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**TAXONOMIC DESCRIPTION**

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# *Alicyclobacillus curvatus* sp. nov. and *Alicyclobacillus mengziensis* sp. nov., two acidophilic bacteria isolated from acid mine drainage

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**Abstract**

Two acidophilic strains, designated as ALEF1$^T$ and S30H14$^T$, were isolated from acid mine drainage sediment. Cells of both strains were Gram-stain-positive, aerobic, endospore-forming rods. Strains ALEF1$^T$ and S30H14$^T$ were acidophilic and mesophilic, the former grew at 20–40 °C (optimum, 30 °C) and pH 2.5–4.5 (optimum, pH 3.5), while the latter grew at 20–45 °C (optimum, 30 °C) and pH 2.0–5.5 (optimum, pH 4.5). The 16S rRNA gene-based sequence analysis revealed that strains ALEF1$^T$ and S30H14$^T$ belonged to the genus *Alicyclobacillus*, and were phylogenetically close to *Alicyclobacillus ferrooxydans* TC-34$^T$ with 97.1 and 97.4% similarity, respectively. The similarity between the two novel strains was 98.6%. The average nucleotide identity value between the genome sequences of ALEF1$^T$ and S30H14$^T$ was 79.5%, and that between each of the two isolates and *A. ferrooxydans* TC-34$^T$ were 72.0 and 74.3%. In addition, the digital DNA–DNA hybridization value between ALEF1$^T$ and S30H14$^T$ was 24.9%, between strain ALEF1$^T$ and *A. ferrooxydans* TC-34$^T$ was 21.7%, and between S30H14$^T$ and *A. ferrooxydans* TC-34$^T$ was 26.3%, far below the interspecies threshold. Both strains could utilize diverse carbon sources for heterotrophic growth; strain ALEF1$^T$ could utilize ferrous iron as the energy source for autotrophic growth. Menaquinone 7 was the only quinone detected in either strain. Both strains contained anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$, while $\omega$-alicyclic fatty acids were not detected. Based on their phylogenetic positions, as well as phenotypic and genomic data, it is considered that strains ALEF1$^T$ and S30H14$^T$ represent two novel species within the genus *Alicyclobacillus*, for which the names *Alicyclobacillus curvatus* sp. nov. (type strain ALEF1$^T$=CGMCC 1.17055$^T$=KCTC 43124$^T$) and *Alicyclobacillus mengziensis* sp. nov. (S30H14$^T$=CGMCC 1.17050$^T$=KCTC 43125$^T$) are proposed.

## INTRODUCTION

The genus *Alicyclobacillus* was proposed by Wisotzkey *et al.* [1] and emended by Goto *et al.* [2] and Karavaiko *et al.* [3]. The genus was characterized by the presence of $\omega$-cyclohexyl or $\omega$-cycloheptyl fatty acids as major cellular fatty acids at first, and then was emended based on the fact that some species of this genus only contain straight- and branched-chain saturated fatty acids [2]. The genus contains species with cells that are rod-shaped, endospore-forming, aerobic or facultatively anaerobic, and obligate acidophilic. Except for several strains inhabiting solfataric environments that can grow mixotrophically by using yeast

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**Keywords:** *Alicyclobacillus curvatus*; *Alicyclobacillus mengziensis*; acid mine drainage; acidophile.

**Abbreviations:** AAI, average amino acid identity; ANI, average nucleotide identity; APGL, aminophosphoglycolipid; APL, aminophospholipid; BSS, basal salts solution; dDDH, digital DNA–DNA hybridization; GL, unidentified glycolipid; L, unidentified lipid; MK-7, menaquinone 7; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; PE, phosphatidylethanolamine; PGL, phosphoglycolipid; PL, phospholipid; PME, phosphatidylmethylethanolamine.

The 16S rRNA gene sequences of *Alicyclobacillus curvatus* ALEF1$^T$ and *Alicyclobacillus mengziensis* S30H14$^T$ were deposited at GenBank/EMBL/DDBJ under accession numbers MW269400 and MT605219, respectively. The genomic data of ALEF1$^T$ and S30H14$^T$ were deposited at eLMSG (www.biosino.org/elmsg/index) which then forward to GenBank under the accession numbers of CP071184 and CP071182, together with the accession numbers of their plasmid sequences of CP071185 and CP071183, respectively.

