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Biofilm formation, communication and interactions of leaching bacteria during colonization of pyrite and sulfur surfaces

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Abstract

Bioleaching of metal sulfides is an interfacial process where biofilm formation is considered to be important in the initial steps of this process. Among the factors regulating biofilm formation, molecular cell-to-cell communication such as quorum sensing is involved. A functional LuxIR-type I quorum sensing system is present in *Acidithiobacillus ferrooxidans*. However, cell-to-cell communication among different species of acidophilic mineral-oxidizing bacteria has not been studied in detail. These aspects were the scope of this study with emphasis on the effects exerted by the external addition of mixtures of synthetic *N*-acyl-homoserine-lactones on pure and binary cultures. Results revealed that some mixtures had inhibitory effects on pyrite leaching. Some of them correlated with changes in biofilm formation patterns on pyrite coupons. We also provide evidence that *A. thiooxidans* and *Acidiferrobacter* spp. produce *N*-acyl-homoserine-lactones. In addition, the observation that *A. thiooxidans* cells attached more readily to pyrite pre-colonized by living iron-oxidizing acidophiles than to heat-inactivated or biofilm-free pyrite grains suggests that other interactions also occur. Our experiments show that pre-cultivation conditions influence *A. ferrooxidans* attachment to pre-colonized pyrite surfaces. The understanding of cell-to-cell communication may consequently be used to develop attempts to influence biomining/bioremediation processes.

Keywords: Quorum sensing; *N*-Acyl-homoserine-lactone; Biofilm formation; Bioleaching; Pyrite

1. Introduction

Bioleaching is the dissolution of metal sulfides such as pyrite (FeS_2) and is driven by bacterial and archaeal oxidation of iron(II)-ions and sulfur compounds as well as biogenic metal chelating organic acids in extracellular polymeric substances (EPS). Coal, minerals and other geological deposits regularly contain metal sulfides such as pyrite or marcarsite. Their natural weathering and dissolution processes cause the formation of acid mine drainage (AMD), which is a serious environmental problem, especially in regions where mining takes place [1]. Bioleaching processes are used in biomining technologies for recovering metals such as copper, nickel or zinc. The term biooxidation is defined as the bacterial oxidation of gold or silver containing sulfide minerals, such as pyrite or arsenopyrite, in order to enrich them for their further chemical extraction [2,3].

Biofilms are communities of microorganisms embedded in a self-produced matrix of EPS, which mainly consist of polysaccharides, proteins, lipids and DNA [4]. Biofilm formation on metal sulfides is considered to be important for bioleaching since the attached microorganisms are the ones

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which start the leaching process by providing an enlarged reaction space between the metal sulfide surface and the cells in which iron(III)-ions are accumulated [5]. In bacteria, biofilm formation and EPS production are complex processes which are, among other mechanisms, regulated by quorum sensing (QS) systems [6] and the second messenger c-di-GMP pathway [7]. QS allows bacterial cells to sense their population density and to modulate gene expression in a cell density-dependent manner due to the secretion of diffusible autoinducers (AIs), which are used to regulate intra- or interspecies processes [8,9].

Acidithiobacillus ferrooxidans^T possesses a canonical type I QS system and produces several *N*-acyl-homoserine-lactone (AHL) AIs [10]. Moreover, the external addition of synthetic long-chain AHLs stimulates its biofilm formation on pyrite or sulfur surfaces [11]. This observation correlated with increased levels of EPS in biofilms formed on the surface of polycarbonate filters floating on medium amended with long-chain AHLs. Recently, a bioinformatic analysis using Hidden Markov Models predicted that at least 75 genes could be regulated by QS in *A. ferrooxidans*^{T}. Among these, some genes involved in the biosynthesis of exopolysaccharides, as well as genes encoding transport and RNA regulatory functions are included [12]. We have previously estimated differences among planktonic and pyrite-attached cell subpopulations after 24 h of biofilm formation by high-throughput proteomics. These accounted for around 16% of the total amount of detected proteins [13]. Functions such as glutathione metabolism, stress responses and EPS biosynthesis seem to be pivotal. As mentioned, biofilm formation and EPS production in many bacterial species is often controlled by the second messenger c-di-GMP. The *A. ferrooxidans*^{T} genome sequence encodes for several proteins involved in the c-di-GMP pathway and biochemical studies showed their functionality [14]. In addition, levels of this second messenger were found to be increased in cells grown on solid substrates such as sulfur prills or pyrite, strongly suggesting its involvement in *A. ferrooxidans* biofilm formation.

Several studies have addressed the biodiversity and changes in the species composition of microbial communities present in bioleaching environments [15–17]. However, cell interactions and communication between different acidophilic mineral-oxidizing species coexisting in biofilms on metal sulfides are largely unknown. It is well known that sulfur-grown *A. ferrooxidans* cells do not attach well to pyrite compared to iron-grown cells. This has been explained due to modifications of their EPS composition [18]. Still it is unknown whether the presence of primary colonizers is required for attachment of sulfur-oxidizing strains. It has been shown that several biofilms from different ecological habitats undergo a succession of colonizers in which the first ones produce EPS which may drive attachment of further species [19,20]. In addition, there are few studies on the presence of antagonistic or synergistic interactions between certain species [21] and whether these may be influenced by QS or other cell-to-cell communication mechanisms is still unknown.

