



Isolation and characterization of ferrous- and sulfur-oxidizing bacteria from Tengchong solfataric region, China

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Abstract

Microbial oxidation and reduction of iron and sulfur are important parts of biogeochemical cycles in acidic environments such as geothermal solfataric regions. Species of *Acidithiobacillus* and *Leptospirillum* are the common ferrous-iron and sulfur oxidizers from such environments. This study focused on the Tengchong solfataric region, located in Yunnan Province, Southwest China. Based on cultivation, 9 strains that grow on ferrous-iron and sulfuric compounds were obtained. Analysis of 16S rRNA genes of the 9 strains indicated that they were affiliated to *Acidithiobacillus*, *Alicyclobacillus*, *Sulfobacillus*, *Leptospirillum* and *Acidiphilium*. Physiological and phylogenetic studies indicated that two strains (TC-34 and TC-71) might represent two novel members of *Alicyclobacillus*. Strain TC-34 and TC-71 showed 94.8%–97.1% 16S rRNA gene identities to other species of *Alicyclobacillus*. Different from the previously described *Alicyclobacillus* species, strains TC-34 and TC-71 were mesophilic and their cellular fatty acids do not contain ω -cyclic fatty acids. Strain TC-71 was obligately dependent on ferrous-iron for growth. It was concluded that the ferrous-iron oxidizers were diversified and *Alicyclobacillus* species were proposed to take part in biochemical geocycling of iron in the Tengchong solfataric region.

Key words: ferrous-iron oxidation; sulfur oxidation; biogeochemical cycle; *Alicyclobacillus*, solfataric region

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Introduction

Geothermal areas are located in various parts of the earth, including terrestrial solfataric regions. Solfataric region is acidic hot springs, mud pots and soils; its low pH derives from the oxidation of sulfur, formed by the condensation of volcanic gases (H_2S and SO_2) (Johnson *et al.*, 2003). The well-studied solfataric region is probably the Yellowstone National Park (Wyoming, USA). The pioneering works on the microbiology of Yellowstone National Park by Brock (1978, 2001) had founded the cornerstone and opened different fields of the current acidothermophilic microbiology.

China has many geothermal regions, as exemplified by the Tengchong solfataric region, located in Yunnan Province, Southwest China. More than hundred solfataroles, thermol vents, and hot springs are distributed in Tengchong area. In the last few years, interest in the microbial community of this area has been increased. Using culture-dependent techniques, some novel species were cultivated and characterized, including *Acidianus tengchongensis* (He *et al.*, 2004), *Sulfolobus tengchongensis* (Xiang *et al.*, 2003), *Thermoanaerobacter tengcongensis* (Xue *et al.*, 2001), *Meiothermus rosaceus* (Chen *et*

al., 2002), *Thermus rehai* (Lin *et al.*, 2002). Culture-independent techniques were also exploited to understand the microbial community of this solfataric region (Cai *et al.*, 2006). The culture-independent studies revealed further that microbial community was very complex and more investigations are needed for understanding the microbial diversity and their ecological/geological functions in the solfataric regions.

Microorganisms living in solfataric region have minimal requirements for nutrients and serve as important producers and consumers in the solfataric microbial ecosystem (Skirnisdottir *et al.*, 2000). Their metabolic activities founded the bases of biogeochemical cycles of elements such as sulfur, arsenic, and iron in solfataric regions (Macur *et al.*, 2004; Kozubal *et al.*, 2008). In Tengchong solfataric region, acidothermophilic sulfur oxidizers such as *A. tengchongensis* and *S. tengchongensis* played important roles in sulfur oxidation (He *et al.*, 2004; Xiang *et al.*, 2003). Other heterotrophic acidophiles such as *Alicyclobacillus* species assimilating various carbon sources were also described (Chen *et al.*, 2004). Recently, a ferric-iron reducing *Acidiphilium* species was isolated (Liu *et al.*, 2007). In this report, we described the isolation and characterization of novel bacterial strains from Tengchong solfataric regions that could oxidize ferrous-iron and sulfur.