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Four supplementary tables and five supplementary figures are available with the online version of this article.

extract and inorganic substances, such as sulphur and ferrous iron [4, 5], most members of this genus grow heterotrophically in habitats such as hot springs [6], soil [7], and spoiled fruit-based beverages [8, 9]. At the time of writing, the genus *Alicyclobacillus* accommodates 28 validly named species, including two subspecies at List of Prokaryotic Names with Standing in Nomenclature (https://lpsn.dsmz.de/genus/alicyclobacillus) and 20 genome sequences [10]. The type species is *Alicyclobacillus acidocaldarius* [1, 11]. Recently, *Alicyclobacillus*-like species have been detected in the mine areas, but have rarely been isolated and characterized [12, 13]. These findings indicated that *Alicyclobacillus* species have evolved to adapt to widespread environments with diverse phenotypic characteristics. During the investigation of the microbial community in acid mine drainage, we isolated two strains designated ALEF1$^T$ and S30H14$^T$ from acidic sediment. In this communication, we described their taxonomic characteristics.

Strains ALEF1$^T$ and S30H14$^T$ were isolated via the enrichment of a sediment sample collected from acid mine drainage at a copper mine in Yunnan Province, PR China (23° 28′ 53″ N, 103° 46′ 47″ E, 1847 m). The pH of the sample was pH 3.0. The sample was incubated at 30 °C in the B2M liquid medium consisting of basal salts solution (BSS), 0.8 g $l^{-1}$ yeast extract and 0.05 mmol $l^{-1}$ $FeSO_4{\cdot}7H_2O$. The BSS contained (per litre of distilled, deionized water): 3.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.15 g $Na_2SO_4{\cdot}10H_2O$, 0.1 g KCl, 0.1 g $KH_2PO_4$, 0.014 g $Ca(NO_3)_2{\cdot}4H_2O$ and 1 ml trace elements solution. The trace elements solution consisted of (per litre of distilled, deionized water): 10 g $ZnSO_4{\cdot}7H_2O$, 1 g $CuSO_4{\cdot}5H_2O$, 1 g $MnSO_4{\cdot}4H_2O$, 1 g $CoSO_4{\cdot}7H_2O$, 0.5 g $Cr_2(SO_4)_3{\cdot}15H_2O$, 0.5 g $Na_2B_4O_7{\cdot}10H_2O$ and 0.1 g $NaVO_3$. Before autoclaving, the pH value of BSS was adjusted to pH 2.7 by 3 mol $l^{-1}$ $H_2SO_4$. After autoclaving, the filter-sterilized yeast extract and $FeSO_4{\cdot}7H_2O$ solution were added to the medium.

After incubation at 30 °C for 7 days, 1 ml culture was used for isolation, performed with the standard dilution plate method on B2M solid medium. The B2M solid plate was prepared by mixing equal volumes of twice-concentrated B2M liquid medium (pH adjusted to pH 2.7) and gelrite (10 g $l^{-1}$, Sigma-Aldrich). After the colonies were observed, their purity was checked by 16S rRNA gene-based sequence analysis and the phenotypic homogeneity of cultures examined using a phase-contrast microscope (Axiostar plus, Zeiss). The type strains *A. ferrooxydans* TC-34$^T$ and *A. acidocaldarius* subsp. *acidocaldarius* 104-1A$^T$ were obtained from the China General Microbiological Culture Collection Center (CGMCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Because strains ALEF1$^T$ and S30H14$^T$ hardly grew on *Bacillus acidocaldarius* medium (BAM) [11, 14], they were cultured in either B2M liquid or solid medium. Unless otherwise indicated, all tests were performed using the B2M medium, and the pH value of the medium indicated the pH of the BSS.

Genomic DNA of strains ALEF1$^T$ and S30H14$^T$ was extracted with the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. The 16S rRNA gene of both strains were amplified by PCR using universal primers 27F and 1492R [15], PCR products were purified and sequenced as described by Haseltine *et al.* [16]. The full-length 16S rRNA gene sequences were BLASTed with the available sequences in the EzBioCloud (www.ezbiocloud.net) [17] and NCBI (www.ncbi.nlm.nih.gov) data libraries and were aligned with those of related type strains using ClustalW in MEGA version X [18]. Phylogenetic trees were reconstructed using three algorithms, neighbour-joining (NJ) [19], maximum-parsimony (MP) and maximum-likelihood (ML) [20], in the software package MEGA version X. The NJ tree was generated using the two-parameter method [21], and the ML tree was reconstructed based on the general time reversible together with gamma distribution and invariable sites model with 1000 replicates. The GenBank accession numbers of 16S rRNA gene sequences used for the phylogenetic analysis are shown in Fig. 1. The phylogenetic tree based on genomes was reconstructed by 92 concatenated single-copy bacterial core genes among strains ALEF1$^T$, S30H14$^T$ and other related type strains using UBCG [22] (Fig. S1, available in the online version of this article).