In order to address these points the influence of the external addition of defined mixtures of AHLs to pyrite leaching assays in pure and binary mixed cultures was tested with several acidophilic mineral-oxidizing bacterial species. Biofilm formation of these strains on pyrite coupons was investigated by epifluorescence microscopy (EFM).

2. Material and methods

2.1. Strains and media

A. ferrooxidans ATCC 23270^T, *A. ferrivorans* SS3 [22], *Acidiferrobacter* sp. SPIII/3 [23], *Acidiferrobacter thiooxydans* DSM 2392^T and *Leptospirillum ferrooxidans* DSM 2391 [24] were cultivated in Mackintosh (MAC) basal salt solution [25] at pH 1.8 with 3 g/l iron(II)-ions or 2–5% pyrite as energy sources. *A. thiooxidans* DSM 14887^T was grown in a modified MAC basal salt solution at pH 4.5 and 1 g/l elemental sulfur (S^0) powder. For pre-colonization experiments, *A. ferrooxidans*^{T} was grown on DSMZ 71 medium (pH 4.5) amended with 5 g/l sodium thiosulfate pentahydrate as described [26].

2.2. Biofilm formation experiments on pyrite and sulfur coupons

Sulfur coupons were produced by melting S^0 and pouring the liquid onto a cover-glass to solidify. Seven coupons were placed in 100-ml wide-neck Erlenmeyer flasks containing 30 ml of MAC medium (pH 4.5) and sterilized by autoclaving at 110 °C for 90 min. Flasks were inoculated with 10^8 cells/ml. AHLs were used at 5 μM. Coupons were withdrawn for microscopic observation. They were washed once with MAC medium (pH 4.5), once with 50 mM Tris–HCl pH 7.4 and twice with double distilled water. Staining of pyrite-attached cells was done with 0.01% 4,6-diamidino-2-phenylindole (DAPI) in 2% formaldehyde for 20 min followed by washing the coupons twice with sterile double distilled water and drying at room temperature for visualization with an epifluorescence microscope (Axiovert-100 MBP microscope, Zeiss®). The microscope was operated with the software AxioVision 4.2 (Zeiss®). In order to prolong the fluorescence of the dye, an anti-fading agent (Citifluor™ AF2) was used when mounting the coupons. Pyrite coupons were prepared as described [11].

2.3. Pyrite dissolution assays

Pure and binary mixed pyrite cultures were prepared in 250-ml Erlenmeyer flasks containing 50 ml MAC medium (pH 1.8), 5% (w/v) pyrite grains (Romania, Baia Mare; 50–100 μm) and an initial cell number of 2.5×10^8 iron-grown cells/ml. Pyrite was washed and sterilized prior use as described [27]. Binary mixed cultures were prepared using equal initial cell numbers of each species. To determine leaching efficiency, iron-ion concentrations were quantified by using the phenanthroline method [28]. Synthetic AHLs were

acquired from the University of Nottingham (http://www.nottingham.ac.uk/quorum/) or Sigma®. AHLs with an acyl chain length of 8, 12 and 14 carbon atoms were added before inoculation as mixtures including the unsubstituted, the 3-oxo- and the 3-hydroxy-AHL (5 μM each). A mixture of the unsubstituted C16- and C18-AHLs was also tested. These AHL-mixtures (AHLm) are hereinafter referred to as C8-, C12-, C14- and C16/18-AHLm. Control assays were done with addition of DMSO, the solvent of AHL stock solutions.

2.4. Pre-colonization experiments

Pyrite grains (50–100 μm) were incubated at 2% (w/v) with 10^8 cells/ml of iron-grown *A. ferrooxidans*^{T} or *L. ferrooxidans* DSM 2391 in 50 ml MAC medium for 18 h. Afterwards, culture supernatants were discarded and the pyrite (with attached cells) was washed three times with 50 ml MAC medium (pH 2.5). To elucidate if live biofilm-forming cells may influence further pyrite colonization one set of pre-colonized pyrite grains were incubated at 80 °C for 2 h. After washing, 10^8 sulfur-grown *A. thiooxidans*^{T} cells/ml were added to all flasks and attachment tests were performed as described [29]. Similarly, this experimental setup was applied to study the influence of the energy source used for growth of *A. ferrooxidans*^{T} on cell attachment to pyrite pre-colonized by *L. ferrooxidans* DSM 2391. For this, 10^8 thiosulfate- or iron-grown cells/ml were added to pre-colonized pyrite flasks as described above. In order to compare cell attachment to cell-free pyrite surfaces, control experiments without pre-colonization were included.

2.5. AHL extraction and identification by mass spectrometry

AHL extraction was carried out using dichloromethane (DCM; p.a). 500-ml cultures of *A. thiooxydans*^{T}, *Acidiferrobacter* sp. SPIII/3, *A. ferrivorans* SS3 and *L. ferrooxidans* DSM 2391 grown on pyrite or iron(II)-ions and *A. thiooxidans*^{T} cultures grown on sulfur were transferred to beakers, including their planktonic cells and biofilms on pyrite or sulfur, respectively. One volume of DCM was added and the mixture was stirred at 300 rpm for 30 min at room temperature. After phase separation, the organic phase was transferred to a glass balloon and rota evaporated at 40 °C and 750 mbar until the volume was reduced to approximately 3 ml. The concentrated organic phase was transferred to a 4 ml glass ampoule, in which further evaporation of the organic phase was done using nitrogen gas stream. Remaining compounds in the glass balloon were recovered by washing it with 2 ml DCM. After complete evaporation of the solvent, extracts were stored at −20 °C. All glass material was previously washed with DCM.

