involving assessment of its physiological, chemotaxonomic and phylogenetic properties are reported here.

Strain ZJ-6^T was isolated from solfataric copper mine drainage using modified Norris broth (Norris *et al.*, 1996; Jiang *et al.*, 2008). The collected samples were first enriched at 30 °C with the modified broth. After enriching three times, the enriched culture was serially diluted (10-fold) in tubes containing 0.9 ml modified Norris broth. Dilutions of 0.2 ml were spread onto plates to cultivate separate colonies. Further purification of single colonies was carried out by repeated streaking on modified Norris plates. The plates were incubated at 30 °C for 7 days. The purity of the strain was checked by 16S rRNA gene sequence analysis and the phenotypic homogeneity of culture.

Cell morphology and flagellation were examined during the exponential growth phase by scanning electron microscopy (Quanta 200; FEI) and transmission electron microscopy (H600; Hitachi). Physiological and chemotaxonomic characterization of strain ZJ-6^T was carried out with *Bacillus acidocaldarius* medium (BAM medium; Deinhard *et al.*, 1987a). Gram-staining reactions were determined according to the method described by Gerhardt *et al.* (1994). Endospore formation was observed after malachite green staining of cells from 6-day-old

cultures in BAM broth. Unless otherwise stated, catalase and oxidase activities, the Voges–Proskauer reaction, carbon source utilization tests and other biochemical characterization tests were performed as described previously (Deinhard *et al.*, 1987a, b; Albuquerque *et al.*, 2000; Goto *et al.*, 2002; Jiang *et al.*, 2008) in BAM media. Biochemical characterization was carried out according to *Bergey's Manual of Systematic Bacteriology* (Claus & Berkeley, 1986) and the *Determinative Manual for Routine Bacteriology* (Dong & Cai, 2001), complemented with the API ZYM, API 20NE and API 50 CH systems (all bioMérieux) using BAM basal salts medium. Cell concentrates were resuspended in 15 ml modified BAM medium (pH 4.0) with 0.03 g bromophenol blue l⁻¹ as indicator and samples were distributed in the API 50 CH test strip wells to test for acid production. The growth temperature range was examined with a TN3F temperature-gradient incubator (Advantec). Growth at various pH (0.5–7.0 at intervals of 0.5, adjusted by the addition of 1 M H₂SO₄) was examined in BAM medium. Cell growth was determined by measuring the increase in OD₅₇₈. The following compounds were tested as electron donors: elemental sulfur (5.0 g l⁻¹, sterilized by heating at 100 °C for 1 h, repeated twice on consecutive days); K₂S₄O₆ (10 mmol l⁻¹); Na₂S₂O₃ (5 mmol l⁻¹); FeSO₄ (14.0 g l⁻¹); and FeS₂ (5.0 g l⁻¹). Growth under anaerobic

conditions was examined according to Dong & Cai (2001) in both modified Norris broth and modified liquid BAM medium.

For analysis of cellular fatty acid patterns, strain ZJ-6^T was grown for 5 days at 30 °C on BAM agar. Cells were harvested from the plates and fatty acids were methylated and analysed using the Sherlock Microbial Identification system (MIDI), which is based on GC, 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 TLC. The purified isoprenoid quinones were analysed by reversed-phase HPLC equipped with a Zorbax ODS C₁₈ column (Agilent) and using acetonitrile/isopropylalcohol (2:1.2, v/v) as the mobile phase. The DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962) using DNA from *Escherichia coli* K-12 as a reference.

The nearly complete 16S rRNA gene of strain ZJ-6^T was amplified with the primers 27f (5'-AGAGTTTGATC-TTGGCTCAG-3') and 1492r (5'-GGTACCTTGTTAC-GACTT-3') and sequenced. Alignments of 16S rRNA gene sequences were performed with the program CLUSTAL_X version 1.64b (Thompson *et al.*, 1997) and positions with insertions or deletions were excluded during calculations. Phylogenetic trees were constructed with the neighbour-joining, maximum-parsimony and minimum-evolution tree-making algorithms 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 ZJ-6^T stained Gram-positive or were Gram-variable and were strictly aerobic, endospore-forming, straight rods that possessed peritrichous flagella (Fig. 2). Cells were negative for catalase and oxidase. Colonies of strain ZJ-6^T on modified Norris plates were brown-centred with yellow-orange peripheries, circular, flat and entire with diameters of 0.3–0.5 mm after incubation for 5–6 days. Colonies on BAM plates were creamy white, circular and entire with diameters of 0.5–1.0 mm after incubation for 3–5 days. Carbon compounds that were tested for their ability to support growth and produce acid are given in Table 1 and the species description.

Strain ZJ-6^T was mesophilic and grew at 25–35 °C with optimal growth at 30 °C, thus differing from the known thermophilic species of the genus *Alicyclobacillus*, which grow optimally at 42–60 °C and do not grow below 25 °C (Wisotzkey *et al.*, 1992; Goto *et al.*, 2003) (Table 1). Strain ZJ-6^T grew at pH 2.0–6.0, with optimal growth at pH 3.5. Strain ZJ-6^T oxidized ferrous iron, elemental sulfur and K₂S₄O₆ as electron donors for growth; however, no growth was observed using FeS₂ as the electron donor. Anaerobic growth did not occur. Supplementing Norris broth (mineral salts medium; Norris *et al.*, 1996) containing ferrous iron, elemental sulfur or K₂S₄O₆ as electron donor with yeast extract stimulated growth of strain ZJ-6^T.

