Chapter 6
The Biofilm Lifestyle of Acidophilic Metal/Sulfur-Oxidizing Microorganisms

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1 Introduction

The most common and widespread lifestyle of microbes on earth is in form of biofilms. These are communities of different species of microorganisms embedded in a matrix of extracellular polymeric substances (EPS), which mainly consist of polysaccharides, proteins, nucleic acids and lipophilic compounds. Biofilms can be found as surface-associated or "floating mats", occurring in air-water interfaces. The biofilm lifestyle protects cells from environmental stress like desiccation, nutrient starvation, radiation and/or oxidative stress (Flemming and Wingender 2010).

Bioleaching of metal sulfides (MS) like pyrite (FeS_2) or chalcopyrite ($CuFeS_2$) is accelerated by a diverse group of acidophilic metal/sulfur-oxidizing microorganisms (AMOM). These can effect MS dissolution through the oxidation of iron(II)-ions and/or reduced inorganic sulfur compounds (RISCs). Thereby iron(III)-ions

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and protons, the MS attacking agents, are available. As a consequence, acid mine/rock drainage (AMD/ARD) is generated. This is a serious problem due to water and soil contamination by heavy metals and sulfuric acid. Several species of acidophilic microorganisms from the genera *Acidithiobacillus*, *Leptospirillum*, *Acidiferrobacter*, *Acidiphilium*, *Ferroplasma*, *Acidimicrobium, Ferrimicrobium, Sulfobacillus, Metallosphaera*, *Sulfolobus* and *Acidianus*, among others, are commonly found at AMD sites as well as in biomining operations under mesophilic, moderately- and thermophilic conditions. In the "contact-leaching" mode microorganisms thrive attached to the MS surface and the electrochemical processes resulting in the dissolution of sulfide minerals, occur at the interface between bacterial cells and the MS surface. This space is filled with EPS, which in *Acidithiobacillus ferrooxidans* are mainly composed of polysaccharides, lipids and uronic acids (Gehrke et al. 1998).

The process of biofilm formation by AMOM on MS is not completely understood. Under laboratory conditions, most AMOM form monolayer biofilms on MS, which are also their energy substrate. Environmental acidophilic biofilms are characterized by a relative low species abundance, which is restricted by the acid pH, high concentration of heavy metals as well as the narrow range of electron donors and acceptors available. In general these biofilms are dominated by iron-oxidizing chemolithoautotrophs such as *Leptospirillum* Group II and sub-dominated by mixed populations of heterotrophic or mixotrophic bacteria, archaea and eukaryotes (Denef et al. 2010).

Biofilm formation and EPS biosynthesis in acidophilic metal/sulfur-oxidizing bacteria are regulated at different levels, comprising among others, energy substrates, inorganic phosphate (P_i) limitation, and cell-cell communication mechanisms of "quorum sensing" (QS). This chapter presents an overview about the biofilm lifestyle of AMOM. This includes surface science, microscopy, cell-cell communication, interspecies interactions as well as molecular and high-throughput studies. Future perspectives in this field include the elucidation of EPS biosynthesis pathways and comprehensive analysis of the chemical nature of the EPS polymers. Cell-cell communication and microbial interactions within multispecies biofilms of acidophiles are considered to be crucial determinants of metabolic activity of AMOM. A comprehensive knowledge of these aspects of AMOM biofilms will be beneficial for biotechnological applications such as biomining, in which an enhancement of biofilm formation and leaching rates is desired and for mitigation of AMD generation, in which inhibition of biofilm formation, or inactivation of biofilm cells might contribute significantly.

2 Biofilms of Acidophilic Metal/Sulfur-Oxidizing Microorganisms

The predominant MS dissolving microorganisms include acidophilic bacteria and archaea (i.e. microorganisms that thrive at pH values lower than 3). These oxidize RISCs and/or iron(II)-ions. Leaching bacteria are distributed among the *Proteobacteria* (*Acidithiobacillus*, *Acidiphilium*, *Acidiferrobacter*, *Ferrovum*), *Nitrospirae* (*Leptospirillum*), *Firmicutes* (*Alicyclobacillus*, *Sulfobacillus*) and

Actinobacteria (*Ferrimicrobium*, *Acidimicrobium*, *Ferrithrix*). Several recent reviews have given an overview of the microbial diversity in mining biotopes (Schippers 2007; Hedrich et al. 2011; Dopson and Johnson 2012; Johnson 2014). Within all bacterial groups, mesophilic and moderately-thermophilic microorganisms have been described (Clark and Norris 1996; Norris and Johnson 1998; Norris et al. 2000). Most leaching archaea belong to the *Sulfolobales*, which are thermophilic, sulfur- and/or iron(II)-oxidizers such as *Sulfolobus*, *Acidianus*, *Metallosphaera* and *Sulfurisphaera* (Norris et al. 2000; Wheaton et al. 2015). Also, within the *Thermoplasmales* the mesophilic iron(II)-oxidizing species, *Ferroplasma acidiphilum* (Golyshina et al. 2000), *Ferroplasma acidarmanus* (Edwards et al. 2000b; Dopson et al. 2004) and *Ferroplasma thermophilum* (Zhou et al. 2008) are known.

2.1 Mechanisms of Metal Sulfide Dissolution

Based on the acid solubility of MS, there are two different bioleaching pathways. The thiosulfate pathway is involved in the dissolution of acid-insoluble MS, such as pyrite, molybdenite (MoS_2), and tungstenite (WS_2). The polysulfide pathway is relevant for dissolution of acid-soluble MS, such as sphalerite (ZnS), galena (PbS) or chalcopyrite (Schippers et al. 1996; Schippers and Sand 1999; Sand et al. 2001). These two pathways are indirect mechanisms, since in acidic solution the acid-insoluble MS are solely oxidized chemically by iron(III)-ions, which are supplied by the microbial oxidation of iron(II)-ions (Schippers et al. 1996; Rodriguez et al. 2003; Gleisner et al. 2006). Acid-soluble MS are also dissolved by proton attack. Two leaching modes have been proposed: "contact" and "non-contact" leaching (Sand et al. 2001; Rawlings 2002). Non-contact leaching is carried out by planktonic cells, which oxidize iron(II)-ions. The resulting iron(III)-ions are reduced at the mineral surface, and the sulfide moiety is oxidized. Thus iron(II)-ions enter the cycle again. In a strict sense, this corresponds to the "indirect mechanism" (Sand et al. 1995). In contrast, contact leaching takes into account biofilm forming cells on the surface of MS. In this case the electrochemical processes resulting in MS dissolution are occurring in the EPS located at the interface between the bacterial cell and the MS surface.

The anodic and cathodic reactions have been reviewed for the case of pyrite. In the first step, electrons are transferred from the surface of the pyrite to the aqueous oxidant species, usually O_2 or iron(III)-ions (cathodic reaction). In the second step a charge is transported from the site of an anodic reaction to replace the electron lost from the cathodic site. Later, at an anodic site, the oxygen atom of a water molecule interacts with a sulfur atom to create a sulfoxy species. This step releases an electron into the solid and one or two hydrogen ions to solution (Rimstidt and Vaughan 2003). Probably cells are chemotactically attracted towards these local anodes. The anodes and cathodes may be resulting from imperfections in the crystal lattice where the iron to sulfur ratio is imbalanced due to inclusion of other metal atoms during the process of crystallization and/or from variations of temperature during crystallization (causing amorphous up to highly crystalline structures). Recently, a new proposal was made by

Crundwell (2013). The new hypothesis of mineral dissolution is based on an electrochemical mechanism which updates contents on the kinetics of mineral dissolution. It proposed that: (1) no separation of the surface into anodic sites and cathodic sites exist, (2) no flow of electrons occurs across the bulk of the mineral, (3) the first step of the dissolution reaction is not by acid, (4) the charge exchange across the mineral-solution interface is the rate-controlling step, and (5) bacterial leaching does not change the mechanism of reaction. This means that biological effects are limited to reaction steps which do not affect the rate-determining step.

2.2 Microbial Attachment to Metal Sulfides, EPS

Microbial attachment to ores and subsequent biofilm formation increase leaching activities, since a unique microenvironment is formed between the bacterium and the MS surface (Fig. 6.1). In case of *At. ferrooxidans*, iron(III)-ions, each one probably complexed by two uronic acid residues, are present in the EPS. Thus, the first function of complexed iron(III)-ions is the mediation of cell attachment, in which cells are electrostatically attached to the negatively charged pyrite. The second function of complexed iron(III)-ions is the oxidative dissolution of the MS, similar to the role of free iron(III)-ions in "non-contact leaching". In this species, the electrons extracted from the MS reduce molecular oxygen via a redox chain forming a supercomplex spanning the periplasmic space and connecting both outer and inner membranes (Castelle et al. 2008).

In general, the majority of AMOM can form biofilms on MS. Assuming of a non-limiting surface, up to 80–90 % of a certain inoculum may attach to MS within 24 h (Dispirito et al. 1983; Bagdigian and Myerson 1986; Gehrke et al. 1998; Harneit et al. 2006). It is important to remark that the initial attachment ratios strongly depend on the nature of the species as well as their pre-cultivation conditions. In general *Leptospirillum* spp. show higher attachment rates to pyrite than iron-oxidizing *Acidithiobacillus* spp. and, among the latter ones, iron(II)- or pyrite-grown cells show higher attachment rates to pyrite than sulfur-grown cells due to a considerable chemical modification of their EPS (Gehrke et al. 1998). Nevertheless, some cells always remain in planktonic state, even though the surface area for attachment is not the limiting factor. For example, when *At. ferrooxidans* ATCC 23270^{T} or *Acidithiobacillus ferrivorans* SS3 is incubated with pyrite for 24 h, about 50 % of the cells attached to the ore (Bellenberg et al. 2015). The reason(s) for this remains unknown.

