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ENVIRONMENTAL MICROBIOLOGY

Physiologic Versatility and Growth Flexibility as the Main Characteristics of a Novel Thermoacidophilic *Acidianus* Strain Isolated from Copahue Geothermal Area in Argentina

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Abstract A novel thermoacidophilic archaeal strain has been isolated from three geothermal acidic hot springs in Copahue, Argentina. One of the most striking characteristic of ALE1 isolate is its metabolic versatility. It grows on sulphur, tetrathionate, iron (II) and sucrose under aerobic conditions, but it can also develop under anaerobic conditions using iron (III) or sulphur as electron acceptors and sulphur or hydrogen as electron donors autotrophically. A temperature of 75 °C and a pH between 2.5 and 3.0 are strain ALE1 optimal growth conditions, but it is able to oxidise iron (II) even at pH 1.0. Cells are irregular cocci surrounded by a regularly arrayed glycoprotein layer (S-layer). Phylogenetic analysis shows that strain ALE1 belongs to the family *Sulfolobaceae* in the class *Thermoprotei*, within the phylum *Crenarchaeota*. Based on 16S rRNA gene sequence similarity on NCBI database, ALE1 does not have closely related relatives, neither in culture nor uncultured, which is more surprising. Its closest related species are strains of *Acidianus hospitalis* (91 % of sequence similarity), *Acidianus infernus* (90 %), *Acidianus ambivalens* (90 %) and *Acidianus manzanensis* (90 %). Its DNA base composition of 34.5 %mol C+G is higher than that reported for other *Acidianus* species. Considering physiological and phylogenetic characteristics of strain ALE1, we considered it to represent a novel species of the genus *Acidianus* (*candidatus* "*Acidianus copahuensis*"). The aim of this study is to physiologically characterise this novel archaea in order to understand its role in iron and sulphur geochemical cycles in the Copahue geothermal area and to evaluate its potential applications in bioleaching and biooxidation.

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Introduction

In the past years there have been many scientific publications on microbial diversity, particularly of thermophilic archaea in extreme environments [20, 23, 24]. Most of these reports are based on molecular techniques and new high throughput sequencing techniques. Reports on isolation of novel extremophilic species are rare in comparison [16, 32]. This is mainly because isolation of a pure culture of these kinds of microorganisms is extremely difficult and very time consuming. For the majority of the cases there is no previous information on cultivation media and nutritional requirements, what makes the task of isolation and characterization of new species a matter of proof and error and patience. Incubation times are generally longer for extremophiles, which have a hard time growing in vitro. Isolation of thermophiles has the extra complications of

media evaporation in liquid culture and the fact that agar cannot be used as a gelling agent for purification of strains on solid media.

Due to the environment at which acidic thermophilic archaea have adapted (solphataric areas, acidic hot springs), many of them are chemolithoautotrophs and can use sulphide, sulphur, iron (II) and hydrogen as electron donors. Alternatively, they can also be facultative heterotrophs capable of using organic carbon sources. There are archaea reported with such characteristics and most of them belong to the order *Sulfolobales*. They are described in the literature as extremely thermophilic, chemolithotrophic, sulphur-metabolising and acidophilic cocci. Members of the genera *Sulfolobus* and *Metallosphaera* are obligate aerobes; in contrast, *Acidianus* spp. are facultative anaerobes [10, 25]. This genus includes phylogenetically similar *Acidianus infernus* [29] and *Acidianus ambivalens* [15], the less thermophilic *Acidianus brierleyi* [29] and three recently described species: *Acidianus tengchongensis* [12], *Acidianus manzaensis* [36] and *Acidianus sulfidivorans* [25]. They grow as facultative aerobes with the ability to oxidise or reduce elemental sulphur depending on oxygen availability and they are capable of autotrophic and facultative heterotrophic growth.

Thermophilic microorganisms able to use sulphide, sulphur or iron (III) as electron donors are especially suitable for bioleaching, biooxidation and biodesulphurisation of coal since they can solubilise metals occurring as sulphides at higher rates as they work at higher temperatures. In the particular case of bioleaching and biooxidation, they are capable of solubilising some refractory ores like molybdenite and chalcopyrite which are scarcely dissolved by mesophilic microorganisms [17].

Copahue geothermal area is located on the Northwest of Neuquén Province in Argentina. It is dominated by Copahue Volcano (37°51′ S, 71°10.2′ W; 2,977 m a.s.l.), an andesitic stratovolcano responsible of the thermal activity in Caviahue–Copahue area. It has a small crater lake with an acid hot spring on its east flank that originates Rio Agrio. The acidic river runs along several kilometres, and after seven waterfalls, discharges into the glacial Caviahue Lake (pH 2.1–3.7). Two small tourist villages have settled in the area; Caviahue on the lake shore and Copahue closer to the base of the volcano. Caviahue–Copahue system is characterised by the presence of acidic hot springs that are a potential source of extremophilic microorganisms, many of them not yet discovered. This volcanic geothermal area has been studied in its geology, geochemistry, volcanism and thermalism over the past 20 years [5, 9, 11, 21, 22, 35] and more recently its prokaryotic biodiversity has been assessed [3, 4, 34]. However, no new indigenous extremophilic species have been reported yet from this area. In this study, we present the first isolation and characterisation of a novel, extremely versatile, autochthonous archaeal strain from various hot springs in geothermal Caviahue–Copahue area.

Methods

Origin of the strain and sample collection

Strain ALE1 was isolated from three geothermal hot springs in Caviahue–Copahue system named Baño 9 (B9), Las Máquinas (LMa) and Las Maquinitas (LMi). Table 1 shows coordinates and some of the main physicochemical characteristics of these hydrothermal pools.