For mass spectrometry analysis, the extracts were resuspended in acetonitrile (p.a) containing 0.1% HCOOH (LC-MS grade) and incubated for 20 min at 30 °C with repeated mixing. A tenfold dilution prepared with water:acetonitrile (80:20) containing 0.1% HCOOH was injected with a syringe pump into the electrospray ionization (ESI) ion source (Ion Max, Thermo) of the mass spectrometer (LTQ XL Orbitrap with ETD) at a flow rate of 10 μl/min. Instrument settings were as follows: positive ionization mode; source voltage 4.5 kV; capillary voltage 11 V; capillary temperature 270 °C; tube lens 60 V; sheath gas pressure 5 au (arbitrary units), ion sweep gas pressure 0 au, auxiliary gas pressure 0 au; normalized collision energy for CID:20, isolation width 2.5 Da, activation Q 0.25, activation time 30 ms. Scan cycles were as follows: full scans were done in the orbitrap in the range of 100–600 m/z, resolution 100,000, CID, ion target value 1,000,000. The 10 most intense precursor ions were subjected to CID in the LTQ and fragment ions transferred to the orbitrap for mass analysis with target value 30,000 ions, max inject time 1 s, dynamic exclusion 300 s, mass resolution of 7500. Xcalibur software version 2.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for data analysis.

3. Results

3.1. Effect of AHL mixtures on pyrite leaching by pure and binary cultures

The effects of addition of AHL-mixtures to pure cultures of mineral-oxidizing bacteria growing on pyrite are shown in Fig. 1. Pyrite leaching was expressed as the total amount of iron-ions solubilized within 28 days after inoculation. Under

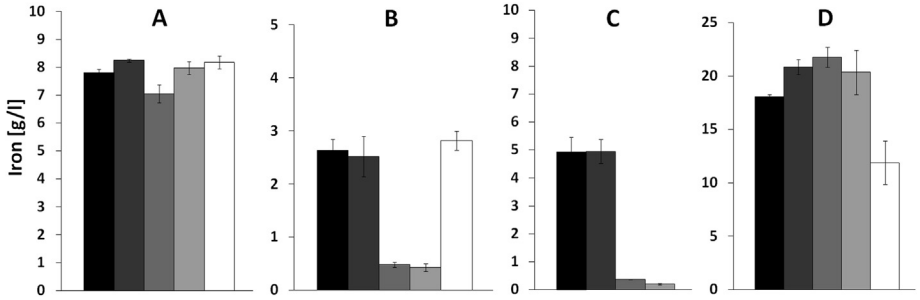

Fig. 1. **Influence of AHL–mixture addition on pyrite dissolution in pure cultures.** Pyrite leaching assays in pure cultures of *A. ferrooxidans*^{T} (A), *A. ferrivorans* SS3 (B), *Acidiferrobacter* sp. SPIII/3 (C) and *L. ferrooxidans* DSM 2391 (D) were incubated at 28 °C with 120 rpm shaking. Total iron concentrations were determined after 28 days of incubation. Mean values from duplicate assays are shown. Black bars correspond to control experiments without addition of AHLs. Colors from left to right correspond to cultures amended with C8-, C12-, C14- and C16/18-AHLm, respectively.

our conditions, the best leaching efficiency was achieved by *L. ferrooxidans* DSM 2391 (18 g/l), followed by *A. ferrooxidans*^{T} (7.9 g/l), *Acidiferrobacter* sp. SPIII/3 (4.9 g/l) and *A. ferrivorans* SS3 (2.6 g/l Fe). These values were in agreement with the described physiological characteristics of these strains [30]. In *A. ferrooxidans*^{T} slight enhancing effects, accounting for less than 6% on pyrite leaching, were measured after addition of C8-, C14- and C16/18-AHLm, while the addition of the C12-AHLm resulted in a decreased pyrite dissolution of 10%. It was also evident that some AHLm caused inhibitory effects in *A. ferrivorans* SS3. In this strain pyrite leaching was decreased by 72 and 74% after addition of C12- and C14-AHLm, respectively. Interestingly, these two mixtures caused also the highest decrease in pyrite leaching by *Acidiferrobacter* sp. SPIII/3 (92 and 96%, respectively). In contrast, the addition of C8-, C12- or C14-AHLm to cultures of *L. ferrooxidans* DSM 2391 resulted in leaching enhancements of 14, 17 or 12% respectively. In this species, a decrease of leaching by 34% was measured after addition of the C16/18-AHLm.

As previously shown, the addition of C12- or C14-AHLm resulted in an increased biofilm formation of *A. ferrooxidans*^{T} on pyrite coupons [11]. In this context it is interesting to remark that the observed enhancement of biofilm formation after addition of these AHLm did not correlate with enhanced pyrite dissolution (Fig. 1A). In contrast, in *A. ferrivorans* SS3 the addition of the C12-AHLm resulted in a very strong inhibition of biofilm formation after 8 days (Fig. 2B), while the addition of C14-AHLm resulted in an enhanced formation of cell aggregates, which presumably were less active, as judged by the leaching values shown in Fig. 1B. In this case the decrease of biofilm formation correlated well with the lowered leaching efficiency observed.