In case of *At. ferrooxidans* strain R1 it was demonstrated that pyrite-grown cells had a similar EPS composition compared to iron(II)-grown cells. These EPS consist of the monosaccharides glucose, rhamnose, fucose, xylose, mannose, C12–C20 saturated fatty acids, glucuronic acid, and iron(III)-ions (Gehrke et al. 1998; Gehrke et al. 2001). However, pyrite-grown cells possessed more than tenfold amount of EPS. As already mentioned, the initial attachment is mainly driven by electrostatic interactions (in which most likely 2 mol negatively charged glucuronic acid residues complex 1 mol positively charged iron(III)-ions resulting in a net positive charge) with the negatively charged

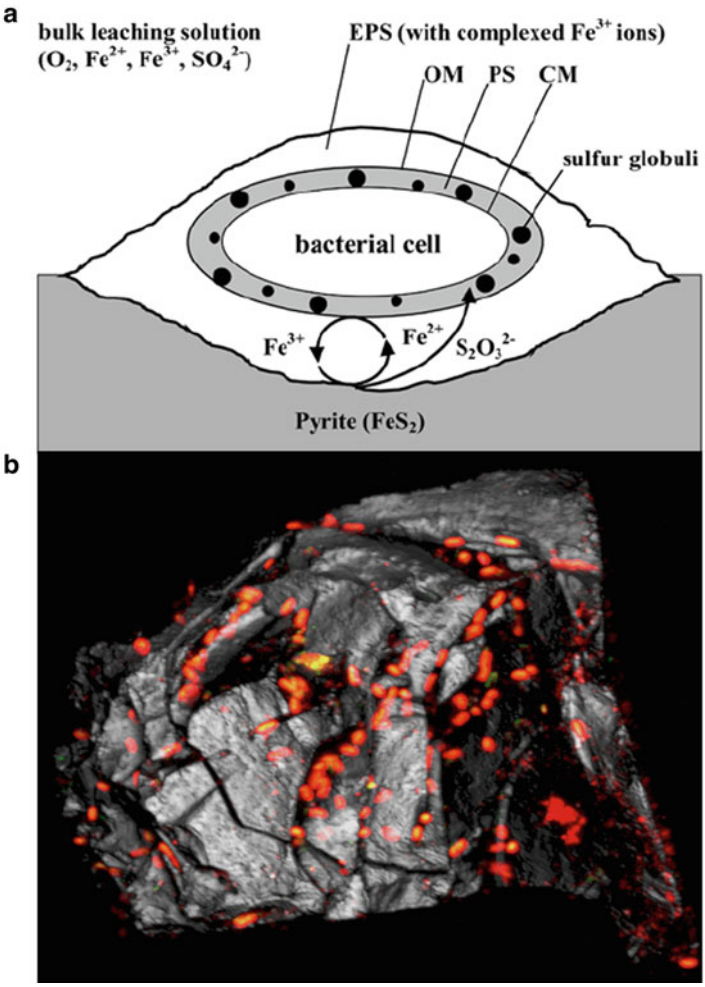

Fig. 6.1 Model for contact leaching catalyzed by a bacterial cell (from Vera et al. 2013b). A: Overview showing a biofilm cell embedded in the EPS layer attached to pyrite. Compounds like Iron(II)/(III)-ions, thiosulfate present during MS dissolution are shown. CM, cytoplasmic membrane; PS, periplasmic space; OM, outer membrane. B: CLSM image showing a 3-D projection of a pyrite grain (50–100 µm) colonized with cells of *At. ferrooxidans*^{T} after 1 week of incubation. Cells were double stained with Syto 9 (*green*) for nucleic acids and Con A. Color allocation: *green* = Syto 9, *red*=Con A-tetramethyl rhodamine isothiocyanate (TRITC), *grey* = reflection. The merged image from all three channels is shown. The bacterial colonization pattern strongly correlates with surface imperfections

pyrite surface (at pH 2) in sulfuric acid solution (Solari et al. 1992; Blake et al. 1994). Also hydrophobic interactions contribute to the attachment to MS surfaces (Gehrke et al. 1998; Sampson et al. 2000b). This applies especially to very hydrophobic surfaces e.g. of elemental sulfur (S^0). Hydrophobic interactions as well as covalent bonds seem to mediate the secondary (tight) surface attachment. Cells grown on S^0 do not attach well

to pyrite, since their EPS composition is different compared to pyrite-grown ones. Their EPS contain considerably less monosaccharides and lack uronic acids, resulting in a complete absence of complexed iron(III)-ions or other positively charged ions. In contrast, EPS from sulfur-grown cells possess much more fatty acids than EPS extracted from pyrite-grown cells. Consequently, it seems that hydrophobic interactions are exclusively relevant for attachment of cells of *At. ferrooxidans* to S^0 (Gehrke et al. 1998). A FTIR analysis indicated that cell surface charge of *At. ferrooxidans* was different from soluble iron(II)-grown cells compared to solid substrate grown ones. Cells possessed a higher amount of protein when grown on insoluble substrates such as pyrite or sulfur compared to cells grown with iron(II)-ions (Sharma et al. 2003). We have shown by confocal laser scanning microscopy (CLSM) together with fluorescent lectin binding analysis (FLBA) that the formation of capsular polysaccharides (CPS) of *At. ferrooxidans*^{T} EPS occurs within the first 24 h of contact with pyrite (Bellenberg et al. 2012).

Isolation of EPS from *Acidiphilum* 3.2Sup(5) was compared by using five extraction methods: EDTA, NaOH, ion exchange resin, heating and centrifugation. The extracted EPS mainly contained carbohydrates and proteins by all methods. However, higher EPS amounts as well as a less degree of cell lysis were obtained by using EDTA. This study confirmed that both, the amount and the chemical composition of EPS strongly depended on the extraction method (Tapia et al. 2009). EPS from a mixed culture predominated by *Acidithiobacillus caldus* and *Leptospirillum ferriphilum* grown on chalcopyrite concentrate were characterized to contain proteins, lipids, carbohydrates and iron(III)-ions (Zeng et al. 2010). Recently, tightly bound EPS of the crenarchaeote *Sulfolobus metallicus* DSM 6482^T were shown to be mainly composed of proteins and carbohydrates. By contrast, the loosely bound EPS were mainly containing carbohydrates. Extracellular DNA and proteins were detected in *S. metallicus*^{T} EPS from biofilms grown on S^0, whereas proteins were characterized as the main components (Zhang et al. 2015b).

EPS production in stirred reactors dominated by *L. ferriphilum* BRGM1 was studied in order to correlate factors for the optimization of bioleaching processes. Under nitrogen limitation, bacterial attachment and leaching efficiency were both decreased in accordance with reduced levels of EPS production. CO_2 limitation caused a significant decrease of exopolysaccharide production (d'Hugues et al. 2008). Another study on EPS composition was done for mixed cultures containing mesophilic, moderate thermophilic and thermophilic leaching strains grown on several MS such as pyrite, sphalerite and chalcopyrite in several continuously operated bioleaching systems. It was found that 70 % of the EPS extracted were mainly composed of carbohydrates and smaller amount of proteins with trace levels of humic and uronic acids (Govender and Gericke 2011). Characterization of two acidophilic microbial biofilms from Iron Mountain, California showed that their EPS were composed of carbohydrates, metals, proteins and minor quantities of DNA and lipids (Jiao et al. 2010). Studies on acidophiles grown on organic compounds were also reported. Two strains of *Sulfolobus* grown with glucose excreted an extracellular polysaccharide containing monosaccharides like of glucose, mannose, glucosamine and galactose (Nicolaus et al. 1993). Proteins and carbohydrates were major components in EPS of *Sulfolobus solfataricus* P2 grown on tryptone, N-Z-amine or glucose (Koerdt et al. 2012).

The site of attachment of AMOM on metal sulfides and how the detection/sensing of this specific location(s) occurs are still open questions. Several evidences from the literature (Andrews 1988; Ohmura et al. 1993; Shrihari et al. 1995; Dziurla et al. 1998; Sanhueza et al. 1999; Edwards et al. 2001) and our own work suggest that microbial attachment to MS does not occur randomly (Gehrke et al. 1998; Sand et al. 1998; Gehrke et al. 2001; Noël et al. 2010; Zhang et al. 2014, 2015a). It has been observed by atomic force microscopy (AFM) as well as CLSM that *At. ferrooxidans* and other AMOM preferentially (>90 %) attach to sites with visible surface imperfections e.g. pores, scratches, etc. (see also Sect. 4). Cell attachment to areas with a low degree of crystallization seems to be favored, resulting in cell orientation along crystallographic axes, in whose direction oxidation fronts propagate (Sanhueza et al. 1999). Cell adhesion to pores and scratches may be explained by contact area enhancement and protection from weak shear forces. In contrast cell attachment to areas with low crystallization and crystallographic axis is not often related to changes in surface topography. Therefore, the presence of some attractants may explain attachment to specific sites on the mineral surface. This is most likely caused by charge imbalances on the surface as caused e.g. by oxidation processes. Several strains of *At. ferrooxidans* and *Leptospirillum ferrooxidans* possess chemosensory systems (Acuña et al. 1992; Meyer et al. 2002). Chemotaxis and attraction to gradients of iron(II)/(III)-ions, thiosulfate and other compounds which may occur compulsorily during MS leaching (Edwards et al. 2000a). Dissolution, occurring at local anodes, brings iron(II)-ions and thiosulfate in solution. Recent work using AFM equipped with a Kelvin probe for force mapping indicates that the cells of *L. ferrooxidans* attached to a pyrite surface are more negatively charged (about 100–200 mV) than the surrounding surface (i.e. extracting electrons from the pyrite) (Vera et al. 2013b). In a similar case, Little and co-workers showed that sulfate-reducing bacteria attached in the immediate vicinity (nanometer range) of the anode on steel surfaces. The latter is negatively charged until a release of iron(II)-ions occurs. As a consequence of bacterial attachment, the anode and the cathode become permanent (manifest), and steel dissolution commences (Little et al. 2000). This observation may be also relevant for bioleaching of MS. In summary, cells seem to be transiently attracted (electrically charged) to dissolution sites by their chemotaxis systems and cause the anodes and cathodes on the MS surface to become permanent. In case of biofilm forming cells the dissolution process occurs within the EPS layer (Fig. 6.1), which can be considered a reaction space filling the void volume between the outer cell membrane and the surface of the MS. Tributsch and co-workers demonstrated that this distance is 10–100 nm wide (Rodriguez-Leiva and Tributsch 1988). The thickness of *At. ferrooxidans* EPS was estimated for iron(II)-grown cells by *in vivo* AFM to be 28.7 nm (±13.5) (Taylor and Lower 2008). The EPS thickness values for S^0- or pyrite-grown cells *in vivo* remains to be elucidated. Presumably these values will be higher than the above mentioned ones since it is already known that EPS levels increase when the bacteria are grown with these solid substrates (Sand et al. 1998).

