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Biofilm formation and interspecies interactions in mixed cultures of thermo-acidophilic archaea *Acidianus* spp. and *Sulfolobus metallicus*

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Abstract

The understanding of biofilm formation by bioleaching microorganisms is of great importance for influencing mineral dissolution rates and to prevent acid mine drainage (AMD). Thermo-acidophilic archaea such as *Acidianus*, *Sulfolobus* and *Metallosphaera* are of special interest due to their ability to perform leaching at high temperatures, thereby enhancing leaching rates. In this work, leaching experiments and visualization by microscopy of cell attachment and biofilm formation patterns of the crenarchaeotes *Sulfolobus metallicus* DSM 6482^T and the *Acidianus* isolates DSM 29038 and DSM 29099 in pure and mixed cultures on sulfur or pyrite were studied. Confocal laser scanning microscopy (CLSM) combined with fluorescent dyes as well as fluorescently labeled lectins were used to visualize different components (e.g. DNA, proteins or glycoconjugates) of the aforementioned species. The data indicate that cell attachment and the subsequently formed biofilms were species- and substrate-dependent. Pyrite leaching experiments coupled with pre-colonization and further inoculation with a second species suggest that both species may negatively influence each other during pyrite leaching with respect to initial attachment and pyrite dissolution rates. In addition, the investigation of binary biofilms on pyrite showed that both species were heterogeneously distributed on pyrite surfaces in the form of individual cells or microcolonies. Physical contact between the two species seems to occur, as revealed by specific lectins able to specifically bind single species within mixed cultures.

Keywords: Biofilm formation; Interspecies interactions; Thermo-acidophilic archaea; *Acidianus* spp.; *Sulfolobus metallicus*

1. Introduction

Bioleaching of metal sulfides (MSs) such as pyrite (FeS_2) or chalcopyrite ($CuFeS_2$) is accelerated by a diverse group of acidophilic iron/sulfur-oxidizing microorganisms, which thrive at a broad range of temperatures [19,23]. Species of *Acidianus* and *Sulfolobus* are thermo-acidophiles frequently isolated from geothermal areas or bioleaching systems at temperatures above 60 °C. Both are capable of oxidizing iron(II)-ions and reduced inorganic sulfur compounds (RISCs) for growth. Under thermophilic conditions, the passivation of

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chalcopyrite ($CuFeS_2$) surfaces by the presence of RISCs can be strongly reduced in comparison with chalcopyrite leaching at lower temperatures. This has importance for the biomining industry, in which thermo-acidophilic archaea such as *Acidianus*, *Sulfolobus* and *Metallosphera* may be considered for applications in which high temperatures are generated.

Detailed knowledge of biofilms formed by thermo-acidophiles, especially of some of their natural substrates, is still rather limited [15]. Nevertheless, some progress in the visualization and characterization of biofilms produced by acidophilic archaea has been recently reported [16,26,27]. Biofilms formed by *Acidianus* sp. DSM 29099 on surfaces of elemental sulfur (S^0) were studied by means of fluorescent lectin binding assays (FLBA) in combination with other cell dyes. It was shown that biofilm cells were heterogeneously distributed and characterized as individual groups of cell clusters and microcolonies. Twenty-one lectins were shown to be useful for the study of EPS glycoconjugates produced by this archaeon. In addition, various glycoconjugates containing monosaccharides such as fucose, glucose, galactose, mannose, N-acetyl glucosamine (GlcNAc) and N-acetyl galactosamine (GalNAc) were detected in these biofilms [26]. As previously mentioned, the combination of several dyes for staining intra- and extracellular DNA, proteins, and lipophilic compounds, led to detailed information about biofilms of thermo-acidophiles growing on different substrates. For instance, biofilms of *Sulfolobus metallicus* DSM 6482^T were shown to be embedded in an EPS matrix containing proteins and eDNA [27]. These studies were done using axenic cultures, and currently, no detailed information on the influence of different species in mixed-species biofilms of thermo-acidophilic leaching archaea has been reported. Several interactions have been shown to occur within acidophilic bacterial leaching species [14]. It has also been shown, for these organisms, that quorum sensing (QS) plays a role in regulation of biofilm formation and leaching behavior. The addition of long chain N-acyl homoserine lactones (AHLs) increased the biofilm formation of *Acidithiobacillus ferrooxidans* ATCC 23270^T on pyrite and sulfur surfaces [7]. Recently, in leaching cultures, it has been shown that the addition of (AHLs) also influenced biofilm formation and pyrite leaching rates of other mesophilic leaching species in pure and mixed cultures [3]. This suggests a QS-based communication between the studied strains of *Acidiferrobacter*, *Acidithiobacillus* and *Leptospirillum*. In addition, it has been shown that the sulfur oxidizer *Acidithiobacillus thiooxidans* is able to attach efficiently to pyrite, which had been pre-colonized by active biofilms of iron oxidizers such as *Leptospirillum ferrooxidans* or *A. ferrooxidans* [3].