Similar experiments carried out with *L. ferrooxidans* DSM 2391 and *Acidiferrobacter* sp. SPIII/3 are shown in Fig. 3. The addition of C14-AHLm resulted in an enhanced biofilm formation of *L. ferrooxidans* DSM 2391 after 8 days, while the addition of C8- and C12-AHLm resulted in cell cluster formation. In *Acidiferrobacter* sp. SPIII/3 the addition of C8-AHLm showed increased pyrite colonization after 24 h compared to the control. In comparison to the results obtained with *A. ferrivorans* SS3 (Fig. 2B), in which an almost complete inhibition of pyrite colonization was observed in presence of C12-AHLm, pyrite coupons incubated with *Acidiferrobacter* sp. SPIII/3 in medium amended with C12- or C14-AHLm showed a presence of attached cells. Presumably, these cells were inhibited by the addition of C12- or C14-AHLm since pyrite dissolution was decreased in presence of these AHLs (Fig. 1C).

Leaching efficiencies in binary cultures containing *A. ferrooxidans*^{T} and *A. ferrivorans* SS3, *L. ferrooxidans* DSM 2391 or *Acidiferrobacter* sp. SPIII/3 were comparable to those observed in pure cultures of *A. ferrooxidans*^{T} (7–8 g/l iron after 28 days), as shown in Supplementary Fig. S1. In this context, it seems possible that due to yet unknown mechanisms, *A. ferrooxidans*^{T} cells could mask the effects of the external addition of AHLs.

Fig. 4 shows the effects of AHLm addition to binary cultures that did not contain *A. ferrooxidans*^{T}. Especially interesting was the mutualistic inhibition observed between cells of *L. ferrooxidans* DSM 2391 and *Acidiferrobacter* sp. SPIII/3 after transfer to cultures with pyrite as energy source

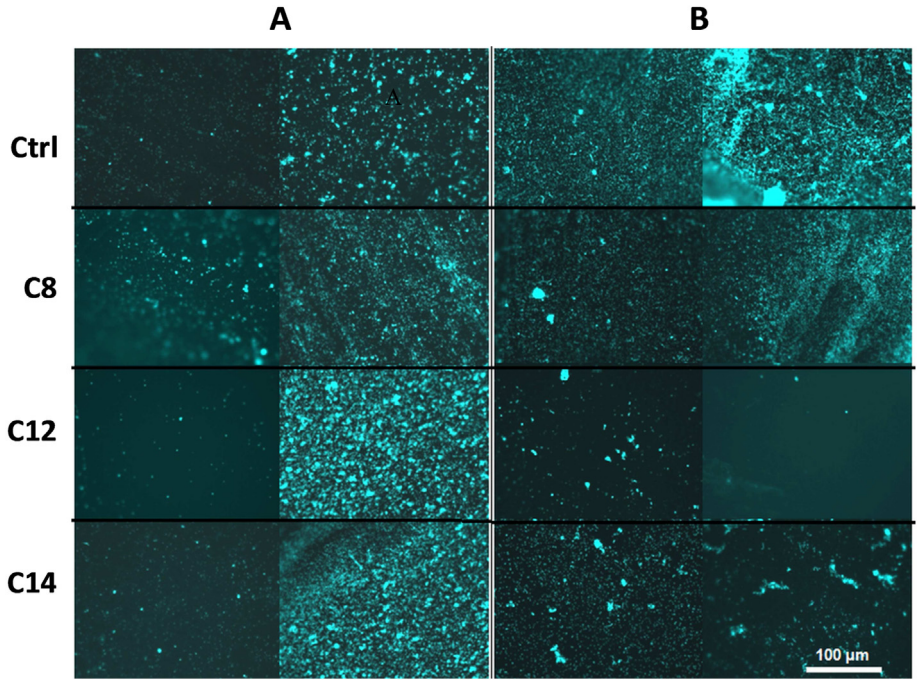

Fig. 2. **Influence of AHL–mixture addition on attachment and biofilm formation on pyrite coupons in pure cultures of *A. ferrooxidans*^{T} (A) and *A. ferrivorans* SS3 (B)**. Coupons were imaged after 1 (left) and 8 (right) days. Size bar represent 100 µm.