Newly developed AFM probes coated with acidophilic bacteria allowed to measure interactive forces between AMOM and mineral surfaces (Diao et al. 2014a, b). It was found that *At. ferrooxidans* grown on chalcopyrite showed the strongest

interaction forces with the same substrate. Those compared with the ones of cells grown with iron(II)-ions or S^0 were considerably lower. Also, chalcopyrite leaching rate and cell attachment capacity were found to correlate positively with the cell adhesion force (Zhu et al. 2015). Among the three species *At. ferrooxidans*, *Acidithiobacillus thiooxidans* and *L. ferrooxidans*, the later one showed the highest adhesion force to chalcopyrite. EPS-deficient cells exhibited reduced adhesion forces and initial cellular attachment to MS surfaces (Zhu et al. 2012).

Still several questions remain open: Are some strains benefiting from the presence of primary colonizers in order to attach to these previously existing biofilms? How is the biochemical/molecular nature of antagonistic or synergistic interactions known to occur among certain species? We have recently shown in binary species biofilms that the presence of active biofilms of iron-oxidizers may influence subsequent cell attachment by other species. *At. thiooxidans* cells attached 40 % more to pyrite precolonized with biofilms of *At. ferrooxidans* or *L. ferrooxidans*. Interestingly, its cell attachment was faster to pyrite precolonized with *L. ferrooxidans* than with *At. ferrooxidans*. As *L. ferrooxidans* leach pyrite more efficiently than *At. ferrooxidans*, the faster attachment observed for *At. thiooxidans* may be related to a chemotactic response towards RISCs like thiosulfate which are known to be released after pyrite leaching (Bellenberg et al. 2014). The analysis of the complete *At. thiooxidans* genome sequence revealed a complete suite of genes for flagellar formation and chemotaxis (Valdes et al. 2011). In contrast, the cell attachment of *At. ferrooxidans* to pyrite grains precolonized with *L. ferrooxidans* was strongly dependent on its pre-cultivation. Thiosulfate-grown cells were influenced by the presence of *L. ferrooxidans*, while iron(II)-grown cells were not (Bellenberg et al. 2014). Summarizing, the presence of iron oxidizers, which have been described as primary colonizers in natural AMD biofilms (Wilmes et al. 2009) may be a relevant factor for sulfur-oxidizers to efficiently attach to MS.

3 Physiological Adaptations of Iron-Oxidizing Acidithiobacilli to the Biofilm Lifestyle

3.1 Responses Against Oxidative Stress

AMOM are confronted to high levels of extracellular reactive oxygen species (ROS), which are formed at the mineral/liquid interface. Hydrogen peroxide (H_2O_2) is formed in reactions of oxygen (in acidic aqueous solutions) with mineral lattice-bound iron (Nooshabadi and Rao 2014) and it is generated by pyrite, among other MS, especially when ores are crushed before biohydrometallurgical processing (Jones et al. 2011). When pyrite is present, H_2O_2 reacts with iron(II)-ions and highly reactive hydroxyl radicals are formed by the Fenton reaction. In addition, ROS such as O_2^- and H_2O_2 are easily penetrating cell membranes at acid pH values at diffusion limited rates, likely causing damage to DNA and enzymes when elevated concentrations of these compounds occur (Imlay 2013). We have recently shown that pyrite-grown *At.*

ferrooxidans or *At. ferrivorans* cells, in contrast to iron(II)-grown cells, were able to oxidize iron(II)-ions or pyrite after 24 h iron starvation and incubation with 1 mM H_2O_2. This indicates that these cells were already adapted to enhanced levels of ROS, which are generated on MS surfaces. We have also shown that the mere presence of pyrite has a clear inhibitory effect on the iron oxidation activity of both, the planktonic sub-population (PP) and the mineral-attached, biofilm cell sub-population (BP), after transfer of iron(II)-grown cells to a medium containing pyrite. The inhibition measured was especially higher in the BP compared to PP (Bellenberg et al. 2015). The reasons for this phenomenon may be manifold. Among these (1) the metabolic transition of BP cells to a biofilm lifestyle and (2) the release of inhibitory ROS, which cause a metabolic inhibition or a reduction of the amount of actively metabolizing cells (Jones et al. 2011), can be included. In this context, another function of biofilm formation and EPS production in AMOM might be related to confer an enhanced resistance against the damages caused by ROS. The enhanced EPS levels of biofilm cells might act by blocking reactive sites on the mineral where generation of ROS might occur as well by acting as a ROS scavenger (Bellenberg et al. 2015).

3.2 Inorganic Phosphate and Polyphosphate Metabolism

Every microbial biofilm is a consequence of physical interactions of microorganisms with a surface via EPS or other attachment factors. EPS biosynthesis is mediated by environmental signals, such as nutrient availability and stress influence (Janczarek 2011). P_i is an essential nutrient for all living cells. Acidophilic leaching bacteria must be able to deal with the problem of P_i scarcity due to its precipitation in environments, where iron(III)-ions are present (Tuovinen 1990). For survival under P_i starvation, *E. coli* and many other bacteria induce the "Pho regulon", which is a genetic system controlled by the PhoB/PhoR two-component regulatory system and plays a key role in P_i homeostasis (Wanner 1996a, b). The *At. ferrooxidans* P_i starvation response and the presence of a Pho regulon have been described (Seeger and Jerez 1993; Vera et al. 2003; Alvarez and Jerez 2004). Besides its role in bacterial P_i homeostasis, this system is part of a complex regulatory network connecting stationary phase responses, virulence, QS and especially biofilm formation, which is known to be induced in several bacterial species as a response against P_i starvation (Lamarche et al. 2008). E.g. in *Sinorizobium melliloti* the DNA-binding transcriptional factor PhoB positively regulates the synthesis of exopolysaccharides involved in bacterial attachment and infection of root nodules, when P_i is limiting (Rüberg et al. 1999). Also in *Agrobacterium tumefaciens* an enhancement of biofilm formation under P_i limited growth conditions has been described (Danhorn et al. 2004). It has been observed that *At. ferrooxidans* cells, which were sub-cultured in iron(II) medium lacking P_i attach more efficiently to MS and S^0 surfaces (Amaro et al. 1993). We have shown by epifluorescence microscopy (EFM) and FLBA that *At. ferrooxidans* cells grown under P_i starvation showed an enhanced amount of CPS compared with cells grown with sufficient P_i (Bellenberg et al. 2012).

AMOM accumulate high levels of inorganic polyphosphates (polyP), which are polymers of phosphoanhydride-linked phosphate residues, found as chains up to 1000 residues long in cells from all three domains of life. Among its several functions, polyP can serve as P_i reservoir, modulator of stress responses as well as a contributing factor to the high copper resistance shown by AMOM (Alvarez and Jerez 2004; Remonsellez et al. 2006; Kanao et al. 2007; Orell et al. 2010). However, probably the most important of its functions is to participate in the physiological adjustments of bacteria to environmental changes and stress conditions. Mutant strains lacking polyP are more sensitive in the stationary phase and show decreased resistance to heat, oxidants, osmotic challenge, antibiotics and UV radiation (Rao and Kornberg 1996; Kim et al. 2002). Recent evidence has pointed out new roles of polyP in protection against oxidative stress. These can be achieved by direct and indirect mechanisms such as (1) protein chaperone, since unlike proteins, polyP does not react with ROS, (2) to participate in the formation of Mn^{2+}/polyP complexes, which can detoxify O_2^- after their hydrolysis or through a non-catalytic mechanism involving the formation of Mn^{3+}-polyP, (3) to provide defense against the Fenton reaction, in which the most probable mechanism involved is facilitating the chelation and export of copper(II)-ions (Grillo-Puertas et al. 2014), (4) to play an essential role in the molecular mechanism by which bacteria enter to a persistent state, in which they become highly stress-resistant and (5) to participate directly in the regulation of general stress response networks (Gray and Jakob 2015). Future studies of changes in polyP levels of leaching bacteria upon biofilm formation as well as the construction of polyP deficient strains will contribute to a better understanding of the role of its versatile polymer in the biofilm lifestyle of AMOM.