Temperature, electrical conductivity and pH were measured in situ with a Hanna portable instrument properly calibrated against calibration standards. Water samples were collected in 1-l sterile plastic jars and kept on ice until further processing. Jars destined to isolation were kept at room temperature until inoculation on specific mediums.

Enrichment and culture media

Culture media used for enrichment and isolation of strain ALE1 were:

M88. It is a selective medium for thermophilic, acidophilic archaea recommended by DSMZ (http:/dsmz.de/media/media88.htm). Table 2 lists the composition (stock solution 10×) of a modified version of the medium originally proposed by Brock [2]. pH of the stock solution was adjusted to 2.0 with sulphuric acid 10 N. Stock solution was sterilised by autoclaving at 2 atm for 20 min. Yeast extract (1.0 gL^{-1}) was sterilised separately and added to the medium when dilution was made.

M88+sulphur (M88+S). M88 medium was supplemented with 5.0 gL^{-1} of sulphur and sterilised separately at 1.5 atm for three periods of 15 min.

Solid media. Gellan Gum was used as gelling agent in thermophilic isolation plate cultures [6, 18]. To improve culture growth, double-layer technique was used [19]. Medium composition is listed in Table 3.

Optimum growth temperature and pH

Assays were done on agitated 250-mL Erlenmeyer flasks with medium M88 supplemented with sucrose (see below). For determination of optimum temperature, M88 was adjusted initially to pH 3.0. For determination of optimum pH growth assays were done at 75 °C. Culture development was monitored by direct cell counting using a Petroff-Hausser counting chamber under phase-contrast microscope.

Table 1 Physicochemical characteristics of hydrothermal pools from where strain ALE1 was isolated

Sampling site name	Coordinates	T (°C)	pH	Conductivity (mS/cm)	Fe (ppm)
Baño 9 (B9)	37°48′59.8″ S, 71°5′48.52″ W	50	2.0	3.38	8
Las Máquinas (LMa)	37°50′2.61″ S, 71°5′3.20″ W	39	1.8	3.81	11
Las Maquinitas (LMi)	37°49′09″ S, 71°05′12″ W	85	2.5	8.60	43

Generation time was calculated using the algorithm proposed in http:doubling-time.com [27].

Carbon and energy sources

In order to study strain ALE1 ability to grow on heterotrophic and autotrophic conditions, different growth media were tested:

M88+glucose (M88+Glu); M88+sucrose (M88+Suc). M88 medium supplemented with glucose and sucrose 1.0 gL^{-1}, respectively

M88+tetrathionate (M88+T). M88 medium supplemented with 3.0 gL^{-1} of potassium tetrathionate

M88+iron (II) (M88+Fe(II)). M88 medium supplemented with iron(II). $FeSO_4{\cdot}7H_2O$ was prepared separately according assay requirements and sterilised by filtration through 0.22-μm size pore membranes.

OK. 3.0 gL^{-1} $(NH_4)_2SO_4$; 0.1 gL^{-1} KCl; 0.5 gL^{-1} KH_2PO_4; 0.5 gL^{-1} $MgSO_4{\cdot}7H_2O$; 0.144 gL^{-1} $Ca(NO_3)_2$, 3.0 gL^{-1} $K_2S_4O_6$.

MP. It was used to measure iron (II) oxidation in autotrophic or heterotrophic conditions as well as the use of different energy sources under anaerobic conditions. Basal medium as proposed by Plumb et al. [25] is 1.50 gL^{-1} $(NH_4)_2SO_4$, 0.25 gL^{-1} K_2HPO_4 and 0.25 $g.L^{-1}$ $MgSO_4{\cdot}7H_2O$. It was supplemented with 0.1 gL^{-1} of yeast extract, 5.0 gL^{-1} of sulphur (MP+S), $FeSO_4{\cdot}7H_2O$ (MP+Fe(II)) or $Fe_2(SO_4)_3{\cdot}H_2O$ (MP+Fe(III)) as required.

In all cases, medium final pH was adjusted as required by the assay.

Table 2 Medium 88 (M88) modified for thermophilic acidophilic archaea growth

	10× (gL^{-1})		10× (mgL^{-1})
$(NH_4)_2SO_4$	13.00	$ZnSO_4{\cdot}7H_2O$	2.20
KH_2PO_4	2.80	$CuSO_4{\cdot}7H_2O$	0.84
$MgSO_4{\cdot}7H_2O$	2.50	$Na_2MoO_4{\cdot}2H_2O$	0.10
$CaCl_2{\cdot}2H_2O$	0.70	$VOSO_4{\cdot}2H_2O$	0.10
$FeCl_3{\cdot}6H_2O$	0.20	$CoSO_4{\cdot}7H_2O$	0.18
$MnSO_4{\cdot}H_2O$	0.15	$Na_2B_4O_7{\cdot}10H_2O$	45.00
Yeast extract (Difco)	1.00		

Anaerobic growth

Hydrogen (H_2) or elemental sulphur (S) were tested as electron donors and iron (III) or S as electron acceptors under anaerobic conditions. Assays were carried out in 25-mL serum bottles filled with MP media (pH 2) supplemented as required with 10 gL^{-1} of sterile powdered S, 4.0 gL^{-1} of iron(III) or 120 mgL^{-1} of zero valent iron, sterilised at 160 °C, as presented before [13] to provide an indirect supply of hydrogen according to the equation:

$$Fe^{\circ} + 2H^{+} \rightarrow Fe^{2+} + H_2 \quad (1)$$

Bottles were sealed, de-aerated by sparging with oxygen-free nitrogen, injected with a 1-M sodium bicarbonate solution to reach a final concentration of 0.01 M CO_2 in the bottle. Bottles were inoculated with active ALE1 culture, grown aerobically on M88+T media and incubated at 75 °C without shaking.

