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Acidithiobacillus sulfuriphilus sp. nov.: an extremely acidophilic sulfur-oxidizing chemolithotroph isolated from a neutral pH environment

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Abstract

The genus *Acidithiobacillus* currently includes seven species with validly published names, which fall into two major groups, those that can oxidize ferrous iron and those that do not. All seven species can use zero-valent sulfur and reduced sulfur oxy-anions as electron donors, are obligately chemolithotrophic and acidophilic bacteria with pH growth optima below 3.0. The 16S rRNA gene of a novel strain (CJ-2^T) isolated from circum-neutral pH mine drainage showed 95–97 % relatedness to members of the genus *Acidithiobacillus*. Digital DNA–DNA hybridization (dDDH) values between strains and whole-genome pairwise comparisons between the CJ-2^T strain and the reference genomes available for members of the genus *Acidithiobacillus* confirmed that CJ-2^{T}represents a novel species of this genus. CJ-2^T is a strict aerobe, oxidizes zero-valent sulfur and reduced inorganic sulfur compounds but does not use ferrous iron or hydrogen as electron donors. The isolate is mesophilic (optimum growth temperature 25–28 °C) and extremely acidophilic (optimum growth pH 3.0), though its pH optimum and maximum were significantly higher than those of non-iron-oxidising acidithiobacilli with validly published names. The major fatty acids of CJ-2^T were C18 : 1ω7*c*, C:16 : 1ω7*c*/iso-C15 : 0 2-OH, C16 : 0 and C19 : 0 cyclo ω8*c* and the major respiratory quinone present was Q8. The name *Acidithiobacillus sulfuriphilus* sp. nov. is proposed, the type strain is CJ-2^T (=DSM 105150^T=KCTC 4683^T).

The first acidophilic sulfur-oxidizing chemolithotroph, named originally as *Thiobacillus thiooxidans*, was described in 1922 by Waksman and Joffe [1]. The genus renamed as *Acidithiobacillus* [2], includes seven species with validly published names, three of them described during the last eight years [2–7]. The acidithiobacilli are chemolithotrophic acidophilic bacteria with pH growth optima below 3.0 and grow between pH 0.5 and 5, although individual species have narrower pH ranges [3–7]. The majority of the known species of the genus *Acidithiobacillus* are mesophiles, with temperature optima at around 30 °C, with the exception of the moderately thermophilic *Acidithiobacillus caldus*, which has an optimum temperature for growth of 45 °C [3]; and *Acidithiobacillus ferrivorans* and some strains of *Acidithiobacillus ferriphilus* that can also to grow at temperatures as low as 4 °C and, therefore, are psychrotolerant [5, 7]. Physiologically, the acidithiobacilli can be divided into two groups: species that can use ferrous iron as an electron donor (*A. ferrooxidans, A. ferrivorans, A. ferridurans,* and *A. ferriphilus*; [5–8]) and those that cannot (*A. thiooxidans*, *A. albertensis* and *A. caldus*; [1, 3, 9]). All species can use zero-valent (elemental) sulfur and reduced sulfur oxy-anions as electron donors and some can also use hydrogen [10]. Results from a plethora of studies carried out during the last 20 years have indicated that the inherent phenotypic and genotypic diversity within the genus *Acidithiobacillus* was higher than first suspected (e.g. [11]). Recently, a study on the hierarchical relationships among members of the genus used a variety of molecular markers and typing approaches to show that the genus potentially includes unrecognized genera and species [12].

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Abbreviation: dDDH, digital DNA–DNA hybridization.
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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and whole-genome shotgun sequences of strain CJ-2^T are MK193868 and RIZI01000000, respectively.
Three supplementary figures and three supplementary tables are available with the online version of this article.

A novel member of the genus *Acidithiobacillus*, strain CJ-2^T, isolated from a circum-neutral pH mine water draining an abandoned lead mine in Wales (UK) is described in this paper. The isolate clusters into the acidithiobacilli, the binomial *Acidithiobacillus sulfuriphilus* sp. nov. is proposed for this isolate.

