AHL communication is a widespread phenomenon in biomining bacteria and seems to be involved in mineral-adhesion efficiency

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A B S T R A C T

Biofilm development plays a pivotal role in the bioleaching process. Quorum sensing (QS) is recognized as one of the main regulatory ways of extracellular polymeric substances (EPS) production and biofilm formation in bacteria. Previous works revealed that *Acidithiobacillus ferrooxidans* ATCC 23270 strain possesses a functional QS type AI-1 system. However, there is neither available data concerning the presence of a QS system in other *A. ferrooxidans* strains nor any other biomining bacterial species. Thus, in this work, different strains of *A. ferrooxidans* (ATCC 19859, ATCC 1927, ATCC 33020, DSMZ 583, DSMZ 9464), *Acidithiobacillus thiooxidans* (DSMZ 504, DSMZ 9463, DSMZ 11478) and *Leptospirillum ferrooxidans* (DSMZ 2391, DSMZ 2705) were screened for their ability to produce QS-signaling molecules [acyl-homoserine lactone (AHLs)]. Thin-layer chromatography (TLC) analysis revealed that all the *A. ferrooxidans* and *A. thiooxidans* strains produced AHL-signaling molecules while both tested *L. ferrooxidans* strains did not. Nevertheless, by using bioinformatic tools to screen the genome sequence of a *Leptospirillum* sp. type III belonging to an AMD biofilm, a QS type AI-1 locus in which the structural organization is different from that of *A. ferrooxidans* has been identified. The effect of synthetic AHLs and AHL-analogues on attachment of *A. ferrooxidans* ATCC 23270 to pyrite was analyzed. Interestingly, preliminary results suggested that some of these molecules change the rate and extent of bacterial attachment to pyrite. The production of AHLs by *A. ferrooxidans* and *A. thiooxidans* strains, the identification of a QS type AI-1 locus in *Leptospirillum* sp. type III and the impact of AHLs and AHL-analogues on the pyrite adhesion of *A. ferrooxidans* cells open new perspectives for the (bio)mining industry to eventually improve bioleaching process and control the acid mine drainage (AMD) contamination.

1. Introduction

The attachment and adhesion of acidophilic bacteria to the sulfidic energy source and the subsequent biofilm formation are prerequisites for mineral dissolution in natural environments and industrial operations. Therefore, a thorough understanding of the molecular mechanisms involved in the control and regulation of biofilm formation would be of great importance to control the bioleaching process from the biomining industry and the acid mine drainage (AMD) damages.

Biofilm formation is a complex process that can be regulated at different levels through diverse mechanisms. The most studied mechanism that controls biofilm development is quorum sensing (QS) signaling (Waters and Bassler, 2005). QS is a cell-to-cell signaling system that functions by means of diffusible autoinducer (AI) signal molecules that allow the regulation of cellular processes in a manner that depends on the density of the microbial population.

Recently, a functional QS system type AI-1 involving two divergent genes *afeI* and *afeR* has been identified in the acidophilic bacterium *Acidithiobacillus ferrooxidans* (Farah et al., 2005; Rivas et al., 2005). Overexpression of the protein AfeI in *Escherichia coli* and the characterization of associated synthesis of AHLs demonstrated that AfeI is an AHL synthase (Farah et al., 2005). *A. ferrooxidans* produces AHLs with acyl chains whose length oscillates between 8 and 16 carbons and presents substitutions of the type oxo- and hydroxyl- in C-3 (3-hydroxy-C8-AHL, 3-hydroxy-C10-AHL, C12-AHL, 3-oxo-C12-AHL, 3-hydroxy-C12-AHL, C14-AHL, 3-oxo-C14-AHL, 3-hydroxy-C14-AHL, and 3-hydroxy-C16-AHL). The AHL hydroxy substitution was independent of the nature of the culture medium whereas the keto substitution was displayed only in cells grown on sulfur and thiosulfate. Besides, the transcription of *afeI* gene is higher in sulfur- and thiosulfate-grown cells with respect to iron-grown cells and appears to be related to phosphate metabolism (Farah et al., 2005). Based on this experimental background,

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different authors suggested that the QS system type AI-1 could regulate biofilm formation in *A. ferrooxidans* (Farah et al., 2005; Rivas et al., 2005; Valenzuela et al., 2006; Valenzuela et al., 2007).