Microscopic techniques

Cells growing in M88+T medium were used to analyse morphological characteristics using a LEO EVO 40 XVP scanning electron microscope (SEM) equipped with secondary electron detector. Cells were harvested and fixed with glutaraldehyde 2.5 % (pH=7.2). Samples were placed in poly-L-lysine stubs and were dehydrated in a graded series of alcohol (25–100 %) and dried in a critical point chamber (Polaron, England). The stubs were coated with a thin layer of gold in a shutter coater (Pelco 9100).

Microorganisms ultra structure was studied by transmission electron microscopy (TEM) using a JEOL 100 CXII microscope. Planktonic cells were fixed in a 2.5 % glutaraldehyde (pH=7.2), post fixed in 2 % osmium tetraoxide and dehydrated in a graded series of alcohol (25–100 %). Samples were embedded in Spur resin and cut with a microtome (LKB) to obtain thin layer sections which were placed in a stub and stained with uranyl acetate and lead citrate to improve image contrast.

To provide a three-dimensional surface image of the cells, atomic force microscopy (AFM) was used (NanoWizard II atomic force microscope, JPK Instruments, Germany). Cells were immobilised in a poly-L-lysine-coated glass slide and were placed in BioMaterial TM Workstation equipped with JPK SPM software for data acquisition. The JPK Image

Table 3 Double layer medium composition [19]

Solution A dense layer (0.8 % *p*/*v*)	Solution B light layer (0.4 % *p*/*v*)	Solution C 0K medium supplemented (gL^{-1})			
		$(NH_4)_2SO_4$	6.0	$K_2S_4O_6$·20 mM	6.05
		$Ca(NO_3)_2$·$4H_2O$	0.028	$MgCl_2$·8 mM	0.76
1.6 g/100 mL Gelrite	0.8 g/100 mL Gelrite	KCl	0.2	NaCl 2 mM	0.12
		K_2HPO_4	1.0	Casamino acids 2 %	2.0
		$MgSO_4$·$7H_2O$	1.0	pH	2.0

Processing software was used for image processing. Measures were done in liquid and contact mode, using cantilevers without coating (μMASCH® CSC38 B, typical spring constant: 0.03 Nm^{-1}, resonance frequency, 10 kHz). AFM images were obtained with a set point of 1.0 V and a line-rate of 0.8 Hz.

DNA Base Composition

It was determined by Identification Service of DSMZ by HPLC.

Polar Lipid Analysis

Polar lipid analysis was carried out by Identification Service of DSMZ and Dr. B.J. Tindall, DSMZ, Braunschweig, Germany.

DNA extraction

Rawlings modified technique was used for DNA extraction [26]. Cultures were filtered to retain solid rests and centrifuged 5 min at 13,000 rpm. Cells pellet was washed with pH 1.5 (H_2SO_4) water, resuspended in 1 mL TE buffer (10 mM tris–HCl, pH 8.0, 1.0 mM EDTA) and incubated over night at −20 °C. Cells were centrifuged 5 min at 13,000 rpm and250 μL 10 % sucrose in TE buffer were added and kept 5 min at −20 °C. To improve cell lysis 50 μL of protease K 5 $mgmL^{-1}$ were added and incubated 1 h at 37 °C and then 50 μL of SDS 10 % solution were added and incubated 30 min at 50 °C. In order to separate aqueous and organic phases 35 μL sodium acetate 3.0 M (pH 3.4) and 350 μL phenol–tris were added and centrifuged 15 min at 13,500 rpm. Aqueous phase was extracted using 100 μL of chlorophorm and centrifuged 15 min at 13,500 rpm for decanting cellular rests. Chlorophorm extraction was repeated once. Cold isopropyl alcohol was used for DNA precipitation by 15 min centrifugation at 13,500 rpm. DNA pellet was washed with 70 % cold ethanol and air dried. DNA was resuspended in 50 μL of MiliQ water and incubated at −20 °C for 24 h. To verify DNA extraction 0.8 % agarose gel was run at 90 V for 30 min.

16S amplification, cloning and sequencing

Archaeal general primers 25F: 5′-TCYGGTTGATCC YGCCRG-3 and 1492r: 5′-TACCTTGTTACGACTT-3′ were used for amplifying DNA extracted from isolation cultures [1]. PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 38 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. Amplification reactions contained 20–30 ng of DNA per 50 mL reaction volume, 1× PCR buffer (Promega Biotech), 2.5 mM of each of the deoxynucleotides, 2.5 mM $MgCl_2$, 500 mM of the forward and reverse primers and 0.025 UmL^{-1} of Taq DNA polymerase (Promega Biotech). PCR amplification was checked by 1 % agarose gel electrophoresis stained with ethidium bromide.

Amplified 16S rRNA gene products (>1,400 bp) were cloned using the Topo Ta Cloning Kit (Invitrogen, CA, USA) and sequenced using a Big-Dye sequencing kit (Applied Biosystem) following the manufacturer's instructions.

16S rRNA sequences of strain ALE1 and representative members of the family *Sulfolobales* were analysed and compared using BLAST at the NCBI database (http://ncbi.nlm.nih.gov/BLAST). They were also checked for potential chimeras using Bellerophon Chimera Check program (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi) and Maillard software. Alignments were performed using MEGA 5.05 [31] and were manually corrected. Alignment gaps and unidentified base positions were not taken into account for these calculations. A phylogenetic reconstruction was done using neighbour-joining [28] based on Kimura's two-parameter model [14]. The robustness of individual branches was estimated by bootstrapping with 1,000 replicates [8]. The NCBI accession number of strain ALE1 16S rRNA gene sequence is JQ513288.