ISOLATION AND CULTIVATION

CJ-2^T was isolated from a mine-drainage sample obtained on April 2016 from the adit that originates at the Catherine and Jane Consols lead mine (Fig. S1, available in the online version of this article) located in Wales (52° 56′ 59.28″ N, 4° 02′ 09.92″ W), which has been closed since the 19th century. The pH of the water was 6.07. No iron was detected in solution and clear evidence of iron precipitates was found in the adit. No other transition metals analysed were detected in the mine waters, the concentrations of Zn, Ni, Cu, Co and Mn were less than the detection limit for the analytical method used (ion chromatography; data not shown). The strain was isolated on an 'overlay' solid medium developed for the growth of acidophilic microorganisms [13]. A small drop of the water sample collected from the adit was spread on solid medium containing ferrous iron, potassium tetrathionate and tryptone soya broth, pH 2.8, and was incubated at 30 °C for three weeks. CJ-2^T was purified by repeated single colony re-isolation. The isolate was transferred to a basal salt medium with trace element solution (ABS-TE; [14]) amended with 2.5 mM tetrathionate (as $K_2S_4O_6$) and adjusted to an initial pH value of 4.0. The isolate was maintained in ABS-TE media containing zero-valent sulfur (1 % w/v) (pH 4.0) at room temperature and transferred once every five weeks to fresh sulfur medium. CJ-2^T forms circular white colonies on solid medium. The presence of straight motile rods (1.5–2.5 µm long and 0.5 µm wide) and absence of endospores was observed by phase contrast microscopy. Presence of a single polar flagellum and a thick glycocalyx was observed by transmission electron microscopy on cells grown on liquid media with tetrathionate as an energy source, while fimbriae-like structures were observed by scanning electron microscopy on surface-attached cells (Fig. S2).