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1 Materials and methods

1.1 Samples and strains

Twenty samples were collected from 3 hot springs areas, namely Da-Gun-Guo (Site 1), Huai-Tai-Quan (Site 2) and Zhen-Zhu-Quan (Site 3), located at the Tengchong geothermal region. Sediment, sludge, water and soils from surroundings (48–50°C, pH 4.5–5.5) were collected and placed into 50-mL sterile polypropylene tubes or bags, transferred to the Beijing laboratory at room temperature. All samples were kept at 4°C until processed.

The type strains of *Alicyclobacillus hesperidum* (DSM12489), *Alicyclobacillus tolerans* (DSM16297) and *Alicyclobacillus cycloheptanicus* (NBRC 15310) were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Germany) and NBRC (NITE Biological Resource Center, Japan).

1.2 Cultivation, enrichment and isolation

Norris broth (Norris *et al.*, 1996) was modified for the purpose of growing ferrous-iron and sulfur oxidizing microorganism by supplementing yeast extract and using gelrite for solid medium. The modified Norris broth (pH 2.0) contains (per liter): MgSO₄·7H₂O 0.5 g, (NH₄)₂SO₄ 0.4 g, K₂HPO₄ 0.2 g, K₂S₄O₆ 0.15 g, KCl 0.1 g and yeast extract 0.2 g and 70 mL 20% (W/V) FeSO₄·7H₂O solution (dissolved in 0.1 mol/L H₂SO₄ and filter sterilized). Solid Norris medium/plates were made by adding 14 g/L Gelrite Gellan Gum (Sigma-Aldrich Co., China). Norris and modified Norris broths were used for enrichment, isolation and cultivation of ferrous-iron and sulfur oxidizing microorganisms. BAM medium (pH 4.0) (Deinhard *et al.*, 1987a) modified by adding FeSO₄·7H₂O (10 mg/L, final concentration) and soluble starch (2.0 g/L) to obtain the final concentrations of yeast extract and glucose 0.2 and 0.5 g/L, respectively, was used for phenotypic characterization of the isolated bacterial strains.

Unless otherwise stated, all enrichment and cultivation were conducted at 30 or 37°C for the purpose of obtaining mesophilic microorganisms.

Enrichment of ferrous-iron and sulfur oxidizing microorganisms was carried out with 50 mL modified Norris broth in 300-mL Erlenmeyer flasks on a rotary shaker at 200 r/min. Growth and microbial activity were visually observed from the color change of broth to a yellow or reddish-brown color, which resulted from the oxidation of ferrous-iron to ferric-iron. After three times repetition, the enrichments were serially 10-fold diluted with modified Norris broth. Dilutions of 0.2 mL were spread on modified Norris plates to obtain individual colony from single cell. Further purification of cultures was carried out by repeatedly streaking on the modified Norris plates.

1.3 Morphological observation and biochemical determination

Cell morphology and flagellation were examined using transmission electron microscopy (H-600, Hitachi, Japan) for the exponentially growing cells in modified Norris

broth. Endospore formation was observed after malachite green staining of 6 day-old cultures. Unless otherwise stated, catalase and oxidase activities, the Voges-Proskauer reaction, carbon source utilization, and other biochemical tests were performed as described in literature (Deinhard *et al.*, 1987a, 1987b; Albuquerque *et al.*, 2000; Goto *et al.*, 2002) in modified BAM broth and plates. Acid production from various carbon sources was examined with API 50 CH test strips (bioMérieux, Co., China) using an incomplete BAM medium (all organic carbon compound are removed). The concentrated cell suspension was mixed with 15 mL incomplete BAM medium (pH 4.0) containing 0.03 g/L of Bromophenol Blue as pH indicator, and then distributed into the API 50 CH test strip wells. Acid production was recorded as positive when the color changed from blue to yellow after 48–96 h cultivation at 30°C.

1.4 Determination of growth temperature and pH range

The growth temperature ranges of all isolated strains were examined with a TN3F temperature-gradient incubator (Advantec, Japan) and modified Norris broth. Temperatures range 10–60°C were examined. The pH range of 0.5–7.0 with interval 0.5, was adjusted by adding 1 mol/L H₂SO₄, and examined in modified BAM medium. The cell growth was determined by measuring the increase of optical density at 460 nm (OD₄₆₀) (BioSpec-1601, Shimadzu, Japan).