Results and discussion

Enrichment culture and solid media isolation of strain ALE1

Natural samples from different hot springs of Copahue geothermal area were inoculated in M88, a selective media

for thermophilic, acidophilic archaea recommended by DSMZ (http:/dsmz.de/media/media88.htm). M88 was supplemented with sulphur and incubated at 65 °C. The enrichment cultures that showed signs of microbial development were plated on solid media supplemented with tetrathionate. Part of the success of this technique was the use of Gellan Gum as gellinig agent, which is specially indicated for thermophilic isolation in culture plates [6, 18]. To improve culture growth, double-layer technique was used [19]. Although plating efficiency was low, it was possible to observe cell growth after certain incubation time. Colonies were small and white at first, but after 15 days of incubation at 65 °C they turned yellow. Single colonies were inoculated on M88+S medium and passed to new medium regularly. Culture purity was confirmed by homogeneous morphology under optic microscope, a single band in a 16S rRNA DGGE gel (data not shown) and an unambiguous complete 16S rRNA sequence. Three strains were isolated from three independent acidic hot springs in Caviahue geothermal area. All isolates presented the same morphology, physiological behaviour and each 16S rRNA sequence was more than 99 % similar to the others. In a fourth hot spring the same 16S rRNA sequence was also found.

Table 1 summarises GPS position and physicochemical characteristics of the isolation points. It is interesting to notice that, although the three pools are high temperature and acidic, environmental conditions (especially temperature) are different for each, showing strain ALE1 natural flexibility.

Morphology

Strain ALE1 planktonic cells growing in M88+T were successfully immobilised on poly-L-lysine-coated glass slides and visualised with AFM without any desiccation step. Figure 1a shows vertical deflection image of AFM scan acquired by contact mode in water: several coccus-shaped cells of 1 to 2 μm lengths are visible. Due to hydration, cells seemed to be larger and their surfaces smoother than those observed by contact mode imaging in air (data not shown). The white streaks in these images are scanning disturbances; surely caused by rests of exopolysaccharides (EPS) adhered to the AFM tip. Morphological features of ALE1 cells were confirmed by SEM analysis. Rounded and lobed cells of 0.5 to 1.0 μm are shown in Fig. 1b. It was not possible to visualise EPS using this technique. Figure 1c shows a TEM image of planktonic strain ALE1 cells growing in M88+T medium. Figure 1d and f micrographs are high-resolution TEM images (the size of the bar is 210 nm). It is possible to appreciate details of the archaeal cell envelope formed by a single membrane and covered by a paracrystalline glycoprotein layer known as S-layer, in an array already described for other archaeas that serves as protection against a hostile environment [30].

Physiological characterisation

It is already known that *Acidianus* members are acidic thermophilic archaea [29]. However, we were interested in testing our isolate growth performance and flexibility towards temperature and pH. Figure 2a shows growth curves at different temperatures in a range of 55 to 80 °C. Strain ALE1 was able to grow at all temperatures tested when M88 supplemented with sucrose (M88+Suc) was used. However, growth rate and cell numbers were influenced by temperature. As it can be seen in Fig. 2b, faster growth was achieved at 75 °C with a generation time of 6.7 h. As regards pH, six different values form 1 to 5 were tested at 75 °C in M88+Suc. Growth was detected at all pH values (Fig. 3a), but was influenced by initial pH conditions. Optimal growth was detected at pH 3.0 (Fig. 3b) with a generation time close to 7.0 h. All the experiments described above were done in duplicate.

Strain ALE1 cultures were able to grow under heterotrophic and also autotrophic conditions at 75 °C. From all carbon sources tested, glucose and sucrose gave the best cells density yields. On 0K or MP media supplemented only with sulphur or iron (II) cell growth was poor; however, some levels of iron and sulphur oxidation were detected. As sterile flasks remained unchanged, decrease in pH or iron (II) concentration were attributed to autotrophic metabolism of strain ALE1. Besides, cells were able to survive in these autotrophic conditions for more than ten passes to new media.

Kinetic growth assays for strain ALE1 in M88 medium supplemented with sulphur, tetrathionate and sucrose as energy source are shown in Fig. 4a. Assays were carried out in duplicate. The highest cell concentration was achieved with sucrose as an energy source (cultures reached about 4.5×10^8 cells mL^{-1} in 30 h). In this media cells formed clusters and even visible flocks. When using other energy sources growth rate was much slower; to achieve similar cells concentrations on tetrathioanate or on sulphur 12 and 30 days were needed, respectively. pH values at the end of the assays correlated well with what was expected considering metabolism of each energy source (Fig. 4b). For M88+Suc pH maintained nearly the initial value of 3.0 while on tetrathionate or on sulphur pH dropped to 1.6 and less than 1.0, respectively. When tetrathionate was the energy source, a precipitate with sulphur as the main component was detected by SEM and EDAX spectroscopy (data not shown). Although the mechanism of usage of tetrathionate as energy source is still not clear [7], our data seem to agree with a similar result reported for other member of the genus *Acidianus*; for *A. ambivalens* a tetrathionate hydrolase was found [15]. This enzyme produces sulphate, thiosulphate and also elemental sulphur. Assuming a similar mechanism, strain ALE1 could probably continue oxidising thiosulphate (through a mechanism not known yet) rather than sulphur, which accumulates.

