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Visualization of attachment and colonization of pyrite surfaces by a novel species of *Acidianus*

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Abstract: In this work we have studied the attachment and colonization of pyrite surfaces by the recently isolated thermophilic archaeon Candidatus *Acidianus copahuensis* (from the geothermal Caviahue-Copahue system, Argentina). Cells pregrown with sulfur, iron(II) or pyrite were tested. In order to characterize the EPS glycoconjugates of this strain, fluorescent lectins were used. Concanavalin A (ConA) gave the best signal and was selected for further studies. Coupons and grains of pyrite were treated with DAPI (to stain attached cells) and TRITC-ConA (to stain polysaccharides from EPS). Pyrite surfaces were imaged by epifluorescence (EFM) and confocal laser microscopy (CLSM). Initial cell attachment to pyrite grains was estimated to be 26%, 35% and 43% when cells were pregrown with sulfur, iron(II) and pyrite as electron donors, respectively. It was observed that the cell adhesion correlated with an increase of EPS production; both processes were favoured when cells were pregrown with pyrite or iron(II). Also the effect of inorganic phosphate (P*i*) starvation in the attachment of *Acidianus* was studied in similar tests using a base medium without P*i*. An increase of cell attachment under P*i* starvation conditions was detectable.

Introduction

Microorganisms can thrive in planktonic states or forming biolfims. The latter mode of growth consists of surface associated cells embedded in extracellular polymeric substances (EPS). It has been reported that EPS play significant roles in the formation and function of microbial biofilms, including adhesion phenomena, matrix structure formation and physiological process [1]. Studies on microbial biofilms have mainly been conducted in bacteria, but in contrast for the archaeal domain only limited information is available [2]. It is well known that attachment of some leaching bacteria to the mineral surface enhances metal sulfide (MS) dissolution, since most of the leaching reactions occur directly at the mineral surface/interface, with the EPS playing a fundamental role [3-4]. Compared with the knowledge about the interfacial process of mesophilic leaching bacteria such as *At. ferrooxidans*, only a few researches have been done with extremely thermoacidophilic microorganisms, such as the archaeal genera *Acidianus, Metallosphaera* and *Sulfolobus* [5]. Furthermore, it is important to understand biofilm formation processes of these microorgamisms in order to influence their leaching efficiency.
In this work, we used the recently isolated thermophilic archaeon Candidatus *A. copahuensis* [6]. Its ability to build biofilms on pyrite surfaces when the cells were adapted to growth with sulfur, iron(II) or pyrite as electron donor was studied. EFM and CLSM were used to visualise the attachment and EPS production by this strain.

Methodology

Strain and media. Candidatus *A. copahuensis* was cultivated at 65°C, using alternatively 1g/L ferrous iron, 5g/L sulfur, or 1% w/V pyrite as electron donor in M88 basal solution [7]. After growth, cultures were filtered and cells were harvested by centrifugation at 8000 rpm for 10 min. at

20°C. Cell pellets were washed once with Mackintosh basal salt solution (MAC) [8] and cells were inoculated in pyrite coupons or grains as further described.

Biofilm formation on pyrite coupons. Pyrite coupons were cut from pyrite cubes with a diamond saw and then cleaned and sterilized as described [9]. Coupons were incubated in 100 mL Erlenmeyer flasks containing 50 mL of MAC and an inoculum of 10^8 cells/mL of *A. copahuensis*. Coupons were collected after 1, 3 and 6 days of incubation at 65°C with shaking at 120 rpm. The formation of the biofilm was visualized by EFM with the lectin TRITC-ConA (to stain polysaccharides from EPS) and DAPI (to stain attached cells).

Attachment and leaching test with pyrite grains. For the attachment and leaching assays on pyrite grains, crushed pyrite was wet sieved and sterilized as described [9]. The experiments were done in 100 mL Erlenmeyer flasks each containing 50 mL MAC and 1% w/V of sterile pyrite (50-100 mesh). An inoculum of 5×10^8 cells/mL was used. Flasks were incubated at 65°C with shaking at 120 rpm. 1 mL samples from supernatants were taken periodically and pH, ferric and ferrous iron as well as planktonic cell numbers were determined [4]. The effect of P*i* starvation in cell attachment was studied in similar tests with pyrite pregrown cells using MAC medium without P*i*.

Nucleic acids and EPS staining. Cells attached to coupons or pyrite grains were stained by incubation the sample in DAPI solution (0.01% w/V in 2% formaldehyde) for 10 min. After one washing step with sterile deionized water, EPS were stained by covering the coupons or grains with a 10 mM bicarbonate solution (pH 8.3) containing 50 µg/mL TRITC-ConA for 30 min. After two washing steps, stained samples were air-dried in darkness.

Results and discussion

In Fig. 1, EFM images of cells and microcolonies formed directly on pyrite coupons at different times of incubation are shown. Cell attachment on pyrite coupons and EPS production were observed after one day of incubation, and both processes were increased in following days. Cells adapted to growth with iron(II) or pyrite showed a higher adhesion and EPS production than cells adapted to growth with sulfur as energy source. Similar results were obtained in attachment tests with pyrite grains.