3.3 Other Factors Affecting Biofilm Formation of Iron Oxidizing Acidithiobacilli

A broad range of other factors influencing the biofilm formation on pyrite of iron-oxidizing acidithiobacilli have been identified. In general, cultivation at non-optimum growth temperatures or under increased ionic strengths led to a decreased colonization of pyrite. *At. ferrivorans* SS3 showed enhanced biofilm formation on pyrite if compared to *At. ferrooxidans*^{T}. The presence of iron(III)-ions increased pyrite colonization, especially when pyrite-grown cells were used, while the addition of 20 mM copper(II)-ions in case of both above-mentioned species resulted in reduced biofilm formation on pyrite. This observation correlated with a different EPS composition of copper-exposed cells in pyrite cultures. Some attachment effectors were also identified. The addition of 1 mM glucuronic acid, especially in combination with 1 mM iron(III)-ions, had strong enhancing effects on attachment of all tested strains. These positive effects regarding pyrite colonization caused by the addition of 1 mM glucuronic acid to *At. ferrooxidans* correlated with a pyrite dissolution enhancement of around 25 % and significantly elevated planktonic cell numbers (Bellenberg et al. 2015).

Several questions remain open: Which other abiotic and biotic factors influence biofilm formation? Are these factors different among species of iron-oxidizing acidithiobacilli? Does the presence of the PP influence the start of the MS dissolution? Are there differences in the iron oxidation activity between BP and PP in pyrite cultures? Does the increase of the initial amount of attached cells correlate with improved pyrite dissolution? In order to answer these questions, we have compared the contribution of the BP and PP from pyrite cultures to the iron oxidation activity in *At. ferrooxidans*^{T}. It was shown that within the first 4–5 days, only the BP was responsible for pyrite dissolution (Bellenberg et al. 2015).

The molecular mechanisms used by leaching bacteria to adapt the composition and amount of EPS according to the energy source (planktonic cells grown with iron(II)-sulfate, produce almost no EPS), as well as the physiological adjustments to a sessile lifestyle remain to be elucidated in detail. Recent results have highlighted that some similarities with biofilm formation in other bacterial systems might occur in acidophilic metal/sulfur-oxidizing bacteria, e.g. involvement of P*i*/polyP metabolism, EPS biosynthesis pathways, coordination of stress responses, altered metabolic rates and cell-cell signaling networks. Our current knowledge in this area is summarized in Sect. 5.

4 Visualization of Biofilms by Acidophilic Metal/Sulfur-Oxidizing Microorganisms

The microbial biofilm lifestyle generally includes attachment, biofilm development, maturation and dispersal (Stoodley et al. 2002; Hall-Stoodley et al. 2004). The EPS components enable formation of 3-dimensional biofilm structures. Several advanced microscopy techniques have been applied to reveal biofilm formation and dynamics. CLSM in combination with fluorescent probes provide detailed 3-dimensional structure and compositional information (Lawrence et al. 2003; Neu and Lawrence 2014a). EPS represent a crucial part of microbial biofilms and a key element in terms of biofilm functionality (Neu and Lawrence 2009). Due to the complexity of EPS, an *in situ* approach to analyze the EPS glycoconjugates by means of FLBA has been developed (Staudt et al. 2003; Peltola et al. 2008; Zippel and Neu 2011; Bennke et al. 2013; Castro et al. 2014). FLBA allows simultaneous visualization and characterization of EPS glycoconjugates. If combined with other stains specific for proteins, nucleic acids or lipids, among others, additional EPS components may be visually characterized (Neu and Lawrence 2014a, b). Transmission electron microscopy (TEM) as well as AFM, offer the highest resolution for biofilm structural inspection (Dufrêne 2003; Lawrence et al. 2003). In addition, scanning electron microscopy (SEM) has been widely used in the field for biofilm visualization and monitoring (Baldensperger et al. 1974; Bryant et al. 1984; Mikkelsen et al. 2007). Also, environmental scanning electron microscopy (ESEM) can minimize biofilm dehydration and preserve native biofilm morphology and support surfaces (Priester et al. 2007). These techniques, as well as magnetic resonance imaging

(MRI), scanning transmission X-ray microscopy (STXM), Raman microscopy (RM) and surface-enhanced Raman scattering (SERS) allow *in situ* and non-destructive analysis of the structure, species and EPS composition as well as dynamic processes within microbial biofilms (Lawrence et al. 2003; Manz et al. 2003; Ivleva et al. 2009; Wagner et al. 2009). RM provides single-organism fingerprints for biological samples with spatial resolution in the nanoscale range and enables correlations between optical and chemical images to be made. Since water is the major component of the biofilm matrix, RM is ideal for *in situ* studies. In contrast, STXM allows visualization of biofilm structures by biochemical and elemental imaging. Especially, these techniques do not involve the use of additional probes. Nevertheless, although STXM reveals unique data sets, it requires synchrotron beam time and has limitations in sample mounting.

As mentioned above, AMOM live in an extremely special niche, usually characterized by a high content of metal ions, low pH and high temperature gradients. Although biofilm occurrence and architecture have been studied for decades, detailed knowledge on acidophilic biofilms is still rather limited if compared with microbial biofilms related to medical and industrial fields. Nevertheless, in recent years progress has been made for visualization and characterization of biofilms produced by acidophilic microorganisms.

4.1 Biofilm Formation by Acidophilic Metal/Sulfur-Oxidizing Microorganisms at Laboratory Scale

4.1.1 Attachment to and Biofilm Formation on Elemental Sulfur

S^0 has a very low solubility in water (<5 μg/L). Hence, a direct contact is prerequisite for microbial oxidation (Vogler and Umbreit 1941). Waksman first noticed that S^0 particles in a growing culture of *At. thiooxidans* were surrounded by bacteria using light microscopy (Waksman 1932). Visualization of biofilms formed by acidophiles on S^0 revealed that cells are forming monolayers and erosion sites associated with cells were evenly distributed on S^0 surface. In general, acidophilic bacteria and archaea showed preferential attachment to defect sites present on S^0 surface. This was observed for *At. thiooxidans* (Schaeffer et al. 1963), *Sulfolobus* sp. (Weiss 1973), *Thiobacillus denitrificans* (Baldensperger et al. 1974) and *At. ferrooxidans* (Espejo and Romero 1987). In addition, pili and cell wall components like a glycocalyx were found to be involved in connecting cells of *Sulfolobus* sp. and *Thiobacillus albertis* and S^0 (Weiss 1973; Bryant et al. 1983; Bryant et al. 1984; Laishley et al. 1986). The presence of mucous polysaccharides in EPS was visualized by ruthenium red staining (Bryant et al. 1983). Additionally, membrane blebs were visualized and these were hypothesized to aid the cells overcoming the hydrophobic barrier necessary for their growth on S^0 (Knickerbocker et al. 2000; Crescenzi et al.

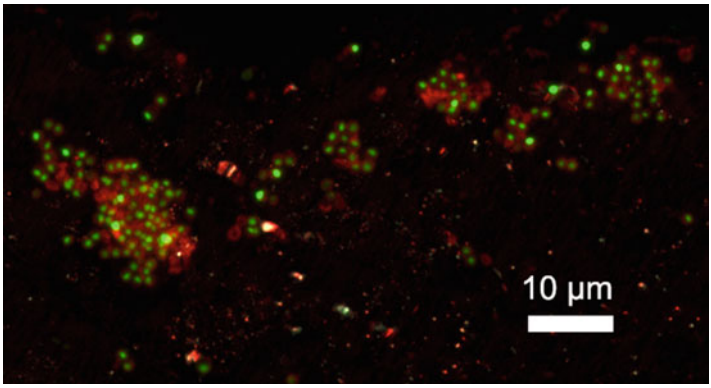

Fig. 6.2 Maximum intensity projection of biofilms by *Acidianus* sp. DSM 29099 on S^0. Cells and biofilm matrix were double stained by Con A and SybrGreen. Color allocation: *green* = SybrGreen, *red*=Con A-tetramethyl rhodamine isothiocyanate (TRITC), *grey* = reflection. Biofilm cells in the form of microcolonies were embedded in EPS matrix containing monosaccharides mannose and glucose

2006). Recently, we studied the biofilm formation of the thermophilic archaeon *Acidianus* sp. DSM 29099 on S^0 by means of FLBA. It was found that biofilm cells were heterogeneously distributed as individual groups of cell clusters and microcolonies. 21 lectins were shown to be useful for the study of EPS glycoconjugates produced by this archaeon on S^0 surfaces. 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 (Zhang et al. 2015a). Figure 6.2 shows an example of biofilm matrix of *Acidianus* sp. DSM 29099 on S^0 visualized by CLSM.

Apart from labelled lectins, other fluorochromes may be simultaneously applied to visualize and characterize additional EPS compounds. For instance, the Syto and Sypro series are used to detect cells via their nucleic acids and cellular proteins, respectively. FM dyes (FM1-43 and FM4-64) and Nile red are specific to stain membranes and lipophilic compounds. These stains can be used also to stain extracellular compounds in biofilms (Lawrence et al. 2007; Neu and Lawrence 2014a). DDAO (7-hydroxy-9H-1,3-dichloro-9,9-dimethylacridin-2-one) stains nucleic acids and normally does not penetrate cell membranes. Thus, it has been selected as the preferred fluorochrome for staining extracellular DNA (eDNA) (Koerdt et al. 2010). Combination of several types of stains allowed us to get detailed information about biofilms of acidophiles on different energetic substrates. For instance, biofilms of *S. metallicus*^{T} were shown to be embedded in an EPS matrix containing proteins and eDNA by combination of Sypro, DDAO and several lectins (Zhang et al. 2015b). In Fig. 6.3 the presence of eDNA in biofilms of *S. metallicus* grown on S^0 is shown.