Fig. 2. Scanning (a; bar, 5 µm) and transmission (b; bar, 1 µm) electron micrographs showing the morphology of strain ZJ-6^T and peritrichous flagella.

The predominant cellular fatty acids of strain ZJ-6^T were anteiso-C_{15:0} (67.1 %), iso-C_{16:0} (7.7 %), anteiso-C_{17:0} (7.4 %), iso-C_{14:0} (5.1 %) and iso-C_{15:0} (3.7 %). *ω*-Alicyclic fatty acid was not detected; this was also the case in *A. pomorum*, *A. macrosporangiidus*, *A. contaminans*, *A. pohliae* and *A. ferrooxydans*, but not in the other *Alicyclobacillus* species, which contain *ω*-alicyclic fatty acid as a characteristic fatty acid. The fatty acid profiles of strain ZJ-6^T and other *Alicyclobacillus* strains are provided in Supplementary Table S1 (available in IJSEM Online). Strain ZJ-6^T had MK-7 as the major respiratory quinone, which accounted for 97 % of the total, and MK-6 as a minor component (3 %). The DNA G+C content of strain ZJ-6^T was 51.2 mol%, which is within the range for recognized *Alicyclobacillus* species (48.7–62.7 mol%) (Karavaiko *et al.*, 2005).

16S rRNA gene sequence analysis showed that strain ZJ-6^T was related phylogenetically to members of the genus *Alicyclobacillus* (similarities of 89.5–94.2 % to type strains of species with validly published names), with the highest similarity (94.2 %) to the type strain of *A. ferrooxydans*. The neighbour-joining tree (Fig. 1) showed that strain ZJ-6^T clustered with strains of *Alicyclobacillus* species. This cluster was also supported topologically by the minimum-evolution and maximum-parsimony trees (see Supplementary Fig. S1 available in IJSEM Online).

Strain ZJ-6^T showed a range of phenotypic characteristics that differ from those of previously described

Table 1. Differential phenotypic characteristics of strain ZJ-6^T and strains of other *Alicyclobacillus* species

Strains: 1, ZJ-6^T (*A. aeris* sp. nov.); 2, *A. ferrooxydans* TC-34^T; 3, *A. pomorum* 3A^T; 4, *A. contaminans* 3-A191^T; 5, *A. tolerans* K1^T; 6, *A. hesperidum* DSM 12489^T; 7, *A. macrosporangiidus* DSM 17980^T; 8, *A. acidoterrestris* DSM 3922^T; 9, *A. sacchari* DSM 17974^T; 10, *A. cycloheptanicus* DSM 4006^T; 11, *A. acidiphilus* TA-67^T; 12, *A. fastidiosus* DSM 17978^T; 13, *A. kakegawensis* DSM 17979^T; 14, *A. shizuokensis* DSM 17981^T; 15, *A. herbarius* DSM 13609^T; 16, *A. disulfidooxidans* DSM 12064^T; 17, *A. vulcanalis* CsHg2^T; 18, *A. sendaiensis* JCM 11817^T; 19, *A. pohliae* MP4^T; 20, *A. acidocaldarius* subsp. *acidocaldarius* DSM 446^T. For all tests, *A. cycloheptanicus* DSM 4006^T was run in parallel with strain ZJ-6^T. When data for *A. cycloheptanicus* DSM 4006^T obtained in this study differed from that reported previously (Albuquerque *et al.*, 2000; Goto *et al.*, 2007), the results from this study are given and the previous results are provided in parentheses. Symbols: +, positive; −, negative; ND, not determined; w, weakly positive; v, variable between strains.