The 16S rRNA gene sequence lengths of strains ALEF1$^T$ and S30H14$^T$ obtained by PCR were 1524 and 1458 bp, respectively, and the completeness compared with the full-length of 1536 bp were 99 and 95%, respectively. The 16S rRNA gene-based sequence analysis showed that strains ALEF1$^T$ and S30H14$^T$ were phylogenetically related to members of the genus *Alicyclobacillus* with similarities in the range of 90.5–97.1%. The similarities of two strains with the closest strain *A. ferrooxydans* TC-34$^T$ were 97.1 and 97.4%, respectively, the similarity between strains ALEF1$^T$ and S30H14$^T$ was 98.6%. The NJ tree showed that strains ALEF1$^T$ and S30H14$^T$ formed a distinct and stable clade with *A. ferrooxydans* TC-34$^T$ (Fig. 1). Most branches of the tree were recovered in the ML (Fig. S2) and MP (Fig. S3) trees, especially for ALEF1$^T$, S30H14$^T$ and TC-34$^T$, which supported the phylogenetic relationships between the two strains and *A. ferrooxydans* TC-34$^T$.

## GENOME FEATURES

After checking the quality, the genomes of two strains were sequenced by the PacBio Sequel (PacBio) sequence platform at Guangdong Magigen Biotechnology (www.magigen.com). Sequence assembly was performed with the PacBio SMRT Analysis version 5.1.0 platform using Unicycler software (https://github.com/rrwick/Unicycler/) [23]. The genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline [24]. Whole-genome average nucleotide identity (ANI) was estimated by using the JSpeciesWS online service (http://jspecies.ribohost.com/jspeciesws/) [25], average amino acid identity (AAI) was estimated by using the Kostas lab AAI calculator online service (http://enve-omics.ce.gatech.edu/aai/) [26]. Digital DNA–DNA hybridization (dDDH) and G+C difference values for whole genome sequences between the two strains and TC-34$^T$ were conducted using DSMZ's online service (http://ggdc.dsmz.de) [27, 28]. Pathway analyses were performed at the KEGG