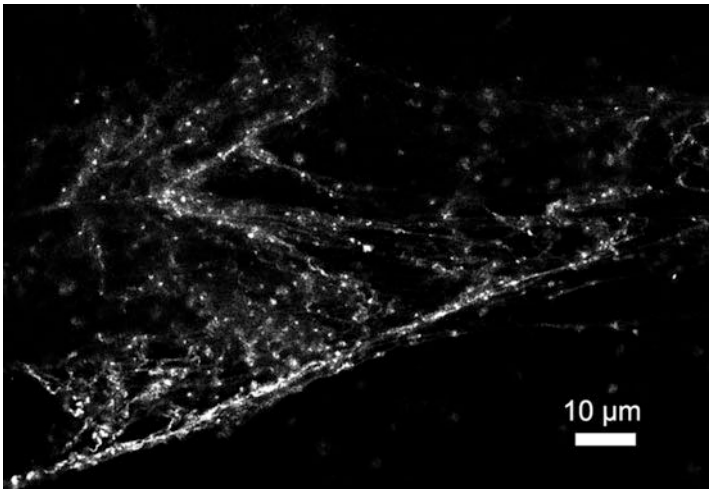

Fig. 6.3 CLSM image of *S. metallicus*^{T} biofilms on S^0. Cells and biofilm matrix were stained by SybrGreen. Diffuse and thread DNA signals around/connecting cells are visible

4.1.2 Biofilm Formation on Mineral Sulfides

In general, cells are forming monolayer biofilms and show selective attachment, preferentially to sites with crystal defects, fractures and pores. Examples of these observations include visualization of cells of *Caldariella* (a thermo-acidophilic archaeon) on pyrite or chalcopyrite phases of a low-grade copper ore (Murr and Berry 1976), *At. ferrooxidans* on pyrite or chalcopyrite (Wakao et al. 1984; Gehrke et al. 1998; Sanhueza et al. 1999; Sampson et al. 2000a; Tributsch and Rojas-Chapana 2000; Lei et al. 2009; Noël et al. 2010), *A. caldus* on pyrite (Edwards et al. 2000a) and enrichment cultures obtained from the Iron Mountain in California on pyrite surfaces (Edwards et al. 1998, 1999b). In contrast, an *in situ* AFM analysis of *Sulfobacillus thermosulfidooxidans* attached to pyrite indicated that cells on the surface were distributed in small clusters instead of forming a continuous biofilm. No evidence was found to suggest a preferential attachment to certain sites or a preferred orientation (Becker et al. 2011). Also, cell attachment of *Metallosphaera sedula* and *S. metallicus* show no preferential orientation. However, pyrite oxidation and pit etching were influenced by surface symmetries (Etzel et al. 2008). Interestingly, two distinct biofilm morphologies were described for a moderate thermophilic archaeon *F. acidarmanus* Fer1. A multilayer biofilm was developed on pyrite surfaces, and up to 5 mm-long filaments were found on sintered glass spargers taken from gas lift bioreactors (Baker-Austin et al. 2010). Cells of *M. sedula* were found to be wiggling along the metal ore by EFM, suggesting that cell appendages were involved in cell attachment to the ore (Huber et al. 1989). Production of EPS (slime or an organic capsule) was often observed. Such phenomenon has been

reported for cells of *Sb. thermosulfidooxidans* (Golovacheva 1978), *At. ferrooxidans*, *At. thiooxidans* and/or *L. ferrooxidans* (Rojas-Chapana et al. 1996; Telegdi et al. 1998; Tributsch and Rojas-Chapana 2000; Bellenberg et al. 2012; González et al. 2012). The production of EPS can be induced by several factors like direct contact with solid substrates or with its dissolution products (Bellenberg et al. 2012; Zhang et al. 2015a). Interactions of three axenic cultures of thermophiles *Acidianus brierleyi*, *M. sedula* and *S. metallicus* with pyrite were first documented using SEM and TEM. Several of deposited structures were formed on the pyrite surface, including sub-micron precipitates and disc-shaped structures (Mikkelsen et al. 2007).

The combination of AFM and EFM allows for confirmation of biological or chemical origins of structures at a high resolution (Mangold et al. 2008). Surface properties of minerals and their modification due to microbial activities can be recorded. In addition, the probes used (e.g. fluorescently labelled lectins) can provide additional biochemical information of the biofilm cells. The formation of monolayer biofilms as well as EPS production were found for cells of *F. acidiphilum* on pyrite (Zhang et al. 2014), *Metallosphaera hakonensis* on chalcopyrite (Africa et al. 2013), *At. ferrooxidans* and mixed cultures of *At. thiooxidans* and *At. ferrooxidans* on pyrite (Harneit et al. 2006; Florian et al. 2010; Noël et al. 2010; Florian et al. 2011; Gonzalez et al. 2013). The biofilm formation was accompanied with the production of EPS containing mannose or glucose. By combination with other techniques, e.g. a Kelvin probe, relative surface potential differences and charge distributions on the surface can be measured (Vera et al. 2013b). We have applied FLBA to analyze biofilms of axenic cultures of *F. acidiphilum*, *S. metallicus* and *Acidianus* sp. DSM 29099 on pyrite. Glycoconjugates containing monosaccharides such as fucose, glucose, galactose, mannose, sialic acids, GlcNAc and GalNAc were detected in these biofilms. The two main binding patterns, e.g. tightly or loosely bound EPS were detected (Zhang et al. 2015a). Cells showed preferential attachment to specific sites of the pyrite surfaces (Fig. 6.4).

The molecular mechanisms controlling biofilm formation in acidophilic metal/sulfur-oxidizing archaea are far less explored (Orell et al. 2013). Preliminary work on crenarchaeal biofilms on other surfaces with respect to their morphology, architecture and chemical components has been done. The first crenarchaeal biofilm analysis was described for three closely related *Sulfolobus* sp. Biofilms with "carpet-like" structures by *S. solfataricus* and *S. tokodaii* and high-density "tower-like" structures by *S. acidocaldarius* were detected (Koerdt et al. 2010). Cell appendages such as pili or flagella were shown to be involved in initial attachment of *S. solfataricus* to various surfaces, including glass, mica, pyrite and carbon-coated gold grids (Zolghadr et al. 2010). Taking advantage of the expression of fluorescent proteins in archaea, three type IV pili-like cell appendages of *S. acidocaldarius* were found to be involved, but possessed different functions in cell colonization and biofilm formation on glass surfaces (Henche et al. 2012). By using comparative fluorescence microscopy and CSLM, the enzyme mannosidase in *S. solfataricus* was found to be important in archaeal biofilm formation and modulation of EPS composition (Koerdt et al. 2012).

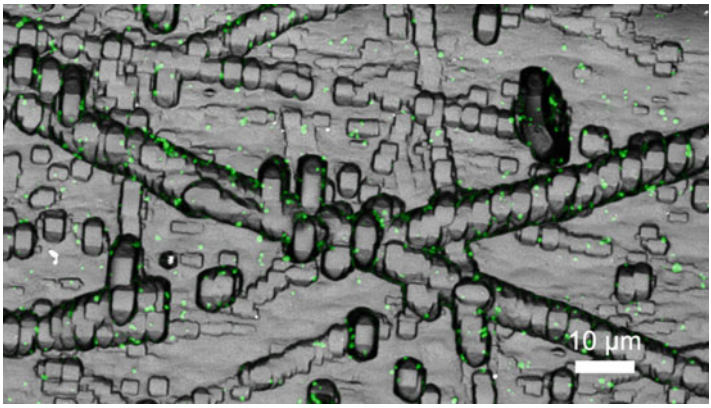

Fig. 6.4 CLSM image of biofilms by *Acidianus* sp. DSM 29099 on pyrite. Cells were stained by the lectin AAL. Color allocation: *green*=AAL-fluorescein isothiocyanate (FITC). The pyrite surface is shown in reflection mode (=*grey*). Cells preferentially attach to pyrite surfaces along surface defective patterns

4.2 Biofilms of Acidophiles in Natural Environments

Microbial biofilms associated with low pH and high metal concentration are often found in natural environments, like AMD sites at Rio Tinto, Spain or the Iron Mountain (Richmond, California). In contrast to laboratory biofilms, environmental ones usually form extended, macroscopic structures of different morphology. In these biofilms, bacteria, archaea and eukaryotic microorganisms have been detected e.g. in the slimes at the Iron mountain AMD site (Edwards et al. 1999a, 2000b). Macroscopic biofilm streamers of around 1 cm in diameter and dominated by *Leptospirillum* sp. have been found (Bond et al. 2000b). Several studies have focused on the microbial communities in biofilm streamers and snottites (Tyson et al. 2004; Wilmes et al. 2009; Jones et al. 2012; Yelton et al. 2013; Aliaga Goltsman et al. 2015). A benthic community in an acidic ($pH \leq 2$) stream was analyzed by CLSM. The dominating microbes *Gloeochrysis* were observed as a brown, mucilaginous biofilm growing on stones within a dense matrix containing also inorganic particles and fungal hyphae, all held together by EPS (Baffico et al. 2004). Macroscopic filaments studied at Rio Tinto identified *At. ferrooxidans*, *L. ferrooxidans* and *Acidiphilum* spp. as the dominant species (García-Moyano et al. 2007). Biolims (streamers and snottites) obtained from a subsurface mine in Königstein (Saxony, Germany) were visualized after staining with four acid stable fluorescent dyes. These dyes were proven to be suitable for staining acidophilic biofilms of acidophilic microorganisms under ambient pH conditions (Brockmann et al. 2010).