1.5 Determination of energy and carbon sources

Energy sources for strains growth were performed with the basal salts solution of modified Norris broth (any sulfur compounds except sulfate and ferrous-iron were deleted). The following compounds were individually added to the basal salt solution (g/L): sulfur 5.0, K₂S₄O₆ 1.5, Na₂S₂O₃ 1.5 and FeS₂ 5.0. The growth of strains in various carbon sources was observed with minimal BAM broth which contained the added sole carbon source.

1.6 16S rRNA gene sequences and phylogenetic analyses

The nearly complete 16S rRNA genes of all isolated strains were amplified with the general primers of 27F and 1492R and sequenced. Alignments of 16S rRNA gene sequences were performed with the CLUSTAL_X program, version 1.64b (Thompson *et al.*, 1994). A neighbour-joining phylogenetic tree was constructed using MEGA3.1 (Saitou and Nei, 1987) based on evolutionary distances that were calculated with the Kimura two-parameter model (Kimura, 1980).

1.7 GenBank accession numbers

The GenBank accession numbers for the nearly complete 16S rRNA genes of TC-34 and TC-71 are EU137838 and EU137837, respectively. Other GenBank accession numbers of partial 16S rRNA genes for bacterial isolates are AM937236–AM937240.

2 Results

2.1 Bacteria isolation

The temperature at the Tengchong hot springs usually is in the range of 50–90°C, and gradient from thermophilic to mesophilic temperatures occurs at the Tengchong geothermal region. On-site determined pH and temperature were 8.0 and 80°C for Da-Gun-Guo (Site 1), 6.0 and 70°C for Huai-Tai Quan (Site 2), and 6.0 and 65°C for Zhen-Zhu-Quan (Site 3). Considering that the previous work had been focused on the thermophiles (Chen *et al.*, 2002, 2004; Liu *et al.*, 2002; He *et al.*, 2004; Xiang *et al.*, 2003), this current study was focused on mesophiles, with particular interests in the sulfur and ferrous-iron oxidizers. For this purpose, the collected samples were firstly enriched at 30, 37, and 45°C with modified Norris broth. By means of dilution to extinction and plating the dilutions onto Norris plates, 9 pure cultures from the different samples were obtained (Table 1). The purity was checked by 16S rRNA gene sequence analysis and the phenotypic homogeneity of cultures.

2.2 Electron donors for growth

Except strain TCZ-33, all other bacterial isolates (Table 1) grew autochemotrophically and could oxidize sulfur, other sulfur compounds and ferrous-iron for energy, which reflect their adaptation to living environment. TCZ-30 grew only in the oxidation of Fe²⁺. The growth of strain TC-71 was obligately dependent on the occurrence of Fe²⁺ in medium, and could also take K₂S₄O₆ and pyrite as electron donors. Figure 1 shows clearly that the biomass production of strain TC-71 was proportional to the oxidation of Fe²⁺ at each tested temperature, and TC-34 showed similar features (Jiang *et al.*, 2008). It also indicated that the oxidation of Fe²⁺ by strains was affected by temperature, and it showed that the maximal oxidation of Fe²⁺ for both bacteria were in the range of 28–30°C.

2.3 Phylogenetic analysis of ferrous-iron and sulfur-oxidizing isolates

The nearly complete 16S rRNA gene of the 9 isolates were PCR-amplified and sequenced. BLAST search, alignments and analysis of these 16S rRNA gene sequences indicated that the 9 bacterial strains belonged to phylogenetically different groups (Table 1). Among them, TCZ-2-8, TCS-5 and TCZ-31 are closely related to

