Reclassification of '*Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans*' strain K1 as *Alicyclobacillus tolerans* sp. nov. and *Sulfobacillus disulfidooxidans* Dufresne *et al.* 1996 as *Alicyclobacillus disulfidooxidans* comb. nov., and emended description of the genus *Alicyclobacillus*

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Comparative analysis of 16S rRNA gene sequences, DNA–DNA hybridization data and phenotypic properties revealed that '*Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans*' strain K1 is not a member of the genus *Sulfobacillus*. Phylogenetically, strain K1 is closely related to unclassified strains of the genus *Alicyclobacillus*: the 16S rRNA gene sequence of strain K1 is similar to that of *Alicyclobacillus* sp. AGC-2 (99.6%), *Alicyclobacillus* sp. 5C (98.9%) and *Alicyclobacillus* sp. CLG (98.6%) and bacterium GSM (99.1%). The 16S rRNA gene sequence similarity values for strain K1 and species of the genus *Alicyclobacillus* with validly published names were in the range 92.1–94.6%, and for *S. thermosulfidooxidans* VKM B-1269^T the value was 87.7%. *Sulfobacillus disulfidooxidans* SD-11^T was also phylogenetically related to strain K1 (92.6% sequence similarity) and thus belonged to the genus *Alicyclobacillus*. Chemotaxonomic data, such as the major cell-membrane lipid components of strains K1 and SD-11^T (ω -alicyclic fatty acids) and the major isoprenoid quinone (menaquinone MK-7) of strain K1, supported the affiliation of strains K1 and SD-11^T to the genus *Alicyclobacillus*. Physiological and molecular biological tests allowed genotypic and phenotypic differentiation of strains K1 and SD-11^T from the nine *Alicyclobacillus* species with validly published names. The G+C content of the DNA of strain K1 was 48.7 $\pm$ 0.6 mol%; that of strain SD-11^T was 53 $\pm$ 1 mol%. DNA–DNA reassociation studies showed low relatedness (22%) between strains K1 and SD-11^T, and even lower relatedness (3–5%) between these strains and *Alicyclobacillus acidocaldarius* subsp. *acidocaldarius* ATCC 27009^T, DSM 446^T. DNA reassociation of strains K1 and SD-11^T with *Alicyclobacillus cycloheptanicus* DSM 4006^T gave values of 15 and 21, respectively. Based on the phenotypic and phylogenetic characteristics of strains K1 and SD-11^T, *Alicyclobacillus tolerans* sp. nov. (type strain, K1^T = VKM B-2304^T = DSM 16297^T) and *Alicyclobacillus disulfidooxidans* comb. nov. (type strain, SD-11^T = ATCC 51911^T = DSM 12064^T) are proposed.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Alicyclobacillus tolerans* K1^T is AF137502.

Graphs showing the growth of and oxidation of ferrous iron and glucose consumption by strains VKM B-1269^T and K1^T, and tables listing the DNA base compositions of various sulfobacilli and alicyclobacilli and the ¹⁴CO₂ fixation by cell suspensions of strain K1^T are available as supplementary material in IJSEM Online.