In this work, leaching experiments, as well as cell attachment and biofilm formation patterns of the crenarchaeotes *S. metallicus* DSM 6482^T and the *Acidianus* isolates DSM 29038 and DSM 29099, in pure and mixed cultures on pyrite or sulfur, has been studied. CLSM combined with fluorescent dyes has been used in combination with strain-specific fluorescently labeled lectins, which enabled identification of single species on binary biofilms in a non-invasive way.

2. Materials and methods

2.1. Strains and media

S. metallicus DSM 6482^T and the strains *Acidianus* sp. DSM 29038, also named "*candidatus Acidianus copahuensis*" [6] and *Acidianus* sp. DSM 29099 (=JCM 30227) [26] were used. All strains were cultivated in Mackintosh (MAC) medium [10]. Initial pH was adjusted to 1.8 when pyrite (1 or 5%) was used as energy source. In case of cultures grown with S^0 (1%), the initial pH was 2.5. Cells were grown aerobically at 65 °C with 120 rpm shaking.

2.2. Preparation of pyrite grains and sulfur prills

Pyrite grains with sizes between 50 and 100 μm or 200–500 μm were selected after grinding and sieving of pyrite cubes (Navajun Mine, Rioja, Spain). Pyrite grains were cleaned and sterilized as previously described [20]. Sulfur prills (1–3 mm in diameter) were prepared according to a previous report [27], and sterilized at 110 °C for 90 min.

2.3. Leaching experiments

Leaching experiments were performed in 100 mL Erlenmeyer flasks, each containing 50 mL MAC medium (pH 2.5), 1% (*w/v*) pyrite and an initial cell number of 5×10^8 cells/mL. Flasks were incubated at 65 °C with shaking at 120 rpm. Samples from supernatants (1 mL) were routinely withdrawn from each flask for determination of planktonic cell numbers, pH and iron-ion concentration [4]. After growth, cells were harvested by centrifugation at 8000 rpm for 10 min. Cell pellets were washed twice with MAC solution in order to remove trapped ions, resuspended in fresh MAC solution and then used as inoculum for the assays. To study the net contribution of the biofilm population to pyrite bioleaching, removal of planktonic cells was done by daily sterile filtration through 0.22 μm pore size polycarbonate filters (Millipore) and return of the cell-free supernatant medium to the assay, or by daily replacement of the MAC culture medium with fresh medium. In this case, the concentration of iron ions was measured in each supernatant sample removed. Consequently, leaching values are given by the sum of iron concentrations in all exchanged supernatant medium samples plus the remaining one at the end of the experiment. Each experimental condition was repeated twice in duplicate ($n = 4$).

2.4. Pre-colonization experiments

Flasks containing 50 mL MAC medium and 1% pyrite were inoculated with 5×10^8 cells/mL of *Acidianus* sp. DSM 29038 or *S. metallicus*^{T} and incubated at 120 rpm orbital shaking and 65 °C for 18 h. After this, culture supernatants were discarded to remove planktonic cells. In order to inactivate biofilm-forming cells, a subset of samples was incubated at 120 °C for 1 h. Afterwards, pyrite was washed three times with 50 mL MAC medium. After washing, 5×10^8 cells/mL of *S.*

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metallicus^T were added to the flasks containing pre-colonized pyrite with heat-inactivated biofilm cells of *Acidianus* sp. DSM 29038. In the same way, flasks with pyrite pre-colonized with *Acidianus* sp. DSM 29038 were treated as described above and inoculated with 5×10^8 cells/mL of *S. metallicus*^{T}. Flasks were incubated at 65 °C with 120 rpm orbital shaking. Periodically, samples were withdrawn from each flask for the quantification of the planktonic cell numbers and for the measurement of pH and iron ions. Control experiments to verify the inactivation of biofilms and controls without pre-colonization were included. Each experiment was carried out twice in duplicate.

2.5. Fluorescence staining of samples

Sulfur prills or pyrite grains with biofilm cells were mounted in a Petri dish. The cell biomass and spatial distribution were visualized after staining with the nucleic acid stains: DAPI (diamidino-2-phenylindole) and SybrGreen (Molecular Probes). Dyes were added to the samples and directly visualized. Based on previous results for FLBA staining of EPS glycoconjugates [26], FITC- or TRITC-labeled lectins were tested first on axenic cultures in order to select suitable ones binding single species. These were tested in binding assays with mixed cultures containing biofilms of *S. metallicus*^{T} and *Acidianus* spp. When necessary, samples were counterstained with nucleic acid stains, as previously mentioned, and directly observed using CLSM without any further treatment.