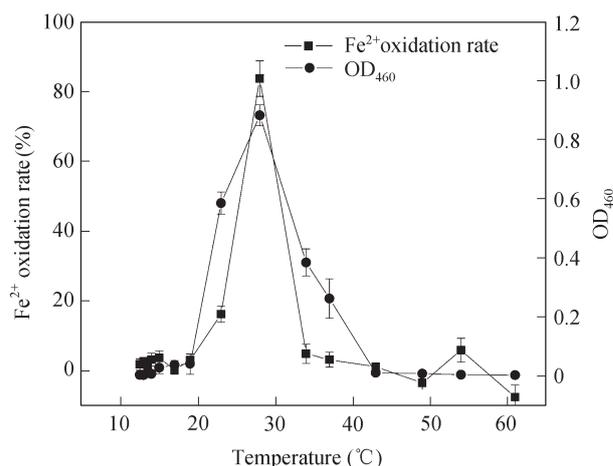


Fig. 1 Biomass production (OD₄₆₀) and Fe²⁺ oxidation by the strain TC-71.

members of the genus *Acidithiobacillus* (99.6%–99.9%); TCS-5-6 and TCZ-33 respectively, represented members of the genera *Sulfobacillus* and *Leptospirillum*; Teng-A is related to *Acidiphilium cryptum* (99.8%); and TC-34, TC-71, TCZ-30 are related to members of the genus *Alicyclobacillus* (94.8%–99.8%).

Strain TC-34 had 16S rRNA gene similarities of 90.8%–94.8% to members of the genus *Alicyclobacillus*, with the highest similarity to *Alicyclobacillus pomorum*. Strain TC-71 had 16S rRNA gene similarities of 91.5%–97.2% to members of the genus *Alicyclobacillus*, with the highest similarity to *A. cycloheptanicus*. The neighbour-joining tree (Fig. 2) showed that strain TC-34^T and TC-71^T clustered with *Alicyclobacillus* species and this cluster was strongly supported. The GenBank accession Nos. EU137838 and EU137837 are respectively for TC-34 and TC-71.

2.4 Morphology, growth optima and physiological properties of TC-34 and TC-71

Due to the phylogenetic novelty, strains TC-34 and TC-71 were subjected to more detailed studies on their phenotypes and phylogenetic relationship to other *Alicyclobacillus* species. As mentioned above, both strains were able to grow autotrophically, it was further found that their growth was enhanced when yeast extract was complemented in medium. Strain TC-34 also grew organotrophically in BAM medium, but strain TC-71 could not.

Cells of strains TC-34 and TC-71 were Gram-positive,

Table 1 Acidophilic and sulfur and/or ferrous-iron oxidizing isolates from solfataric regions of Tengchong County, Yunnan Province, China

Strain	Energy source for growth	Growth temperature (°C)	Closest relatives and 16S rRNA identities (%)	Sampling site
TCZ-2-8	S ⁰ , Na ₂ S, K ₂ S ₄ O ₆ , Na ₂ S ₂ O ₃ , pyrite	30–40	<i>Acidithiobacillus ferrooxidans</i> (99.6)	Sludge surrounding site 2
TCS-5	S ⁰ , Na ₂ S, K ₂ S ₄ O ₆ , Na ₂ S ₂ O ₃	25–37	<i>Acidithiobacillus thiooxidans</i> (99.9)	Sludge surrounding site 2
TCZ-31	Fe ²⁺ , S ⁰ , K ₂ S ₄ O ₆ , pyrite	30–55	<i>Acidithiobacillus ferrooxidans</i> (99.7)	Sludge surrounding site 3
TCZ-30	Fe ²⁺	Unknown	<i>Alicyclobacillus</i> sp. CLD (99.8)	Sludge surrounding site 1
TC-34	Fe ²⁺ , K ₂ S ₄ O ₆ , pyrite	17–40	<i>Alicyclobacillus pomorum</i> (94.8)	Sludge surrounding site 2
TC-71	Fe ²⁺ , K ₂ S ₄ O ₆ , pyrite	22–40	<i>Alicyclobacillus cycloheptanicum</i> (97.2)	Sludge surrounding site 2
TCS-5-6	Fe ²⁺ , K ₂ S ₄ O ₆ , pyrite	30–55	<i>Sulfobacillus thermosulfidooxidans</i> (99.4)	Sludge surrounding site 2
TCZ-33	Fe ²⁺ , pyrite	30–45	<i>Leptospirillum ferriphilum</i> (99.6)	Sludge surrounding site 1
Teng-A	Glucose, S ⁰ , Fe ³⁺ , K ₂ S ₄ O ₆ , Na ₂ S ₂ O ₃	20–45	<i>Acidiphilium cryptum</i> (99.8)	Sludge surrounding site 2