This work attempts to examine if the quorum sensing type AI-1 system identified in *A. ferrooxidans* ATCC 23270 is a widespread phenomenon in *A. ferrooxidans* species and other biomining bacteria. Knowledge of the molecular mechanisms that regulate biofilm formation in biomining bacteria such as *A. ferrooxidans* is limited. Therefore, the involvement of the AHL-signaling molecules in the attachment of the type strain *A. ferrooxidans* ATCC 23270 to mineral surfaces was examined. Since marker exchange mutagenesis for the construction of QS mutants in *A. ferrooxidans* is still very difficult to carry out, we decided to use different synthetic AHLs and AHL-analogues that have been evaluated as potential inhibitors and/or activators of QS in other microorganisms (Castang et al., 2004; Geske et al., 2005; Frezza et al., 2006).

2. Materials and methods

2.1. Bacteria

The following strains of biomining bacteria obtained from culture collections were used: (i) six strains of *A. ferrooxidans* ATCC 23270 type strain, ATCC 19859, ATCC 1927, ATCC 33020, DSMZ 583, DSMZ 9464; (ii) three strains of *Acidithiobacillus thiooxidans* DSMZ 504, DSMZ 9463, DSMZ 11478; two strains of *Leptospirillum ferrooxidans* DSMZ 2391, DSMZ 2705.

2.2. Growth media and culture conditions

A. ferrooxidans strains were grown in ferrous iron-containing modified 9K medium at pH 1.5 as described previously, and growth on elemental sulfur was done at pH 2.5 with 5% (wt/v) sulfur prills (Amaro et al., 1991). Growth of *A. ferrooxidans* strains in thiosulfate was done at pH 4.6 in DSMZ medium 71 containing 20 mM thiosulfate as described before (Ramírez et al., 2004). *A. thiooxidans* and *L. ferrooxidans* strains were grown on their corresponding DSMZ medium.

2.3. Southern analysis

Genomic DNAs were obtained by using the Wizard Genomic DNA Purification Kit for Gram-negative bacteria, as described by Promega. 5 μg of purified genomic DNA was digested by EcoRI and HindIII restriction enzymes (New England Biolabs) at 37 °C during 4 h. After separation of the restriction enzyme-digested DNA fragments by electrophoresis, they were denatured and transferred to a positively charged nylon membrane (Immobilon-NY+; Millipore) by the semi-dry capillary method (Sambrook and Russel, 1989). ^{32}P labelled-*afeI* probe was obtained by using the NEBlot kit (New England Biolabs) according to the manufacturer's recommendations. Prehybridizations and hybridizations were accomplished as described by Sambrook and Russel (1989). ^{32}P-signal detection was carried out by using the PhosphoImager (Molecular Imager FX; Biorad).

2.4. AHL-signaling molecules determination

Extracts for analytical thin-layer chromatography (TLC) and LC–MS–MS were prepared from 600-ml cultures. Bacteria were removed by centrifugation and the supernatant was extracted twice with equal volumes of dichloromethane (DCM) as described previously (Farah et al., 2005). Extracts were then dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness.

For TLC plate analysis, dried samples were dissolved in 30–60 μl of HPLC-grade ethyl acetate. Samples (20–40 μl) were spotted onto C_{18} reversed-phase TLC plates (Merck) along with a set of standards and developed with methanol/water (60:40; vol/vol). After development, the TLC plates were air-dried and AHL-signaling molecules were detected by using as a bioreporter *Agrobacterium tumefaciens* NTL4 (pZLR4) as described previously (Shaw et al., 1997). Briefly, 250 μl of an overnight culture of *A. tumefaciens* NTL4(pZLR4) AHL reporter strain grown in 25-ml LB medium with gentamicin 25 μg/ml was inoculated in AB minimal glucose medium (ABm) and grown during 8 h at 30 °C with shaking. The culture was then mixed with an equal volume of 1.5% TOP agar containing 80 μg/ml 5-bromo-4chloro-3-indolyl-β-D-galactopyranoside (X-gal), and the preparation was spread over the surface of developed TLC plates. Overlaid TLC plates were incubated overnight at 30 °C in a closed plastic container. Production of AHL is visualized by the development of a blue color.