2.6. CLSM

Examination of stained samples was performed by CLSM using a TCS SP5X AOBS (Leica, Heidelberg, Germany), controlled by the LASAF 2.4.1 build 6384. The system was equipped with an upright microscope and a super continuum light source (470–670 nm) as well as a 405 nm pulsed laser diode. Images were collected with a 63× water immersion lens with a numerical aperture (NA) of 1.2 and a 63× water immersible lens with a NA of 0.9. In addition, a laser scanning module (LSM 510 Carl Zeiss® Jena) coupled to an inverted Axiovert100 M BP microscope (Zeiss®) was used. Appropriate laser excitation wavelengths and optical filters were used for specific detection of fluorophore signals (Argon laser: 488 nm, 505–550 nm bandpass filter) using constant excitation laser energy and detector settings. Images were collected with 20× and 40× air lenses with NA of 0.45 and 0.64, respectively. The microscope was operated with the software LSM 510 Release 3.2 (Zeiss®). CLSM data sets were recorded in sequential mode to avoid cross-talk of the fluorochromes between two different channels. Surface topography and texture of the pyrite as well as of the S^0 surface were recorded by using the CLSM in reflection mode. For all microscopic images, at least 5 spots from each sample were visualized. For each sample, more than three pyrite grains or sulfur prills were checked by eye under the microscope after staining. Biological replicates were re-checked for data reproducibility.

2.7. Digital image analysis

Fluorescence images were analyzed using an extended version of the software ImageJ. Maximum intensity (MIP) and XYZ projections of 3-dimensional data sets were produced with the software IMARIS version 7.3.1 (Bitplane AG, Zurich, Switzerland).

3. Results

3.1. Influence of biofilm and planktonic cell populations of Acidianus sp. DSM 29038 on pyrite dissolution

Pyrite leaching assays were performed to evaluate the contribution of the biofilm and planktonic cell subpopulations of *Acidianus* sp. DSM 29038 to pyrite dissolution. For this, assays with filtration of culture supernatants and return of cell-free supernatants to the assay, as well as assays with exchange of the culture supernatants with fresh medium, were done (see Material and methods). Both assays are similar in the removal of planktonic cells, but differ in the chemical composition of culture supernatants. Results were compared to assays without separation of biofilm and planktonic cell subpopulations (Fig. 1). Changes in total iron concentrations were similar among assays with or without the separation of planktonic cells during the first 3 days of incubation (Fig. 1B). Afterwards, assays without planktonic cells showed a greater increase in iron-ion dissolution compared to assays with both cell subpopulations. When the planktonic cell subpopulation was daily removed, by replacing the culture medium with fresh medium, the oxidation activity reached around 1063 (±15) ppm of iron dissolution after 10 days of incubation. This value was 72% (±2) higher than the one reached in flasks containing both cell subpopulations (Fig. 1B). A similar trend was observed in assays with *S. metallicus*^{T} (Supplementary Fig. 1).

Interestingly, some differences in iron(II)-ion concentrations were observed (Fig. 1C). In assays with both cell populations, iron(II)-ion levels reached 27% (±2) and 4.8% (±0.4) of iron-ions at 4 and 10 days of incubation, respectively. In assays with daily removal of planktonic cells by filtration, about 28% (±2) of the dissolved iron was present as iron(II)-ions after 4 days of incubation. Afterwards, the iron(II)-ion concentration decreased to 16% (±1) at the end of the assay. A similar tendency was observed in assays with daily replacement of the culture supernatant medium. The concentration of iron(II)-ions was similar for the first 7 days (41–45%) and decreased to 26% (±1) after 10 days of incubation. In all assays, pH decreased over the time of the experiment. The pH dropped from 2.50 ± 0.02 to 1.38 ± 0.02 in assays with both populations, while in assays with daily removal of planktonic cells by cell filtration or by replacement of the MAC medium, the pH decreased to 1.61 ± 0.01 and 2.02 ± 0.02, respectively (Fig. 1A).

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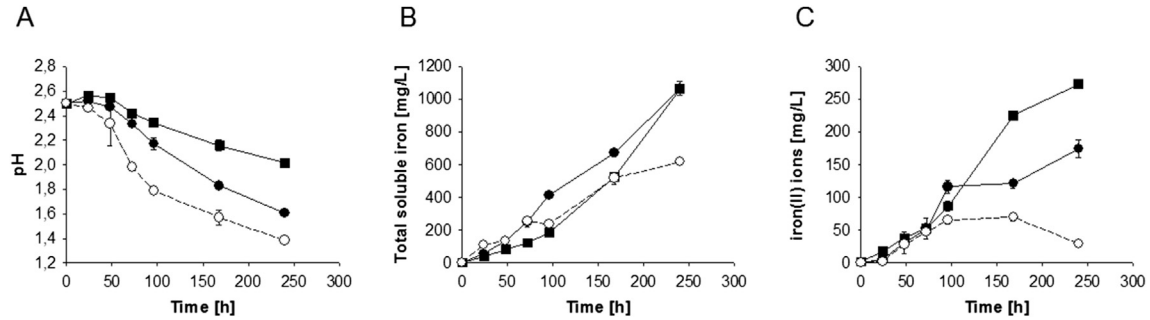

Fig. 1. **Pyrite bioleaching by *Acidianus* sp. DSM 29038 with and without removal of planktonic cells**. Changes in pH (A), iron-ion concentration (B), and concentration of iron(II)-ions (C) during assays performed without (open circles) or with daily removal of the planktonic cell subpopulation (filled symbols), by sterile filtration and return of the cell-free supernatant medium to the assay (filled circles), or by replacement of the culture medium (filled squares). Assays were carried out twice in duplicate (n = 4). Error bars represent the standard deviation of the mean of replicates.