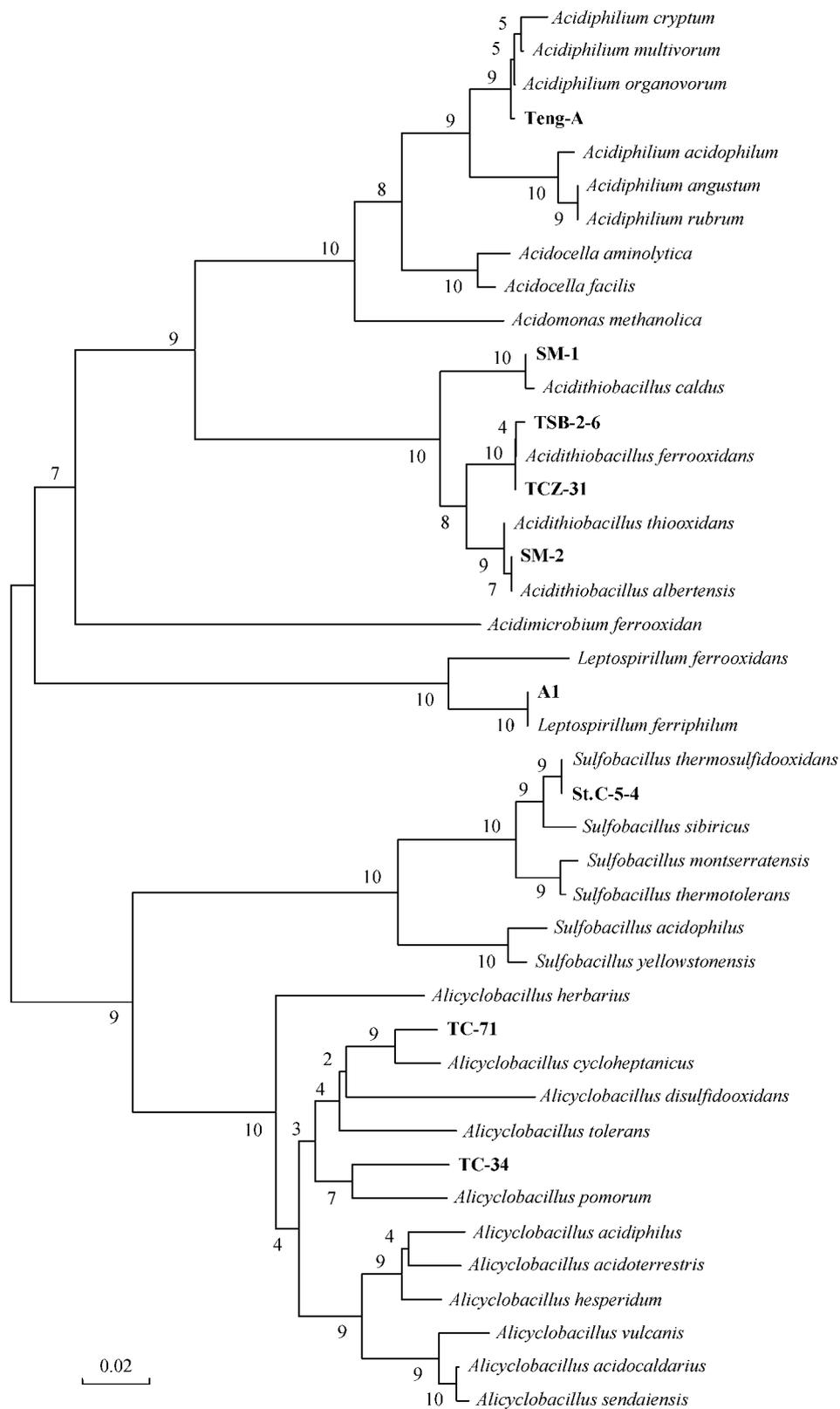


Fig. 2 Phylogenetic analysis of ferrous-iron and/or sulfur-oxidizing isolates from Tengchong solfataric region. Strains from this study are shown in bold.