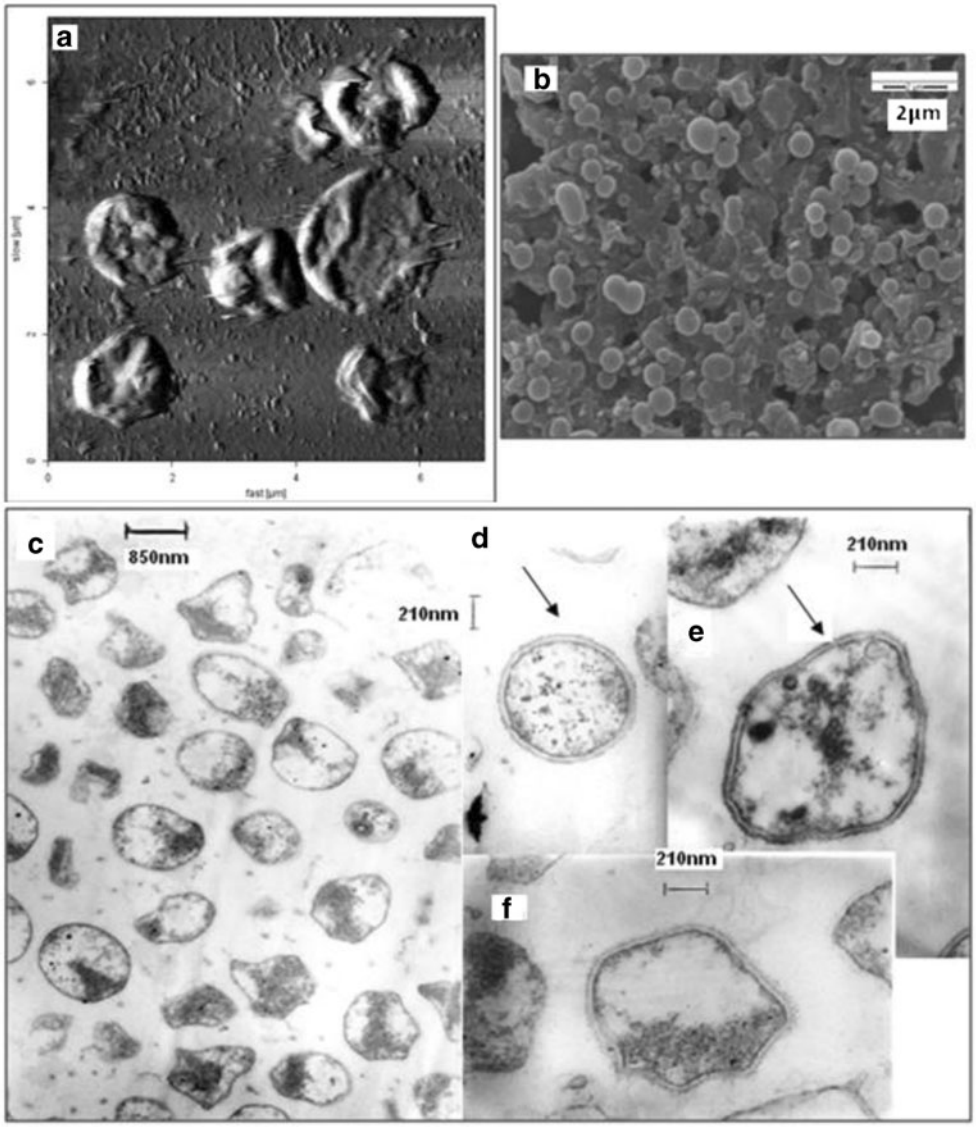

Figure 1 Images of strain ALE1 growing in M88+T medium. **a** Vertical deflection image of an AFM scan acquired by contact mode in water (7 by 7 µm; line rate, 0.8 Hz for both trace and retrace; over scan time, 0.063 s; total time, 3.51 min); **b** SEM micrograph (resolution, 20 HV; magnification, ×10,400; bar: 2 µm). **c**–**f** TEM micrographs *arrows* denote typical S-layer present in archaeal cells (*scale bars*: **c** 850 nm; **d**–**f** 210 nm)

Iron (II) oxidation

Strain ALE1 oxidised iron (II) autotrophically at 75 °C and pH 2.0 for more than ten passes to new medium; however, cell counts were very low and lots of iron precipitates were formed. To improve cell growth iron oxidation assays were done on MP+Fe(II) medium supplemented with 0.1 gL^{-1} yeast extract. Cells were able to oxidise iron (II) at all pH values tested (Fig. 4c) being the optimal pH lower than the optimal one found for culture growth on sucrose. The experiments were carried out in duplicate. Due to its capability of oxidising iron this strain could be used in bioleaching processes even at very low pH values.

Anaerobic growth

Various conditions and different redox couples were assayed for anaerobic growth and gave positive results proving once more the metabolic versatility of this isolate. Iron (III) or sulphur were used as electron acceptor and sulphur or hydrogen as electron donor under autotrophic conditions. All the experiments were done in duplicate.

Initial pH of 2.0 and Eh of 590 mV were used in a culture under anaerobic conditions with Fe(III)/S as redox couple. After 7 days at 75 °C, pH and Eh decreased to 1.3 and 417 mV respectively and iron (II) concentration reached a value of 3.4 gL^{-1}. The abiotic controls did not show any significant change, proving that strain ALE1 under anaerobic conditions catalysed the reaction represented by:

$$6\,Fe^{3+} + S^0 + 4\,H_2O \rightarrow 6\,Fe^{2+} + HSO_4^- + 7\,H^+ \qquad (2)$$

On a different assay hydrogen was used as electron donor and ferric iron as electron acceptor at 75 °C. Figure 5 shows the results obtained in this experiment. Iron (III)