3.2. Influence of pyrite pre-colonization on its subsequent colonization and leaching

To elucidate whether the presence of a pre-colonizer biofilm may influence subsequent pyrite colonization and leaching by a second species, pyrite samples were pre-colonized with *S. metallicus*^{T} and one subset of samples was heat-inactivated. Afterwards, flasks were inoculated with *Acidianus* sp. DSM 29038 (Fig. 2). This experimental set-up was also applied in a reverse way, to study the attachment and leaching behavior of *S. metallicus*^{T} to pyrite pre-colonized by *Acidianus* sp. DSM 29038, with heat-inactivated biofilms (Fig. 3). In control assays performed without pyrite pre-colonization, both strains showed a high attachment rate during the first minutes of contact between the cells and the pyrite particles. Cell attachment was estimated to be 78% (±1) and >99% for *Acidianus* sp. DSM 29038 and *S. metallicus*^{T}, respectively. As shown in Fig. 2A, the presence of an inactivated biofilm of *S. metallicus*^{T} reduced attachment to pyrite by *Acidianus* sp. DSM 29038 to 19% (±4). Also, for this condition, pyrite bioleaching was 77% (±3) less than in control cultures without pre-colonization (Fig. 2B). Similarly, the presence of an inactivated biofilm of *Acidianus* sp. DSM 29038 inhibited attachment of *S. metallicus*^{T} to pyrite by 35% (±1) within the first 360 min (Fig. 3A). In this condition, iron dissolution was 39% (±6) slower compared to control experiments, reaching 323 ppm (±30) after 10 days of incubation (Fig. 3B).

3.3. Cell interactions within mixed cultures

Fig. 4A shows the iron-ion concentration in pyrite leaching by axenic cultures of *Acidianus* sp. DSM 29038 or *S. metallicus*^{T} and mixed cultures containing both species. Iron-ion concentration values were similar in *Acidianus* sp. DSM 29038 and *S. metallicus*^{T} axenic cultures while, in mixed cultures, final values were reduced 16% (±1). Interestingly, in axenic cultures, iron(II)-ion concentrations were relatively constant with values below 100 ppm. However, in mixed cultures, 380 ppm (±46) of the iron-ions were detected in the form of iron(II)-ions (Fig. 4B), clearly suggesting inhibition of iron-oxidizing activity in case of co-existence of both species.

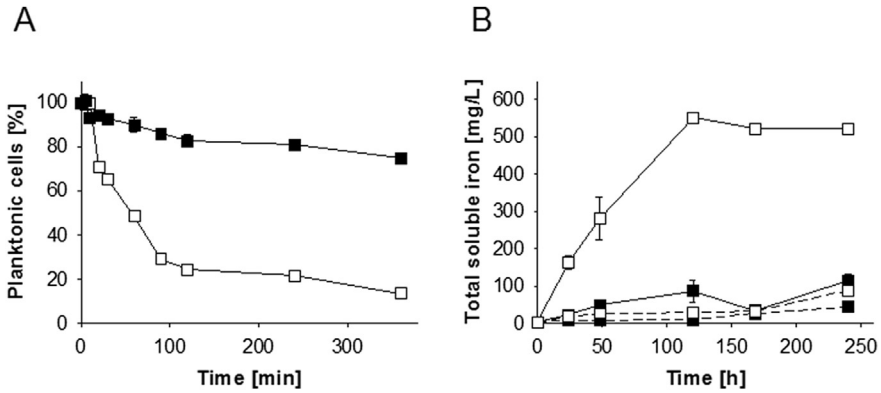

Fig. 2. Attachment and pyrite leaching by *Acidianus* sp. DSM 29038. Effect of the presence of precolonizing *S. metallicus*^{T} biofilm cells. Cell attachment of *Acidianus* sp. DSM 29038 to pyrite is shown in (A). Assays were performed with pyrite grains pre-colonized with *S. metallicus*^{T} (filled boxes), or without pre-colonization (open boxes). Pyrite leaching results are shown in (B). After removal of *S. metallicus*^{T} planktonic cells and further heat-inactivation of biofilms cells of a subset of samples, all flasks were inoculated with *Acidianus* sp. Dashed lines show abiotic pyrite leaching of control experiments without (open boxes) or with heat-inactivated *S. metallicus*^{T} biofilms (filled boxes). Assays were carried out twice in duplicate (n = 4). Error bars represent the standard deviation of the mean of replicates.