aerobic, spore-forming coccoid rods or rods. Flagella were not observed. Colonies of TC-34 on modified Norris plates were brown centered with yellow-orange peripheries, circular, flat, entire with a diameter of 0.3–0.5 mm after incubation for 5–6 d. Colonies of TC-71 on modified Norris plates were ferruginous, circular, flat, entire with a diameter of 0.4–0.6 mm after incubation for 4–5 d. The strain TC-34 grew at 17–40°C and at pH 1.0–5.0. The optimal conditions are at 28°C and pH 2.0. Strain TC-71 grew at 22–40°C and pH 1.0–5.0 with optimum at 28°C and pH 2.0.

The phylogenetic studies clearly indicate that strains TC-34 and TC-71 represent two new members of the genus *Alicyclobacillus* (Fig. 2). When compared to the previously described species of *Alicyclobacillus*, strains TC-34 and TC-71 showed a range of distinctive features that phenotypically differentiated them from other *Alicyclobacillus* species. The most distinctive features are that they were mesophilic, and oxidizing ferrous-iron and sulfuric compounds, and cells did not contain ω -alicyclic fatty.

3 Discussion

The microbe-mediated iron oxidation and reduction under acidic conditions are important processes which govern geochemical cycles of elements in environments such as acid mine drainage (Küsel *et al.*, 2002; Blöthe *et al.*, 2008), deep-sea thermal vents (Reysenbach *et al.*, 2006), and geothermal solfataric region like Yellowstone National Park (Kozubal *et al.*, 2008). Previous research revealed that *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* were the common inhabitants and played the major roles in the biogeochemical cycling of iron in these environments (Goebel and Stackebrandt, 1994; Johnson *et al.*, 2001). In addition to these Fe²⁺ oxidizing species, this study revealed that novel members of *Alicyclobacillus* oxidizing Fe²⁺ were also universal inhabitants at the Tengchong solfataric region. The ability of strains TC-34 and TC-71 to thrive on wide range of conditions and to take Fe²⁺ and various sulfur compounds as electron donors suggested that they are well adapted to the variables of pH, temperature and alterations of sulfur and iron contents. Although this study did not quantify the population of the Fe²⁺ oxidizing *Alicyclobacillus* species, the culture-independent survey on the microbial diversity revealed that 16S rRNA gene sequences representing members of the phylum Firmicutes were the most abundant sequences of the Tengchong solfataric samples (data not shown). Moreover, a previous work reported the isolation of other strains of *Alicyclobacillus* from the same regions (Chen *et al.*, 2004). We deduced that the Fe²⁺ oxidizing *Alicyclobacillus* population might be significant for the solfataric ecosystems. Given the results that strain TC-71 growth was obligately dependent on Fe²⁺, we further proposed that *Alicyclobacillus* population play an important role in the biogeochemical cycling of iron in the Tengchong solfataric region.

Currently, the genus *Alicyclobacillus* contains 17

species and 2 subspecies (Euzéby, 1997, updated website at <http://www.bacterio.cict.fr>) and many others detected but not yet described members (as represented by GenBank acc. No. AY371273 and AY371274). *Alicyclobacillus* species live in very diversified environments including geothermal spring water and acidic soil (Simbahan *et al.*, 2004; Albuquerque *et al.*, 2000; Chen *et al.*, 2004), spoiled fruit juice (Goto *et al.*, 2003), and herbal tea (Goto *et al.*, 2002). Not only ecologically diversified, this community is also phenotypically heterologous and covers both autotrophiles and heterotrophiles. Members of this community may contain ω -alicyclic fatty acid or may not (Goto *et al.*, 2003). The previously described *Alicyclobacillus* species are thermophilic, but the strains TC-34 and TC-71 are mesophilic. Strain TC-34 and TC-71 have DNA G+C molar contents of 48.6% and 53.6%, respectively, which fall into the range of DNA G+C molar contents described for the genus *Alicyclobacillus* (48%–62%). Strains TC-34 and TC-71 do not have ω -alicyclic fatty acid, which are similar to *Alicyclobacillus pomorum* (Goto *et al.*, 2003) but different from the majority of *Alicyclobacillus* species. It is deduced that strain TC-34 and TC-71 represent two novel *Alicyclobacillus* species, but more detailed studies on their phenotypic and phylogenetic properties are needed for valid specific descriptions. Strain TC-34 has recently been identified as a novel species of the genus *Alicyclobacillus*, and was named *Alicyclobacillus ferrooxidans* (Jiang *et al.*, 2008).

