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Colonization and biofilm formation of the extremely acidophilic archaeon *Ferroplasma acidiphilum*

Ruiyong Zhang [a], Sören Bellenberg [a], Laura Castro [b], Thomas R. Neu [c], Wolfgang Sand [a], Mario Vera [a,*]

[a] Aquatische Biotechnologie, Biofilm Centre, Universität Duisburg-Essen, 45141 Essen, Germany
[b] Department of Material Science and Metallurgical Engineering, Complutense University of Madrid, Av. Complutense s/n, 28040 Madrid, Spain
[c] Department of River Ecology, Helmholtz Centre for Environmental Research-UFZ, 39114 Magdeburg, Germany

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ABSTRACT

Ferroplasma spp. are widely distributed in acid mine drainage (AMD) and biomining environments at mesophilic and moderately elevated temperatures, at low pH and high concentrations of iron and other metal ions. Microbial attachment and biofilm formation on metal sulfides are of great importance during bioleaching. In this work, several cultivation and microscopical techniques were applied to investigate the biofilm development of *Ferroplasma acidiphilum*. Biofilms were heterogeneously distributed on filters over time, and varied within the different growth conditions such as supplementation with glucose. Additionally, cell distribution, biofilm formation as well as EPS production of *F. acidiphilum* cells forming biofilms on pyrite were observed by confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) combined with epifluorescence microscopy (EFM). Cells formed a monolayer biofilm and were preferably attached to the cracks/defects of pyrite surfaces. Biofilm and planktonic cells exhibited significant morphological differences. Capsular EPS were observed in both biofilm and planktonic cells.

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1. Introduction

The mobilization of metal cations from often almost insoluble ores by biological oxidation and complexation processes is referred to as bioleaching (Rohwerder et al., 2003). The recovery of heavy metals such as zinc, cobalt, copper and nickel by an application of microorganisms is now a widely used technique (Rawlings and Johnson, 2007). Around 20% of copper production worldwide was done by biohydrometallurgical operations in 2010 (Schippers et al., 2013). However, bioleaching can occur spontaneously in nature and cause serious environmental problems like acid mine drainage (AMD) (Hallberg, 2010). There are many species of prokaryotes that have been reported in AMD and relevant environments (Johnson and Hallberg, 2003; Schippers et al., 2010). Acidophilic archaea belonging to *Thermoplasmatales* including *Thermoplamsa*, *Picrophilus*, *Ferroplasma* and *Acidiplasma* are the most common acidophiles of all known microorganisms.

Ferroplasma acidiphilum was first isolated from a semi-industrial bioleaching reactor processing arsenopyrite in Kazakhstan (Golyshina et al., 2000). *Ferroplasma* spp. are present in various acidic man-made operations such as leaching tanks/heaps and also natural habitats, which are considered as hostile environments. The family *Ferroplasmaceae* received scientific attention for its lifestyle in extremely acidic environments and new insights into acid and metal tolerance for cells without a protective cell wall. A key explanation for *Ferroplasma* spp. flourishing in acidic environments are their membrane lipids which are mainly composed of caldarchaetidylglycerol tetraether-linked monolayers (Macalady et al., 2004). *Ferroplasma* spp. are considered to be major players in global iron and sulfur cycling (Edwards et al., 2000; Golyshina, 2011).

Biofilms are communities of microorganisms attached to a surface embedded in extracellular polymeric substances (EPS). EPS mainly contain carbohydrates, proteins, lipids, nucleic acids and complexed metal ions (Flemming and Wingender, 2010). Attachment of microorganisms to mineral surfaces is of great importance for the process of mineral dissolution (Vera et al., 2013). Evidence showed that attachment of cells of *Acidithiobacillus ferrooxidans* to pyrite correlated with the degree of pyrite crystallization (Sanhueza et al., 1999). EPS mediate the contact between cells and metal sulfide (MS) and help to facilitate the dissolution of a MS due to ferric iron complexation, thus providing a reaction space for the chemical attack on a MS (Sand and Gehrke, 2006). EPS of *At. ferrooxidans* contain the carbohydrates glucose, rhamnose, fucose, xylose, mannose, C12–C20 saturated fatty acids, glucuronic acid, and Fe(III) ions (Gehrke et al., 1998). More than 80% of bacterial cells were attached to the non-limiting surfaces within 24 h, although less than 5% of the available surface area was colonized (Sand et al., 1998).