Acidophilic, aerobic, endospore-forming, thermotolerant strain K1 was isolated from oxidizable lead–zinc ores of the Kurgashinkan deposit (Uzbekistan). On the basis of several phenotypic properties (the ability to oxidize iron, elemental sulfur and sulfides), the strain was described as a subspecies of the type species of the genus *Sulfobacillus*, '*Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans*' (Kovalenko & Malakhova, 1983). However, the assignment of the strain to a subspecies of *S. thermosulfidooxidans* was questionable as its genotypic and chemotaxonomic characteristics were not studied. A comparative analysis of the number and size of restriction fragments generated from the chromosomal DNA of strain K1 and of the type strain of *S. thermosulfidooxidans* (VKM B-1269^T=DSM 9293^T) attested to the lack of intraspecies similarity between the two strains (Kondrat'eva *et al.*, 1998). In addition, phylogenetic analysis based on partial 16S rRNA gene sequencing (500 bp) showed that strain K1 was more closely related to the genus *Alicyclobacillus* than to the genus *Sulfobacillus* (Karavaiko *et al.*, 2000). Strain SD-11^T was isolated from a mixed culture obtained after enrichment of waste water sludge of Blake Lake City, Quebec, Canada (Dufresne *et al.*, 1993). On the basis of its ability to use elemental sulfur and pyrite as sole energy sources, the strain was described as the type strain of *Sulfobacillus disulfidooxidans* (Dufresne *et al.*, 1996). Strain SD-11^T is phylogenetically related to strain K1 (Karavaiko *et al.*, 2000) and to *Alicyclobacillus cycloheptanicus* DSM 4006^T (Dufresne *et al.*, 1996); 16S rRNA gene sequence similarity values were 92.6 and 95.5%, respectively. Phenotypically, strains of the genus *Alicyclobacillus* are spore-forming, thermoacidophilic, Gram-positive bacteria. In common with most alicyclobacilli, strains K1 and SD-11^T contained ω -alicyclic fatty acids as the major cell-membrane lipid component (Tsaplina *et al.*, 1994; Dufresne *et al.*, 1996). In this report, we propose the classification of '*S. thermosulfidooxidans* subsp. *thermotolerans*' strain K1 as the type strain (K1^T) of *Alicyclobacillus tolerans* sp. nov. and the reclassification of *S. disulfidooxidans* as *Alicyclobacillus disulfidooxidans* comb. nov.

For experiments, strain K1^T was grown at 40 °C on modified Manning medium (Manning, 1975) containing 50–70 mM Fe²⁺, 0.2 g yeast extract l⁻¹ and mineral salts [g l⁻¹: (NH₄)₂SO₄, 6.0; KCl, 0.2; K₂HPO₄, 0.2; MgSO₄·7H₂O, 1.0; Ca(NO₃)₂·7H₂O, 0.1; pH 2.5–2.7]. There was no growth on the BAM agar of Deinhard *et al.* (1987a). Strain K1^T grew organotrophically in liquid and solid modified BAM that contained less yeast extract and glucose (to 0.2 and 0.5 g l⁻¹, respectively).

The preliminary screening for 16S rRNA gene sequences similar to that of strain K1^T was carried out by using BLAST (<http://www.ncbi.nlm.nih.gov/blast>). Then, the 16S rRNA gene sequence of strain K1^T was aligned with a representative set of 16S rRNA gene sequences obtained from recent GenBank releases by using CLUSTAL W. Positions of sequence and alignment uncertainties were omitted; in total

1308 nucleotides were used in the analysis. Phylogenetic trees were constructed by using various algorithms implemented in TREECON (Van de Peer & De Wachter, 1994). The almost-complete 16S rRNA gene sequence of strain K1^T (1409 bp) was used in the analyses, corresponding to positions 15 to 1435 of *Escherichia coli* numbering. The comparative phylogenetic analysis showed that strain K1^T clustered with species of the genus *Alicyclobacillus*. However, in this cluster, the similarity levels between the 16S rRNA gene sequence of strain K1^T and those of strains (including the type strains) of species of the genus *Alicyclobacillus* with validly published names did not exceed 94.5%. Therefore, according to current concepts (Stackebrandt & Goebel, 1994), strain K1^T could not be assigned to any known species of the genus *Alicyclobacillus*. The 16S rRNA gene sequence of strain K1^T also showed a high degree of similarity to sequences of the following unidentified strains that have been tentatively assigned to the genus *Alicyclobacillus* (Fig. 1): *Alicyclobacillus* sp. AGC-2 (99.6%); two strains of halophilic, acidophilic, iron-oxidizing bacteria, *Alicyclobacillus* sp. 5C (98.9%) and *Alicyclobacillus* sp. CLG (98.6%); and bacterium GSM (99.1%), a moderately thermophilic strain that oxidizes elemental sulfur and pyrite (in the presence organic C) and is capable of oxidizing and reducing iron (Johnson *et al.*, 2001a, 2003; A. Yahya, K. B. Hallberg & D. B. Johnson, unpublished data). Within the genus *Alicyclobacillus*, these unclassified strains formed a separate phylogenetic cluster with an intraspecies similarity level of 98.6–99.6%. Based on the results of the phylogenetic analysis, it may be concluded that strain K1^T, together with several as-yet-unclassified strains, composes a new species of the genus *Alicyclobacillus*. The 16S rRNA gene sequences of strains K1^T and SD-11^T exhibited a low level of similarity (83.3–87.7%)