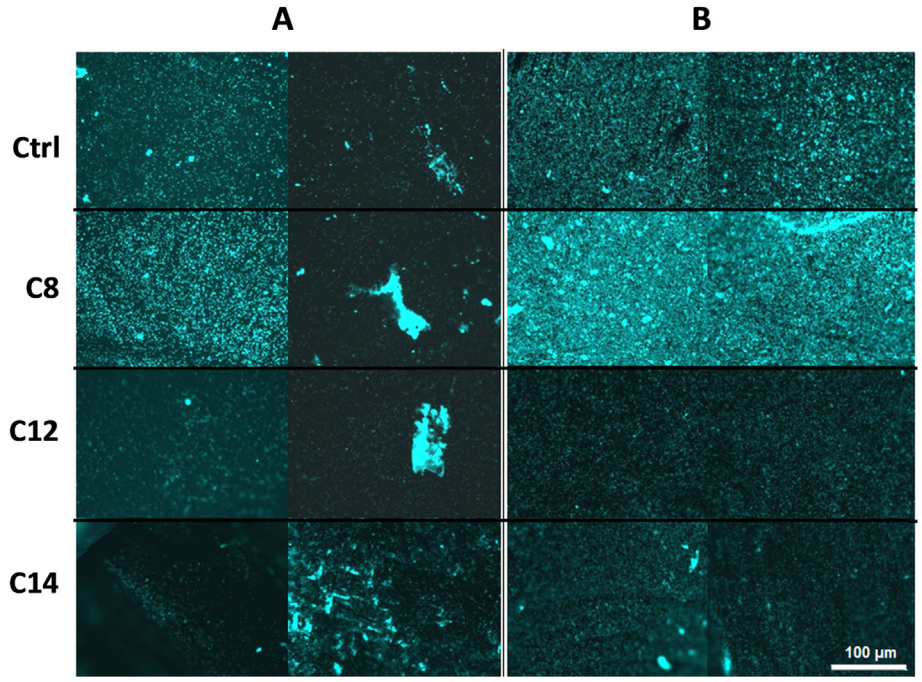

Fig. 3. **Influence of AHL–mixture addition on attachment and biofilm formation on pyrite coupons in pure cultures of *L. ferrooxidans* DSM 2391 (A) *Acidiferrobacter* sp. SPIII/3 (B)**. Coupons were imaged after 1 (left) and 8 (right) days. Size-bar represents 100 µm.

(Fig. 4A). In control experiments without addition of AHLs, pyrite leaching was inhibited by 95% compared to the iron concentrations observed in pure cultures of *L. ferrooxidans* DSM 2391 (Fig. 1D) or by 20% compared to those in pure cultures of *Acidiferrobacter* sp. SPIII/3. This leaching inhibition was reduced after addition of C8-, C14- or C16/18-AHLm to 16, 39 or 35% of the values observed in pure cultures of *L. ferrooxidans* DSM 2391 without AHL addition, respectively. In contrast, when *L. ferrooxidans* DSM 2391 was co-cultured with *A. ferrivorans* SS3, no clear effect of AHLm addition was observed (Fig. 4B). Pyrite leaching values by these mixed cultures did not differ by more than 10% compared to the values achieved for pure cultures of *L. ferrooxidans* DSM 2391. In case of the mixture *Acidiferrobacter* sp. SPIII/3/*A. ferrivorans* SS3, pyrite leaching values were similar to those observed for pure cultures of *Acidiferrobacter* sp. SPIII/3. As expected from the values observed in pure cultures of both species, the addition of C12-AHLm inhibited pyrite leaching by this binary culture. However, the inhibition upon addition of C14-AHLm observed for pure cultures of *Acidiferrobacter* sp. SPIII/3 did not occur in the mixed cultures.

3.2. Presence of AHLs in cultures of mesophilic, mineral-oxidizing acidophiles

It has been shown previously that *A. ferrooxidans*^{T} produces long-chain AHLs [10,11]. Communication phenomena mediated by AHLs in acidophilic bacteria may not be

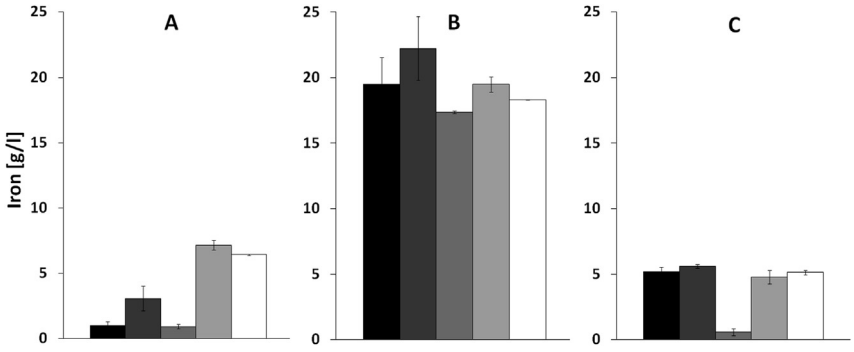

Fig. 4. **Influence of AHL–mixture addition on pyrite dissolution in mixed cultures without the presence of *A. ferrooxidans*^{T}**. Pyrite leaching assays in binary mixed cultures of *L. ferrooxidans* DSM 2391/*Acidiferrobacter* sp. SPIII/3 (A), *L. ferrooxidans* DSM 2391/*A. ferrivorans* SS3 (B) and *Acidiferrobacter* sp. SPIII/3/*At. ferrivorans* SS3 (C) were incubated at 28 °C with 120 rpm shaking. Total iron concentrations were determined after 28 days of incubation. Mean values from duplicate assays are shown. Black bars correspond to control experiments without addition of AHLs. Colors from left to right correspond to cultures amended with C8-, C12-, C14- and C16/18-AHLm, respectively.

Table 1
AHLs detected by mass spectrometry in cultures of mesophilic leaching bacteria.