For LC–MS–MS analysis, residues were dissolved in 0.5 ml of HPLC-grade acetonitrile and analyzed by using reverse-phase liquid chromatography coupled with positive-ion electrospray ionization and ion trap mass spectrometry (LC–MS–MS) (Morin et al., 2003). In the case of 3-hydroxy-AHLs, their identification was done by comparison with synthetic 3-hydroxy-AHLs based on three criteria: MS-MS fragmentation product ions ($[M+H-H_2O]^+$ and m/z 102), their relative intensities and HPLC retention times.

2.5. Attachment assay

This assay was carried out only with the type strain of *A. ferrooxidans*, ATCC 23270. It is based on the reduction of the number of planktonic cells in the solution due to the attachment of the bacteria to a mineral sulfide substratum (Gehrke et al., 1998; Harneit et al., 2006). Pyrite (FeS_2) (Suior mine in Baia Mare, Romania) was the substratum used for the attachment assay. Ground FeS_2 was wet sieved to a grain size of 50–100 μm (Test sieves, Retsch, Germany). To remove ferric iron, the FeS_2 was boiled in 6 N HCl with stirring for 1 h. Then, the HCl was removed by washing extensively with distilled H_2O. Next, sulfur compounds were extracted from the FeS_2 by washing twice with acetone. Finally, FeS_2 aliquots were put under vacuum and gassed with nitrogen and sterilized at 115 °C for 48 h (Schippers and Sand, 1999). For the attachment assay, 5×10^9 cells were incubated with 20% (w/v) FeS_2 in a final volume of 50-ml mineral salt solution in 100-ml Erlenmeyer flask. *A. ferrooxidans* ATCC 23270 grown in $FeSO_4$ medium was harvested in the late exponential growth phase. Harvesting was performed by cross flow filtration (Hemoflow F60S, Fresenius, Germany) or centrifugation for 20 min at 6000 ×*g*, at 10 °C and the cells thus obtained were resuspended in 50 ml of mineral salts solution. Then, cell concentration was determined using a Thoma counting chamber (depth 0.02 mm, smallest square area 0.0025 mm^2). Subsequently, cell concentration was adjusted at 1×10^8 cells per ml. Next, this cell suspension was distributed among 100-ml Erlenmeyer flasks. Bacteria harvested by centrifugation were supplemented with 0.2 g of ferric iron l^{-1} and kept overnight at 17 °C to regenerate their EPS. Afterwards, AHLs or AHL-analogues were added and the mix was incubated for 1 h at 30 °C, at 180 rpm. Subsequently, 10 g of FeS_2 was added to each Erlenmeyer flask containing the assay mixture and agitated on a benchtop shaker (Type TR, Infors HT, Switzerland) at 120 rpm and room temperature for 8 h. Samples (1 ml) of the culture supernatant were taken at specific time intervals and the cell concentration was determined using a Thoma counting chamber. Cell counting was done with a light microscope (Zeiss) in phase contrast mode with 400-fold magnification.