Fig. 1. Phylogenetic tree showing the position of strain K1^T among members of the genus *Alicyclobacillus* of the *Bacillus*–*Clostridium* phylum of the Gram-positive bacteria. Bootstrap values (expressed as a percentage of 100 replications) are shown at branch points; values greater than 95% were considered significant. Bar, Jukes–Cantor distance (5 nucleotide substitutions per 100 nucleotides).

to the sequences of strains belonging to the genus *Sulfobacillus* and a high level of similarity (92.2–95.5 %) to the sequences of strains belonging to the genus *Alicyclobacillus* (Dufresne *et al.*, 1996; Karavaiko *et al.*, 2000).

In Table 1 the main phenotypic and genomic properties of strains K1^T and SD-11^T and alicyclobacilli are compared.

The G+C content of the DNA of *Alicyclobacillus* species varies from 51.6 to 62.7 mol% (Wisotzkey *et al.*, 1992; Karavaiko *et al.*, 2000; Goto *et al.*, 2003). The G+C content of the DNA of strain K1^T, determined by the methods of Marmur (1961) and Owen *et al.* (1969), and that of bacterium GSM were 48.7 ± 0.6 and 50.5 mol% (Johnson *et al.*, 2001a), respectively, i.e. less than the minimum G+C values determined for identified alicyclobacilli (Table 1). The G+C content of the DNA of strain SD-11^T was 52.7 mol% (our data) and 53 ± 1 mol% (Dufresne *et al.*, 1996) and close to the value for *Alicyclobacillus acidoterrestris* DSM 3922^T (52.2 mol%) (Wisotzkey *et al.*, 1992). DNA–DNA hybridization was performed by using the optical reassociation method (De Ley *et al.*, 1970). The results confirmed the interspecies level of relatedness between strain K1^T and the tested sulfobacilli strains (*S. thermosulfidooxidans*, *Sulfobacillus acidophilus* and '*Sulfobacillus sibiricus*') and a very low reassociation level (3–5 %) between strains K1^T and SD-11^T and *A. acidocaldarius* subsp. *acidocaldarius* ATCC 27009^T, DSM 446^T grown on medium described by Darland & Brock (1971) at 63 °C and pH 3.5 (see Table A, available as supplementary material in IJSEM Online). Higher levels of DNA reassociation were determined for strains K1^T and SD-11^T with *A. cycloheptanicus* DSM 4006^T (15 and 21 %, respectively).

The membrane lipid composition of cells of strain K1^T organotrophically grown in liquid modified BAM medium and in liquid modified Manning medium (with Fe²⁺/FeS₂) was determined after acid methanolysis. To 10 mg of a cell pellet dried in a nitrogen flow was added 400 µl of absolute methanol containing 2.6 M HCl for 4 h at 70 °C. Methyl esters of fatty acids and dimethylacetals, formed as a result of methanolysis, were twice extracted with 100 µl hexane, dried, and treated with 30 µl *N,O*-bis(trimethylsilyl)-trifluoroacetamide for 15 min at 80 °C to obtain trimethylsilyl esters of hydroxyl acids. The obtained mixture (2–5 µl) was fed into the injector of an HP-5973 gas chromatograph mass spectrometer (Hewlett Packard). Regular software supplied with a quadruple mass-analyser was used for control of the apparatus and data processing. The mass spectrometer used electrons with energy of 70 eV for ionization and had a resolution of 0.5 amu and a working mass range of 2–100 amu; the sensitivity threshold was 0.01 ng of methylstearate. Chromatographic separation of the mixture was performed on an HP-5ms methyl silica capillary column (Hewlett Packard) (30 m; inner diameter, 0.2 mm, coated with immobile methylsilicone, 0.2 µm – width phase). Helium, the carrier gas, passed through the column at a flow rate of 1.5 ml min⁻¹. The initial temperature was set at 120 °C for 2 min and then increased to