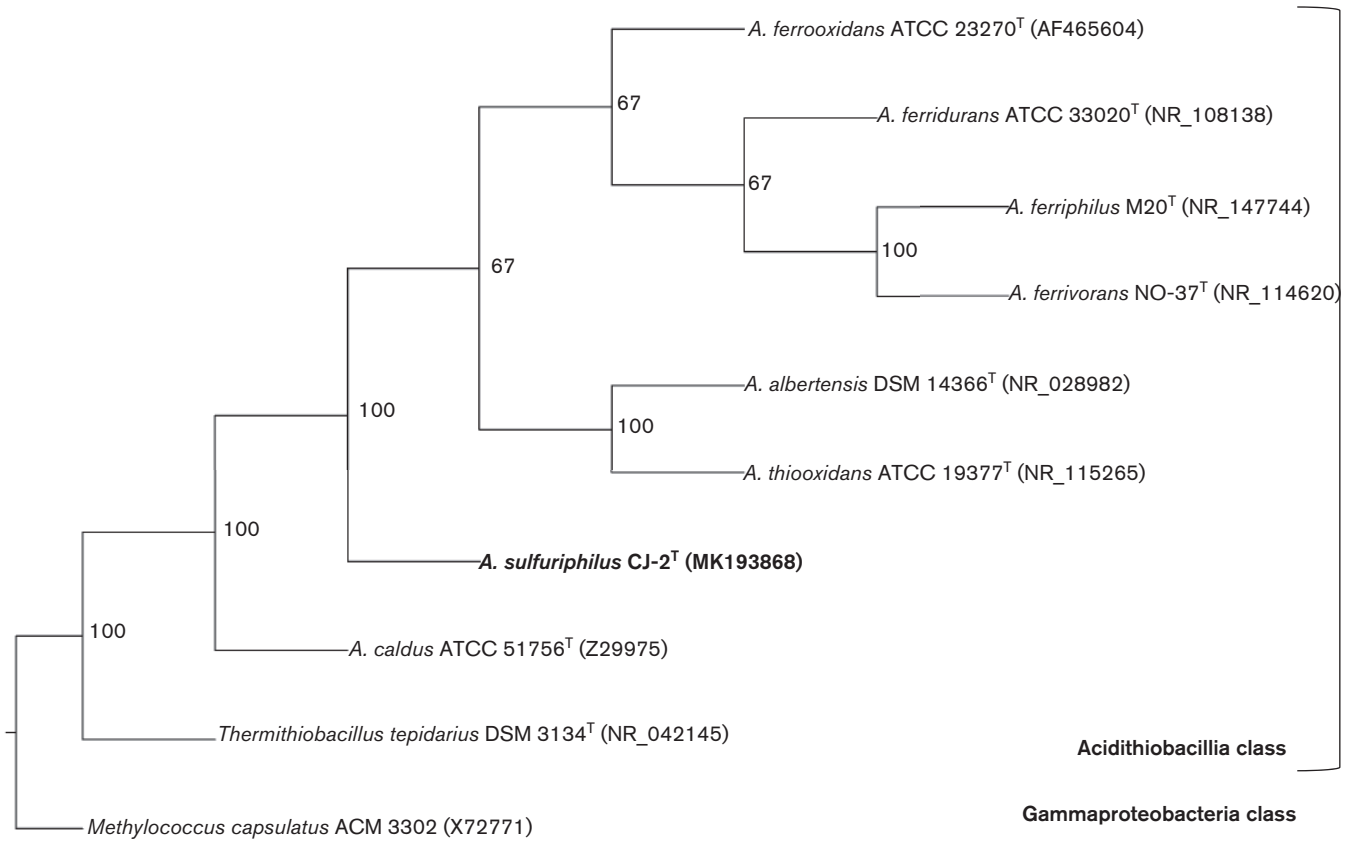

Fig. 1. Consensus phylogenetic tree derived from the 16S rRNA gene sequences showing the relationship of CJ-2^T with the type strains of the species with validly published names of the genus *Acidithiobacillus* and the genus *Thermithiobacillus*, the only other known genus in the class. The Gammaproteobacterium *Methylococcus capsulatus* ACM 3302 (X72771) was used as outgroup. Bootstrap values are indicated at the respective nodes in the consensus tree derived using the ML, NJ and BI phylogenetic treeing algorithms. Individual trees with branch length information are provided in Fig. S3.

GENOME AND PHYLOGENY

Total DNA was extracted from a tetrathionate-grown culture of CJ-2^T at mid-exponential growth phase using conventional methods [15]. The genome of CJ-2^T was sequenced using Illumina sequencing technology (MiSeq platform) and paired-end libraries with insert sizes of ~460 bp (Nextera DNA Sample Preparation kit). Sequencing reads were processed, assembled *de novo* and scaffolded as described by Castro *et al.* [16]. The final draft assembly contained 195 contigs (length >500 bp; depth of coverage >110×) and is based on 2.2 Gbp of Illumina data. The total size of the draft genome is ~2.8 Mbp and the DNA G+C content is 61.5 mol%. This whole-genome shotgun project has been deposited at GenBank under the accession number RIZI01000000. The version described in this paper is version RIZI01000000.1.

The complete 16S rRNA gene sequence was extracted from the draft genome sequence using the Barrnap (BAsic Rapid Ribosomal RNA Predictor version 0.9-dev). The GenBank accession number for the 16S rRNA gene sequence of CJ-2^T is MK193868. The most conserved region (nucleotide coordinates 113 to 1300) was compared with the 16S rRNA gene sequences available in GenBank nr and RefSeq databases (as of June 2018) using BLASTN 2.8.0 with default parameters. The sequence identity of the isolate against a previous 16S rRNA gene sequence deposited in GenBank for CJ-2^T (KX426303) was 99 %, validating its identity. Also, high identity (>98 %) scores were obtained between CJ-2^T and a number of uncultured clones of geo/hydro-thermal origin related to the members of the genus *Acidithiobacillus* (Table S1). Identity against all sequenced type strains or reference strains of members of the genus *Acidithiobacillus* fell below the conventional species cut-off values (Table S2), making specific assignment of the isolate uncertain.