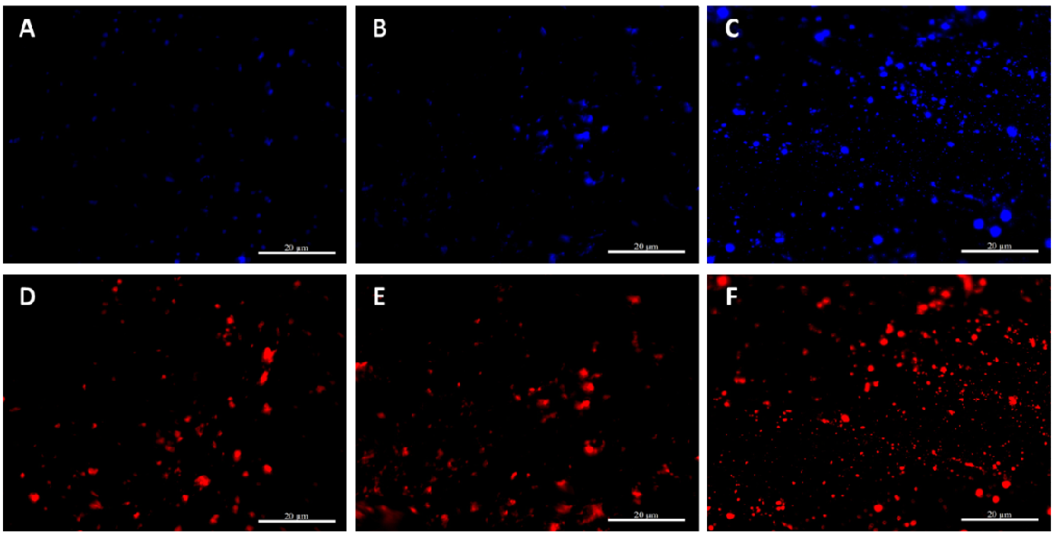

Figure 1. EFM images of Candidatus *A. copahu*ensis cells pregrown with pyrite attached to pyrite coupons. Cells were stained with DAPI (A-C) and TRITC-ConA, specifically bound to the EPS of *Acidianus* cells (D-F) on pyrite coupons at day 1 (A and D), day 3 (B and E) and day 6 (C and F). Size bars represent 20 µm.

Pyrite leaching tests were performed. Samples were taken at different times of incubation for the direct determination of total cells and iron species determination. No significant changes in the number of planktonic cells were observed during the experiment. In all inoculated flasks pH values decreased over the time of the experiment (Fig. 2). In the flasks inoculated with cells pregrown with sulfur pH dropped from 2 to 1.6; while in the flasks inoculated with cells adapted to growth with iron(II) or pyrite the pH showed a decrease from 2 to 1.2. These values suggest an enhanced oxidation of the reduced sulfur compounds released from the pyrite.

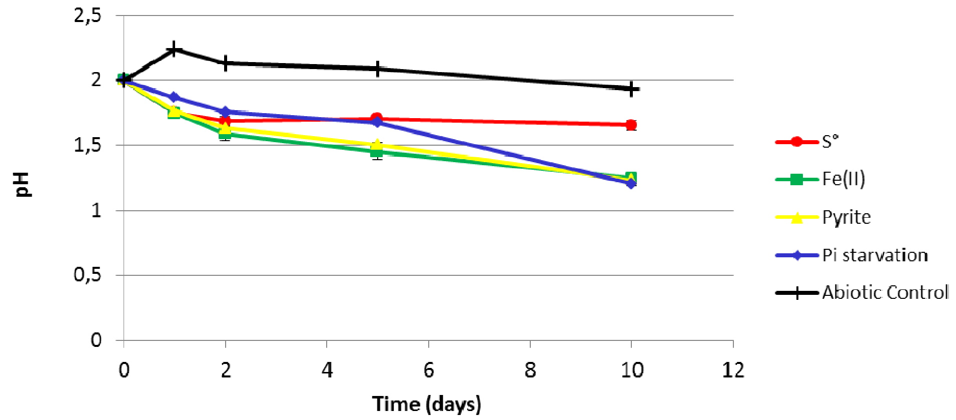

Figure 2. Changes in pH values during attachment experiments on pyrite grains with Candidatus *A. copahuensis* cells pregrown using sulfur (●), iron(II) (■), pyrite (▲), cells pregrown with pyrite under P*i* starvation conditions (u) and abiotic control (+) (average values from duplicates; variation <1%).

The most iron species measured were iron(III) ions, while iron(II) concentrations in all samples were low (approximately 6 ppm) and remained constant over the time of the experiment (not shown). Cells pregrown with sulfur did not show an increase of iron(III) ions during the experiment compared to iron or pyrite pregrown cells (Fig. 3). After 10 days of the experiment, values of 3300 ppm and 4500 ppm of total iron were obtained with cells adapted to growth with pyrite or iron(II) as electron donors, respectively.