300 °C at a rate of 5 °C min⁻¹. Separated substances were identified on known features of their mass spectra and relative retention times using the mass-spectral databases nbs75k and wiley275. Simple ω -cyclohexane acids were also identified from the published data (Oshima & Ariga, 1975) and oxyacids by specific fragmentation of Me-TMS derivatives (methyl-*O*-trimethylsilyl derivatives) and methyl esters (without silylation). It was shown that strain K1^T differed only slightly from *A. acidocaldarius*, *A. acidoterrestris*, *Alicyclobacillus acidiphilus*, *Alicyclobacillus sendaiensis*, *Alicyclobacillus hesperidum* (Wisotzkey *et al.*, 1992; Goto *et al.*, 2003; Matsubara *et al.*, 2002; Tsuruoka *et al.*, 2003; Albuquerque *et al.*, 2000), *S. disulfidooxidans* (Dufresne *et al.*, 1996) and strains of *S. thermosulfidooxidans* (Tsaplina *et al.*, 1994). All these strains contained ω -alicyclic (cyclohexanoic) fatty acids as the major lipid component. Under heterotrophic conditions, the percentage of particular ω -alicyclic acids in the total fatty acid content of strain K1^T comprised: ω -cyclohexane-C_{17:0}, from 27.5 (under autotrophic conditions) to 60 %; ω -cyclohexane-C_{19:0}, from 2.1 (under mixotrophic conditions) to 7.9 %; ω -cyclohexane-C_{17:0} 2OH (never before revealed in alicyclobacilli), from 8.2 (under autotrophic and mixotrophic conditions) to 11.3 %; and ω -cyclohexane-C_{18:0}, from 0.7 (under mixotrophic conditions) to 1.2 %. When grown organotrophically, strain K1^T could be differentiated from other species of the genus *Alicyclobacillus* by the amounts of ω -cyclohexane-C_{17:0} (60.0 %) and ω -cyclohexane-C_{19:0} (7.9 %) present: values for *A. acidocaldarius* were 47.6–52.8 % and 25.2–42.6 %, respectively; values for strains of *A. acidoterrestris* were 62.6–68.4 % and 18.9–23.9 %, respectively (Goto *et al.*, 2003); values for *A. hesperidum* were 56.8 and 13.3 %, respectively; and values for *A. sendaiensis* were 44.1 and 30.2 %, respectively.

The main isoprenoid quinone in strain K1^T, as determined by the method of Collins (1985), was menaquinone with seven isoprene units, MK-7 (90 % from common sum), while MK-6 was a minor component. Standard mixtures of menaquinones were used for identification purposes.

Morphologically, strains K1^T and SD-11^T differed from species of *Alicyclobacillus* and *Sulfobacillus* by a slightly larger cell size [3.0–6.0 × 0.9–1.0 µm (K1^T) and 1.0–6.0 × 0.6–1.0 µm (SD-11^T)], the lack of flagella and the thermal resistance of their spores (remain viable after 30 min heating at 110 °C) (Kovalenko & Malakhova, 1983; Bogdanova *et al.*, 1990). The cell wall consists of a thin murein layer (50–60 Å in thickness); cells exhibited a surface S-layer comprising large hexagonally packed (p6-symmetry) rod-shaped subunits of 150–160 Å in diameter and 180–200 Å in height (unpublished data). On a solid medium containing 0.5 % (w/v) agarose, strain K1^T grew slowly: small colonies of 0.3–0.5 mm in diameter were observed after 7–10 days incubation.

Strain K1^T grew optimally at 37–42 °C, differing from the known thermophilic bacteria of the genus *Alicyclobacillus*, which are characterized by optimal growth at 42–60 °C and