Small subunit ribosomal RNA gene sequences of CJ-2^T and the type strains of species of the genus *Acidithiobacillus* with validly published names were aligned using MAFFT v (7.229) [17]. The resulting alignments were trimmed and masked (>50 %) manually. Phylogenetic trees were reconstructed with (1) the neighbour-joining (NJ) algorithm [18], (2) the maximum-likelihood (ML) algorithm [19] based on the best-fit model of nucleotide substitution using a generalized time-reversible (GTR) model [20] and bootstrap of 1,000 replicates, and (3) using Bayesian Inference (BI) as implemented in MrBayes v.3.0b4 [21], run for 1,000,000 generations, saving trees every 10 ,000 generations, and calculating posterior probabilities after discarding the first 25 % of trees. Limited disagreement in topology was observed between trees reconstructed using the three methods (Fig. S3). The consensus tree, reconstructed with PHYLIP [22], is shown in Fig. 1. Phylogenetic analysis of the 16S rRNA gene sequence placed CJ-2^T outside the clade grouping all the other mesophilic members of the genus *Acidithiobacillus*, yet clearly separated from the *A. caldus* branch (Fig. 1).

The genomic taxonomy of CJ-2^T was evaluated using well-acknowledged genomic relatedness indexes, including the average nucleotide identity (ANI) [23] and digital DNA–DNA hybridization (dDDH) [24]. The average nucleotide identity between the CJ-2^T draft genome and those of the reference type strains of species of the genus *Acidithiobacillus* were calculated using pyani [25] and the dDDH values between strains were calculated using the Genome-to-Genome Distance Calculator (GGDC) web server [26]. Whole-genome pairwise comparisons between CJ-2^T and the reference genomes available for this study using these indexes (Table 1) were, in both cases, well below the established thresholds used for prokaryotic species delimitation. Both indexes strongly indicate that the genomic divergence between the CJ-2^T isolate and sequenced type strains of the taxon warrants its recognition as representing a novel species within the genus.

To further support these findings, phenotypic and chemotaxonomic differentiating characteristics between CJ-2^T and other acidithiobacilli were investigated (Table 2).

PHYSIOLOGY AND CHEMOTAXONOMY

To test the ability of CJ-2^T to grow autotrophically on reduced-sulfur inorganic compounds duplicate cultures were grown in a basal salt medium with trace elements solution [14] containing zero-valent sulfur, 2.5 mM tetrathionate or 5 mM thiosulfate, and pH adjusted to 3.0. Cultures were incubated aerobically at 30 °C and shaken at 100 r.p.m.

Table 1. Genomic relatedness indexes (%) calculated between CJ-2^T and other strains of species of the genus *Acidithiobacillus*

Accession	Strain	dDDH*	ANIb†	ANIm†
RIZI01	*Acidithiobacillus* sp. CJ-2^T	100.0	100.00	100.00
NC_011761	*A. ferrooxidans* ATCC 23270^T	23.7	76.11	88.38
AF0H01	*A. thiooxidans* ATCC 19377^T	23.5	72.90	92.11
MOAD01	*A. albertensis* DSM 14366^T	19.6	72.54	86.77
CO005986	*A. caldus* ATCC 51756^T	19.0	73.58	84.18
AUIS01	*Thermithiobacillus tepidarius* DSM 3134^T	18.0	73.24	83.22

*If dDDH >70 % strains represent the same species; [23].

†If ANI >96 % strains represent the same species; [24].

Table 2. Growth phenotypic features of species of the genera *Acidithiobacillus* and *Thermithiobacillus*: 1, CJ-2^T; 2, *A. ferrooxidans*^{T} [5, 6]; 3, *A. ferridurans*^{T} [6]; 4, *A. ferrivorans*^{T} [5]; 5, *A. ferriphilus*^{T} [7]; 6, *A. thiooxidans*^{T} [1, 2, 5, 10]; 7, *A. albertensis*^{T} [2, 9, 28]; 8, *A. caldus*^{T} [3]; and 9, *T. tepidarius*^{T} [33]. +, positive; −, negative; ND; no data.

Abbreviations for polar lipids: AL, aminolipid; PE, phosphatidylethanolamine; PG, phophatidylglycerol; PL, phospholipid; PME, phosphatidylmethylethanolamine; UA, unknown aminolipids.