4.3 Detachment of Acidophilic Biofilms

Biofilm detachment as well as cell dispersal from acidophilic biofilms is still an unexplored field and these processes are not well understood. However, there is evidence that AMOM show such a behavior since microbial footprints on MS have been observed. These are mainly composed of organic substances which are left behind by microbes during transient contact with mineral surfaces or due to a cell programmed detachment as part of the biofilm lifestyle cycle. This ability of microbes to label relevant surfaces was first reported as 'footprints', for polymeric substances left on a glass surface after cells of *Pseudomonas* detached by a shear force (Marshall et al. 1971). Microbial footprints are mainly composed of EPS remaining on surfaces after cell detachment or mechanical removal (Neu and Marshall 1990, 1991). Footprints have been described for *T. intermedius* on iron (Telegdi et al. 1998), *At. ferrooxidans* (Rojas-Chapana et al. 1996; Mangold et al. 2008), *St. thermosulfidooxidans* (Becker et al. 2011) and a mixed culture of mesophilic acidophilic chemolithotrophs where *L. ferriphilum* was predominant (Ghorbani et al. 2012). We detected that cells of *S. metallicus*^{T} and *Acidianus* sp. DSM 29099 left behind mannose or glucose containing materials on surfaces after cell detachment (Zhang & Vera, unpublished data). We have also observed different dynamics of colonization, biofilm formation and cell detachment of iron-oxidizing acidithiobacilli and leptospirilli. In this case the biofilms formed by the latter species are stable for longer incubation periods than the ones formed by iron-oxidizing acidithiobacilli. Probably these detachment processes form part of a response to oxidative stress caused by the presence of ROS, the increase of the ionic strength and the decrease of the pH. It has been shown that EPS compounds like lipopolysaccharides (LPS) might detach from biofilms during their maturation (Jiao et al. 2010).

Several extracellular enzymes have been found to be present in EPS from environmental biofilms. Many of these are probably involved in the degradation of biopolymers, resulting in cell detachment and biofilm dispersal (Flemming and Wingender 2010). Currently there are no identified enzymes which could be involved in detachment of acidophilic biofilms. However, transcriptomic studies have suggested that several genes could be involved in maintenance and detachment/dispersal of acidophilic biofilms (Moreno-Paz et al. 2010).

4.4 Chemical Mapping of Extracellular Components

Recently, synchrotron radiation based STXM imaging and μ-XRF mapping have been applied for an *in situ* comparative analysis of the extracellular thiol groups (SH) of *At. ferrooxidans* cells. It was found that the SH content of *At. ferrooxidans* grown on S^0 is roughly four times higher than those contents of iron(II)-grown cells. These data suggest that extracellular SH play an important role in sulfur activation prior to its oxidation (Xia et al. 2013). STXM has been used to visualize biofilms

and EPS formed by *At. ferrooxidans* on pyrite. The distribution of polysaccharides and proteins in these biofilms was visually correlated with the optical overview. Polysaccharide-rich biomolecules were abundant at the pyrite-cell boundary, while lipid- and protein-rich regions were detected in the center region of the cells (Mitsunobu et al. 2015).

5 Molecular Studies of Biofilm Lifestyle of Acidophilic Metal/Sulfur-Oxidizing Microorganisms

5.1 Proteomics of Biofilm Formation in At. ferrooxidans^T

As mentioned earlier, the different EPS amount and composition between cells grown with different energy substrates, adaptation to enhanced levels of ROS and the obvious physiological adaptations necessary for the transition to a biofilm lifestyle strongly suggest that planktonic and sessile cells differ in their gene expression and, consequently, in their proteomic patterns. *At. ferrooxidans*^{T} possess homologous systems for CPS biosynthesis and export to the ones described in Gram-negative bacteria (Barreto et al. 2005). The genes *Afe_1339* and *Afe_2975* encode for two outer membrane polysaccharide export proteins, homologous to *E. coli* Wzy and KpsD (Whitfield 2006). The expression levels of *Afe_2975* were found to be enhanced in PP and BP after transfer of iron (II)-grown cells to pyrite cultures (Bellenberg et al. 2011). A bioinformatic analysis of putative CPS synthesis genes in *At. ferrivorans* SS3 complete genome sequence revealed the presence of homologous genes of the Wz CPS biosynthesis and export system, while homologous genes for the Kps system are absent. Most of putative genes including *wza* (polysaccharide biosynthesis export domain; *Acife_1181*), *wzc* (tyrosine-protein-kinase; *Acife_1180*), *wzb* (tyrosine-protein-phosphatase; *Acife_1179*), and *wzx* (polysaccharide biosynthesis protein; *Acife_1172*), were clustered in a possible operon in this bacterium. The *wzy* gene (polysaccharide biosynthesis protein; *Acife_0130*) and two copies of *wbaP* gene (undecapyrenyl-galactose-phosphate transferase; *Acife_1166* and *Acife_1194*) are present but not clustered in operons. This finding allows us to speculate that if these gene clusters are the main responsible ones for the biosynthesis of EPS polysaccharide polymers, presumably these would be different among both species. However, the functionality of the Wz system in *At. ferrivorans* remains to be determined.

The first high-throughput proteomic study of the biofilm formation in *At. ferrooxidans*^{T} was done in order to map the changes during its early biofilm formation process. It compared proteomes from PP and BP after 24 h of biofilm formation on pyrite by using quantitative shotgun proteomics. In total 1319 proteins, (42 % of the predicted *At. ferrooxidans*^{T} proteome) were identified. At least 16 % of the total amount of detected proteins were found to have increased or decreased levels among both cell subpopulations. Functions such as biosynthesis of EPS and electron carrier

containing molecules, transport of reduced inorganic sulfur compounds as well as osmotic stress response were found to be enhanced in the BP.

The presence of three transcriptional factors, which were found to have increased (MerR, IclR) or decreased levels (AbrB) in the BP, suggests their involvement on the regulation of *At. ferrooxidans* biofilm formation process. In addition, membrane and outer membrane transport functions, including increased levels of proteins for CPS biosynthesis, efflux pumps, lipoproteins, ABC transporters and several proteins related to stress responses had increased levels in BP. It is well accepted that bacteria show stress responses during biofilm formation (Otto and Silhavy 2002).

As mentioned in subchapter 3, especially the BP must deal with changes in the osmolarity as well as with the presence of enhanced levels of ROS, which are generated in presence of pyrite. The genome of *At. ferrooxidans*^{T} possesses several genes encoding proteins related to responses against oxidative stress. These include a Mn-dependent superoxide dismutase (SodA), as well as members of the alkylhydroperoxidase family (AhpC and AhpD). Also, several components for non-enzymatic neutralization of ROS are encoded. These include thioredoxins, genes for biosynthesis and export of glutathione (GSH), and disulfide reductases, which maintain the thiol/disulfide balance of other molecules (Valdes et al. 2008). In this context, changes in proteins related to responses against osmotic and oxidative stress were detected. Apart from the osmolarity sensor protein EnvZ, a protein encoding an iron and 2-oxoglutarate (2-OG) dependent dioxygenase (AFE_3138) was also found to have increased levels in biofilm cells. Members of this protein family might be involved in ectoine biosynthesis as a response to high osmolarity (Reuter et al. 2010). Glutathione (GSH) is a major biological antioxidant, which contributes to maintain redox balance in prokaryotes (Smirnova and Oktyabrsky 2005). It is especially important for re-establishing the redox balance of proteins and lipids. In addition, GSH possesses a dual role in *At. ferrooxidans,* since it has been shown that low molecular weight thiol-containing compounds like GSH are relevant for the oxidation of elemental sulfur to sulfite in a reaction catalyzed by a periplasmic sulfur dioxygenase (SDO) (Rohwerder and Sand 2003). Several proteins involved in GSH metabolism were found to be induced in BP. These include AFE_1390 (CydC), a GSH transporter, AFE_0366 and AFE_2773, a GSH reductase (GR), and a protein of the YghU family of GSH S-transferases. This suggests an increase in periplasmic GSH levels, as part of a GSH-driven response against oxidative stress or related sulfur and RISCs oxidation (Vera et al. 2013a). In this context, it has been shown that the GR encoding gene is induced after *At. ferrooxidans* is exposed to copper, suggesting its involvement in recovering GSH pools as a response against oxidative stress (Xia et al. 2011).

Certain molecules can modulate biofilm formation. Polyamines are linear organic polycations widespread in bacteria. A common function of polyamines does not seem to exist but recent evidence has suggested their involvement in biofilm enhancement or inhibition in different bacterial species, acting extra- or intracellularly (Karatan and Michael 2013). The asymmetrical polyamine spermidine is widespread in bacteria. In *E. coli*, GSH is mostly present under aerobic growth and glutathionyl-spermidine (GspSH) is predominating under anaerobic growth condi-

tions and stationary phase (Smith et al. 1995). A protein of the GSH S-transferase YghU family probably related with the synthesis of GspSH (AFE_2773) was found to be enhanced in *At. ferrooxidans*^{T} BP. It has been suggested that YghU belongs to a new family of GSH S-transferases (Stourman et al. 2011). Spermidine has been found ubiquitously in *At. thiooxidans* cells grown with different energy sources such as iron(II)-sulfate, sulfur and chalcopyrite (Martínez et al. 2013).

Biofilm cells are characterized to have "altered metabolic rates" (Seneviratne et al. 2012). In *At. ferrooxidans* BP there was an increased amount of proteins involved in recycling of cellular components and decreased levels of respiratory functions. These include several nucleotidases and ATPases, which may enhance the pools of building blocks for *de novo* macromolecule biosynthesis. Also an enhanced biosynthesis of cofactors and coenzymes was suggested to occur in BP, including proteins containing iron-sulfur clusters, heme, pyrroloquinoline quinone (PQQ) and functions related to ubiquinone metabolism (Vera et al. 2013a). Around one fifth of the total proteins detected were hypothetical proteins (231 proteins, from which 12 % were found with altered levels among BP and PP). Therefore, it is highly likely that still unknown metabolic pathways of *At. ferrooxidans* are relevant during biofilm formation. Among these ones, some "biofilm specific molecules" with potential roles as antibiotics or inhibitors may be considered. The latter ones have been shown to occur in some bacterial species, being probably involved in survival against competitors and predators (Beloin et al. 2004; Rendueles and Ghigo 2012). The increased presence of hypothetical proteins induced in biofilm proteomes, including those from AMD biofilms will require further studies of heterologous expression and subsequent biochemical screening in order to address their functions (Denef et al. 2010).