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References

- Albuquerque L, Rainey F A, Chung A P, Sunna A, Nobre M F, Grote R *et al.*, 2000. *Alicyclobacillus hesperidum* sp. nov. and a related genomic species from solfataric soils of Sao Miguel in the Azores. *International Journal of Systematic and Evolutionary Microbiology*, 50: 451–457.
- Blöthe M, Akob D M, Kostka J E, Göschel K, Drake H L, Küsel K, 2008. Heterogeneity of Fe(III)-reducing microorganisms in coal-mining lake sediments caused by a pH gradient. *Applied and Environmental Microbiology*, 74: 1019–1029.
- Brock T D, 1978. *Thermophilic Microorganism and Life at High Temperatures*. New York: Springer-Verlag.
- Brock T D, 2001. The origins of research on thermophiles. In: *Thermophiles: Biodiversity, Ecology and Evolution* (Reysenbach A L, Voytek A, eds.). New York: Kluwer Academic/Plenum 1–9.
- Cai Y, Chen X Z, Yang K Q, Huang L, Dong Z Y, 2006. Construction and analysis of a metagenomic library from Tengchong hot spring soil in Yunnan Province. *Acta Microbiologica Sinica*, 46: 427–431.
- Chen C Y, Lin L B, Peng Q, Ben K L, Zhou Z M, 2002. *Meiothermus rosaceus* sp. nov. isolated from Tengchong hot spring in Yunnan, China. *FEMS Microbiology Letters*, 216: 263–268.
- Chen Z, Jiang C, Liu S, 2004. Survey on and phylogeny of *Alicyclobacillus* species in hot springs of southern China's Guangdong and Yunnan Provinces. *Microbiology*, 31: 50–

54.