	1	2	3	4	5	6	7	8	9
Gram staining	−	−	−	−	−	−	−	−	−
Motility	+	−	−	+	+	+	+	+	+
Temperature									
Growth at 4 °C	−	−	−	+	−	−	ND	ND	−
Growth at 10 °C	+ (15 °C)	−	+	+	+	+	ND	ND	−
Growth at 30 °C	+	+	+	+	+	+	+	ND	+
Growth at 45 °C	−	−	−	−	−	−	ND	+	+
Optimum (°C)	25–28	30–35	29	28–33	30	28–30	25–30	45	43–45
pH									
Lowest pH for growth	1.8	1.5	1.4	1.9	1.5	0.5	2.0	1.0*	5.2
Growth at pH 3	+	+	+	+	+	+	+	+	+
Highest pH for growth	7.0	ND	ND	ND	ND	5.5	4.5	3.5	8.0
Optimum	3.0	2.5	2.1	2.5	2.0	2.0–3.0	3.5-4-0	2.0–2.5	6.8–7.5
Electron donors (Electron acceptor)									
Sulfur (O_2)	+	+	+	+	+	+	+	+	+
Tetrathionate (O_2)	+	+	+	+	+	+	+	+	+
Thiosulfate (O_2)	+	+	+	+	+	+	+	+	+
Ferrous iron (O_2)	−	+	+	+	+	−	−	−	−
Pyrite (O_2)	−	+	+	+	+	−	−	−	−
Hydrogen (O_2)	−	+	+	−	−	−	ND	+	ND
Tetrathionate (Fe^{3+})	−	+	+	+	+	+	ND	ND	ND
Usage of organic carbon as a carbon source	−	−	−	−	−	−	−	+	−
Chemotaxonomic characteristics									
DNA G+C content (mol %)	61.5	58–59	58	55–56	57.4	52	61.5	63.9	66.6
Respiratory quinones	Q8 (98 %) Q7 (2 %)	ND	Q8	Q8	Q8 (94 %) Q9 (3 %) Q7 (2 %)	ND	ND	ND	Q8
Polar lipids	AL PG PME	ND	PG PE UA	ND	AL PL PG	ND	ND	ND	ND

*Growth at pH 1.0, but not at pH 0.5. Values between those figures have not been tested.

The pH of the cultures decreased in all three cultures (cell numbers increased only in cultures grown in the presence of tetrathionate and thiosulfate) confirming growth of the isolate on all these electron donors. CJ-2^T was unable to oxidize molecular hydrogen or ferrous iron or catalyse the dissolution of pyrite in aerobically-grown cultures, unlike some other species and strains of the genus *Acidithiobacillus* [2, 3, 10, 27, 28]. CJ-2^T was grown under anaerobic conditions [oxygen was removed by activated carbon using AnaeroGen sachets (Fisher)] on tetrathionate using ferric iron as a potential electron acceptor. Cell numbers achieved after four weeks of incubation at 30 °C (data not shown) indicated that this isolate was unable to reduce ferric iron coupled to the oxidation of tetrathionate under anaerobic, conditions confirming that CJ-2^T is an obligate aerobe.

The capacity of CJ-2^T to grow as a chemolitho-heterotroph, using an organic carbon source and an inorganic electron donor, was tested by cultivating the isolate in media (pH 3.0) containing 2.5 mM tetrathionate and 0.02 % yeast extract, 5 mM glucose or 0.02 % tryptone, together with organic carbon-free medium (control). After 30 days of incubation at 30 °C, cell numbers were similar in all the cultures, confirming that CJ-2^T was an obligate autotroph, as are other acidithiobacilli.

Optimum temperature and optimum pH for growth were determined by growing CJ-2^T in batch mode in a reactor vessel coupled with FerMac unit (Electrolab Biotech) and with pH and temperature controls. The isolate was grown on 4 mM tetrathionate as an electron donor, oxygen as an electron acceptor and stirred at 100 r.p.m., the pH was

maintained by automated addition of 250 mM H_2SO_4. To determine the optimum pH for growth, the temperature was fixed at 28 °C, and for the optimum temperature pH was maintained at 2.5. Mean generation time was determined by direct cell counting during the exponential phase of growth. CJ-2^T had its optimum temperature for growth between 25 and 28 °C (Fig. 2), typical of mesophiles, and was unable to grow at 4 °C or at 45 °C. The optimum pH value for growth of the isolate was 3.0 (Fig. 2), which is within the pH limit that circumscribes extreme extremophiles [28, 29]. CJ-2^T was less tolerant of extreme acidity as it did not grow at pH values lower than 1.8, unlike other species of non-iron oxidizing members of the genus *Acidithiobacillus*, which can grow at pH values of 0.5–1.0. CJ-2^T was able to grow at pH 7 (although not at pH 8), making it more alkali-tolerant than most other acidithiobacilli.