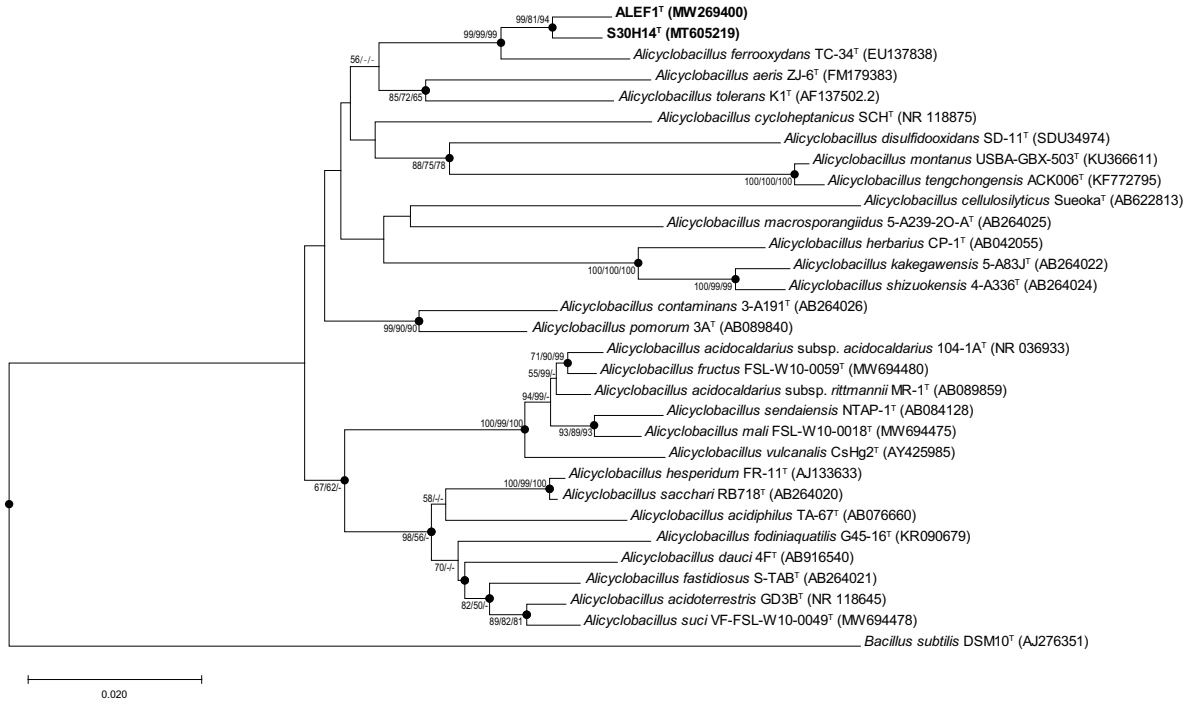


**Fig. 1.** Phylogenetic tree reconstructed using the neighbour-joining method based on 16S rRNA gene sequences of strains ALEF1$^T$, S30H14$^T$ and other related type strains of recognized species within the genus *Alicyclobacillus*. Bacillus subtilis DSM10$^T$ (AJ276351) was used as an outgroup. Numbers at branch nodes represent confidence levels (values ≥50% are shown, '–' represents <50% or differ in the corresponding trees) based on 1000 replicates bootstrap samplings, in the order of neighbour-joining/maximum-likelihood/maximum-parsimony trees. GenBank accession numbers are given in parentheses. Filled cycles indicates branchs that also found in maximum-likelihood and maximum-parsimony trees. Bar, 0.02, represents the number of substitutions per site.

website (www.genome.jp/kegg/). The final assembled genome sequences of ALEF1$^T$ and S30H14$^T$ contained only one contig each, the sizes of which were 5.97 and 5.24 Mbp with sequencing depths of 156 and 270, and the G+C contents of 52.1 and 51.0mol%, respectively. The genomes of ALEF1$^T$ and S30H14$^T$ contained 5478 and 4892 genes, respectively, in which there were 5306 and 4737 protein-encoding genes, 76 and 74 tRNA genes, and 21 and 24 rRNA genes (including seven and eight 16S rRNA genes), were identified, respectively. Among the seven 16S rRNA genes of ALEF1$^T$, five were the same, and the other two had one and three bases difference from the other five, respectively; and no differences existed between the eight 16S rRNA genes of strain S30H14$^T$. The 16S rRNA gene sequence from PCR of ALEF1$^T$ was the same as the five identical ones from the genome, and that of S30H14$^T$ was the same as all of the 16S rRNA gene sequences from the genome. Detailed sequencing information is shown in Table S1. The ANI value between ALEF1$^T$ and S30H14$^T$ was 79.5%, and that between ALEF1$^T$ and *A. ferrooxydans* TC-34$^T$ was 72.0%, and between S30H14$^T$ and *A. ferrooxydans* TC-34$^T$ was 74.3%, far below the 95–96% interspecies threshold [29]. The AAI values between ALEF1$^T$and S30H14$^T$ was 81.28%, and that between ALEF1$^T$ and *A. ferrooxydans* TC-34$^T$ was 71.5%, and between S30H14$^T$ and *A. ferrooxydans* TC-34$^T$ was 73.3%, far below the 95% interspecies threshold [30]. The dDDH value between ALEF1$^T$ and S30H14$^T$ was 24.9%, between ALEF1$^T$ and *A. ferrooxydans* TC-34$^T$ was 21.7%, between S30H14$^T$ and *A. ferrooxydans* TC-34$^T$ was 26.3%, which were below the threshold of 70% proposed by Moore *et al.* [31] for bacterial species classification. This implied the novel isolates ALEF1$^T$ and S30H14$^T$ represented two distinct genospecies of the genus *Alicyclobacillus*. Pathway analysed by KEGG showed that the genes encoding flagellar biosynthesis proteins FlhF (JZ785_RS08095 in ALEF1$^T$, JZ786_RS12210 in S30H14$^T$), FlhA (JZ785_RS08100 in ALEF1$^T$ and JZ786_RS12205 in S30H14$^T$), FliK (JZ785_RS08165 in ALEF1$^T$ and JZ786_RS12140 in S30H14$^T$) and spore-forming associated proteins CotJA (JZ785_RS00040 in ALEF1$^T$ and JZ786_RS17655 in S30H14$^T$), CotJB (JZ785_RS00045 in ALEF1$^T$ and JZ786_RS17650 in S30H14$^T$), Ger(x)C (JZ785_RS02225 in ALEF1$^T$ and JZ786_RS19510 in S30H14$^T$) presented in the genomes of both strains, which were agreement with their morphological characteristics described below. Genome annotation showed that both strains have the complete TCA cycle for carbon source utilization and energy acquisition. Although only ALEF1$^T$ showed the ability to oxidize sulphur and ferrous iron, both strains possessed cytochrome *c* genes (JZ785_RS23605 in ALEF1$^T$ and JZ786_RS21675 in S30H14$^T$) which might be involved in Fe(II) oxidation [32], *sseA* genes (thiosulfate/3-mercaptopyruvate sulfurtransferase gene, JZ785_RS12100 in ALEF1$^T$ and JZ786_RS08920 in S30H14$^T$) and