3. Results

3.1. Quorum sensing type AI-1 system and biomining bacteria

In previous experiments, we reported that *A. ferrooxidans* type strain possesses a functional QS type AI-1 system and is able to produce nine different kinds of AHL-signaling molecules (Farah et al., 2005). To examine if the QS type AI-1 system is a widespread phenomenon in biomining bacteria, we analyzed if other strains of *A.*

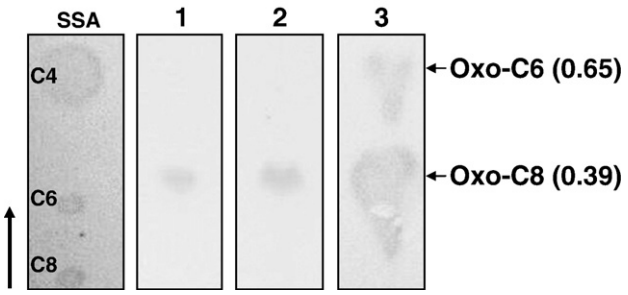

Fig. 1. Thin-layer chromatograms of the AHL-signaling molecules purified from spent culture supernatant of *A. thiooxidans* strains DSMZ 504 (1), DSMZ 9463 (2) DSMZ 11478 (3). Detected molecules are indicated by arrows and respective Rf values are noted. SSA, Standard of synthetic AHLs composed of C4-AHL, C6-AHL and C8-AHL. The direction of solvent migration is marked with a vertical arrow.

ferrooxidans and other members of the biomining community, such as *A. thiooxidans* and *L. ferrooxidans*, are capable of producing AHL-signaling molecules. Dichloromethane extracts of spent culture supernatants were subjected to C_{18} reversed-phase TLC and tested for their ability to activate the *A. tumefaciens* reporter strain, as described in Materials and methods. This experiment revealed that all the tested *A. ferrooxidans* and *A. thiooxidans* strains produce AHL-signaling molecules while no reporter activity was detected in the *L. ferrooxidans* extracts suggesting that DSMZ 2391 and DSMZ 2705 strains of this bacterial species which belongs to *Leptospirillum* sp. type I, do not produce detectable amount of AHL-signaling molecules. However, a bioinformatic analysis of the complete and partial genome sequences of microorganisms present in an AMD biofilm (Tyson et al., 2004) allowed us to identify a QS type AI-1 locus for *Leptospirillum* sp. type III, as previously reported (Valenzuela et al., 2007). This locus is composed of two convergent genes, *lttR* and *lttI* (for *Leptospirillum* sp. type *t*hree), which code for putative proteins that are 50% and 59% similar to R and I proteins from *Geobacter uraniumreducens* Rf4. A third ORF was identified in the *lttIR* intergenic region and it coded for a protein with 42% similarity to a transposase from *Bacillus halodurans* C-125.

The identified AHL-signaling molecules obtained for the three *A. thiooxidans* strains are shown in Fig. 1. The characteristic tailing spot (Shaw et al., 1997) and the estimated Rf values allowed us to determine that all the *A. thiooxidans* strains synthesize 3-oxo-C8-AHL (Fig. 1, lanes 1–3). Moreover, the DSMZ 11478 strain also produced 3-oxo-C6-AHL (Fig. 1, lane 3). As the same relative supernatant quantities were spotted onto the TLC plates, in our experimental conditions, the strain DSMZ 11478 appeared to overproduce oxo-C6-AHL and oxo-C8-AHL compared with the other two strains. Since no *A. thiooxidans* genome sequence is currently available in the data base, it is actually impossible to know what kind of AHLs synthase family(ies) is(are) in charge of the production of oxo-C6-AHL and oxo-C8-AHL.