To test the ability of CJ-2^T to tolerate the presence of selected metals, the isolate was grown (in duplicate) in basal salt media and trace elements containing 2.5 mM tetrathionate and amended with different concentrations of copper, ferric iron, ferrous iron or zinc, all added as their sulfate salts (Table 3). The initial pH of the media used was 3.0 except for ferric and ferrous iron where it was set at 2.0 to avoid precipitation of iron. Salt-tolerance (NaCl) and osmotolerance were tested using a similar approach, with media containing increasing concentrations of sodium chloride or magnesium sulfate; initial pH of the cultures was set at 4.0. Growth was determined from cell counts and compared with control assays, free of metal, NaCl or $MgSO_4$. CJ-2^T was more sensitive to copper than any of the other metals tested (Table 3), similar to *A. thiooxidans*, which is very sensitive to copper (tolerance $\leq$20 mM, *A. thiooxidans* strain SFR01 [30]), and *A. caldus* which can tolerate up to 23 mM copper [31]. CJ-2^T was highly tolerant to ferrous iron; concentrations higher than 500 mM were not tested due to chemical oxidation of this metal at pH>3. CJ-2^T was able to grow in media containing 300 mM NaCl, but showed greater tolerance (500 mM) to $MgSO_4$. Although metal and salt tolerances exhibited by acidophiles are pH-dependant [32], the sulfur- and iron-oxidizing species of the genus *Acidithiobacillus* can, in general, grow at higher concentrations of copper than of NaCl, except for some strains of *A. ferriphilus* [5–7], while non-iron-oxidizing species of the genus *Acidithiobacillus* tend to be more tolerant of NaCl than of copper [32].

To compare the chemotaxonomic characteristics of CJ-2^T with those of other acidithiobacilli, the isolate was grown on a bench scale reactor on tetrathionate under the optimal growth conditions (pH 3.0 and 28 °C). Biomass was harvested and freeze dried. Analyses of respiratory quinones, fatty acids and polar lipids were carried out by the Identification Service and Dr. Brian Tindall at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. The major polar lipids found in CJ-2^T grown on tetrathionate were aminolipids, phosphatidylglycerol and phosphatidylmethylethanolamine (Table 2). The major fatty acids profile of CJ-2^T (C18 : 1ω7*c*, C16 : 1ω7*c*/iso-C15 : 0 2-OH, C16 : 0 and C19 : 0 cyclo ω8*c*) was distinct from those reported previously for members of the taxon (Table S3). The major respiratory quinone present in CJ-2^T is Q8 (98 %) with a smaller amount of Q7 (2 %), which is also the case for most other acidithiobacilli.

In summary, whole-genome pairwise comparisons indexes strongly indicate that the genomic divergence between CJ-2^T and sequenced genomes of type strains of members of the taxon warrants its recognition as representing a novel species within the genus *Acidithiobacillus*. CJ-2^T can be discriminated from other species in the taxon by its higher optimal pH for growth. Unlike the type strains of iron-oxidising members of the genus *Acidithiobacillus*, CJ-2^T is not able to oxidise ferrous iron, or catalyse the dissolution of pyrite in aerobically-grown cultures and unlike other sulfur-oxidising acidithiobacilli it is not able to oxidise molecular hydrogen. The major fatty acids profile of CJ-2^T was distinct from those reported previously for the taxon. In conclusion, phylogenetic, physiological and phenotypic tests carried out