It is well accepted that biofilm development is an organized series of sequential events: in which planktonic microorganisms adhere to a surface; later on these adhered organisms produce a discrete set of microbial colonies in which EPS production starts together with the appearance of a three-dimensional community structure. At mature stages, some of the biofilm cells leave to seek for new surfaces (Flemming and Wingender 2010). As mentioned before, in acidophilic biofilms we have microscopic evidence pointing out the existence of cell detachment processes (see Sect. 4). However, to the best of our knowledge, there is no detailed knowledge of the physical factors as well as the physiological responses and molecular events probably involved in cell detachment processes.

5.2 *Molecular Diversity Studies in Environmental Acid Mine Drainage Biofilms*

A major contribution to the ecology and molecular study of AMD biofilms has been published by Jillian Banfield & co-workers. These studies include extensive metagenomic, metaproteomic and proteogenomic analyses of natural AMD communities and their biofilms (Ram et al. 2005; Denef et al. 2009; Wilmes et al. 2009).

Sequencing of metagenomes obtained from the Richmond Mine at the Iron Mountain in northern California allowed the reconstruction of near complete genomes of *Leptospirillum* Group II (which is the dominating microorganism), later characterized as *Leptospirillum rubarum* (Goltsman et al. 2009) and a *Ferroplasma* type II (Tyson et al. 2004). Subsequent studies have allowed to perform genomic reconstructions of some other microorganisms present within these communities such as *Leptospirillum* group III, later characterized as *L. ferrodiazotrophum* (Goltsman et al. 2009) and *Leptospirillum* group IV UBA BS (Goltsman et al. 2013), as well as new uncultivated species of archaea (Yelton et al. 2013).

AMD biofilms grow at the air-liquid interfaces of sites like streams and pools that overlay pyrite containing sediments. Biofilm growth (up to hundred microns thick) generates iron(III), which subsequently catalyzes pyrite oxidation, coupling inorganic and biological oxidation processes. The main characteristics of this system is low species diversity and reproducible stages of biofilm development and succession over time (Wilmes et al. 2009; Denef et al. 2010). In the earliest developmental stages of AMD biofilms *Leptospirillum* group II dominates in presence of a small proportion of *Leptospirillum* group III and some archaea. When the biofilms enter into a mature stage, the proportion of *Leptospirillum* group III and archaea increases and some eukaryotes appear. Interestingly, although *Leptospirillum* groups II and III are present through all the biofilm development, they strongly differ in their spatial arrangement. *Leptospirillum* group II was observed to form tight agglomerations of cells while *Leptospirillum* group III was mostly observed as microcolonies or single cells (Wilmes et al. 2009).

5.3 Proteomics in Acid Mine Drainage Biofilms

The genomic types of natural biofilm populations in a community sample were inferred from proteomics data. Samples from two locations (UBA and 5-way GC) within the Richmond mine were used, each one dominated by a distinct *Leptospirillum* group II type, with 0.3 % differences at the 16S rRNA sequence level. Both genomes possessed 80 % identity at the gene level with an average amino acid identity of 95.2 %. A proteomics inferred genome typing (PIGT) provided evidence of recombination among two closely related *Leptospirillum* group II populations (Lo et al. 2007). Later on, PIGT was used to genotype the dominant *Leptospirillum* group II population by analysis of 27 biofilm samples taken from the Richmond mine over a 4-year period. Six distinct phenotypes, which are recombinants derived from two parental genotypes were identified, confirming that homologous recombination is the main strategy used for fine scale environmental adaptation within those biofilms (Denef et al. 2009).

Comparative proteomic analyses of AMD biofilms have been also performed with laboratory-cultivated biofilms, inoculated from environmental samples (Belnap et al. 2010, 2011). Interestingly, laboratory-grown biofilms were also dominated by *Leptospirillum* group II, with a lower abundance of *Leptospirillum* group III. Biofilm

field samples showed a higher abundance of proteins related to functional categories such as energy production and conversion, cell motility, cell wall, membrane and envelope biogenesis, intracellular and secretion and vesicular transport functions. In contrast, transcriptional proteins as well as proteins probably involved in defence mechanisms were more abundant in laboratory-grown biofilms (Belnap et al. 2010). Further studies determined that *Leptospirillum* group II proteins involved in amino acid and nucleotide metabolism, together with proteins involved in cell membrane and envelope biogenesis were overrepresented at high pH. In addition, a pH-specific niche partitioning was shown to occur for some low abundance bacteria and archaea, since *Leptospirillum* group III was more abundant in biofilms grown at higher pH values, whereas archaeal species were more abundant at lower pH values (Belnap et al. 2011).

By high-throughput proteomics and metabolomics it has been shown that the identified proteins and metabolites from *Leptospirillum* groups II and III exhibit organism related correlation patterns, which suggest a restructuration of their metabolic and/or regulatory networks reducing their competition and allowing them to occupy distinct niches (Wilmes et al. 2010). After evaluation of the reproducibility of AMD community proteomes, a set of reliable classifier proteins was identified which may be used to predict growth stages of biofilm communities. During early stages of biofilm growth, *Leptospirillum* group II cell population responded to some abiotic stresses by reorganizing their metabolism, since functions such as protein biosynthesis, cell division as well as metabolism of 1 or 2 carbon compounds were more abundant. Stress responses were abundant in early stage biofilms, since proteins involved in metal efflux were found to be increased. Among the increased proteins in this growth stage, a cytochrome 572, a cytochrome oxidase with high O_2 affinity as well as a cytochrome c-553 were found. Cell division functions were also more abundant in early stage biofilms and these seem to further decrease due to accumulation of DNA mutations. This is in agreement with a 26-fold increase in the abundance of ribosomal proteins detected in early stage biofilms, compared to the mature ones. Several enzymes involved in the metabolism of more complex carbohydrates, amino acid biosynthesis, amino- and nucleotide-sugar metabolism, lipopolysaccharide biosynthesis, starch and trehalose metabolism were more abundant in mature biofilms. Proteomes from late stage biofilms also showed an increased level of proteins involved in P*i* and molybdenum transport, suggesting that essential nutrients such as oxygen, phosphorous, nitrogen and molybdenum become limiting in those biofilms (Mueller et al. 2011).

The response of AMD microbial biofilm communities to temperature gradients has been recently studied. Cultivation at elevated temperatures e.g. increase from 40 °C to 46 °C repressed carbon fixation in two *Leptospirillum* genotypes while a third one was probably subjected to a viral stress, which would increase the carbon turnover through the release of the viral lysate (Mosier et al. 2015). Several enzymes upregulated could probably be involved in changes in the EPS composition in these AMD biofilms, which have been shown to possess carbohydrates such as glucose,

galactose, rhamnose, heptose and mannose (Jiao et al. 2010). Recently it has been shown that approximately 29 % of the proteins reliably detected in the dominant *Leptospirillum* type II biofilm population carry post-translational modifications (PTMs), among these, 43 % carry more than one PTM. The PTMs profile strongly differs between early and mature biofilms, as well as between orthologous of two ecologically differentiated *Leptospirillum* group II bacteria (Li et al. 2014). By metabolomic analysis it has been found that natural acidophilic microbial biofilms dominated by bacteria of the genus *Leptospirillum* contained unusual lysophosphatidylethanolamine (PE) lipids in high abundance. The unusual polar head group structure of these lipids may be related to their affinity for iron and calcium ions (Fischer et al. 2012). Around 3500 metabolic features were identified in biofilms grown at AMD solutions at pH 0.9. By stable isotope labeling, several unrepresented metabolites were found in these biofilm communities, which may represent novel compounds. Among the known metabolites found, taurine, ectoine and hydroxyectoine were suggested to provide protection against osmotic stress. As taurine is not known to be synthesized by bacteria or archaea (but probably assimilated by some bacterial members of the AMD community), it was speculated that *Acydomyces richmondensis*, the dominant fungus in the Richmond mine AMD community, could be responsible for its biosynthesis (Mosier et al. 2013). The genomes of *Leptospirillum* group II possess genes for ectoine or hydroxyectoine biosynthesis. The production of ectoine and hydroxyectoine decreased in mature biofilms, suggesting that during the early biofilm development ectoine and hydroxyectoine are more abundant in order to help bacteria to the exposure to the a high ionic strength AMD solution. In later stages, the biofilm structure may provide some protection against high metal and proton concentrations (Mosier et al. 2013).

6 Cell-Cell Communication in Acidophilic Metal/Sulfur-Oxidizing Bacteria

Microbial biofilm development is a complex process which is regulated at different levels by a diverse set of mechanisms. Microbes do not only exist as single cells and they are able to coordinate their activities in a concerted manner, in a similar way to multicellular organisms. Several bacterial species employ sophisticated intercellular communication systems, which rely on the secretion and sensing of small signal molecules which allow to control the expression of multiple target genes. In many Gram-negative bacteria biofilm formation and EPS production are modulated by cell-cell communication mechanisms of Quorum Sensing (QS) (Marketon et al. 2003). QS cell-cell signalling is mediated by diffusible molecules named autoinducers (AIs), which facilitate the regulation of cellular processes affected in a cell-density dependent way (Labbate et al. 2007).