Archaeal biofilms are a common phenomenon and as complex as bacterial ones (Fröls, 2013; Orell et al., 2013). Some data have shown characteristics of attachment, biofilms and EPS composition of

* Corresponding author at: Universität Duisburg-Essen, Fakultät für Chemie, Biofilm Centre, Aquatic Biotechnology., Universitätsstr. 5, 45141 Essen, Germany. Tel.: +49 201 1837080; fax: +49 201 1837090.
E-mail address: mario.vera@uni-due.de (M. Vera).

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Sulfolobus spp. (Koerdt et al., 2010, 2012). However, in these studies cells were grown with organic substrates and no applicable data are available for bioleaching processes. A biofilm of *Sulfolobus metallicus* was reported to develop on the support of a biotrickling filter during hydrogen sulfide/sulfur oxidation (Morales et al., 2011). A genome analysis indicated that several genes may be involved in adhesion and biofilm formation in *Metallosphaera sedula* (Auernik et al., 2008). Cells of *M. hakonensis* adhered to different MS and have maximum surface coverage at their optimal growth temperature. In addition, cells showed selective attachment to different sulfide minerals (Africa et al., 2013).

Although archaea are almost ubiquitously present in biomining ecosystems, very few investigations on acidophilic archaea for their role in bioleaching and AMD have been conducted (Brune and Bayer, 2012). In this study biofilm development of *F. acidiphilum* was examined in order to get an improved knowledge of the interaction of iron-oxidizing archaea and surfaces as well as colonization and growth on pyrite. Several microscopical techniques, including confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) combined with epifluorescence microscopy (EFM) as well as the floating filter technique were used.

2. Materials and methods

2.1. Strain and cultivation

F. acidiphilum BRGM4 (DSM 28986) was isolated from a pilot-scale bioreactor (d'Hugues et al., 2008). 16S rRNA gene sequence analysis demonstrated that strain BRGM4 (GenBank accession no. KJ847278) showed sequence identity of 99% with the type strain *F. acidiphilum* Y (Golyshina et al., 2000). *F. acidiphilum* BRGM4 was cultivated in Mackintosh (MAC) medium (Mackintosh, 1978). Cells were grown at an initial pH of 1.7 in 5 L bottles containing 4 g/L iron (II) ions and 0.2 g/L yeast extract at 37 °C with agitation and aeration. For bioleaching, attachment and floating filter experiments, cells in late exponential phase were harvested by centrifugation at 8000 rpm for 15 min.

2.2. Pyrite preparation

Pyrite coupons with a size of approx. 1 cm × 1 cm × 2 mm were cut from cubes (origin Navajun, Spain). Grains with a size of 50–200 μm were selected after grinding and sieving. Both, slices and grains, were washed with boiling 6 M HCl for 30 min, rinsed with deionized water until neutral pH and three times with acetone. After cleaning, pyrite was dried at 80 °C for 12 h and sterilized for 24 h at 120 °C under a nitrogen atmosphere.

2.3. Leaching experiments

300-mL Erlenmeyer flasks containing 5 g of pyrite grains (50–100 μm grain sizes) and 100 mL of MAC medium (pH 1.7) and 0.2 g/L yeast extract were inoculated with *F. acidiphilum* BRGM4 at an initial cell number of 1.2×10^8 cells/mL. Abiotic controls were also done. Cell numbers were determined by direct microscopic counts and pH of the leachates was measured using a digital pH meter (Model pH 537, WTW). Iron ions were quantified using the phenanthroline method (according to DIN 38406-1).