Table 1. Characteristics of strains of species of the genus *Alicyclobacillus*

Strains: 1, *A. tolerans* K1^T; 2, *A. disulfidooxidans* SD-11^T (data from Dufresne *et al.*, 1996); 3, *A. acidocaldarius* subsp. *acidocaldarius* DSM 446^T (Wisotzkey *et al.*, 1992); 4, *A. acidoterrestris* DSM 3922^T (Wisotzkey *et al.*, 1992); 5, *A. cycloheptanicus* DSM 4006^T (Wisotzkey *et al.*, 1992); 6, *A. hesperidum* DSM 12489^T (Albuquerque *et al.*, 2000); 7, *A. acidiphilus* TA-67^T (Matsubara *et al.*, 2002); 8, *A. herbarius* DSM 13609^T (Goto *et al.*, 2002); 9, *A. pomorum* DSM 14955^T (Goto *et al.*, 2003); 10, *A. sendaiensis* ATCC BAA-609^T (Tsuruoka *et al.*, 2003). +, Positive result; –, negative result; W, weakly positive; ND, not determined. All cells were rod-shaped.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell size (µm)	0.9–1.0 × 3.0–6.0	0.6–1.0 × 1.0–6.0	0.7–0.8 × 2.0–3.0	0.6–0.8 × 2.9–4.3	0.35–0.55 × 2.5–4.5	–	0.9–1.1 × 4.8–6.3	–	0.8–1.0 × 2.0–4.0	0.8 × 2.0–3.0
Growth pH range (optimum)	1.5–5.0 (2.0–2.7)	0.5–6.0 (1.5–2.5)	2.0–6.0 (4.0)	2.2–5.8 (4.0)	3.0–5.5 (4.5)	(4.0)	2.5–5.5 (3.0)	3.0–6.5 (4.5–5.0)	3.0–6.0 (4.0–4.5)	2.5–6.5 (5.5)
Growth temp. (°C) range (optimum)	<20–55 (38–42)	4–40 (35)	45–70 (60)	<35–55 (42–53)	40–53 (48)	(50)	20–55 (50)	35–65 (55–60)	30–60 (45–50)	40–65 (55)
G + C content (mol%)	48.1–49.3	53	60.3	52.2	55.6	54.0	54.1	56.2	53.1	62.3
Genome size (Da)	3.9 × 10 ⁹	3.0 × 10 ⁹	ND	ND	ND	ND	ND	ND	ND	ND
Fatty acid composition	<i>ω</i> -Cyclohexane-C _{17:0} , <i>ω</i> -cyclohexane-C _{19:0} , <i>ω</i> -cyclohexane-C _{17:0} 2OH	<i>ω</i> -Cyclohexanoic acids	<i>ω</i> -Cyclohexane-C _{17:0} , <i>ω</i> -cyclohexane-C _{19:0}	<i>ω</i> -Cyclohexane-C _{17:0} , <i>ω</i> -cyclohexane-C _{19:0}	<i>ω</i> -Cycloheptane-C _{18:0} , <i>ω</i> -cycloheptane-C _{18:0} 2OH	<i>ω</i> -Cyclohexane-C _{17:0} , <i>ω</i> -cyclohexane-C _{19:0}	<i>ω</i> -Cyclohexane-C _{17:0} , <i>ω</i> -cyclohexane-C _{19:0}	<i>ω</i> -Cycloheptane-C _{18:0} , <i>ω</i> -cycloheptane-C _{20:0} , <i>ω</i> -cycloheptane-C _{18:0} 2OH	iso-C _{15:0} , anteiso-C _{15:0} , iso-C _{16:0} , anteiso-C _{17:0}	<i>ω</i> -Cyclohexane-C _{17:0} , <i>ω</i> -cyclohexane-C _{19:0}
Mineral substrates	Fe ²⁺ , S ⁰ , sulfide minerals	S ⁰ , FeS ₂ , S ₂ O ₃ ²⁻ , Fe ²⁺	ND	ND	ND	ND	ND	ND	ND	ND
Differential tests										
Glycerol	W	+	+	+	–	+	–	+	+	+
Erythritol	–	–	–	+	–	–	–	–	–	–
Inositol	–	–	–	+	–	–	–	–	–	–
Mannitol	+	–	+	+	+	+	–	+	+	ND
Sorbitol	–	+	–	+	+	–	+	–	–	–
Lactose	W	–	+	+	–	–	+	+	–	+
D-Raffinose	W	+	+	–	–	–	+	+	–	+
Mannose	+	–	ND	ND	+	ND	ND	ND	+	ND
Rhamnose	–	–	+	+	+	–	–	+	–	–
Ribose	+	+	ND	ND	+	ND	–	–	+	ND
D-Fructose	+	–	+	ND	ND	ND	ND	ND	+	ND
Cellobiose	–	+	+	+	–	+	+	+	–	+
Trehalose	+	–	+	+	–	+	+	+	+	+
Sucrose	+	+	+	+	–	+	+	+	+	+
Melibiose	–	+	+	W	–	–	–	+	–	–
5-Ketogluconate	+	+	–	–	+	–	–	+	+	–
L-Sorbose	+	+	–	–	+	–	+	–	+	ND
Methyl α-D-glucoside	+	+	+	–	–	–	+	+	+	+
Amygdalin	+	+	–	–	–	–	–	+	+	–
Arbutin	–	–	+	–	–	–	+	+	–	+
Aesculin	+	+	+	–	+	–	+	+	+	–
Salicin	–	–	+	–	–	–	+	+	+	+
Melezitose	+	+	–	–	–	–	+	+	–	–
Glycogen	+	+	+	–	–	+	–	–	–	+
D-Turanose	W	–	–	–	–	+	+	+	+	+
D-Tagatose	W	+	–	–	+	–	–	–	+	ND
D-Fucose	–	+	–	–	–	–	–	+	–	–
Starch	+	+	+	–	–	W	–	–	–	ND
D- and L-arabinose	W	+	+	+	+	+	+	+	–	+
D-Galactose	+	–	+	+	–	+	+	+	–	+
Glucose	+	+	+	+	+	ND	ND	ND	+	ND
D- and L-xylose	+	+	+	–	+	–	+	+	–	ND
Maltose	W	+	+	+	–	+	+	+	+	+