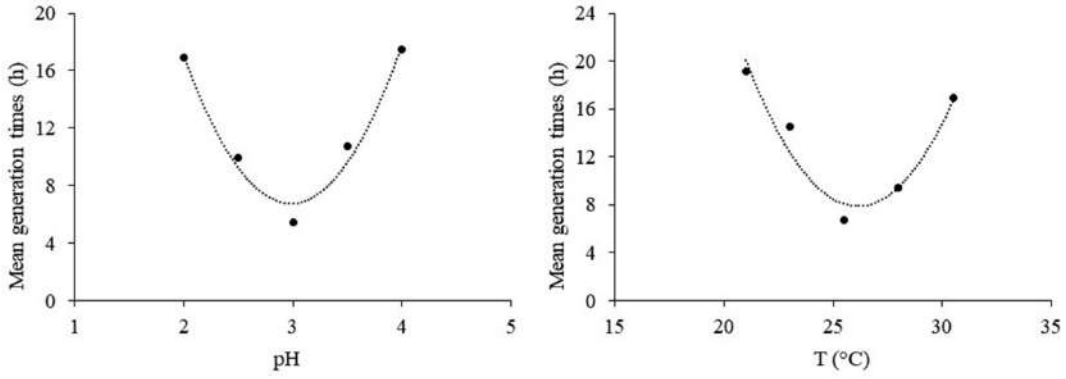

Fig. 2. Mean generation times of CJ-2^T in ABS-TE medium supplemented with 4.0 mM tetrathionate at different pH values (left) and fixed temperature (28 °C); and at different temperatures (right) and pH fixed at 2.5.

Table 3. Minimum inhibitory concentrations and maximum concentrations (in parenthesis) at which growth was detected for CJ-2^T and other acidithiobacilli [1–5, 33]. Metals were provided as sulfate salts. All concentrations are given in mM. ND, no data.

	Initial pH	Fe (II)	Fe(III)	Zn	Cu	$MgSO_4$	NaCl
CJ-2^T	4.0	>500*	500 (300)	300 (200)	50 (25)	700 (500)	500 (300)
***A. caldus*^{T}**	1.8	ND	>34	>99	>23	ND	685 (513)†
***A. thiooxidans*^{T}**	3.0	500 (300)	>500	1000 (700)	10 (5)	1000 (700)	800 (500)‡
***A. ferrooxidans*^{T}**	2.0	400 (200)	400 (200)	1000 (800)	500 (400)	1000 (800)	500 (250)‡
***A. ferridurans*^{T}**	2.0	600 (400)	300 (200)	1000 (800)	300 (200)	1200 (1000)	800 (700)‡
***A. ferrivorans*^{T}**	2.0	400 (200)	<100	300 (200)	<50	1000 (800)	ND
***A. ferriphilus*^{T}**	1.8–2.0	1000 (900)	500 (300)	800 (700)	500 (300)	1000 (900)	500 (250)

*Higher concentration prevented cell counting as iron precipitates at pH (4.0) of the media used.

†The initial pH of the media used was 2.5.

‡The initial pH of the media used was 3.0.

with this isolate confirmed that it represents a novel species of the genus *Acidithiobacillus*, for which the name *Acidithiobacillus sulfuriphilus* sp. nov. is proposed.

DESCRIPTION OF *ACIDITHIOBACILLUS SULFURIPHILUS* SP. NOV.

Acidithiobacillus sulfuriphilus (sul.fu.ri'phi.lus. L. n. *sulfur* sulfur; N.L. masc. adj. *philus* (from Gr. masc. adj. *philos*) friend, loving; N.L. masc. adj. *sulfuriphilus* sulfur-loving, referring to its ability to grow only using reduced forms of sulfur).

Strain CJ-2 forms circular white colonies on solid medium. Straight, motile, flagellated and fimbriated rods (1.5–2.5 µm long and 0.5 µm wide), does not form endospores, stains Gram-negative. Obligate chemolitho-autotroph, uses zero-valent sulfur and reduced inorganic sulfur anions as electron donors. Strict aerobe, uses only molecular oxygen as electron acceptor. Mesophilic and acid-tolerant; the optimum growth pH is approximately 3.0 (minimum pH value for growth is 1.8) and optimum growth temperature is 25–28 °C.

The type strain, CJ-2^T (=DSM 105150^T=KCTC 4683^T) was isolated from an adit draining a former lead mine located in Wales (UK). The only other isolate belonging to this novel species was isolated from mine tailings in China, and clones of the species have been detected in geothermal and hydrothermal habitats. The G+C content of the chromosomal DNA of the type strain is 61.5 mol%. The GenBank/EMBL/DDBJ accession numbers of strain CJ-2^T are MK193868 and RIZI01000000.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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