*soxA* genes (L-cysteine *S*-thiosulfotransferase gene, JZ785_RS26360 in ALEF1$^T$ and JZ786_RS18570 in S30H14$^T$), which were supposed to associate with sulphur metabolism in their genomes. The molecular mechanism of their sulphur and Fe(II) oxidation needs further exploration. Potassium-transporting ATPase subunits encoding genes (*kdpA*, *kdpB*, *kdpC* and *kdpF*) exist in both genomes, which may prevent the entry of hydrogen ions into the cytoplasm by maintaining the intracellular electrochemical gradient of potassium ions for acid adaptation. In addition, the *pstSCAB*, *gadAB*, *adi*, *speA* and *cadA* genes for phosphate uptake and glutamate, arginine, and lysine decarboxylation were also distributed in the genomes of both strains, which could help maintain the cytoplasmic pH by protons metabolism.

## PHYSIOLOGY AND CHEMOTAXONOMY

Cell morphology and flagella were examined using transmission electron microscope (JEM-1400, JEOL) with cells grown at 30 °C for 5–7 days on B2M solid medium. Motility was observed by optical microscopy. The presence of spores and endospores was checked microscopically by phase contrast microscope (Axio Imager.A2, Zeiss) and the Gram-reaction was carried out using the method described by Hucker *et al.* [33]. The temperature range for growth was tested at 15–50 °C with 5 °C unit intervals with B2M liquid medium at pH 3.0. The pH range for growth was examined at pH 1.0–5.0 with 0.5 pH unit intervals (pH was adjusted by 3 mol l$^{-1}$ $H_2SO_4$ and 1 mol l$^{-1}$ NaOH) in B2M broth at 30 °C. NaCl tolerance was determined at 0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0% (w/v) NaCl concentrations in B2M broth (pH 3.0) at 30 °C. Cell growth was estimated by measuring turbidity at a wavelength of 600 nm using the microplate reader (Victor Nivo). Enzyme activities were determined using the API ZYM system according to the manufacturer's instructions. API 50CH and API 20NE were used for detecting the utilization of carbon sources, acid production, assimilation tests, and other conventional tests. The cells collected by centrifugation were resuspended in BSS with 0.2 g l$^{-1}$ yeast extract (pH adjusted to pH 4.0) for inoculation sources and using 0.02 g l$^{-1}$ bromocresol green as the indicator for acid production. The utilization of carbon sources was estimated by the turbidity change of test strip wells. Acid production was judged by the colour change of the medium from blue to yellow. The cultures in BSS with 0.2 g l$^{-1}$ yeast extract without other carbon source were used as the control [34]. No growth and acid production of ALEF1$^T$ and S30H14$^T$ were observed in control wells. Catalase and oxidase activities were performed as described previously [34]. Other biochemical characteristics were also determined by the API 20NE system according to the manual. Oxidation of ferrous iron, elemental sulphur and tetrathionate was performed by adding 10 mmol l$^{-1}$ $FeSO_4{\cdot}7H_2O$, 0.5% (w/v) $S^0$, and 5 mmol l$^{-1}$ potassium tetrathionate into BSS with or without 0.2 g l$^{-1}$ yeast extract (final pH was adjusted to pH 4.0), respectively. Oxidation of ferrous iron was determined by the decrease of ferrous iron in broth using 1,10-phenanthroline spectrophotometry assay [35], oxidation of sulphur and tetrathionate was determined by detecting the reduction of pH and increase of sulphate using the barium sulphate turbidimetric method [36]. Anaerobic growth was tested on B2M or BSS supplemented with 0.2 g l$^{-1}$ yeast extract and ferrous iron (10 mmol l$^{-1}$), elemental sulphur (5.0 g l$^{-1}$), or potassium tetrathionate (5 mmol l$^{-1}$). Filter-paper discs treated with different antibiotics were used to examine the antibiotic resistance of both strains. The specific types and concentrations of antibiotics were shown in Table S2.