an inability to grow at 25 °C (Wisotzkey *et al.*, 1992; Goto *et al.*, 2003) with the exception of *A. acidiphilus* DSM 14558^T (Matsubara *et al.*, 2002). Bacterium GSM has optimal growth at 46 °C and does not grow below 35 °C (Johnson *et al.*, 2001a). Strain SD-11^T is a mesophile with optimal growth at 35 °C (Dufresne *et al.*, 1993, 1996); however, authors have observed spore germination at 4 °C for this strain and regard this temperature as the lower growth limit. Strain K1^T grew between pH 1.5 and 5.0, with the optimum at pH 2.5–2.7, while the alicyclobacilli with validly published names grew in the range pH 2.0–6.5, with the optimum at pH 3.0–5.5. Growth of strain SD-11^T was optimal at pH 1.5–2.5 (Table 1).

Physiological characteristics of strain K1^T studied with the use of the API 50 CH system (BioMérieux), described by Deinhard *et al.* (1987a, b) for the biochemical profiling of *Alicyclobacillus* species, are compared with the respective data reported for several species of sulfobacilli and alicyclobacilli (Table 1). The growth of strain K1^T and acidification of the medium were monitored every 2 days for 10 days. All tests were repeated in triplicate. Compared with *S. thermosulfidooxidans* VKM B-1269^T, strain K1^T utilized a greater number of organic substrates (Tsaplina *et al.*, 1991) and accumulated 1.5–2 times greater biomass (up to 20 mg protein l⁻¹, as determined by the Lowry method). Mannose, ribose, fructose, galactose, sucrose, trehalose, starch, glycine, cysteine, aspartic acid and glutamic acid could support rapid growth. Mannitol, glucose, xylose, sorbose, melezitose, amygdalin, methyl α -D-glucoside, aesculin and glycogen could support slower growth. Weak growth was observed on glycerol, lactose, raffinose, turanose, arabinose and maltose. Acidification of the medium did not exceed 0.2 of a pH unit. All other carbohydrates tested did not support growth. Strain K1^T and all other strains except *Alicyclobacillus herbarius* do not reduce nitrate to nitrite. *Alicyclobacillus pomorum* and *A. cycloheptanicus* were oxidase-positive; strain K1^T was weakly oxidase-positive. *A. pomorum* was catalase-positive; strain K1^T was weakly catalase-positive. Gelatin was hydrolysed by *A. acidiphilus*, *A. cycloheptanicus* and *A. herbarius*. Starch was hydrolysed by *A. acidocaldarius*, *A. hesperidum*, strain K1^T and strain SD-11^T. Most noticeable growth of strain K1^T (up to 30 mg protein l⁻¹) was observed in the presence of 0.1 % (w/v) complex organic substrates – peptone, yeast extract and casein hydrolysate – which was accompanied by the excretion of $0.5\text{--}1.2 \times 10^{-3}$ units proteolytic enzymes ml⁻¹, determined according to Nomoto & Narahashi (1956). Much like with the sulfobacilli (Wood & Kelly, 1983; Zakharchuk *et al.*, 1994, 2003; Tsaplina *et al.*, 1994, 2000), mixotrophic growth conditions were optimal for strain K1^T; the most active growth and the highest biomass yield were observed under these conditions (see Fig. A, available as supplementary material in IJSEM Online). The specific growth rate reached values of 0.28 h⁻¹ with glucose (1.2 mM), yeast extract (0.02 %) and 70 mM Fe²⁺ present in the medium. Fe²⁺ oxidation (determined by method of Reznikov *et al.*, 1970) occurred at a rate of 0.62 mM h⁻¹