6.1 *QS Studies in* At. ferrooxidans^T

At. ferrooxidans^T has one AI-1-type QS system, involving the production of several AIs of the acyl-homoserine lactone (AHL) type (Farah et al. 2005; Rivas et al. 2005). The system is composed of an AHL synthase (AfeI), a transcriptional regulator (AfeR) that binds AHLs, an *afe*-box which is the target of the binary complex [AfeR-AHL] and different AHL signalling molecules. It has been shown that at least 9 different AHLs with diverse C-3 substitutions (oxo or hydroxy) and a range of carbon atoms in the acyl chain oscillating between 8 and 16 are synthesized in *At. ferrooxidans*^{T}. P_i starvation increased the transcription of *afeI* and AHL levels (Farah et al. 2005; Valenzuela et al. 2007). Moreover, compared to iron(II)-grown cells, *afeI*-transcript levels were also increased in cells grown with S^0. Altogether, these information allowed to propose that the *At. ferrooxidans*^{T} QS system (*afeI/afeR*) could be involved in the regulation of EPS production and consequently in the regulation of biofilm formation, as it has been demonstrated in other Gram-negative bacteria (Marketon et al. 2003; Parsek and Greenberg 2005; Labbate et al. 2007; Decho et al. 2010). If so, synthetic AHLs and AHL-analogues (Choudhary and Schmidt-Dannert 2010; Stevens et al. 2010; Galloway et al. 2012) could be used in cells of *At. ferrooxidans*^{T} to enhance or inhibit QS-regulated phenotypes, such as EPS production and biofilm formation. This has been demonstrated, since biofilm formation on pyrite or S^0 surfaces was stimulated by the external addition of synthetic long-chain AHLs (Gonzalez et al. 2013). Concominantly with this, increased levels of CPS were observed in biofilms grown on polycarbonate filters in presence of long-chain AHLs. In agreement with these findings, a bioinformatic study predicted that at least 75 genes possess *afe*-boxes, suggesting their expression to be regulated by AfeR-AHL complexes in *At. ferrooxidans*^{T}. Among these, genes encoding glycosyltransferases, metallo-beta lactamases, proteins probably related to RNA metabolism and active transport-related proteins were found, suggesting these to be directly related in EPS biosynthesis and export (Banderas and Guiliani 2013).

Cell attachment to MS during bioleaching is a selective process in which microorganisms attach preferentially to certain sites on the pyrite surface. In consequence, increased cell densities may occur at these interfaces. Such kind of cell trapping may consequently result in QS auto-induction in attached cells but not in planktonic ones. This activation presumably contributes to the establishment of the EPS/CPS matrix and the biofilm phenotype which can be observed in solid substrate cultures. In contrast, in cultures with iron(II)-ions as soluble substrate AHL detection was either unsuccessful or it indicates levels below auto-induction threshold. A similar mechanism of regulation during the initiation of biofilm formation has been postulated to exist in *Vibrio cholerae* (Waters et al. 2008).

6.2 Cell-Cell Communication in Other Acidophilic Leaching Bacteria

Interaction(s) of bacterial species in natural environments represents a complex research field. In AMOM this field is poorly understood. However, microbial interactions, such as competitive and cooperative traits were demonstrated with laboratory strains and are hypothesized to have strong influences on the propagation of single species in laboratory cultures or microbial community compositions in natural ecological niches (Johnson 1998). These interactions are supposed to act in addition to specific adaptation or strain specific fitness towards abiotic factors, such as ranges of suitable temperatures, nutrients, electron acceptors, concentrations of heavy metals or enhanced osmotic pressure. As previously mentioned, natural habitats of AMOM are characterized by a low microbial diversity compared to neutrophilic environments due to their extremes regarding pH, heavy metal concentration and electron acceptors (Bond et al. 2000a). However, microbial intra- and interspecies interactions are complex due to the amount of environmental variables which are undoubtedly involved. These include temperature, hydration, pH, presence of diverse inorganic ions, organic compounds, such as secreted metabolites, EPS, proteins, secondary metabolites with antibiotic activity or other compounds which may originate from cell lysis or allochthonous biomass. Niche specialization of AMOM is of particular relevance, since in mixed species cultures these are supposed to compete for common nutrients, electron acceptors and carbon sources. The array of possibilities also extends when considering that acidophilic heterotrophs, fungi and algae as well as bacteriophages are also involved in ecological processes such as synergy, mutualism and predation in nature, which may affect the bacterial community composition.

In order to gain insights about cell communication between different species coexisting in bioleaching habitats, the presence of AHLs by mass spectrometry and *Agrobacterium tumefaciens* NTL4 bioreporter assays (Cha et al. 1998) was analyzed. Cultures of *At. ferrivorans* SS3, *At. thiooxidans* DSM 14887, *L. ferrooxidans* DSM 2391 and two strains of *Acidiferrobacter* spp. were assayed. Interestingly, similar as described for *At. ferrooxidans*^{T}, *Acidiferrobacter* strains produced a great diversity of AHLs: C10-AHL, C12-AHL, 3-hydroxy-C12-AHL, C14-AHL, 3-hydroxy-C14-AHL and C16-AHL. In *At. thiooxidans* two kinds of unsubstituted AHLs were detected (C10 and C12-AHL), whereas no AHLs were detected in cultures of *L. ferrooxidans* DSM 2391 and *At. ferrivorans* SS3 (Bellenberg et al. 2014).

The influence of defined mixtures of synthetic AHLs added to pyrite leaching assays in pure and binary mixed cultures containing *At. ferrooxidans*^{T}, *At. ferrivorans* SS3, *Acidiferrobacter* sp. SPIII/3 and *L. ferrooxidans* DSM 2391 was tested. Reduced cell attachment to pyrite upon AHL mixture additions correlated with lowered mineral dissolution. Consequently AHL-based interspecies interactions are likely to occur in natural habitats and they were also demonstrated in binary mixed cultures of *L. ferrooxidans* DSM 2391 and *Acidiferrobacter* sp. SPIII/3, in which an interdependent inhibition was observed.

A search for the presence of QS systems in the complete genomes of leaching bacteria revealed the presence genes encoding for LuxR-like proteins in *At. ferrivorans* SS3 (Acife_1471, 56 % identity) and *L. ferrooxidans* C2-3 (LFE_1606, 29 % identity). Interestingly, no AHLs were detected in cultures of *L. ferrooxidans* or *At. ferrivorans* SS3. The fact that these strains possess LuxR-like receptors may explain in part some of the inhibitory effects observed after addition of certain AHL mixtures. Interestingly, *At. thiooxidans* strains produced different AHLs, in a range of C8 to C10, and could also sense them since the addition of C8, oxo-C8 and C10-AHLs resulted in an enhanced biofilm formation on S^0. However no homologous genes to *luxI/R* are encoded in the genome of *At. thiooxidans* DSM 14887^T (Valdes et al. 2008). At the moment it is unknown how *At. thiooxidans* may sense AHLs and respond differentially after their addition without any LuxR-like receptor. This strongly suggests the presence of novel pathways probably involved in AHL biosynthesis and sensing. Probably these pathways may be widespread among some species of *Acidithiobacillus*. Taken together, these results clearly suggest that cell-cell communication mechanisms and their connection with biofilm formation phenotypes must be thoroughly understood in order to further influence biotechnological processes and develop countermeasures against unwanted natural leaching of MS.

6.3 The c-di-GMP Pathway in Acidithiobacilli

In many bacterial species the transition to a biofilm lifestyle is controlled by the second messenger c-di-GMP. It is synthesized from two GTP molecules by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). Several proteins are known as c-di-GMP effectors, being the ones possessing a PilZ domain one the most characterized to date (Amikam and Galperin 2006; Hengge 2009). These regulate phenotypes such flagellar and twitching motility and EPS biosynthesis. In *At. ferrooxidans*^{T}, c-di-GMP levels were increased in biofilm cells on S^0 or pyrite, strongly suggesting its involvement in *At. ferrooxidans* biofilm formation (Ruiz et al. 2011). Very recently it has been shown that the c-di-GMP pathway is also functional in *At. caldus* ATCC 51756^T. Several genes encoding DGC and PDE effector proteins were identified in its genome sequence and the presence of c-di-GMP by mass spectrometry was confirmed. In addition, genes for several of the enzymes with DGC domains were functional as shown by heterologous genetic complementation in *Salmonella enterica* serovar Typhimurium mutants. A deletion mutant lacking the DGC encoded by the gene ACAty_C1319, which is presumably the main DGC enzyme in *At. caldus*, was recently constructed. This mutant strain had almost 14-fold less c-di-GMP levels than the wild type strain. Interestingly, it showed an increased motility and a reduced capacity to attach to S^0, indicating that the c-di-GMP pathway is involved in the regulation of swarming motility and cell attachment to S^0 surfaces by *At. caldus* (Castro et al. 2015).

7 Future Perspectives

Our current knowledge on the chemical compositions and microbial dynamics of acidophilic biofilms is still limited. The presence and functional roles of macromolecules and metabolites within EPS from these biofilms remain to be clarified. High-throughput FLBA lectin studies may allow a deeper understanding of detailed biofilm formation and interfacial interactions. Acid-stable fluorescent stains, e.g. isolation of lectins from metal/sulfur-oxidizing acidophiles shall be developed. A combination of novel physical and chemical microscopic techniques, e.g. Raman microscopy and nanoscopy techniques such as STED and blink microscopy may allow a detailed investigation. Together with molecular techniques it may greatly extend the understanding of acidophilic biofilm function, chemical structure and dynamics at different scales (Neu and Lawrence 2015). Future molecular studies shall include polymer chemistry, metabolomics as well as and molecular cloning and protein chemistry of proteins remaining as "unknown". These may have important roles in controlling the dynamics of acidophilic biofilm phenotypes and interactions with other microbial populations. The detailed studies of QS, cell signaling and development of QS analogs chemically stable and with enhanced activities could also contribute to develop future methods to influence bioleaching of metal sulfides. The detailed understanding of biofilm lifestyle and cell-cell interactions will be helpful to develop strategies for bioleaching manipulation both in metal recovery where enhancement of leaching rates is necessary and mitigation of AMD, where reduction or inactivation of biofilms might reduce environmental problems.

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