After 7 days incubation on B2M solid medium, colonies of ALEF1$^T$ were light yellow, smooth, flat with entire margins, and circular with the diameter of 1.0–2.0 mm, while colonies of S30H14$^T$ were light yellow, rough, circular with the diameter of 0.5–1.0 mm. Cells of strains ALEF1$^T$ and S30H14$^T$ were Gram-stain-positive, slightly curved rods with the size of 2.3–3.3×0.6–0.9 and 1.8–2.1×0.5–0.6, respectively, endospores were subterminal and swollen ellipsoid (Fig. S4). Both ALEF1$^T$ and S30H14$^T$ were motile with 1–3 lateral flagella (Fig. S4). Oxidase activity of ALEF1$^T$ was positive, while S30H14$^T$ was negative. Indole production and catalase activity of both strains were negative. Strain ALEF1$^T$ was able to grow at the pH range of pH 2.5–4.5 with the optimum at pH 3.5 in B2M, and strain S30H14$^T$ grew at 2.0–5.5 with the optimum at 4.5 in B2M. Both of them could grow in the presence of 0–0.5% (w/v) NaCl. The temperature ranges for growth of ALEF1$^T$ and S30H14$^T$ were 20–40 °C and 20–45 °C with optimum growth at 30 °C for both of them. Both strains can utilize a variety of organic substances to grow or produce acid. The results of the antibiotic resistances assay are shown in Table S2. Strain ALEF1$^T$ could oxidize ferrous iron and elemental sulphur with the ferrous oxidation rate of 129 mg l$^{-1}$ per day and sulphate production rate of 200 mg l$^{-1}$ per day, meanwhile pH of broths with ferrous and sulphur decrease 0.7 (pH dropped from pH 3.5 to 2.8) and 1.4 (pH dropped from pH 4.5 to 3.1), respectively, after 2 and 8 days. Strain S30H14$^T$ did not oxidize either of them. Both strains cannot grow anaerobically. Detailed basic phenotypic characteristics, enzyme activities, utilization of carbon sources, acid production, and other biochemical characteristics of both novel species and related reference species are shown in Tables 1 and S3.

For analysis of *N*-acyl type of muramyl residues and diamino acids of the cell wall, cellular fatty acid, polar lipids and isoprenoid quinones, the strains were grown for 7 days at 30 °C on B2M medium. The muramyl residues in the peptidoglycan of the bacteria cell wall were extracted and detected by glycolate test as described by Uchida *et al.* [37]. Diamino acids of the cell wall were extracted from freeze-dried cells (10 mg) with 10% v/v trichloroacetic acid at 100 °C and detected according to the methods of McKerrow *et al.* [38]. The fatty acids were methylated and analysed by the gas chromatography (HP 6890 Series GC System; Hewlett Packard), using the TSBA6 database of Sherlock Microbial Identification System according to the manufacturer's instructions (Microbial ID) [39]. Total lipids of the cells were extracted according to the method of Minnikin *et al.* [40], and separated by two-dimensional TLC plates (Merck silica gel 60), with chloroform–methanol–water (65:25:4; v/v/v) and chloroform–methanol–acetic acid–water (80:12:15:4, v/v/v/v) for the first and the second dimension developing solvents, respectively. The plates were

**Table 1.** Phenotypic characteristics of ALEF1$^T$, S30H14$^T$ and related species

Strains: 1, ALEF1$^T$; 2, S30H14$^T$; 3, *Alicyclobacillus ferrooxydans* TC-34$^T$; 4, *Alicyclobacillus acidocaldarius* subsp. *acidocaldarius* 104-1A$^T$; 5, *Alicyclobacillus aeris* ZJ-6$^T$. +, Positive; –, negative; v, variable between tests. All data from this study.