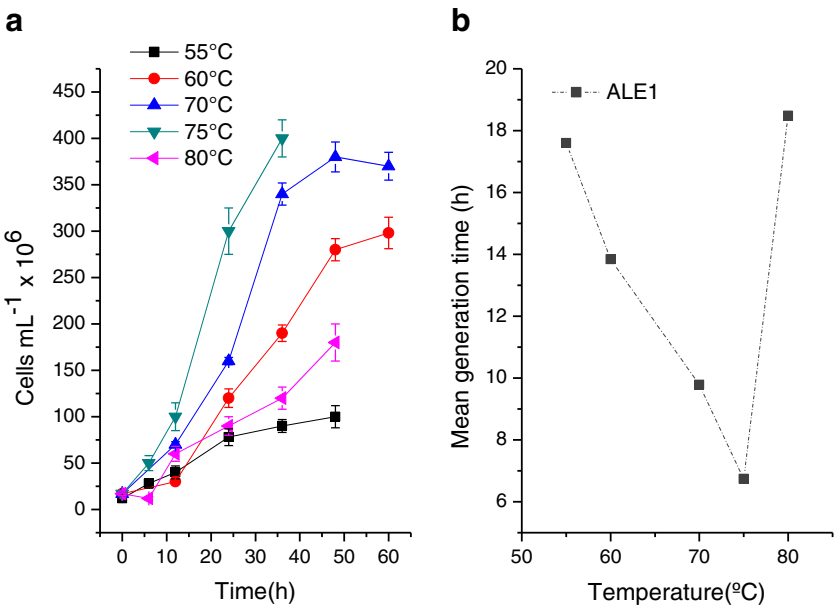

Figure 2 **a** Growth curves of strain ALE1 growing on M88+ Suc at different temperatures; **b** effect of temperature on growth rates (expressed as generation times) in temperature-controlled assays at initial pH of 3 and without any posterior regulation. *Error bars* show standard deviation from two independent experiment (n=2)

concentration decreased from 4.0 to 0.3 gL^{-1} after 8 days while an increase of cells number and a decrease in pH and Eh values were detected. In the same period of time no significant reduction of iron (III) was detected in the abiotic controls.

$$2\,Fe^{3+} + H_2 \rightarrow 2\,Fe^{2+} + 2\,H^{+} \quad (3)$$

Finally, in a third assay under anaerobic conditions $S°/H_2$ couple was tested. After 4–5 days a precipitate (later identified as iron (II) sulphide) was detected in the inoculated vials but not in the sterile controls; iron was present due to the in situ generation of hydrogen (Eq. 1). These results suggest strain ALE1 is also capable of biosulphidogenesis (oxidation of hydrogen and reduction of sulphur) as shown in the following equation:

$$S^{\circ} + H_{2(g)} \rightarrow H_2S \quad (4)$$

Molecular biology characterisation

Phylogenetic analysis based on 16S rRNA gene sequence (Fig. 6) shows that strain ALE1 clusters together with member of the family *Sulfolobales*, in a separate branch from the other members of the genus *Acidianus* reported so far. The position of strain ALE1 as regards other *Acidianus* species was well supported according to bootstrap analysis. This

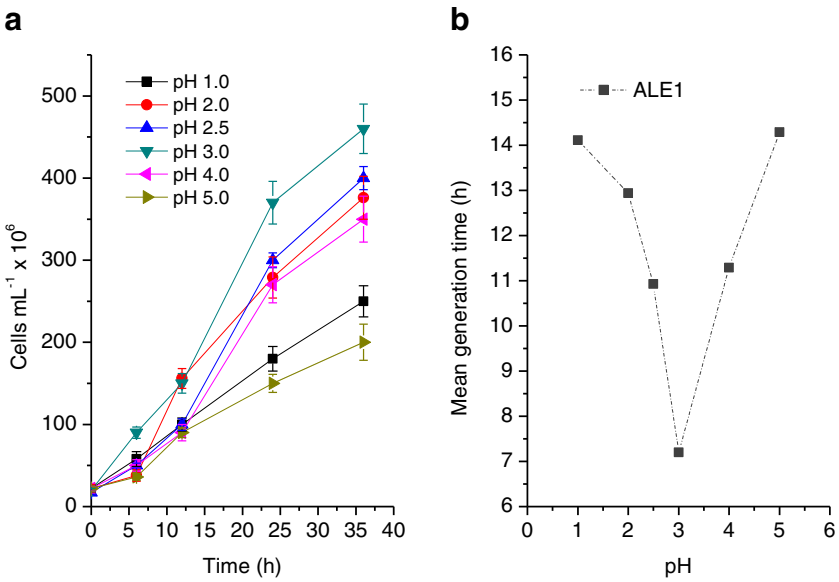

Figure 3 **a** Growth curves for strain ALE1 on M88+Suc at different pH values; **b** effect of pH on growth rates (expressed as generation times) of the isolate strain ALE1 in temperature-controlled assays (75 °C). *Error bars* show standard deviation from two independent experiment (n=2)