and the amount of glucose consumed was 80 % (by the method of Ashwell, 1957) of its initial content (see Fig. B, available as supplementary material in IJSEM Online).

After a change in the cultivation conditions from mixotrophic to heterotrophic in the presence of glucose and yeast extract in the medium (first culture transfer), strain K1^T used 60 % of the glucose initially present (1.2 mM) in the medium (see Fig. B, available in IJSEM Online). The specific growth rate reached values of 0.13 h⁻¹. After a change in the cultivation conditions from mixotrophic to autotrophic without organic compounds, strain K1^T exhibited lower growth rates in the first culture transfer ($\mu = 0.035\text{ h}^{-1}$). The rate of iron oxidation was 0.27 mM h⁻¹. Strain K1^T oxidized only 15 % of the ferrous iron (70 mM) added to the medium. Chemo-organoheterotrophic and chemolithoautotrophic growth ceased after six and two culture transfers, respectively. Yeast extract is needed as growth factor for strain SD-11^T (Dufresne *et al.*, 1996).

Of all the alicyclobacilli, only *A. cycloheptanicus*, phylogenetically most closely related to strains K1^T and SD-11^T, required growth factors (certain vitamins and amino acids), but growth of all alicyclobacilli was stimulated by the addition of yeast extract to the medium (Wisotzkey *et al.*, 1992). As with alicyclobacilli, ammonium served as the best source of nitrogen for strain K1^T.

In cells of strain K1^T grown mixotrophically in the presence of glucose, yeast extract and Fe²⁺, the activities of the key enzymes of carbohydrate metabolism were several times higher than in organotrophically grown cells (Karavaiko *et al.*, 2001). The activity of almost all enzymes sharply decreased under autotrophic and heterotrophic conditions. Moreover, only enzymes of the Embden–Meyerhof–Parnas pathway could be detected in the cells grown autotrophically, whereas the key enzymes of the Entner–Doudoroff and pentose-phosphate pathways could not. The activity of pentose-phosphate-pathway enzymes was not detected under organotrophic growth (Karavaiko *et al.*, 2001). The highest protein yield observed in cells of strain K1^T under mixotrophic conditions, together with the highest activity for most carbohydrate metabolism enzymes under these conditions, allows an assumption to be made: that strain K1^T can use additional energy derived from glucose oxidation during mixotrophic growth. Under the mixotrophic conditions the growth was stable. A higher capacity for glucose utilization was characteristic of the thermo-tolerant strain K1^T (as compared with *S. thermosulfidooxidans* VKM B-1269^T) (see Fig. B, available in IJSEM Online) and of the mesophilic strain SD-11^T (Dufresne *et al.*, 1996).

A significant biochemical characteristic of strain K1^T that distinguishes it from sulfobacilli is its complete TCA cycle (Karavaiko *et al.*, 2002). In sulfobacilli, the TCA cycle is blocked at the level of 2-oxoglutarate dehydrogenase, thus merely fulfilling a biosynthetic role (Zakharchuk *et al.*, 1994, 2003; Tsaplina *et al.*, 2000). Some strains of sulfobacilli are