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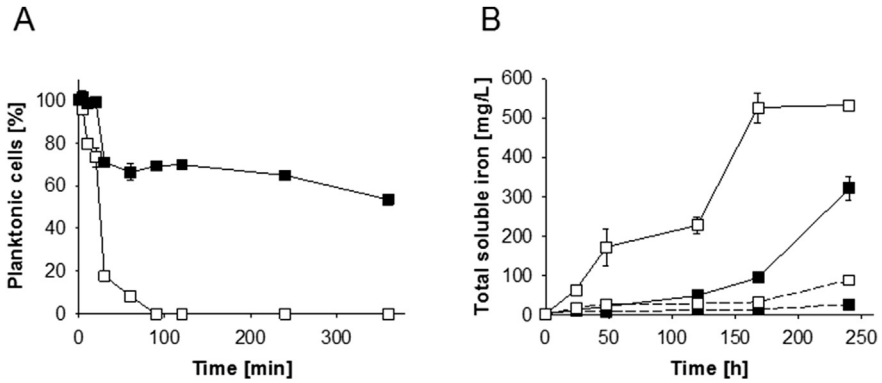

Fig. 3. Attachment and pyrite leaching by *S. metallicus*^{T}. Effect of the presence of precolonizing *Acidianus* sp. DSM 29038 biofilm cells. Cell attachment of *S. metallicus*^{T} to pyrite is shown in (A). Assays were performed with pyrite grains pre-colonized with *Acidianus* sp. DSM 29038 cells (filled boxes), or without pre-colonization (open boxes). Pyrite leaching results are shown in (B). After removal of *Acidianus* sp. DSM 29038 planktonic cells and further heat inactivation of biofilm cells of a subset of samples, all flasks were inoculated with *S. metallicus*^{T} (solid lines). Dashed lines show abiotic pyrite leaching of control experiments without (open boxes) or with heat-inactivated *Acidianus* sp. DSM 29038 biofilms (filled boxes). Assays were carried out twice in duplicate (n = 4). Error bars represent the standard deviation of the mean of replicates.

3.4. Visualization of biofilms in pure and mixed cultures of thermo-acidophilic archaea on pyrite and elemental sulfur surfaces

Biofilms of *S. metallicus*^{T} and *Acidianus* sp. DSM 29099 on sulfur prills or pyrite surfaces are shown in Fig. 5. Thin-layer biofilms were found on the sulfur surface with both strains. Microcolonies were, in some cases, also visible. In case of *S. metallicus*^{T}, apart from DNA signals from single cells, filamentous/thread-like DNA signals connecting individual cells were detected on the sulfur surface (Fig. 5A). This indicates that extracellular nucleic acids of *S. metallicus*^{T} were present in these biofilms. Cells of both species were mainly found in the valleys, cracks or grooves of the sulfur prills and, in some cases, microcolonies with several micrometers in diameter were visible. It may be possible that under shaking conditions, crushing among sulfur prills caused partial cell detachment (see further). When cells were grown on pyrite grains, both species colonized pyrite surfaces in the form of individual cells. For both species, the majority of biofilm cells were found on defect sites on the pyrite. In addition, no extracellular nucleic acids were evident in these biofilm samples on pyrite (Fig. 5C–D).

As earlier observed, individual cells were mainly found attached on areas with pores, scratches and imperfections. After 10 days of incubation, pyrite grains showed a high degree of corrosion. Biofilm cells and glycoconjugates from EPS were made visible by the binding of the fluorescently labeled lectin ConA-TRITC (Supplementary Fig. 2).

Previous studies have suggested that some lectins could be potentially useful for studying mixed biofilms of acidophilic archaea on pyrite or elemental sulfur by distinguishing single species [25]. Several lectins were tested in FLBA assays with axenic cultures of the aforementioned species on pyrite in order to find lectins binding to single species (Supplementary Table 1). Although a variety of lectins could be potentially used to distinguish single species in mixed biofilms of *Acidianus* sp. DSM 29038 and *S. metallicus*^{T}, several of them gave weak binding signals. The lectin PSA (*Pisum sativum* agglutinin) reacted with biofilm cells of *S. metallicus*^{T}, but not with cells of *Acidianus* sp. DSM 29038 on pyrite. In contrast, lectin HHL (*Amaryllis lectin*) bound specifically to *Acidianus* sp. DSM 29038 biofilm cells on pyrite, but did not show binding to *S. metallicus*^{T} biofilm cells. These lectins, in combination with the lectin ConA, which binds glycoconjugates produced by both species, were selected to visualize mixed biofilms of

