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TAXONOMIC DESCRIPTION

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Acidiferrimicrobium australe gen. nov., sp. nov., an acidophilic and obligately heterotrophic, member of the Actinobacteria that catalyses dissimilatory oxido-reduction of iron isolated from metal-rich acidic water in Chile

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Abstract

A novel acidophilic member of the phylum *Actinobacteria* was isolated from an acidic, metal-contaminated stream draining from an abandoned underground coal mine (Trongol mine), situated close to Curanilahue, Biobío Region, Chile. The isolate (USS-CCA1^T) was demonstrated to be a heterotroph that catalysed under aerobic conditions the oxidation of ferrous iron and the reduction of ferric iron under anaerobic conditions, but not the oxidation of sulfur nor hydrogen. USS-CCA1^T is a Gram-positive, motile, short rod-shaped, mesophilic bacterium with a temperature growth optimum at 30 °C (range 20–39 °C). It was categorized as an extreme acidophile growing between 1.7 and 4.5 and optimally at pH 3.0. The G+C content of the chromosomal DNA of the isolate was 74.1 mol%, which is highly related to *Aciditerrimonas ferrireducens* IC-180^T, (the most closely related genus; 94.4% 16S rRNA gene identity), and higher than other acidophilic actinobacteria. The isolate (USS-CCA1^T) was shown to form a distinct 16S rRNA clade from characterized acidophilic actinobacteria, well separated from the genera *Acidimicrobium*, *Ferrimicrobium*, *Ferrithrix*, *'Acidithrix'* and *Aciditerrimonas*. Genomic indexes (ANIb, DDH, AAI, POCP) derived from the USS-CCA1^T draft genome sequence (deposited at DDBJ/ENA/GenBank under the accession WJHE00000000) support assignment of the isolate to a new species and a new genus within the *Acidimicrobiaceae* family. Isolate USS-CCA1^T is the designated type strain of the novel species *Acidiferrimicrobium australe* (=DSM 106828^T,=RGM 2506^T).

Acidic environments in which sulfidic minerals are subjected to bacterially-accelerated oxidative dissolution, contain significant concentrations of sulfate and transition metals [1]. The biodiversity of life forms that can grow in such waters comprise a large variety of different species distributed in all three domains. Although, the greatest number of known acidophilic bacteria are members of the phylum *Proteobacteria* that grow in low pH environments, such as areas impacted by mining activities, other phyla including *Firmicutes*, *Nitrospirae*, *Aquificae* and *Actinobacteria* live in the same environments forming complex associations [2]. Known species of Gram-positive acidophilic bacteria are facultative autotrophs or obligate heterotrophs, divided into two phyla: *Firmicutes* of genera *Sulfobacillus*, *Alicyclobacillus and Acidibacillus* [2] and *Actinobacteria* . The latter comprises four classified genera with validly published names, all of them containing a single species: *Acidimicrobium* (*Am.*) *ferrooxidans* ICP^T [3], *Ferrimicrobium* (*Fm.*) *acidiphilum* T23^T, *Ferrithrix* (*Fx.*) *thermotolerans* Y005^T [4], *Aciditerrimonas* (*Act.*) *ferrireducens* IC-180^T (JCM 15389^T) [5]. In addition, *'Acidithrix* (*Acx.*) *ferrooxidans'* Py-F3 [6] and the candidatus genus '*Acidithiomicrobium*' [7] were described as novel acidophilic members of the phylum *Actinobacteria*. During a study of microbial diversity from an acidic stream draining from an abandoned coal mine, in the

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One supplementary table and four supplementary figures are available with the online version of this article.

south of Chile, a novel strain of an acidophilic, iron-oxidizing microorganism was obtained on solid media. Based on physiological, chemotaxonomic and genomic characteristics of strain USS-CCA1^T we propose the novel species in the novel genus *Acidiferrimicrobium australe* within the phylum *Actinobacteria*.

