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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.
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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 10% 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 in cultures of 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

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