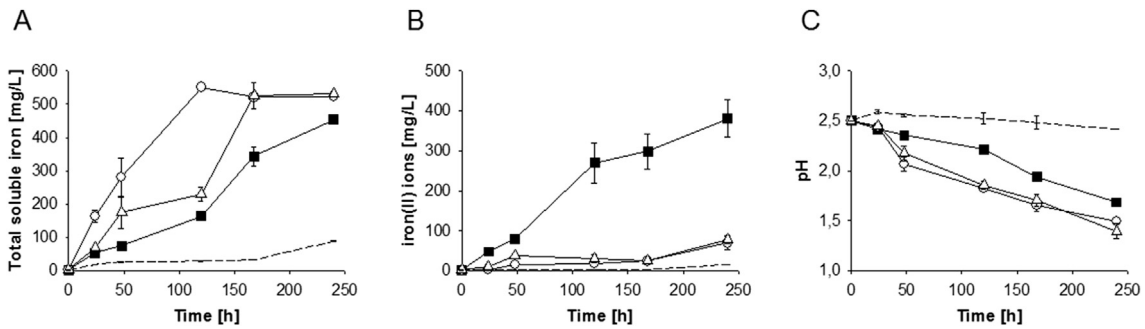

Fig. 4. **Pyrite leaching by pure and mixed cultures of *S. metallicus*^{T} and *Acidianus* sp. DSM 29038**. Development of iron-ion concentration (A), concentration of iron(II)-ions (B) and pH (C) during pyrite bioleaching with pure cultures of *Acidianus* sp. DSM 29038 (open circles), *S. metallicus*^{T} (open triangles) and a mixed culture of *Acidianus* sp. DSM 29038 and *S. metallicus*^{T} (filled boxes). Dashed lines represent abiotic controls. Assays were carried out twice in duplicate (n = 4). Error bars represent the standard deviation of the mean of replicates.

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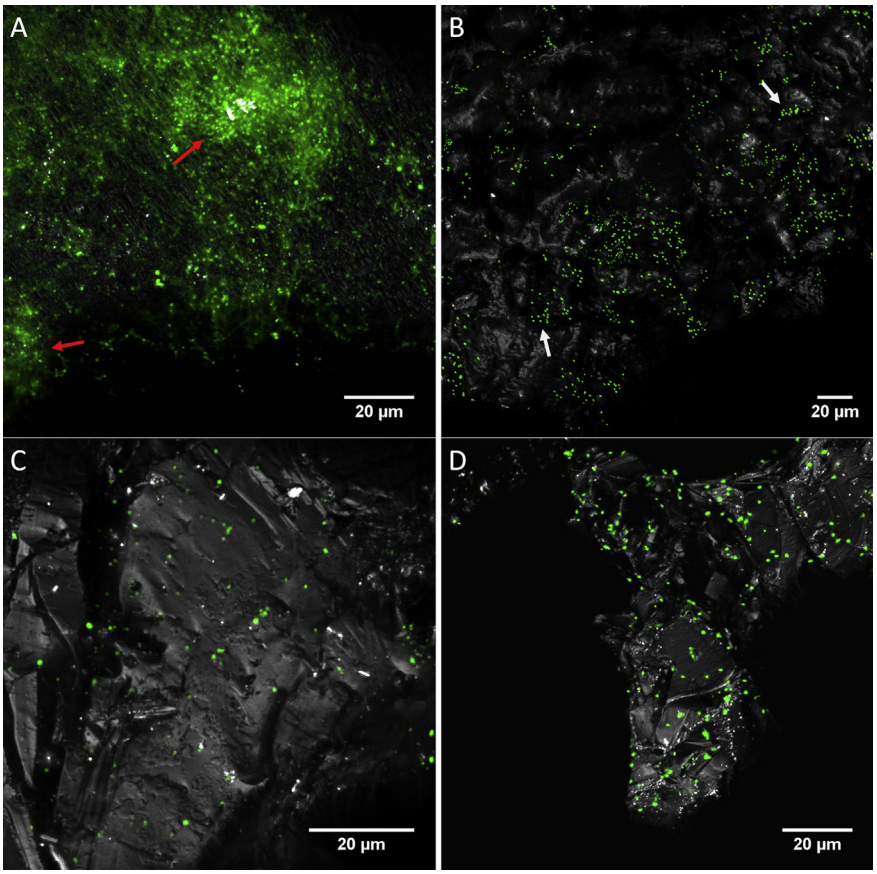

Fig. 5. **CLSM visualization**. Biofilms of *S. metallicus*^{T} (A, C) and *Acidianus* sp. DSM 29099 (B, D) on sulfur prills (A, B) or pyrite (C, D) are shown. Cells were stained by SybrGreen. The sulfur or pyrite surface is shown in reflection mode (in gray). When cells were grown on sulfur grains, both species colonized surfaces in the form of individual cells. In some cases, microcolonies with several micrometers in diameter were visible (red arrows in A). Biofilm cells on sulfur were in some cases found on grooves or cracks as indicated by arrows in B. In addition, no extracellular nucleic acids were evident in biofilms on pyrite (C, D).

Acidianus sp. DSM 29038 and *S. metallicus*^{T} on pyrite. As shown in Fig. 6, DAPI (blue) signals revealed the distribution of both species within mixed biofilms on pyrite. PSA-FITC stained part of the biofilms containing *S. metallicus*^{T}, while *Acidianus* sp. DSM 29038 was stained by HHL-FITC. In addition, cells of *S. metallicus*^{T} formed microcolonies surrounded by a few cells of *Acidianus* sp. DSM 29038. Also, some areas were devoid of DAPI signals, but clearly contained microbial footprints, as evidenced by either PSA, HHL or ConA binding patterns. These indicate that detachment processes are playing an active role during biofilm development of thermo-acidophiles under our experimental conditions. The physical contact (Supplementary Fig. 3) observed in these experiments also suggests the existence of interspecies interactions between these two species.