ISOLATION AND CULTIVATION

Strain USS-CCA1^T was isolated from macroscopic streamer growths in an acidic, metal-contaminated stream draining from an abandoned underground coal mine (Trongol mine), situated close to Curanilahue, in the southern Biobío Region of Chile (37° 33′ 07.5′ S, 73° 22′ 45.2′ W). The acidic mine waters (pH 4.2) that drain from the Trongol mine contained a mixture of elevated transition metals such as soluble iron (ca. 47.5 mg l^{-1}), manganese (ca. 37.2 mg l^{-1}) and zinc (ca. 9 mg l^{-1}). Streamers were collected (ca. 1 to 5 cm^3) and taken to the laboratory within 2 h of sampling. After disruption, the streamer was serially diluted in autotrophic basal salt media [8] and spread onto ferrous iron/tetrathionate/ tryptone soya broth solid overlay plates [9]. Plates were incubated under oxic conditions at 30 °C for up to 3 weeks. The isolate was purified by repeated single-colony streaking using the same solid medium and incubation conditions. After the purity of the isolate was confirmed by 16S rRNA gene sequencing, it was transferred into a liquid medium, pH 3.0, containing 0.01% (w/v) yeast extract, 5 mM glucose and basal salts [8], and incubated at 30 °C shaken at 100 rpm.

When viewed under the phase contrast microscope, cells of strain USS-CCA1^T proved to be short motile rods. Cells are 0.5–0.7 µm in width and 0.6–0.9 µm in length as observed by scanning electron microscopy (Fig. S1, available in the online version of this article). Numbers of individual cells increased during early phases of incubation (2 days) but declined subsequently, which appeared to be related to cells aggregating as incubation progressed. Endospores were not observed using phase-contrast microscopy. Colonies on solid media (~2 mm in diameter) had a distinct 'fried egg' -like morphology [9], typical of heterotrophic iron-oxidizing acidophiles with orange coloration of the colony centres resulting from the accumulation of oxidized iron.

PHENOTYPIC AND CHEMOTAXONOMIC CHARACTERIZATION

Optimum pH values and temperatures for growth were determined by growing the bacterium in a pH- and temperature-controlled 2L bioreactor as described elsewhere [6] using the defined medium described above. The bioreactor was stirred at 100 rpm. and aerated with ~0.5 L min^{-1} of sterile atmospheric air. To determine optimum pH, the temperature was maintained at 30 °C and pH fixed at set values between 2.0 and 4.0; to determine the optimum temperature for growth, pH was maintained at 3.0 and temperature varied from between 25 and 37 °C. Samples were collected at regular intervals and their optical densities measured at 600 nm (OD_{600}). Culture doubling times were evaluated from semi-logarithmic plots of OD_{600} values against time (Fig. S2). Optimum growth of strain USS-CCA1^T was observed at pH 3.0 and 30 °C, where its doubling time was about 6 h. By culturing the isolate in shake flasks, the lowest pH at which growth was recorded was pH 1.7 and no growth was observed at pH 1.6. In addition, the isolate was unable to grow above pH 4.5 and 39 °C and below 20 °C. Therefore, strain USS-CCA1^T fits the definition of extreme acidophiles (microorganisms that have pH optima at 3.0 or below [10],). Like *Fm. acidiphilum* T23^T, strain USS-CCA1^T appears to be a mesophilic and the absence of growth in an organic-free medium suggests that this acidophile is an obligate heterotroph. The isolate did not grow on defined single carbon sources, however the addition of small amounts of yeast extract to a defined medium was required for growth. A comparing summary of organic substrates utilized by strain USS-CCA1^T and other acidophilic actinobacteria is given in Table 1. The ability to oxidize ferrous iron (10 mM) or catalyse the dissolution of pyrite (1%) under aerobic conditions was tested by cultivating the isolate in media (pH 2.0) with yeast extract (0.01%) and basal salts [8], together with a non-inoculated control and incubated at 30 °C. Iron oxidation was found to be coupled to increased cell numbers on ferrous iron and pyrite and confirms that acidophilic microorganisms that catalyse the dissimilatory oxidation of ferrous iron should catalyse the dissolution of pyrite, as ferric iron is the main oxidant of this sulfide mineral under acidic conditions [2]. Iron oxidation has been demonstrated for other acidophilic actinobacteria, but not for *Act. ferrireducens* IC-180^T [5]. Dissimilatory reduction of ferric iron by the isolate was tested. Duplicate flasks containing amorphous ferric hydroxide were filled with liquid medium pH 2.0 containing glucose (5 mM) and yeast extract (0.01%). A sterile control was set up in parallel. The flasks were placed in sealed 2.5 L jars in which an anoxic environment was generated (using an Anaerogen sachet; Oxoid, UK) and another three flasks in a separate jar under micro-aerobic conditions (CampyGen sachet; Oxoid, UK). In common with other acidophilic actinobacteria, strain USS-CCA1^T was able to catalyse the dissimilatory iron reduction under anaerobic conditions, though no reduction was observed in parallel cultures under micro-aerobic conditions. Strain USS-CCA1^T was unable to oxidize hydrogen and elemental sulfur.

