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Final Report for Funding by Mary-Hegeler Stipendium

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1 Description of Habilitation Project

1.1 State of the Art and Preliminary Work

Numerous species of acidophilic Bacteria and Archaea thrive in natural or man-made environments where weathering of metal sulfides such as pyrite (FeS_2) or chalcopyrite ($CuFeS_2$) emerge (Brierley and Brierley 2013). The metal-oxidizing microorganisms have the ability to oxidize iron (II)-ions (and/or reduced inorganic sulfur compounds) producing iron(III)-ions and protons as sole attacking agents for the oxidation of metal sulfides (Sand et al. 1995; Rohwerder et al. 2003). This process plays an important role in the geochemical iron cycle (e.g. the weathering of metal sulfides), the formation of secondary iron(III) minerals (e.g. schwertmannite), the unwanted formation of acid mine drainage (Sand et al. 2007), and the industrial applied winning of metals in heap or tank bioleaching operations (Brierley and Brierley 2013).
Biofilms are surface-associated microbial communities and represent a common and widespread lifestyle on earth. Within a biofilm, the microorganisms are embedded in a self-produced slimy matrix of extracellular polymeric substances (EPS) surrounding the cells. They consist mainly of water, macromolecules such as polysaccharides, proteins, (extracellular) nucleic acids, and lipophilic compounds (Flemming et al. 2016). The EPS form a protective barrier, provide a source of nutrients, and a reaction room for cell-cell interactions, redox reactions, and the transfer of genetic material (Fleming and Wingender 2010). A biofilm is spatial heterogeneous system because of gradients in terms of nutrients, oxygen, CO_2, pH, or temperature which leads to genetically different subpopulations even in single species biofilm structures (Stewart and Franklin 2008). Several studies have highlighted an altered metabolic activity or the presence of resting cells in upper and bottom layers of biofilms (Dolan and Costerton 2002; Fux et al. 2005; Seneviratne et al. 2012). Furthermore, biofilm cells show a strikingly different gene expression profile as compared to their planktonic counterparts. The development of a mature (mineral attached) biofilm is a well-organized sequential event (Stoodley et al. 2002; Seneviratne et al. 2012) including the i) initial reversible attachment of cells by active or passive movement (Fig. 1, 1-2); ii) EPS production resulting in irreversible attached cells, formation of microcolonies, and the beginning cell differentiation (Fig.1, 3), and iii) the maturation of the biofilm and dispersal of “streamer“ cells (Fig. 1, 4).

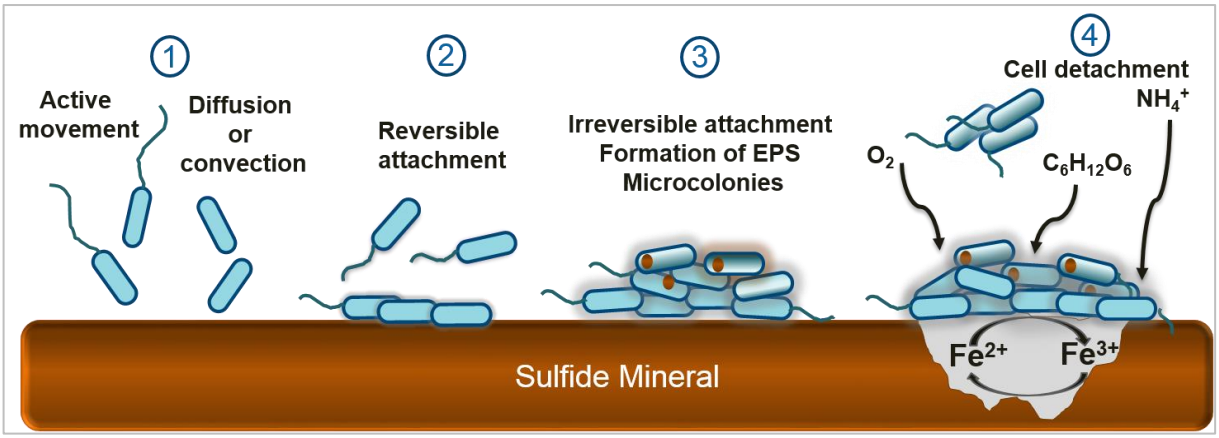

Fig. 1: Stages of biofilm development on a sulphide mineral (e.g. pyrite) as surface. Compared to attachment of well-investigated *Acidithiobacillus* strains, the metal-oxidizing bacteria shown here form multi-layered biofilms instead of monolayers.