4. Discussion

Our results clearly show, for leaching assays with axenic *Acidianus* sp. DSM 29038 cultures, that biofilm cells are unable to oxidize all iron(II)-ions which arise in the course of pyrite dissolution. Their oxidation as well as the oxidation of RISCs (also arising from pyrite) seem to be carried out to a considerable extent by the planktonic cell subpopulation. A similar trend was observed in assays in which separation of the biofilm from planktonic cell subpopulations during pyrite leaching by the mesophilic bacterium *A. ferrooxidans*^{T} [2] was carried out.

Results showed that pre-colonization (either by *Acidianus* sp. DSM 29038 or by *S. metallicus*^{T} of pyrite surfaces) has negative effects on cell attachment and subsequent leaching by the second species used in this study. During initial attachment tests, it was also observed that cell attachment of *S. metallicus*^{T} to sulfur prills pre-colonized by *Acidianus* sp. DSM 29099 was considerably reduced compared to its cell attachment to clean sulfur prills (not shown). The reasons for these phenomena are unknown. Interestingly, several *Sulfolobus* strains seem to produce proteinaceous toxins, called sulfolobicins, which may kill cells of other strains [18]. Similar compounds are also produced by haloarchaeal species (halocins) [22]. Their activity is apparently associated with small particles derived from the cell S- layer [13]. The activity spectrum of sulfolobicins appears to be restricted to other members of the sulfolobales [13,18,22]. The production of antibiotic peptides and/or proteins has not been screened for other sulfolobales. Their potential presence may help to explain the antagonistic interaction(s) between *Acidianus* sp. DSM 29038 and *S. metallicus*^{T}, which was noted in our work.

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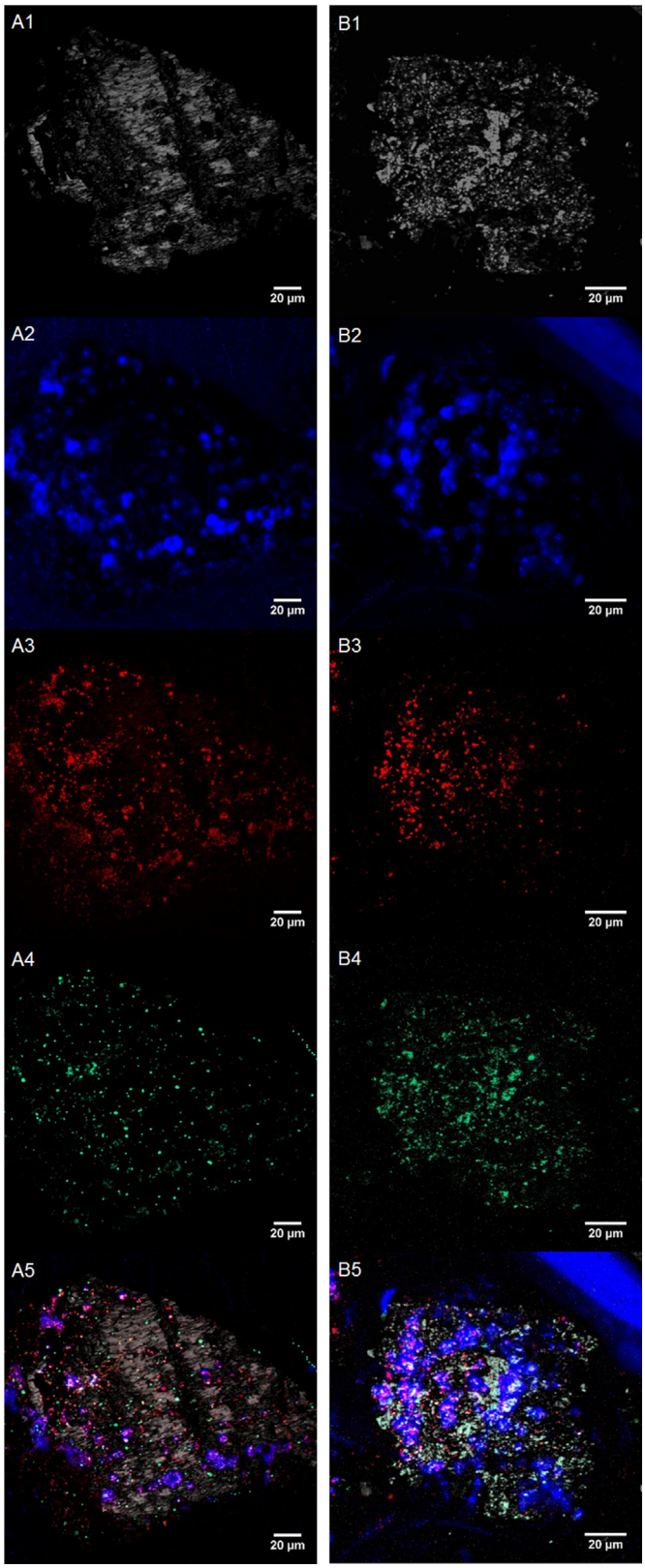