For chemotaxonomic analyses, biomass was prepared by growing the isolate in liquid medium (pH 3.0, 28 °C) and cells were harvested by centrifugation at 16000 ***g***. Analysis of diaminopimelic acid isomers was performed according to the procedure described elsewhere [11–13]. Polar lipids profiles were examined by two-dimensional thin-layer chromatography [14–17]. Cellular fatty acid composition was determined using the Microbial Identification System (MIDI Inc.; version 6.1). Isoprenoid quinones were extracted from dried biomass with chloroform/methanol (2:1, v/v) [18] and analysed by HPLC [15]. DNA G+C content was determined by HPLC as described by Tamaoka and Komagata [19]. The cell wall of strain USS-CCA1^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid of its peptidoglycan. Previous

Table 1. Comparison of major characteristics between strain USS-CCA1^T and phylogenetically closely related acidophilic actinobacteria. ++, strong growth; +weak growth, – no growth

	USS- CCA1^T	***Act. ferrireducens* IC-180^{T}***	***'Acx. ferrooxidans' Py-F3*†**	***Am. ferrooxidans* ICP^T‡**	***Fm. acidiphilum* T23^T§**	***Fx. thermotolerans* Y005^T§**
Growth temperature (°C)‖	30	50	25	45–50	35	43
Growth pH‖	3.0	3.0	3.0–3.2	2.0	2.0	1.8
DNA G+C content (mol%)	74.1	74	57.4	67–69	55	50
Major fatty acids¶	i-$C_{16:0}$, ai-$C_{17:0}$	i-$C_{16:0}$, ai-$C_{17:0}$, i-$C_{18:0}$	i-$C_{16:0}$, i-$C_{16:1}$	i-$C_{16:0}$	i-$C_{16:0}$, i-$C_{14:0}$	i-$C_{16:0}$
Major metaquinone¶	MK-9 (H_4), MK-9 (H_6)	MK-9 (H_8)	MK-9 (H_4)	MK-9 (H_8)	MK-8(H_{10})	ND
Oxidation of ferrous iron	++	–	++	++	++	++
Reduction of ferric iron	++	++	++	ND	++	++
Utilization of organic substrates:						
Glucose	++	++	++	++	-	-
Galactose	++	++	–	ND	ND	ND
Fructose	++	++	++	+	–	–
Xylose	–	++	–	ND	ND	ND
Arabinose	+	–	–	ND	ND	ND
Maltose	+	++	–	ND	ND	ND
Sucrose	++	++	++	ND	ND	ND
Lactose	+	++	++	ND	ND	ND
Cellobiose	++	ND	++	ND	ND	ND
Methanol	–	ND	–	–	ND	ND
Ethanol	–	ND	–	++	–	++
Glycerol	+	ND	–	+	+	++
Mannitol	–	ND	–	ND	ND	ND
Citric acid	–	–	–	++	++	–
Lactic acid	–	–	–	ND	ND	ND
Butyric acid	–	–	–	ND	ND	ND
Propionic acid	–	ND	–	ND	ND	ND
Acetic acid	–	ND	–	–	ND	ND
Glycine	–	ND	–	ND	–	–
Glutamic acid	–	ND	–	ND	++	–
Asparagine	–	ND	–	ND	ND	ND
Benzoate	–	ND	–	ND	ND	ND
Phenol	–	ND	–	–	ND	ND
Peptone	+	ND	–	ND	ND	ND
Tryptone	+	ND	–	ND	ND	ND
Tryptone soy broth	+	ND	++	++	ND	ND
Yeast extract	++	++	++	++	++	++

*[5].
†[6].
‡[3].
§[4].
‖Optimum values are given.
¶Fatty acids and metaquinones present at more 10% are given. ND,not determined.