N-acyl-homoserine-lactone	C10	C12	3-OH-C12	C14	3-OH-C14	C16
Calculated monoisotopic m/z $[M+H]^+$	256.19072	284.22202	300.21693	312.25332	328.24824	340.28462
Measured m/z $[M+H]^+$	256.19113	284.22229	300.21713	312.25327	328.24817	340.28464
Error (ppm)	+1.6	+0.9	+0.7	−0.2	−0.2	+0.1
A. thiooxidans^T	+	+	ND	ND	ND	ND
Acidiferrobacter thiooxydans^T	+	+	+	+	+	+
Acidiferrobacter sp. SPIII/3	+	+	+	+	+	+
L. ferrooxidans DSM 2391	ND	ND	ND	ND	ND	ND
A. ferrivorans SS3	ND	ND	ND	ND	ND	ND

ND, Not detected.

restricted to iron-oxidizers. By thin layer chromatography and biosensor assays (TLC-BA) using *Agrobacterium tumefaciens* NTL4 [31] it was also shown that three *A. thiooxidans* strains (DSMZ 504, DSMZ 9463 and DSMZ 11478) produce short-chain AHLs which could correspond to 3-oxo-C8-AHL. In order to better understand if communication between different species which presumably coexist in leaching habitats occurs, we analyzed the presence of AHLs by mass spectrometry in cultures of *A. thiooxidans*^{T}, *A. thiooxydans*^{T}, *Acidiferrobacter* sp. SPIII/3, *L. ferrooxidans* DSM 2391 and *A. ferrivorans* SS3 (Table 1). Interestingly, as described for *A. ferrooxidans*^{T} [32], both *Acidiferrobacter* species produced a great diversity of AHLs: C10-AHL, C12-AHL, 3-hydroxy-C12-AHL, C14-AHL, 3-hydroxy-C14-AHL and C16-AHL. These compounds were detected in pyrite cultures but not in iron-grown cultures of both strains. In sulfur-grown cultures of *A. thiooxidans*^{T} C10- and C12-AHLs were detected, whereas AHLs were not detected in iron- nor in pyrite-grown pure cultures of *L. ferrooxidans* DSM 2391 or *A. ferrivorans* SS3.

3.3. C8- and C10-AHLs increase attachment to sulfur coupons by *A. thiooxidans*^{T} cells

In order to expand our knowledge about QS communication, experiments with the sulfur-oxidizer *A. thiooxidans*^{T} were carried out. Cultures were incubated with single AHLs on sulfur coupons. As shown in Fig. 5, the addition of C8-AHL, 3-oxo-C8-AHL and C10-AHL increased *A. thiooxidans*^{T} attachment to sulfur coupons. Biofilm morphologies were different after addition of 3-oxo-C8-AHL, where the formation of micro-colonies was observed on the sulfur surfaces, while this phenotype was not observed after addition of C8-AHL or C10-AHL, which only promoted an increased cell attachment (Fig. 5).

3.4. Influence of a pre-colonization by iron-oxidizers on *A. thiooxidans*^{T} attachment to pyrite

It has been shown that *A. ferrooxidans*^{T} cells previously grown on sulfur do not attach well to pyrite compared to iron- or pyrite-grown cells. This has been attributed to a considerable modification in their EPS composition, including increased amounts of fatty acids, absence of complexed iron(III)-ions and glucuronic acid as well as changes in the sugar monomer composition [18]. To ascertain whether *A. thiooxidans*^{T} required pre-colonization of pyrite by iron-oxidizing acidophiles to enable them to attach to the mineral, *A. thiooxidans*^{T} was grown on sulfur and added to cultures containing pyrite grains, which were pre-colonized for 18 h with *A. ferrooxidans*^{T} or *L. ferrooxidans* DSM 2391 cells. Within this time 50 or 80% of these initial inocula of *A. ferrooxidans*^{T} or *L. ferrooxidans* DSM 2391 attached to the pyrite, respectively (not shown). After removal of planktonic cells and heat-inactivation of one set of samples per condition, sulfur-grown *A. thiooxidans*^{T} cells were added. As shown in Fig. 6, *A. thiooxidans*^{T} cells attached to pyrite only when it

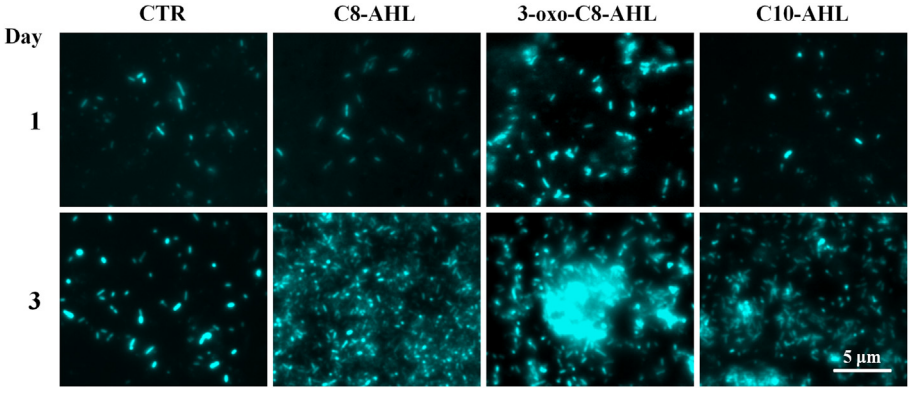

Fig. 5. **Effect of the addition of selected AHLs on *A. thiooxidans*^{T} biofilm formation on sulfur coupons**. Cultures were incubated at 28 °C and 120 rpm. Size-bar represents 5 µm.