Characteristic	1*	2*	3†	4†	5‡	6‡	7†	8‡	9†	10*‡	11‡	12†	13†	14†	15‡	16‡	17§	18	19¶	20‡
Growth temperature (°C)	25–35	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
Growth pH range	2.0–6.0	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%)	51.2	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 reduction to nitrite	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	ND	+	ND	–
Growth in 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-xyloside	–	+	–	–	+	–	–	–	+	–	–	+	–	–	–	ND	–	ND	–	–
D-Galactose	+	–	–	+	+	+	+	+	+	–	+	+	+	+	+	–	ND	+	+	+
L-Sorbose	–	–	+	v	+	–	+	–	–	+	+	–	+	–	–	+	ND	ND	+	–
Rhamnose	+	–	–	v	–	–	+	+	+	+	–	+	+	–	+	–	–	–	+	+
Mannitol	+	–	+	+	+	+	–	+	+	+	–	+	+	+	+	+	+	ND	–	+
Sorbitol	+	+	–	–	–	–	+	+	–	+	+	–	+	–	–	–	–	–	–	–
Methyl α-D-mannoside	–	+	–	–	+	–	+	–	–	–	–	–	+	–	+	ND	ND	ND	–	–
Amygdalin	–	–	+	–	+	–	–	–	–	–	–	–	+	–	+	+	ND	–	–	–
Arbutin	–	–	–	+	–	+	–	–	+	–	+	–	+	+	+	–	w	+	w	+
Aesculin	+	+	+	+	+	+	+	–	–	+	+	–	+	+	+	+	ND	–	+	+
Salicin	–	–	+	v	–	–	+	–	+	–	+	–	+	+	+	–	–	+	w	+
Cellobiose	–	–	–	+	–	+	–	+	+	–	+	–	+	+	+	+	ND	+	+	+
Maltose	–	–	+	+	w	+	+	+	+	–	+	–	+	+	+	+	ND	+	+	+
Lactose	–	–	–	v	w	–	+	+	+	–	+	–	+	–	+	–	–	+	+	+
Melibiose	–	–	–	–	–	–	–	w	+	–	–	+	–	+	+	+	+	–	+	+
Sucrose	–	–	+	+	+	+	+	+	+	–	+	–	+	+	+	+	+	+	+	+
Trehalose	–	+	+	+	+	+	+	+	+	–(+)	+	+	+	+	+	–	+	+	w	+
β-Gentiobiose	–	–	–	v	ND	w	+	–	+	–	+	–	+	+	+	ND	–	ND	+	–
Turanose	–	+	+	–	w	+	–	–	+	–	+	–	+	–	+	–	+	+	+	–
D-Lyxose	–	+	–	–	ND	–	+	–	–	+	–	+	+	–	–	ND	ND	ND	+	–
D-Tagatose	–	+	+	v	w	–	–	–	–	+	–	+	v	–	–	+	ND	ND	+	–
5-Ketogluconate	+	+	+	–	+	+	–	–	–	+	–	–	–	–	+	+	w	–	+	–

*Data from this study.

†Data from Goto *et al.* (2007).‡Data from Karavaiko *et al.* (2005).§Data from Simbahan *et al.* (2004).||Data from Tsuruoka *et al.* (2003).¶Data from Imperio *et al.* (2008).

Alicyclobacillus species. These included differences in the ability to assimilate various carbon sources, produce acids, reduce nitrate, and oxidize ferrous iron and inorganic sulfuric compounds (Table 1). The major cellular fatty acids of strain ZJ-6^T were also significantly different from those of other *Alicyclobacillus* species.

Based on the above phenotypic and phylogenetic studies, it is clear that strain ZJ-6^T represents a novel species of the genus *Alicyclobacillus*, for which the name *Alicyclobacillus aeris* sp. nov. is proposed.

Description of *Alicyclobacillus aeris* sp. nov.

Alicyclobacillus aeris (ae'ris. L. gen. neut. n. *aeris* of ore/copper).

Cells are rods with rounded ends (0.4–0.6 × 1.5–2.5 µm), stain Gram-positive or are Gram-variable, and are strictly aerobic, motile and endospore-forming. Colonies on modified Norris broth are brown-centred with yellow–orange peripheries, circular, flat and entire with diameters of 0.3–0.5 mm after incubation for 5–6 days. Colonies on BAM plates are creamy white, circular and entire with diameters of 0.5–1.0 mm after incubation for 3–5 days. Temperature range for growth is 25–35 °C; optimum growth temperature is 30 °C. Optimum pH for growth is 3.5; growth occurs at pH 2.0–6.0. Cells grow well in BAM medium containing 0–2 % (w/v) NaCl, but are inhibited by 3 % NaCl. Oxidase, catalase and Voges–Proskauer reactions are negative. Indole and H₂S are not produced. Reduces nitrate. Gelatin and aesculin are hydrolysed, but casein, starch and Tween 80 are not. Leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase are positive; alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Grows on the carbon sources peptone, starch, raffinose, D-galactose, D-glucose, L-arabinose and adipate, but not on D-glucitol, L-rhamnose, D-sorbose, D-mannose, maltose, sucrose, D-fructose, D-xylose, cellobiose, inulin, mannitol, phenylacetate or caprate. Acid is produced from glycerol, erythritol, L-arabinose, ribose, D-xylose, L-xylose, adonitol, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, aesculin, xylitol, fucose, arabitol and 5-ketogluconate, but not from D-arabinose, methyl β-D-xyloside, L-sorbose, rhamnose, dulcitol, inositol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, β-gentiobiose, turanose, D-lyxose, D-tagatose, gluconate or 2-ketogluconate. Can grow chemoautotrophically by using ferrous iron, elemental sulfur and K₂S₄O₆ as electron donors. Major fatty acids are anteiso-C_{15:0}, iso-C_{16:0}, anteiso-C_{17:0}, iso-C_{14:0} and iso-C_{15:0}. The major isoprenoid quinone is MK-7.

The type strain is ZJ-6^T (=CGMCC 1.7072^T=NBRC 104953^T), isolated from Zi-Jin copper mine, Inner Mongolia, China. The DNA G+C content of the type strain is 51.2 mol%.

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