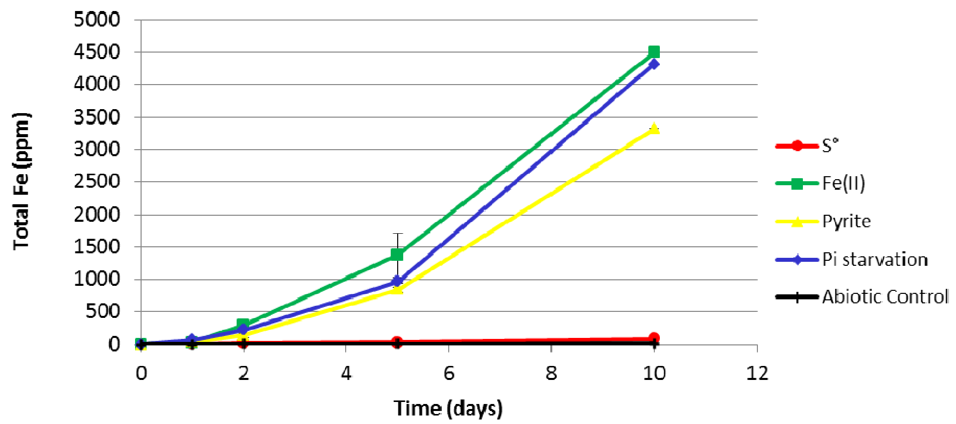

Figure 3. Changes in total iron ions concentration during attachment tests on pyrite grains with Candidatus *A. copahuensis* cells pregrown using sulfur (●), iron(II) (■), pyrite (▲), cells pregrown with pyrite under P*i* starvation conditions (u) and abiotic control (+). Error bars represent the standard deviation of the mean of two replicates.

This finding is in agreement with similar observations reported by other researches for other bioleaching microorganisms, confirming that also in this case the cell attachment to the surface can greatly improve the dissolution of metal sulfides [4].

Several biomining microorganisms have also been shown to accumulate electron-dense granules composed of inorganic polyphosphates (polyP) as seen in the bacterium *At. ferrooxidans* and the archaeon *Sulfolobus metallicus* [10]. PolyP granules constitute an intracellular P*i* reserve, allowing microbial development under P*i* starvation conditions. In our experiments, Candidatus *A. copahuensis* cells were able to grow and leach pyrite under P*i* starvation conditions. These results suggest the presence of P*i* reserves in this strain. Also, an increase of cell adhesion under P*i* starvation conditions was observed (Fig. 4). The oxidation activity under P*i* starvation conditions reached 4500 ppm of iron dissolution after 10 days of incubation (Fig. 3); this dissolution was ~27% higher than in the control. These results suggest that the increase on cell attachment observed under P*i* starvation results in an enhanced pyrite leaching.

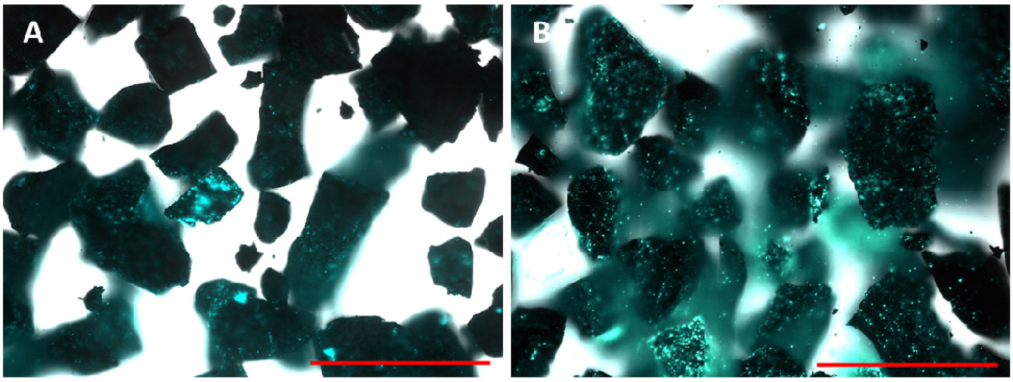

Figure 4. CLSM images of DAPI staining showing Candidatus *A. copahuensis* cells attached to pyrite grains after 5 days of incubation. (A) Attachment of cells pregrown with pyrite under P*i* sufficient conditions. (B) Attachment of cells pregrown with pyrite under P*i* starvation conditions. Size bars represent 50 µm.

Conclusions

According with the results obtained, the adhesion properties of Candidatus *Acidianus copahuensis* were dependent on the growth media used. Based on the evidence presented in this study, it is possible to conclude preliminarly that cells adapted to growth with iron(II) or pyrite showed higher attachment to pyrite surface and oxidation activity than those adapted to growth on sulfur. That new evidence allow suggests that the higher cell attachment to the surface can increase the dissolution of metal sulfides. Also, the ability to grow under P*i* starvation conditions, suggests the presence of P*i* reserves in this strain.

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