capable of oxidoreduction of iron (Bridge & Johnson, 1998). Similar to the phylogenetically related bacteria bacterium GSM, *A. acidocaldarius* and *Alicyclobacillus*-like strain Y004 (Johnson *et al.*, 2001a, b, 2003), strain K1^T was able also to reduce Fe³⁺: 10 mM for 7–10 days. Similar to bacterium GSM (Johnson *et al.*, 2001a), strain K1^T could weakly assimilate ¹⁴CO₂ under different conditions with Fe²⁺ or S⁰ as the electron donors (see Table B, available as supplementary material in IJSEM Online). Thus, strains K1^T and SD-11^T differ from strains of the genus *Sulfobacillus* by a more-efficient organotrophic growth and from *Alicyclobacillus* strains by the requirement for inorganic electron donors. Judging from a number of the foregoing features, strain K1^T occupies an intermediate position between the genera *Sulfobacillus* and *Alicyclobacillus*. Presumably, the well described bacilli GSM and SD-11^T (Johnson *et al.*, 2001a, 2003; Dufresne *et al.*, 1996) and some unclassified strains may also group together with strain K1^T.

On the basis of the above-mentioned morphological, physiological, chemotaxonomic and phylogenetic characteristics of strains K1^T and SD-11^T, two novel species are proposed, *Alicyclobacillus tolerans* sp. nov. and *Alicyclobacillus disulfidooxidans* comb. nov., respectively. Comparative characteristics of strains K1^T and SD-11^T and alicyclobacilli are given in Table 1.

Emended description of the genus

Alicyclobacillus Wisotzkey *et al.* 1992 emend. Goto *et al.* 2003

The description of the genus *Alicyclobacillus* (Wisotzkey *et al.*, 1992; Goto *et al.*, 2003) should be supplemented with the following phenotypic characteristics. Temperature growth range is broader (<20–70 °C). Optimum growth temperature range is 35–60 °C. Species have a more diverse type of nutrition than previously thought: can also grow mixotrophically. The G+C content of the DNA ranges from 48.7 to 62.7 mol% (*T_m*).

The type species is *Alicyclobacillus acidocaldarius* (Darland and Brock 1971) Wisotzkey *et al.* 1992.

Description of *Alicyclobacillus tolerans* sp. nov.

Alicyclobacillus tolerans (tol.er'ans. L. part. adj. *tolerans* tolerant to changes in the growth temperature and pH).

Cells are non-motile, aerobic, Gram-positive, spore-forming rods, often occurring in short chains composed of two to four cells. Rods are either straight or slightly curved, 3–6 µm long and 0.9–1.0 µm wide. Reproduction is by binary fission with the transverse septum formation. The cytoplasm contains numerous vesicular inclusions, polyphosphate and poly-β-hydroxybutyrate granules. The location of oval spores is terminal or subterminal, enlarging the sporangium. Mixotroph, slowly oxidizing Fe²⁺, S⁰ or sulfide minerals in the presence of 0.2 g yeast extract l⁻¹ or organic substrates (Table 1). Capable of reducing Fe³⁺. Facultative chemo-organoheterotroph. Organotrophic

growth is supported by the substrates indicated in Table 1. The best source of nitrogen is ammonium. Thermotolerant: temperature range <20–55 °C; optimum 37–42 °C. Obligate acidophile: pH range for growth 1.5–5.0; optimum 2.5–2.7. The G+C content of the DNA is 48.7 ± 0.6 mol%. Fatty acid profile is mainly composed of ω-cyclohexane-C_{17:0}, ω-cyclohexane-C_{19:0}, ω-cyclohexane-C_{17:0} 2OH and ω-cyclohexane-C_{18:0}. Normal and branched-chain iso- and anteiso-acids are also present. The main isoprenoid quinone is menaquinone MK-7 (90 %); minor component is MK-6. Isolated from oxidizable lead–zinc ores (Uzbekistan).

The type strain is K1^T (= VKM B-2304^T = DSM 16297^T).

Description of *Alicyclobacillus disulfidooxidans* comb. nov.

Alicyclobacillus disulfidooxidans (di.sul.fi.do.ox'i.dans. L. adj. *duplus* double; L. n. *sulfur* sulfur; N.L. v. *oxido* oxidize; N.L. adj. *disulfidooxidans* disulfide-oxidizing bacterium).

Basonym: *Sulfobacillus disulfidooxidans* Dufresne *et al.* 1996.

The description is that given by Dufresne *et al.* (1996).

The type strain is SD-11^T (= ATCC 51911^T = DSM 12064^T).

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