| Characteristics | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Temperature range for growth (°C) | 20–40 | 20–45 | 17–40 | 45–70 | 25–35 |
| Optimum growth temperature (°C) | 30 | 30 | 28 | 60 | 30 |
| pH range for growth | 2.5–4.5 | 2.0–5.5 | 2.0–6.0 | 2.0–6.0 | 2.0–6.0 |
| Optimum growth pH | 3.5 | 4.5 | 3.0 | 4.0 | 3.5 |
| Growth at 5% NaCl | – | – | – | – | – |
| Motility | + | + | – | + | + |
| Oxidase | + | – | + | – | – |
| Catalase | – | – | + | – | – |
| Hydrolysis of: | | | | | |
| Gelatin | + | + | – | + | – |
| Starch | – | – | + | + | – |
| Nitrate reduced to nitrite | – | – | – | – | – |
| Acid production from: | | | | | |
| Glycerol | + | + | – | + | – |
| D-Arabinose | – | – | – | – | – |
| L-Arabinose | + | + | + | + | – |
| D-Xylose | + | + | + | + | + |
| L-Xylose | – | – | – | – | – |
| Methyl $\beta$-D-xylopyranoside | – | – | – | – | – |
| D-Galactose | + | – | – | + | – |
| L-Sorbose | + | – | – | – | – |
| L-Rhamnose | – | – | – | + | – |
| D-Mannitol | + | + | – | + | – |
| D-Sorbitol | + | – | – | – | – |
| Methyl $\alpha$-D-mannopyranoside | – | – | – | + | – |
| Amygdalin | – | – | – | – | – |
| Arbutin | – | + | – | + | – |
| Aesculin | + | – | + | + | + |
| Salicin | – | – | – | – | – |
| Cellobiose | + | + | – | + | – |
| Maltose | – | + | – | + | – |
| Lactose | – | – | – | + | – |
| Melibiose | – | – | – | + | – |
| Sucrose | + | + | + | + | – |
| Trehalose | + | + | + | + | – |
| Gentiobiose | + | + | – | + | – |

Continued

**Table 1.** Continued

| Characteristics | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Turanose | – | – | – | + | – |
| D-Lyxose | – | – | – | – | + |
| D-Tagatose | – | – | – | – | v |
| 5-Ketogluconate | – | – | – | + | + |
| DNA G+C content (mol%) | 52.1 | 51.0 | 48.6 | 60.3 | 51.2 |

sprayed with 10% (w/v) ethanolic molybdatophosphoric acid (Sigma) for detection of the total lipids, $\alpha$-ninhydrin (Sigma) for aminolipids, molybdenum blue (Sigma) for phospholipids, and naphthol for glycolipids [41]. Isoprenoid quinones were extracted from freeze-dried cells (200 mg) with chloroform–methanol (2:1, v/v), and thin-layer chromatography was used to separate from other components. The purified quinones were identified by HPLC apparatus equipped with a ZOBAX ODS C18 column (4.6×250 mm; Agilent) as described previously [5].

The *N*-acyl type of muramyl residues in the peptidoglycan of the cell wall of the isolated strains ALEF1$^T$, S30H14$^T$, and the reference type strain TC-34$^T$ were all *N*-glycolyl type, and their diamino acid was L-lysine. The predominant cellular fatty acids (≥10%) of ALEF1$^T$ were anteiso-$C_{15:0}$ (44.2%), anteiso-$C_{17:0}$ (29.6%) and iso-$C_{16:0}$ (11.0%), which for S30H14$^T$ were anteiso-$C_{15:0}$ (62.9%) and anteiso-$C_{17:0}$ (10.0%). The cellular fatty acid components of these two strains were similar to that of strain TC-34$^T$, but with different contents. Detailed information on the cellular fatty acids components is shown in Table S4. The isoprenoid quinone of ALEF1$^T$ and S30H14$^T$ was MK-7, the same as the major respiratory quinone of the other members within the genus *Alicyclobacillus* [1]. Similar to the type strain TC-34$^T$, both strains contained phosphatidylethanolamine (PE), aminophosphoglycolipid (APGL), phospholipid (PL), aminophospholipid (APL) and phosphoglycolipid (PGL), while only ALEF1$^T$ contained phosphatidylmethylethanolamine (PME), unidentified lipid (L) and unidentified glycolipid (GL). Detailed information on polar lipids is shown in Fig. S5.