Table 2. Sequencing, assembly and annotation statistics for USS-CCA1^T

USS-CCA1^T (WJHE00000000)	
Sequencing technology	Illumina MiSeq
Assembly_method	Velvet v. 1.2.10
Genome_coverage	30.0×
N50	4025
# contigs	1697
Total length	4073972
G+C (mol%)	73.16
Genes (total)	4397
Genes (coding)	4277
rRNAs	1, 1, 3 (5S, 16S, 23S)
complete rRNAs	1, 1 (5S, 16S)
partial rRNAs	3 (23S)
tRNAs	49
ncRNAs	3

reports have confirmed that *meso*-diaminopimelic is characteristic of peptidoglycan type (A1γ) [12] found in related '*Acx. ferrooxidans*' Py-F3, *Fm. acidiphilum* T23^T and *Fx. thermotolerans* Y005^T and *Act. ferrireducens* IC-180^T. Major cellular fatty acids (≥10%) were iso-$C_{16:0}$ (60.1%), reported as dominant fatty acid in related strains *Am. ferrooxidans* ICP^T, *Fm. acidiphilum* T23^T, *Fx. thermotolerans* Y005^T, *Act. ferrireducens* IC-180^T and '*Acx. ferrooxidans*' Py-F3 [3–6] and anteiso-$C_{17:0}$ (26.1%). Also, anteiso-$C_{17:1}$ ω9c (9.7%) and iso-$C_{16:1}$ H (2.1%) were detected. The polar lipid profile of strain USS-CCA1^T mainly consisted of diphosphatidylglycerol, phosphatidylinositol, one dominant phosphoglycolipid and one dominant aminoglycolipid; several glycolipids, phospholipids, aminolipids and lipids were present in lower quantities (Fig. S3).

The major respiratory quinones were MK-9 (H_4) (80.3%), also reported as dominant in '*Acx. ferrooxidans*' Py-F3 and MK-9 (H_6) (13.9%); smaller amounts of MK-9 (H_8) (2.5%), MK-8 (H_4) (2.1%) and MK-9 (H_2) (1.2%) were also detected. The G+C content of the chromosomal DNA of the isolate was 74.1 mol%, which is comparable to that of *Act. ferrireducens* IC-180^T (73.8–74.3) and higher than those of other acidophilic actinobacteria: 67–69 mol% (*Am. ferrooxidans* ICP^T), 54.9 mol% (*Fm. acidiphilum* T23^T) and 50.2 mol% (*Fx. thermotolerans* Y005^T). A comparison of phenotypic and chemotaxonomic characteristics between strain USS-CCA1^T and other acidophilic actinobacteria are summarized in Table 1.

PHYLOGENY AND GENOME FEATURES

Genomic DNA extraction was performed by using a grown culture in glucose/yeast extract medium at mid-exponential