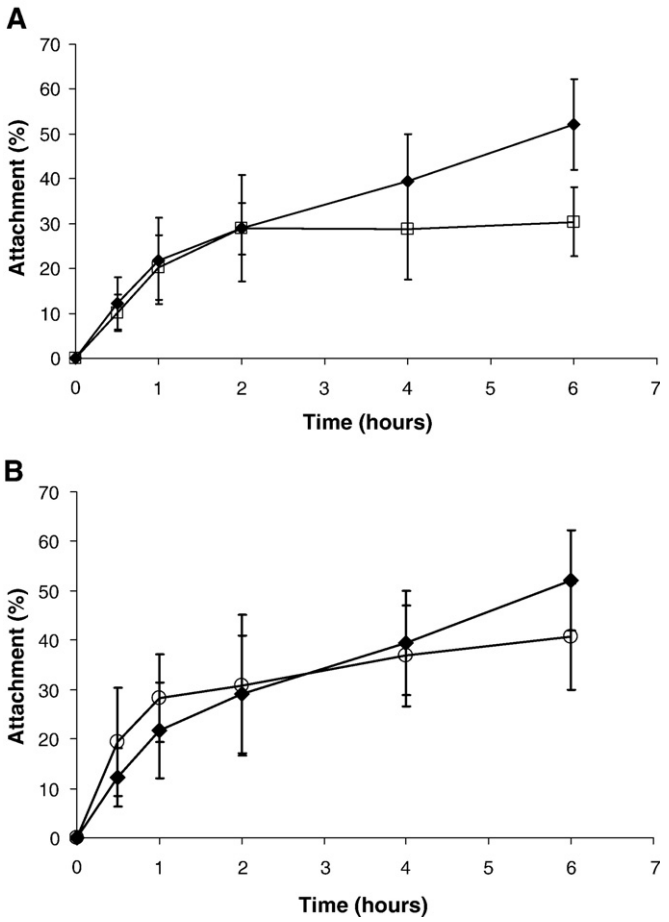

Fig. 3. Effects of an AHL mixture and an AHL-analogue on the attachment of *A. ferrooxidans* to pyrite. A) AHL mixture (□) composed of hydroxyl-AHLs (C10, C12, C14, C16) and oxo-AHLs (C10, C12, C14, C16); B) AHL-analogue, 4-phenyl-3-oxo-HSL (○); (◆), control without AHLs. Each result represents an average of six experiments. The bars show the standard deviation values.

Regarding *A. ferrooxidans* strains, additional studies were performed to characterize more precisely the AHL-signaling molecules of the different strains. Besides, we compared the genomic organization of QS loci from

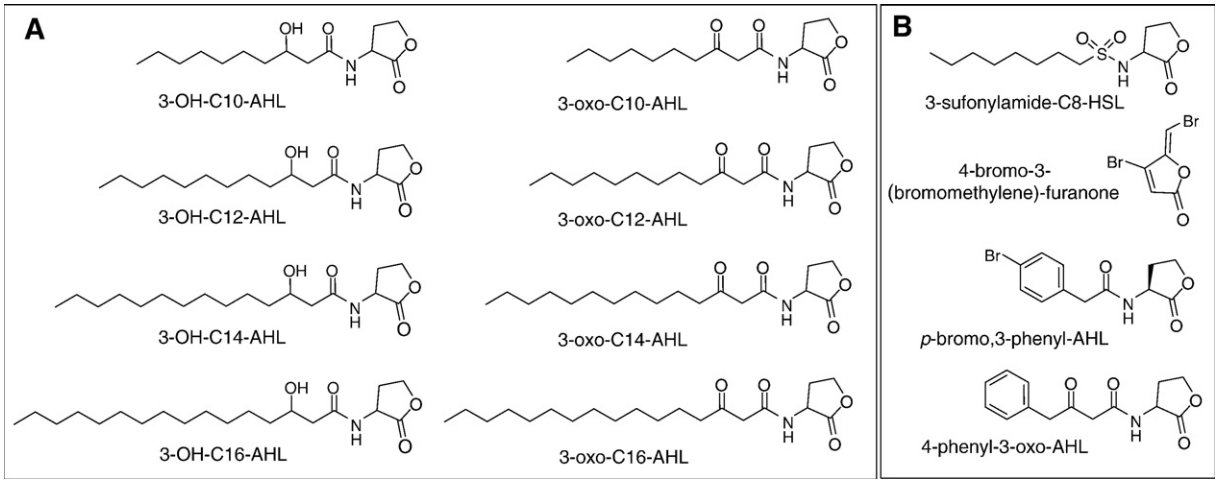

Fig. 2. Structures of AHLs (A) and AHL-analogues (B) used in this work.