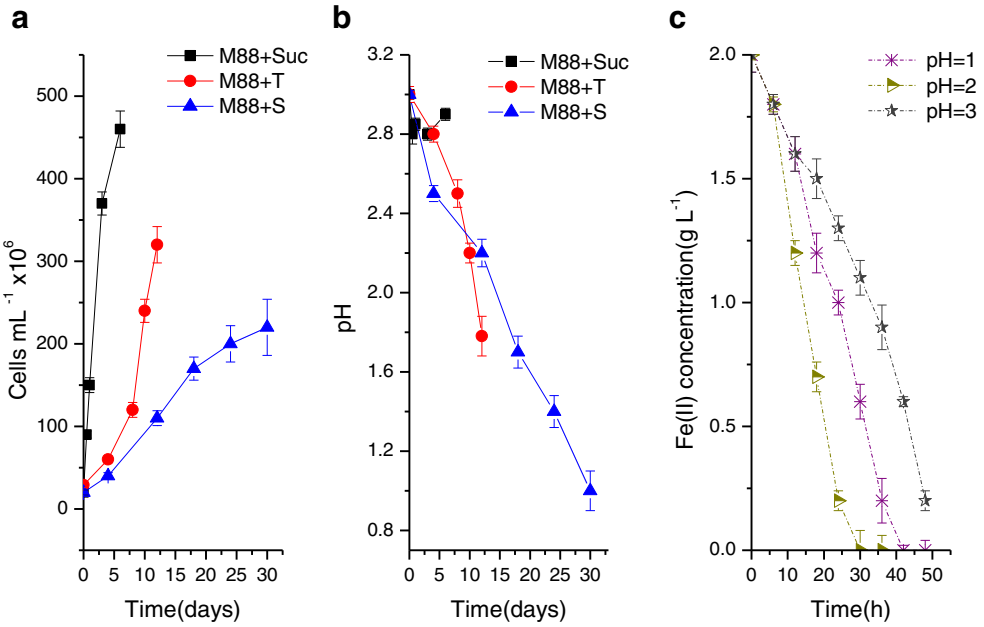

Figure 4 Kinetics growth assays (**a**) and pH evolution over time (**b**) for strain ALE1 using M88 medium supplemented with sucrose (*Suc*), potassium tetrathionate (*T*) and sulphur (*S*). **c** Ferrous iron oxidation rates by strain ALE1 growing in MP medium supplemented with 0.1 g/L of yeast extracts at different pH initial values. *Error bars* show standard deviation from two independent experiment (n=2)

isolate has no close relatives according NCBI sequence database, cultured or uncultured, what is even more surprising. The closest relatives are members of the genus *Acidianus*, but they show low sequence similarity: *Acidianus hospitalis* 91 %, *A. infernus* DSM 3191, *A. ambivalens* DSM 3772, "*A. manzaensis*" NA-1 and *A. sulfidivorans* DSM 18786 share 90 % of sequence similarity.

C+G% DNA base composition of strain ALE1 was 34.5 mol%. This value is higher than the ones reported for other *Acidianus* (*A. manzaensis* 29.9 mol%, *A. ambivalens* 31 mol%, *A. sulfidivorans* 31.1 mol%, *A. brierleyi* 31.5 mol% and *A. infernus A* 32.7 mol%), but lower than 38 mol% C+G described for *A. tengchongensis*. Polar lipid analysis by thin layer chromatography revealed the presence of diphosphatidylglycerol, phosphatidylinositol, phosphoglycolipids and six different chromatographic mobility types of glycolipids.

In spite of having rather low 16S rRNA sequence similarities to other *Acidianus* members reported in NCBI database, Ribosomal Data Base Project (http://rdp.cme.msu.edu/index.jsp) Seqmatch and Taxomatic tools assigned strain ALE1 to *Acidianus* genus with 100 % assertiveness. Together with its physiologic characteristics, this is why we consider strain ALE1 to represent a novel species within the

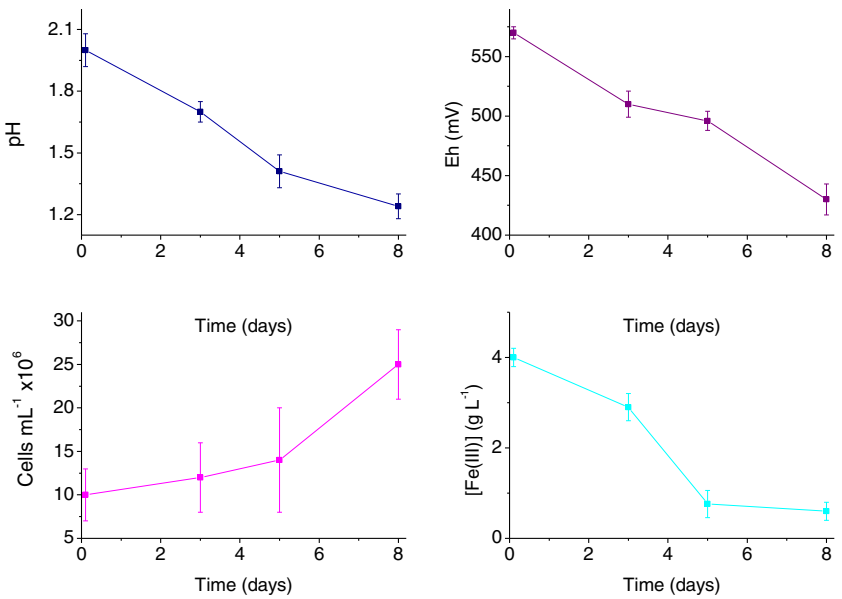

Figure 5 pH, Eh, cell density and Fe (III) concentration evolution over time for strain ALE1 under anaerobic culture at 75 °C using [Fe (III)/H_2] as redox couple. *Error bars* show standard deviation from two independent experiment (n=2)