Fig. 6. **CLSM visualization of biofilms in mixed cultures by species-specific lectins**. Binary biofilms of *Acidianus* sp. DSM 29038 and *S. metallicus*^{T} stained by DAPI (A2; B2), ConA-TRITC (A3; B3), HHL-FITC (A4) and PSA-FITC (B4) are shown. DAPI and ConA-TRITC lectin signals revealed the distribution of both species and their glycoconjugates, respectively, on the pyrite surface. HHL-FITC was bound to biofilm cells of *Acidianus* sp. DSM 29038. In a similar way, *S. metallicus*^{T} cells were stained selectively with PSA-FITC lectin. Pyrite surface is shown in reflection mode (A1; B1). Merged images are shown in A5 and B5. Size bars represent 20 µm.

Further research is necessary to understand biofilm formation and interactions of thermo-acidophiles in multispecies biofilms.

Cell attachment tests were performed to study the influence of pre-colonization with iron-oxidizing bacteria on the subsequent attachment of sulfur-oxidizing bacteria to pyrite [3,11,12]. It has been shown that sulfur oxidizers such as *A. thiooxidans* and thiosulfate-grown *A. ferrooxidans* cells attached more readily to pyrite pre-colonized by living iron-oxidizing biofilms of *L. ferrooxidans* than to heat-inactivated ones or to sterile pyrite grains [3]. Since pyrite dissolution is mediated by the chemical attack of iron(III)-ions, there is a clear benefit for sulfur oxidizers, which may attach to certain sites with an enhanced local concentration of RISCs arising from the dissolution of pyrite. This is supported by the fact that heat-inactivated biofilms of iron oxidizers did not enhance cell attachment of sulfur oxidizers [3]. This seems not to occur within *S. metallicus*^{T} and *Acidianus* spp. which, judging from the attachment inhibition and leaching impairment when a pre-colonizer strain is present, seem to compete for the same attachment sites on the pyrite surface. This effect can be partially dissociated from the potential presence of certain soluble toxins produced by the pre-colonizer strain, since the presence of heat-inactivated biofilms of the precolonizer strain also reduced attachment and leaching efficiencies of the second species.

In general, biofilm structures of thermo-acidophilic archaea in mixed cultures have been little studied. In mixed species biofilms of *Pyrococcus furiosus* with *Methanopyrus kandleri,* it has been shown that the *P. furiosus* cells attach to *M. kandleri* cells, forming some colonies with an unusual shape [21]. Cell contact within these two species has been shown to be mediated by flagella and probably EPS. Another example of archaeal cell interactions is that observed in cells of ARMAN (archaeal Richmond Mine acidophilic nano-organisms) with cells of *Thermoplasmatales*. ARMAN cells do not directly interact with other types of microorganisms; however, some cells showed a direct cytoplasmic connection with Thermoplasmatales. This interaction could involve the injection of nutrients from cells of thermoplasmalates to ARMAN in the form of parasitism [1]. Another close association has been reported between *Ignococcus hospitalis* and *Nanoarchaeum equitans* [9]. In this interaction, *N. equitans* attaches to the outer membrane of *I. hospitalis*, obtaining lipids, amino acids and probably ATP from the former [8]. An antagonistic interaction between hyperhalophilic archaea *Halorubrum* sp. and *Halobacterium* sp. has been described recently. *Halobacterium* sp. can outcompete the pre-established *Halorubrum* sp. biofilms by a mechanism that might include the combined action of tunneling swimmers and antimicrobial compounds [5].

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Interactions between acidophilic archaea during bioleaching processes have been studied to a limited extent. As mentioned before, in bioleaching bacteria, QS signaling and chemotactic responses seem to play a major role. In the domain Archaea, QS is a phenomenon that has received relatively little attention, in contrast to bacteria. Some insight into the occurrence of QS has been evidenced for the halophilic archaeon *Natronococcus occultus* and in methanogenic euryarchaeota such as *Methanosaeta harundinaces, Methanosarcina mazei* and *Methanobrevibacter smithi* [17,24]. These species seem to produce AHLs. The presence of AHL-based QS systems in thermo-acidophiles is difficult to understand, mainly due to the poor heat stability of AHLs. In addition, canonical QS auto-inducers were also sought in the crenarchaeon *Sulfolobus* spp.; however, neither AHL- nor furanone-like molecules were reported in cultures of these thermo-acidophiles [16]. The effect of the addition of cell-free *S. metallicus*^{T} supernatant cultures on bioleaching of pyrite by *Acidianus* sp. was also studied. Our preliminary results have shown that bioleaching activity of *Acidianus* sp. 29038 cells exposed to *S. metallicus*^{T} supernatants was partially inhibited (not shown). In consequence, and taking into account the antagonistic effect observed during pre-colonization tests, the release of some antagonistic compounds by *S. metallicus*^{T} cells cannot be excluded.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2016.06.005.

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