three geographically and phylogenetically different *A. ferrooxidans* strains. LC–MS–MS revealed that all the *A. ferrooxidans* strains produced hydroxy-AHLs with an acyl-chain length ranging from C8 to C16 and in addition, strains ATCC 19859 and DSMZ 583 also synthesized C12-AHL and C14-AHL (data not shown). On the other hand, by using an *afeI* labelled-probe, Southern blot analysis of genomic DNA digested by EcoRI and BamHI restriction enzymes revealed that the *afeI* gene encoding for an AHL synthase was present in strains ATCC 19859, ATCC 33020 and DSMZ 9464 (data not shown). Therefore, in these strains AfeI protein should be also responsible for the synthesis of some AHL molecules. However, as in the case in the *A. ferrooxidans* type strain, the presence of an AHL synthase belonging to the HdtS family cannot be excluded (Farah et al., 2005; Rivas et al., 2007). Despite being isolated from different continents and in different kinds of mines and having different positions in an *A. ferrooxidans* phylogenetic tree (Karavaiko et al., 2003), the size similarities between the different EcoRI (1.3 kb) and HindIII (12 kb) positive restriction fragments revealed that the QS locus genomic organization of the *A. ferrooxidans* strains DSMZ 9464, ATCC 19859 and ATCC 33020 is similar to the type strain. These strongly suggest that the QS Type AI-1 locus was a widespread and conserved phenomenon in *A. ferrooxidans* species.

3.2. Quorum sensing type AI-1 system and mineral attachment

As the QS type AI-1 system appears to be a widespread phenomenon in *A. ferrooxidans* species and, at least, 7 of nine AHLs identified in the *A. ferrooxidans* type strain were characterized in other *A. ferrooxidans* collection strains (this work), all the attachment experiments were performed with the *A. ferrooxidans* type strain. To study the role of QS type AI-1 system in cell attachment to a mineral, we assessed the effects of synthetic AHL and AHL-analogues shown in Fig. 2. *A. ferrooxidans* cells were harvested by centrifugation, a condition in which a partial loss of EPS is induced. For the replenishment of their EPS, cells were left overnight in a mineral salt solution supplemented with ferric iron. In this way, a homogeneous bacterial population was obtained, allowing us to observe clearly the possible effects of AHLs and AHL-analogues.

Indeed, an AHL mixture, composed by hydroxyl-AHLs (C10, C12, C14, C16) and oxo-AHLs (C10, C12, C14, C16), inhibited cell attachment to pyrite by 42% (Fig. 3A). Six hours after the incubation with the AHL mixture, only 30% (30.4 ± 7.6) of the bacterial population was attached compared with 52% (52.2 ± 10.1) of attachment for the control bacterial population (not treated) (Fig. 3A). Similar attachment values (50%) were reported for the strain *A. ferrooxidans* 23270 (Barreto et al., 2005). However, the rate and extent of attachment of *A. ferrooxidans* to pyrite vary depending on the strain (Ghauri et al., 2007) as well as with the batch of pyrite used (unpublished results). So far, we evaluated individually two molecules of the mixture: hydroxy-C16-AHL and the Oxo-C16-AHL; in our experimental conditions, none of these two compounds affected the attachment.

In addition, we analyzed several AHL-analogues. After 6 h of induction, a preliminary result revealed that the 4-phenyl-3-oxo-HSL derivative produced a decrease on the attachment level (22%; from 52.2 ± 10.1 to 40.7 ± 10.8) (Fig. 3B) while some of the AHL-analogues evaluated induced an increase on the attachment levels (Table 1). Variations in control data with the previous experiment (Fig. 3) were due to the use of different pyrite batches.