Based on the results of the phylogenetic analysis, as well as phenotypic and genomic data, strains ALEF1$^T$ and S30H14$^T$ represent two novel species of the genus *Alicyclobacillus*. Both isolates showed many physiological and biochemical characteristics that clearly distinguished them from other species in genus *Alicyclobacillus*, such as hydrolysis of gelatin and starch, acid production from different carbon sources, as well as Fe(II) and $S^0$ oxidation. In addition, the content of major fatty acids, the polar lipid profiles, and the metabolism characteristics also showed the specificity of the two strains. Detailed differences between the two isolates and other members of the genus are listed in Table 1. In conclusion, strains ALEF1$^T$ and S30H14$^T$ are considered two novel species within the genus *Alicyclobacillus*, for which the names *Alicyclobacillus curvatus* sp. nov. (strain ALEF1$^T$) and *Alicyclobacillus mengziensis* sp. nov. (strain S30H14$^T$) are proposed.

## DESCRIPTION OF *ALICYCLOBACILLUS CURVATUS* SP. NOV.

*Alicyclobacillus curvatus* (cur.va′tus. L. masc. part. adj. *curvatus* bent, curved, referring to the curved shape of the cell).

Strain ALEF1$^T$ forms smooth, light yellow and opaque circular colonies with entire margins on B2M medium that are 1.0–2.0 mm in diameter after 7 days incubation. Cells are curved rods (2.3–3.3×0.6–0.9 µm), motile using multiple lateral flagella, endospore-forming and Gram-stain-positive. Cells can grow in 0–0.5% (w/v) NaCl, but not in 5% (w/v) NaCl. The temperature for growth is 20–40 °C, with the optimum temperature at 30 °C. pH for growth is pH 2.5–4.5, with the optimum pH at 3.5. Oxidase is positive, while catalase, nitrate reduction, indole production, and Voges–Proskauer test are negative. Cells can oxidize ferrous iron and elemental sulphur in the presence of 0.2 g $l^{-1}$ yeast extract under aerobic conditions. The *N*-acyl groups of muramyl residue and diamino acid in the peptidoglycan of the cell wall are glycolyl type and L-lysine. Predominant cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. The polar lipids include an APGL, a PE, a PME, five PLs, four GLs, three APLs, four PGLs and two Ls. MK-7 is the only isoprenoid quinone. The genomic DNA G+C content of strain ALEF1$^T$ is 52.1 mol%.

The type strain, ALEF1$^T$ (=CGMCC 1.17055$^T$=KCTC 43124$^T$), was isolated from acid mine drainage sediment.

## DESCRIPTION OF *ALICYCLOBACILLUS MENGZIENSIS* SP. NOV.

*Alicyclobacillus mengziensis* (meng.zi.en′sis. N. L. masc. adj. *mengziensis* of Mengzi, China, referring to the source of the sample from which the type strain was isolated).

Strain S30H14$^T$ forms rough, light yellow and opaque circular colonies with entire margins on B2M medium that are 0.5–1.0 mm in diameter after 7 days incubation. Cells are short rods (1.8–2.1×0.5–0.6 µm), motile by one to three lateral flagella,

endospore-forming and Gram-strain-positive. Cells can grow in 0–0.5% (w/v) NaCl, but not in 5% (w/v) NaCl. The temperature for growth is 20–45 °C, with the optimum temperature at 30 °C. pH for growth is pH 2.0–5.5, with the optimum pH at 4.5. Oxidase, catalase, nitrate reduction and Voges–Proskauer test are negative, weak indole production activity. *N*-Acyl groups of muramyl residue and diamino acid in the peptidoglycan of the cell wall are glycolyl type and L-lysine. Predominant cellular fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The polar lipids include a PE, an APGL, four PL, two APLs and two PGLs. MK-7 is the only isoprenoid quinone. The genomic DNA G+C content of strain S30H14$^T$ is 51.0mol%.

The type strain, S30H14$^T$ (=CGMCC 1.17050$^T$=KCTC 43125$^T$), was isolated from cid mine drainage sediment.

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Author contributions
C.Y.J. and S.J.L. designed and coordinated the study. Z.J., D.X.T.L. and Y.H. identified phenotypic, physiological and chemotaxonomic characteristics. Z.L.L. and N.Z. isolated the strains. Z.H.L., and H.Q.Y. analysed the whole genome sequence and performed ANI and dDDH calculations. G.J.Z. and Y.J. provided the samples. Z.J. and D.X.T.L. wrote the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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