2.4. Floating filter technique and CLSM

5×10^7 cells were filtered on autoclaved polycarbonate filters (GTTB, Ø 2.5 cm, 0.2 μm pore size, Millipore®) and immediately transferred to MAC medium containing 5 g/L iron (II) ions coupled with additional conditions. These included glucose supplementation (1 g/L) and P_i starvation (by incubation in MAC medium prepared without P*i*). Cells grown on filters or pyrite were stained by 4′,6-diamidino-2-phenylindole (DAPI) or Sypro Red (Invitrogen). Polysaccharide moieties were observed by using the fluorescently labeled lectins Concanavalin A (Con A) or *Limulus polyphemus* agglutinin (LPA) (EY Laboratories), respectively. The staining of biofilms on filters was conducted as previously described (Bellenberg et al., 2012). A similar procedure was applied for pyrite samples, except that samples were stained in a coverwell chamber of 20 mm in diameter and 0.5 mm in depth (Invitrogen). Freshly-stained pyrite samples were visualized by CLSM using a TCS SP5X, controlled by the LASAF 2.4.1 build 6384 (Leica, Heidelberg, Germany). The system was equipped with an upright microscope and a super continuum light source (470–670 nm) as well as a 405 nm 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. CLSM data sets were recorded in sequential mode in order to avoid interference of the fluorochrome emission signals between two different channels. Surface topography and texture of the pyrite surface were recorded by using CLSM in reflection mode.

2.5. AFM & EFM

Pyrite slices were rinsed with sterile MAC medium and deionized water. Cells attached on pyrite coupons and their EPS were stained by Syto 9 (Invitrogen) and fluorescently labeled Con A, respectively as mentioned in Section 2.4. Stained samples were dried at room temperature and visualized by EFM (Zeiss, Germany) combined with AFM (BioMaterial™Workstation, JPK Instruments) for the investigation of cell morphology and distribution on the surfaces of pyrite coupons as described previously (González et al., 2012; Mangold et al., 2008).

2.6. SEM observations

Pyrite slices incubated with cells were rinsed with deionized water and then successively dehydrated with increasing concentrations of acetone (60%, 80% and 90%) and stored overnight at 4 °C in 90% acetone. Samples were subjected to critical-point drying and coated with graphite and gold. Specimens were examined with a JEOL JSM-6330F microscope, FE-SEM at 10 kV.

3. Results and discussion

3.1. Effects of substrates on cell morphology

Comparative studies on the cell morphology of *F. acidiphilum*, grown on iron (II) sulfate and yeast extract with or without glucose over time, were performed. Cells showed morphological variability and thus responded to defined growth conditions. As shown in Fig. 1, young cultures (3 days, early exponential phase) grown on iron (II) sulfate were characterized by irregularly shaped spherical cells. In contrast, cells taken from early stationary phase cultures (7 days) were pleomorphic. A substantial proportion of cells showed extensions with a size of 0.3–0.4 μm. These occurred more abundant in cells taken from stationary phase cultures, as compared to those cells from exponential phase cultures. With the addition of glucose (1 g/L), cells appeared to be also pleomorphic and accumulated in chains containing several cells/buds (Fig. 1b). This preference to form aggregates was possibly due to an enhanced EPS production because of glucose supplementation. A similar phenomenon of increased EPS production has been observed in *At. ferrooxidans* after addition of glucose or galactose (Bellenberg et al., 2012). *F. acidiphilum* proliferates via budding (Golyshina et al., 2000). Buds were observed in both, planktonic and biofilm cells. Iron (II) sulfate grown cells in the exponential phase varied from 1.1 to 2.5 μm with bud sizes ranging from 0.3 to 0.6 μm, while cells in stationary phase were between 0.3 and 2.8 μm, with bud sizes ranging from 0.3 to 0.8 μm. Pyrite-attached cells showed morphological differences compared to planktonic cells (Fig. 2). They were slightly rod-shaped with an approximate size of 0.7 × 1.2 μm. *Ferroplasma*, *Acidiplasma* and *Thermoplasma*, unlike other archaeal genera, lack a cell wall (Albers

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