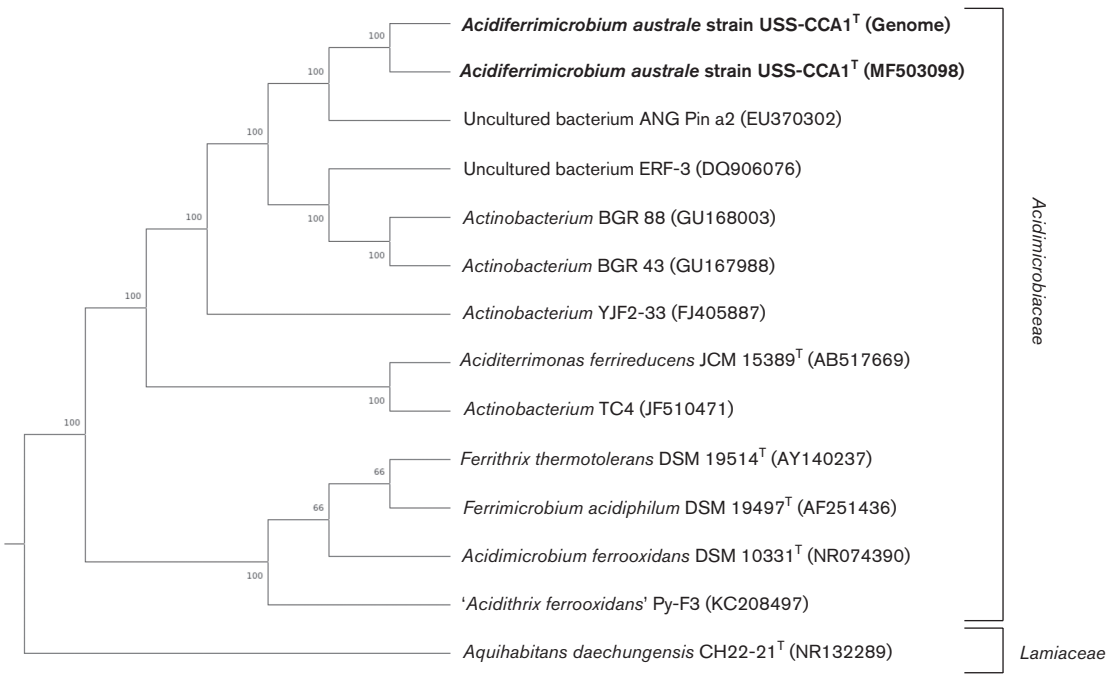

Fig. 1. Consensus phylogenetic tree derived from 16S rRNA gene sequence data showing the relationship of *Acidiferrimicrobium australe* USS-CCA1^T (MF503098) to other acidophilic representatives of the *Acidimicrobiales* order. Bootstrap values are indicated at the respective nodes. *Aquihabitans daechungensis* CH22-21^T (NR132289), a member of the family *Lamiaceae* was used as outgroup to root the tree. Individual trees used to infer the depicted consensus tree are shown in Fig. S4.

Table 3. Genomic relatedness indexes (%) calculated between strain USS-CCA1^T and other *Actinobacteria* reference strains

Accession	Reference genome	dDDH*	ANIb†	AAI‡	POCP§
WJHE01	*Acidiferrimicrobium australe* USS-CCA1^T	100.0	100.0	100.0	100.0
JXYS01	*'Acidithrix ferrooxidans'* Py-F3	32.9	69.8	56.6	19.0
FQUL01	*Ferrithrix thermotolerans* DSM 19514^T	32.4	68.9	57.3	20.9
JQKF01	*Ferrimicrobium acidiphilum* DSM 19497^T	31.8	69.5	56.8	21.1
BBCH01	*Aciditerrimonas ferrireducens* JCM 15389^T	22.3	76.1	65.9	7.6
CP001631	*Acidimicrobium ferrooxidans* DSM 10331^T	19.4	71.8	56.4	21.7
FWWY01	*Sulfobacillus thermosulfidooxidans* DSM 9293^T||	20.6	74.0	49.2	9.3

*If dDDH > 70%, same species [26].
†If ANI > 96, same species [27].
‡If AAI >95, same species, >70 same genus, >50 same family [28, 29].
§If POCP > 50%, same genus [30].
||Outgroup belonging to Firmicutes.

growth phase. The genome of USS-CCA1^T was sequenced using Illumina sequencing technology (MiSeq platform at Genoma Mayor, Universidad Mayor, Santiago, Chile) and paired end libraries with insert sizes of ~500 bp. Sequencing reads (2.4 Gbp of Illumina data) were processed and assembled *de novo* to produce a draft assembly consisting of 1697 contigs (average depth coverage 30 fold). The total size of the draft genome is ~4.07 Mbp and the whole-genome based G+C content is 73.16 mol%. This whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession WJHE00000000 which is the version described in this paper. Sequencing, assembly and genome statistics are summarized in Table 2.

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 predicted sequence (1512 bp) was compared to the 16S rRNA gene sequences available in GenBank nt database (as of August 2019) using BLASTN 2.8.0 with default parameters. The GenBank accession number for the partial 16S rRNA gene sequence of USS-CCA1^T deposited upon initial isolation is MF503098. Sequence identity between the genomic 16S rRNA and that of the previous (partial, 90% coverage) 16S rRNA gene sequence deposit was 100%, validating its identity. Also, identity values between 90 and 97% were obtained between the 16S predicted gene and several partial sequences of uncultured bacterium clones. Identity against genome sequenced type strains or reference strains of the phylum *Actinobacteria* are under 95% (Table S1), making a specific assignment of the isolate uncertain and proofing the status of a new genus within the phylum *Actinobacteria*.