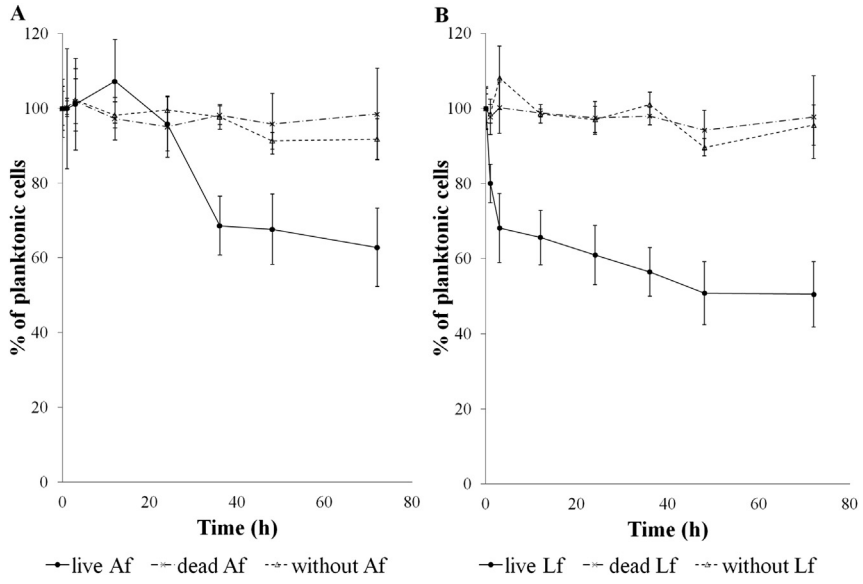

Fig. 6. **The presence of active biofilms of iron–oxidizers increase *A. thiooxidans*^{T} attachment to pyrite**. Pyrite grains (50–100 μm) were pre-colonized for 18 h with 10^8 *A. ferrooxidans*^{T} (A) or *L. ferrooxidans* DSM 2391 (B) cells. After removal of the planktonic cell subpopulation, further cell attachment of *A. thiooxidans*^{T} was assayed by adding 10^8 cells/ml to active biofilms (circles), heat-inactivated biofilms (X) or pyrite grains without pre-colonization (triangles). Mean values from duplicates are shown.

was pre-colonized with active biofilms of *A. ferrooxidans*^{T} or *L. ferrooxidans* DSM 2391. Interestingly, *A. thiooxidans*^{T} attachment to pyrite pre-colonized with *L. ferrooxidans* DSM 2391 was faster than to pyrite pre-colonized with *A. ferrooxidans*^{T}. In the first case, 30% of the cells attached within 3 h, while in the latter case similar attachment values were observed after 25–35 h. The reasons for this phenomenon are unknown. Heat-inactivation did not significantly remove attached cells or their EPS from the pyrite surface (not shown).

3.5. Influence of pyrite pre-colonization by L. ferrooxidans *DSM 2391 on A. ferrooxidans*^{T} *attachment to pyrite*

In a similar way the influence of an active biofilm of *L. ferrooxidans* DSM 2391 on pyrite colonization and biofilm formation by *A. ferrooxidans*^{T} was tested (Fig. 7). As *A. ferrooxidans*^{T} is able to oxidize iron(II)-ions and reduced sulfur compounds, its growth condition was included as a new variable. The presence of an active *L. ferrooxidans* DSM 2391 biofilm enhanced attachment to pyrite by *A. ferrooxidans*^{T} for thiosulfate-grown cells from 50% to 70%. No enhancement of cell attachment was observed with heat-inactivated biofilms (Fig. 7A). Interestingly, with iron-grown *A. ferrooxidans*^{T} cells no significant differences were observed for attachment to pyrite pre-colonized with living or heat-inactivated *L. ferrooxidans* DSM 2391 biofilms (Fig. 7B).

4. Discussion

Results from the current work have shown that the addition of some AHL signalling molecules affect the cell attachment, pyrite leaching and biofilm formation by different species of acidophilic bacteria. A BLAST search in NCBI database revealed the presence of homologous genes encoding for LuxR-like proteins in *A. ferrivorans* SS3 (Acife_1471, 56% identity) and *L. ferrooxidans* C2-3 (LFE_1606, 29% identity). Under our experimental conditions AHLs were not detected in *L. ferrooxidans* DSM 2391 or in *A. ferrivorans* SS3 cultures, however the presence of LuxR-like receptors may explain the inhibitory effects observed after addition of some AHL-mixtures.

Previously, three *A. thiooxidans* strains (DSM 504, DSM 9463 and DSM 11478) were characterized by TLC-BA to produce 3-oxo-C8-AHL [31]. In this study we show by mass spectrometry that *A. thiooxidans*^{T} produces C10- and C12-AHLs. Furthermore this strain is able to sense AHLs, since the addition of C8-, 3-oxo-C8- or C10-AHL resulted in an enhanced biofilm formation on sulfur coupons (Fig. 5). A BLAST search in the NCBI database revealed that the genome of *A. thiooxidans*^{T} does not possess homologous genes to *luxI/R* [33]. This suggests the presence of alternative pathways involved in AHL biosynthesis and sensing in this bacterium, which may also be present in other acidithiobacilli.