Table 1
Effect of different AHL-analogues on attachment of *A. ferrooxidans* ATCC 23270 to pyrite

AHL-analogues	Attachment (%)
Control without AHL-analogues	39.4 ± 10.1
3-sulfonylamid-C8-AHL	47.1 ± 11
4-bromo-3-(bromomethylene)-furanone	49.9 ± 3.5
p-bromo-3-phenyl-HSL	55.4 ± 9.7

4. Discussion

Multiple microorganisms coexist in the environment as communities, competing for or sharing resources and often associated as biofilms. Cell–cell communication through AHL-signaling between different bacterial species inside a mixed biofilm has been reported (Riedel et al., 2001). On the other hand, different works revealed that mixed cultures composed by *A. ferrooxidans*, *A. thiooxidans* and *L. ferrooxidans* increase the yield of pyrite oxidation and, on the other hand, that EPS plays a pivotal role in this bioleaching process (Schippers and Sand, 1999; Gehrke et al., 2001; Harneit et al., 2006; Sand and Gehrke, 2006; Akcil et al., 2007). In many Gram-negative bacteria, EPS production and biofilm formation are regulated by QS type AI-1 (Lynch et al., 2002; Marketon et al., 2003).

Two relevant questions can be addressed for the bioleaching process: i) Does cell–cell communication occur in a mixed biofilm composed by *A. ferrooxidans*, *A. thiooxidans* and *L. ferrooxidans*? ii) Are the AHL-signaling molecules involved in the efficiency of mineral colonization by bacterial cells?

To answer the first question, the production of AHL-signaling molecules by different *A. ferrooxidans*, *A. thiooxidans* and *L. ferrooxidans* strains was analyzed. The results obtained revealed that all tested *A. ferrooxidans* and *A. thiooxidans* strains produce AHL-signaling molecules while both *L. ferrooxidans* strains do not. However, the presence of minor amounts of AHL-signaling molecules cannot be ruled out. On the other hand, it may be possible that even though *L. ferrooxidans* does not synthesize its own AHLs, it could detect exogenous AHLs synthesized by other microbial species belonging to its ecological niche through the expression of a *sdiA*-like gene, as is the case for *Escherichia* and *Salmonella* spp. (Michael et al., 2001). In *Escherichia* and *Salmonella* spp., the SdiA protein responds with the highest sensitivity to AHLs that have a keto modification at the third carbon and an acyl-chain length of 6 or 8 (Michael et al., 2001). Therefore, if a SdiA-like protein was present in *L. ferrooxidans* strains, these microorganisms could detect, at least, oxo-C6-AHLs and oxo-C8-AHLs produced by *A. thiooxidans*. All these data allowed us to hypothesize that an AHL communication could be effective inside bioleaching planktonic and/or biofilm communities. This cell–cell communication could certainly occur between i) different *A. ferrooxidans* strains, ii) different *A. thiooxidans* strains, and iii) *A. ferrooxidans* and *A. thiooxidans* strains. In addition, to date it appears that *L. ferrooxidans* cannot talk through AHL-signaling molecules but there is no data to avoid thinking that it could hear the *A. ferrooxidans*|*A. thiooxidans* conversation.

The understanding of the molecular mechanisms involved in bacterial–mineral attachment and biofilm formation could be relevant to design biological strategies to improve bioleaching timing and efficiency. Thus, to address the second question, we tested the effect of AHL-analogues on the attachment of *A. ferrooxidans* cells to pyrite. Our preliminary results showed a small but significant tendency of the AHL mixture to inhibit attachment of *A. ferrooxidans* to pyrite. However, these tendencies are preliminary results and further experiments will be required to identify which molecules and at what specific concentrations they affect adherence of this bacterium to pyrite. In addition, some AHL-analogues increased attachment of *A. ferrooxidans* to pyrite whereas others inhibited its attachment. The present data suggest that the AHL-signaling molecules and probably AfeR protein from *A. ferrooxidans* – therefore the QS type AI-1 system – may be involved in its attachment to pyrite.

In conclusion, these results strongly suggest that QS interferences could be an appropriate strategy to improve or to prevent the pyrite (mineral) colonization by pure and/or mixed cultures and therefore to affect the timing and efficiency of the bioleaching process. Therefore, cost analysis and studies with different strains and with bacterial communities composed by *A. ferrooxidans*, *A. thiooxidans* and *L. ferrooxidans* will be of great importance.

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