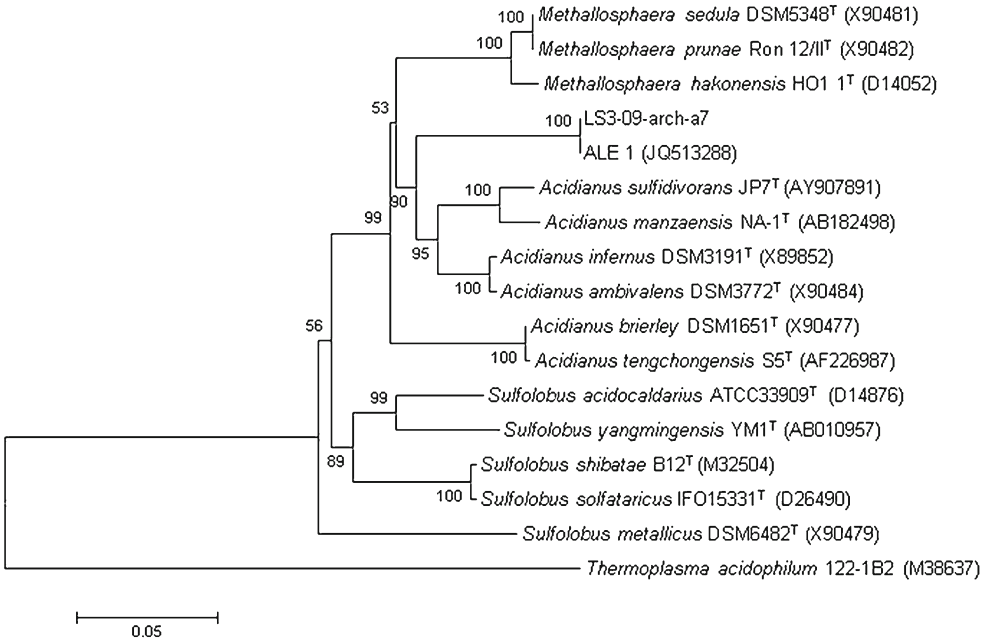

Figure 6 Phylogenetic tree based on 16S rRNA gene sequences showing affiliation of strain ALE1 and selected members of the order *Sulfolobales*. Gene Bank accession numbers are given in parentheses. T means type strain. Tree was constructed using Neighbor-Joining and Kimura 2 parameters methods. Bootstraps values are percentages based on 1,000 resampling. *Scale bar* indicates 0.05 sequence divergence

genus *Acidianus* (*candidatus* "*Acidianus copahuensis*"). Novel strain ALE1 seems to have very interesting metabolic abilities even within the *Acidianus* genus. Table 4 presents a detail comparison of members of *Acidianus* genus. All *Acidianus* reported so far have similar optimal growth temperatures and pH values, being *A. infernus* the most thermophilic and *A. sulfidivorans* the most acidophilic. Strain ALE1 is the most versatile when comparing energy sources for both metabolisms, aerobic and anaerobic.

As regards its potential uses in bioprocesses, a microorganism with ALE1 characteristics could be very important in bioleaching and biooxidation. As iron- and sulphur-oxidising microorganism, it can enhance metal sulphides bioleaching; as thermophile it can bioleach faster and also allow solubilising some refractory ores like molybdenite and chalcopyrite which are scarcely dissolved by mesophilic microorganisms [17]. The ability to survive and to grow within wide ranges of temperatures and pH values and even under anaerobic conditions makes it able to play a significant

Table 4 Comparison of metabolic and molecular characteristics between strain ALE1 and different members of *Acidianus* genus

Acidianus genus members	Growth temperature (optimum) (°C)	Growth pH (optimum)	Electron donor	Electron acceptor	Autotrophic growth	16S rRNA gene sequence similarity (%)	C+G content (%)
Strain ALE1 (this work)	55–80 (75)	1.0–5.0 (2.5–3.0)	H_2, S^0, $Fe^{2+}S_4O_6^{2-}$, MS[a], OC	Fe^{3+}, S^0, O_2	Facultative autotroph	100	34.5
Acidianus manzaensis [36]	60–90 (80)	1.0–5.0 (1.2–1.5)	H_2, S^0, OC	Fe^{3+}, O_2	Facultative autotroph	90	29.9
Acidianus sulfidivorans [25]	45–83 (74)	0.35–3.0 (0.8–1.4)	S^0, Fe^{2+}, FeS_2, MS	Fe^{3+}, S^0, O_2	Facultative autotroph	90	31.1
Acidianus brierleyi [29]	45–75 (70)	1.0–6.0 (1.5–2.0)	H_2, S^0, Fe^{2+}, OC	S^0, O_2	Facultative autotroph	89	31.5
Acidianus infernus [29]	65–96 (90)	1.0–5.5 (2.0)	H_2, S^0	S^0, O_2	Obligate autotroph	90	32.7
Acidianus ambivalens [15]	70–87 (80)	1.0–3.5 (2.5)	H_2, S^0	S^0, O_2	Obligate autotroph	90	31
Acidianus tengchongensis [12]	60–90 (80)	1.0–5.5 (1.5–2.0)	H_2, S^0	S^0, O_2	Obligate autotroph	89	38

MS (metal sulphide): FeS_2, CuS and ZnS, *OC* organic compounds: yeast extract, peptone, glucose and triptone

[a] MS, our own results, data not shown

role in almost all the different local environments that could be created into bioleaching heaps [33]. Currently, our group is carrying laboratory scale assays with promising results.

On the other hand, this species seems to play a significant and unique role in geochemical iron and sulphur cycles, and probably other metal cycles, as it is able to participate in many different redox processes (iron and sulphur reduction and oxidation) under a great variety of the environmental conditions found in the area (aerobiosis and anaerobiosis, wide range of temperature and pH values).

Conclusion

In this study we showed growth flexibility and physiological versatility of a novel isolate, as well as its phylogenetic position in the order *Sulfolobales*. Considering all of its characteristics, we think strain ALE1 represents a novel species within the genus *Acidianus* (*candidatus* "*Acidianus copahuensis*"). The ability of strain ALE1 to use iron and sulphur compounds with different oxidation states, hetero and autotophically, aerobic and anaerobically, points to its crucial role in biogeochemical sulphur and iron cycling in Copahue geothermal area. More studies focused on functional properties would be needed to fully understand this role and its impact in the area biogeochemistry as well as its potential for bioleaching and biooxidation.

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