- Deinhard G, Blanz P, Poralla K, Altan E, 1987a. *Bacillus acidoterrestris* sp. nov., a new thermotolerant acidophile isolated from different soils. *Systematic Applied Microbiology*, 10: 47–53.
- Deinhard G, Saar J, Krischke W, Poralla K, 1987b. *Bacillus cycloheptanicus* sp. nov., a new thermoacidophile containing alicycloheptane fatty acids. *Systematic Applied Microbiology*, 10: 68–73.
- Euzéby J P, 1997. List of bacterial names with standing in nomenclature. *International Journal of Systematic Bacteriology*, 47: 590–592. List of Prokaryotic names with Standing in Nomenclature. Updated on December 01, 2007. <http://www.bacterio.net>.
- Geobel B M, Stackebrandt E, 1994. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. *Applied and Environmental Microbiology*, 60: 1614–1621.
- Goto K, Matsubara H, Mochida K, Matsumura H, Hara K, Niwa M, Yamasato K, 2002. *Alicyclobacillus herbarius* sp. nov., a novel bacterium containing omega-cycloheptane fatty acids, isolated from herbal tea. *International Journal of Systematic and Evolutionary Microbiology*, 52: 109–113.
- Goto K, Mochida K, Asahara M, Suzuki M, Kasai H, Yokota A, 2003. *Alicyclobacillus pomorum* sp. nov., a novel thermoacidophilic, endospore-forming bacterium that does not possess ω -alicyclic fatty acids, and emended description of the genus *Alicyclobacillus*. *International Journal of Systematic and Evolutionary Microbiology*, 53: 1537–1544.
- He Z G, Zhong H, Li Y, 2004. *Acidianus tengchongensis* sp. nov., a new species of acidothermophilic archaeon isolated from an acidothermal spring. *Current Microbiology*, 48: 159–163.
- Jiang C Y, Liu Y, Liu Y Y, You X Y, Guo X, Liu S J, 2008. *Alicyclobacillus ferrooxidans* sp. nov., a novel ferrous-oxidizing bacterium from solfataric soil. *International Journal of Systematic and Evolutionary Microbiology*, 58: 2898–2903.
- Johnson D B, Okibe N, Roberto F F, 2003. Novel thermoacidophilic bacteria isolated from geothermal sites in Yellowstone National Park: physiological and phylogenetic characteristics. *Archives of Microbiology*, 180: 60–68.
- Johnson D B, Rolfe S, Hallberg K B, Iversen E, 2001. Isolation and phylogenetic characterization of acidophilic microorganisms indigenous to acidic drainage waters at an abandoned Norwegian copper mine. *Environmental Microbiology*, 3: 630–637.
- Kimura M, 1980. A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequence. *Journal of Molecular Evolution*, 16: 111–120.
- Kozubal M, Macur R E, Korf S, Taylor W P, Ackerman G G, Nagy A, Inskeep W P, 2008. Isolation and distribution of a novel iron-oxidizing Crenarchaeon from acidic geothermal springs in Yellowstone National Park. *Applied and Environmental Microbiology*, 74: 942–949.
- Küsel K, Roth U, Drake H L, 2002. Microbial reduction of Fe(III) in the presence of oxygen under low pH conditions. *Environmental Microbiology*, 4: 414–421.
- Lin L B, Chen C Y, Peng Q, Ben K L, Zhou Z M, 2002. *Thermus rehai* sp. nov., isolated from Rehai of Tengchong, Yunnan Province, China. *Journal of Basic Microbiology*, 42: 337–344.
- Liu Y Y, Chen Z W, Jiang C Y, Liu S J, 2007. Isolation and characterization of *Acidiphilium* strain teng-A and its metabolism of Fe(III) during pure- and mixed cultivation. *Acta Microbiologica*, 47: 350–354.
- Macur R E, Langner H W, Kocar B D, Inskeep W P, 2004. Linking geochemical processes with microbial community analysis: successional dynamics in an arsenic-rich, acid-sulfate-chloride geothermal spring. *Geobiology*, 2: 163–177.
- Norris P R, Clark D A, Owen J P, 1996. Characteristics of *Sulfobacillus acidophilus* sp. nov. and other moderately thermophilic mineral-sulphide-oxidizing bacteria. *Microbiology*, 142: 775–783.
- Reysenbach A L, Liu Y, Banta A B, Beveridge T J, Kirshtein J D, Schouten S *et al.*, 2006. A ubiquitous thermoacidophilic archaeon from deep-sea hydrothermal vents. *Nature*, 442: 444–447.
- Saitou N, Nei M, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology Evolution*, 4: 406–425.
- Simbahan J, Drijber R, Blum P, 2004. *Alicyclobacillus vulcanalis* sp. nov., a thermophilic, acidophilic bacterium isolated from Coso Hot Springs, California, USA. *International Journal of Systematic and Evolutionary Microbiology*, 54: 1703–1707.
- Skirmisdottir S, Hreggvidsson G O, Hjörleifsdottir S, Marteinson V T, Petursdottir S K, Holst O *et al.*, 2000. Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. *Applied and Environmental Microbiology*, 66: 2835–2841.
- Thompson J D, Higgins D G, Gibson T J, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22: 4673–4680.
- Xiang X Y, Dong X Z, Huang L, 2003. *Sulfolobus tengchongensis* sp. nov., a novel thermoacidophilic archaeon isolated from a hot spring in Tengchong, China. *Extremophiles*, 7: 493–498.
- Xue Y, Xu Y, Liu Y, Ma Y, Zhou P, 2001. *Thermoanaerobacter tengchongensis* sp. nov., a novel anaerobic, saccharolytic, thermophilic bacterium isolated from a hot spring in Tengchong, China. *International Journal of Systematic and Evolutionary Microbiology*, 51: 1335–1341.