Small subunit ribosomal RNA gene sequences alignment was performed using MAFFT (v 7.407) [20]. The alignment was trimmed and masked (>50%) manually and phylogenetic trees (Fig. S4) were calculated using: (i) the Neighbour-Joining (NJ) algorithm [21], (ii) the Maximum-Likelihood (ML) algorithm [22] based on the best-fit model of nucleotide substitution using a generalized time-reversible (GTR) model [23] (iii) using Bayesian Inference (BI) as implemented in MrBayes v.3.2.6 [24], run for 100000 generations, saving trees every 10000 generations, and calculating posterior probabilities after discarding the first 25% of trees. The consensus tree, built with PHYLIP [25] is shown in Fig. 1. Phylogenetic analysis based on the 16S rRNA gene sequenced showed the strain USS-CCA1^T to branch with other *Acidimicrobiales* of the phylum *Actinobacteria* in the phylogenetic tree. The isolate formed a separate clade with respect to characterized taxa (*Fm. acidiphilum* T23^T, *Fx. thermotolerans* Y005^T, *Act. ferrireducens* IC-180^T, *Am. ferrooxidans* ICP^T), yet it grouped with other isolates and uncultured clone sequences lacking further description (Fig. 1). The closest characterized acidophilic member of the *Actinobacteria* is *Act. ferrireducens* IC-180^T (AB517669) with a sequence identity of 94.38% at the 16S rRNA gene level. The described clustering was supported by all three algorithms applied. The distant relationship to described taxa (Table S1) and the distinct clustering support the assignment of strain USS-CCA1^T to a different taxon.

To further assess this contention, well-acknowledged genomic relatedness indexes, including the average nucleotide identity (ANI) [26], the digital DNA–DNA hybridization (dDDH) [27], the average amino acid identity (AAI) [28, 29] and the percentage of conserved proteins (POCP) [30] were evaluated for strain USS-CCA1^T and sequences of relatives within the phylum *Actinobacteria*. Pairwise comparisons between the USS-CCA1^T draft genome and the reference genomes available for this study, using these indexes (Table 3) strongly indicate that USS-CCA1^T belongs to a separate genus (POCP <50% and AAI <70%) and species (ANI <96%; dDDH <70%) within the *Acidimicrobiaceae* family (AAI >50%).

DESCRIPTION OF *ACIDIFERRIMICROBIUM* GEN. NOV.

Acidiferrimicrobium (A.ci.di.fer.ri.mi.cro'bi.um. N.L. neut. n. *acidum* (from L. masc. adj. acidus, sour), an acid; L. neut. n. *ferrum* iron; N.L. neut. n. *microbium* microbe; N.L. neut. n. *Acidiferrimicrobium* acidic iron microbe).

Cells are rod-shaped, motile and do not form endospores. Cells are Gram-stain-positive. Acidophilic, mesophilic and obligately heterotrophic. Capable of both oxidizing ferrous iron and reducing ferric iron. The peptidoglycan contains *meso*-diaminopimelic acid.

The major cellular fatty acids are iso-$C_{16:0}$ and anteiso-$C_{17:0}$. The major respiratory quinones is MK-9 (H_4). Phylogenetically affiliated to the order *Acidimicrobiales* and the phylum *Actinobacteria*. The type species is *Acidiferrimicrobium australe*.

DESCRIPTION OF *ACIDIFERRIMICROBIUM AUSTRALE* SP. NOV.

Acidiferrimicrobium australe (aus.tra'le. L. neut. adj. *australe* southern, relating to the region in which the organism was isolated).

Cell size is 0.5–0.7 µm wide and 0.6–0.9 µm long. Cultural properties, chemotaxonomic and phylogenetic features are as described for the genus. Mesophilic and acidophilic; Grows at 20–39 °C (optimal growth temperature is 30 °C) and pH 1.7–4.5 (optimal at pH 3.0). Yeast extract is required for growth on sugars such as glucose, fructose, galactose, sucrose and cellobiose.

The type strain, USS-CCA1^T (=DSM 106828^T,=RGM 2506^T) was isolated from streamer growths in an acidic stream draining a former coal mine located in Chile. The DNA G+C content of the type strain is 74.1 mol%. The GenBank/ENA/DDBJ accession numbers for the 16S rRNA and whole-genome shotgun sequences of strain USS-CCA1^T are MF503098 and WJHE00000000, respectively.

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

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