On one hand, the addition of long-chain AHLs to cultures of *A. ferrooxidans*^{T} resulted in an enhanced biofilm formation. However, under these experimental conditions, pyrite leaching was not significantly enhanced. On the other hand, the addition of C12- and C14-AHLm to pure cultures of *A. ferrivorans* SS3 and *Acidiferrobacter* sp. SPIII/3 resulted in a significant decrease in leaching efficiency (Figs. 2B and 3B), which must be directly correlated with a decreased pyrite colonization (Fig. 1). Both results indicate that a correlation between mineral colonization and leaching efficiency is species-dependent. In addition, no significant effect of synthetic AHLs on leaching efficiency was observed for mixed cultures with *A. ferrooxidans*^{T} (Supplementary Fig. S1). In

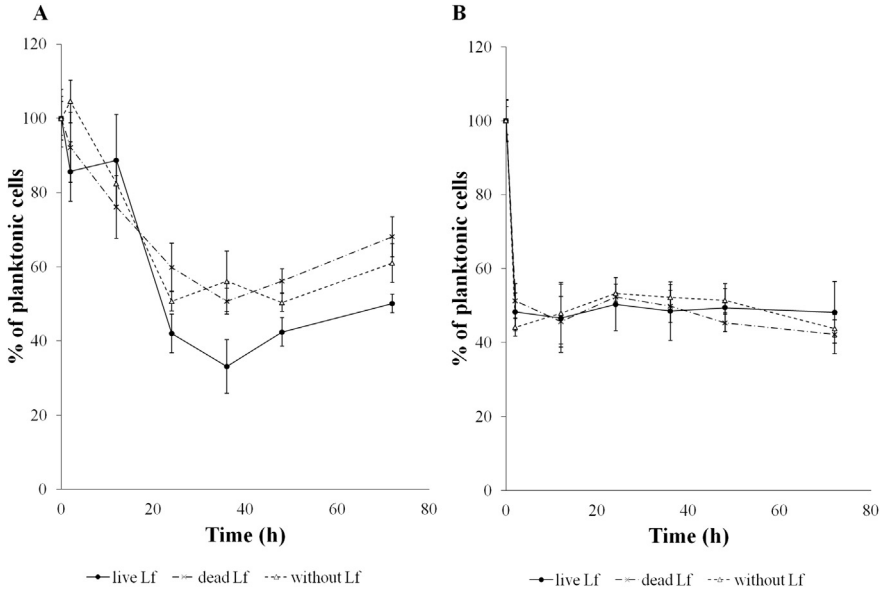

Fig. 7. **Pre–cultivation conditions of *A. ferrooxidans*^{T} influence its attachment to pyrite pre–colonized by active *L. ferrooxidans* DSM 2391 biofilms**. Pyrite grains (50–100 μm) were pre-colonized for 18 h with 10^8 cells/ml of *L. ferrooxidans* DSM 2391. After removal of the planktonic cell subpopulation, further cell attachment of *A. ferrooxidans*^{T} grown on thiosulfate (A) or iron(II)-ions (B) was assayed by adding 10^8 cells/ml to active biofilms (circles), heat-inactivated biofilms (X) or pyrite grains without pre-colonization (triangles). Mean values from duplicates are shown.

this way, the presence of more abundant but less active or dormant biofilms could be related to a "bioshrouding" effect that impairs colonization and pyrite leaching by other strains [34]. *A. thiooxydans*^{T} and *Acidiferrobacter* sp. SPIII/3 produced an identical pattern of AHLs. An incomplete draft genome sequence from the latter strain revealed the presence of a QS system consisting of homologous genes to *lasI* and *luxR* (unpublished data). Interestingly, the observed intrinsic inhibitory interaction in pyrite cultures between *Acidiferrobacter* sp. SPIII/3 and *L. ferrooxidans* DSM 2391 was partially relieved by addition of some AHLs. Taken together, these results suggest that AHLs excreted by *A. ferrooxidans*^{T} or *Acidiferrobacter* spp. may play an important role in their competition with other iron-oxidizers.

We have also shown that pre-colonization of pyrite with active biofilms may influence further cell attachment by other species. The presence of iron-oxidizers may be a relevant factor for sulfur-oxidizers in order to attach more efficiently to pyrite. We provide evidence that attachment of *A. thiooxidans*^{T} cells is related to the presence of active biofilms and not merely an indirect consequence of cell interactions with the EPS from inactivated iron-oxidizers. As *L. ferrooxidans* DSM 2391 leach pyrite more efficiently than *A. ferrooxidans*^{T}, probably the faster attachment by cells of *A. thiooxidans*^{T} to pyrite with previously established biofilms of the former strain may be related to an increased chemotaxis towards reduced inorganic sulfur compounds like thiosulfate which arise on the pyrite surface once leaching takes place. Analysis of the complete *A. thiooxidans*^{T} genome sequence revealed a complete repertoire of genes for flagella formation and chemotaxis [35]. In contrast, *A. ferrooxidans*^{T} attachment to pyrite grains pre-colonized with *L. ferrooxidans* DSM 2391 cells was strongly dependent on its pre-cultivation substrate (Fig. 7). At the moment we cannot prove direct cell interactions between established biofilm cells and new colonizers on the pyrite surface. Currently we are working on testing different fluorescent lectins on biofilms of mineral-oxidizing acidophilic bacterial species that may allow for the identification and direct visualization of mixed species biofilms by fluorescence lectin-binding assays (FLBA) in a non-invasive way [36].

Our results clearly show that cell-to-cell communication mechanisms and biofilm formation processes are interrelated and can determine bioleaching efficiency in laboratory cultures to a great extent. Both processes must be better understood in order to improve the industrial application of bioleaching and to develop countermeasures against AMD.

Conflict of interest

We confirm that there are no conflict of interests for us to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2014.08.006.

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