The majority of acidophilic chemolithoautotrophic iron- and/or sulfur-oxidizing bacteria, hereinafter referred to as metal-oxidizing bacteria, can grow attached to metal sulfides or sulfur surfaces (Rodriguez et al. 2003; Sand and Gehrke 2006). Attachment to sulfide minerals increases the initial leaching activity and enhances the kinetics of mineral dissolution due to the formation of a distinctive microenvironment (reaction room) between the bacterial cells and the mineral surface (Gehke et al. 1998). The EPS mediates the contact between the mineral surface and the bacteria and contains glucuronic acid which complexes ferric iron ions in the matrix. This causes a positive net charge of the EPS resulting in electrochemical interaction with the negatively charged pyrite surface (Gehrke et al. 1998).
Several studies provide evidence that attachment onto metal sulfides does not occur randomly but at sites with imperfections and low crystallinity (Edwards et al. 1998; Edwards and Rutenberg 2001). Furthermore, all well studied chemolithoautotrophic metal-oxidizing bacteria are described to form monolayer biofilms on minerals, which is strikingly different from the three-dimensional microcolonies shown in Fig. 1. Harneit et al. (2006) demonstrated mineral specific attachment on pyrite (FeS_2), chalcopyrite ($CuFeS_2$), sphalerite (ZnS) for different strains of the model bioleaching organisms *Acidithiobacillus. ferrooxidans*, *At. thiooxidans*, and *Leptospirillum ferrooxidans*. While pyrite was colonized rapidly by 90 % of the inoculated *At. ferrooxidans* cells, none of them attached to quartz. The acidophilic Archaea *Ferroplasma acidiphilum*, *Sulfolobus metallicus*, and *Acidianus* sp. also formed monolayer biofilms by heterogeneously distributed cells, whereas colonization of areas with surface cracks was much denser (Zhang et al. 2015).
To date the majority of biofilm studies on metal-oxidizing bacteria have been carried out with i) chemolithoautotrophic gram-negative bacteria (Africa et al. 2010; Bellenberg et al. 2014; Bellenberg et al. 2015); ii) using Confocal Laser Scanning Microscopy (CLSM), Epifluorescence Microscopy, Atomic Force Microscopy (AFM), or a combination of those techniques (e.g. Noël et al. 2010; Florian et al. 2011; Diao et al. 2014), and iii) and use the mineral sulphide pyrite (FeS_2) as model surface (e.g. Becker et al. 2011; Castro et al. 2016; Li and Sand 2017). **For biofilm imaging and analyses CLSM** in combination with fluorescently labelled biological specimens is widely applied. CLSM uses a spatial filter (detector pinhole) to remove unwanted background fluorescence beyond the focal plane of interest, contributing to high-quality and -magnification fluorescence imaging (Paddock 1999). Furthermore, CLSM is carried out to localize cell components with specifically targeted fluorescent dyes, for

multidimensional microscopy and imaging of fully hydrated biofilms, and life-cell imaging of cells *in-situ* (Costerton et al. 1995; Vu et al. 2009). **However, no studies have been published on *in-vivo* imaging developing biofilms on minerals formed by metal sulfide oxidizing bacteria**. This can be achieved by using a flow chamber system for the observation of microbes in a controlled, fully hydrated environment under continuous cultivation.

Although nowadays a variety of genome data of metal-oxidizing bacteria exist - by March 2016 157 genomes of acidophiles are available in public data bases (Cardenas et al. 2016) - many of these microorganisms are recalcitrant to genetic manipulation. Therefore, mining the genomic information is a major route allowing insights into metabolic functions of metal-oxidizing bacteria. The best studied genetic model system is *At. ferrooxidans*, amended by genetic predictions of further bioleaching bacteria, e.g. *At. thiooxidans* (Travisany et al. 2014), *At. caldus* (Chen et al. 2012), and more recently "*Ferrovum*" (Ullrich et al. 2016).

Bioinformatic analysis revealed that the *At. ferrooxidans* genome encodes for quorum sensing (QS) cascades (Farah et al. 2005; Rivas et al. 2007) and the formation of EPS (Baretto et al. 2005). In many gram-negative bacteria, EPS production and biofilm formation are regulated by QS systems using acyl homoserine lactones AHLs (Lynch et al. 2002; Marketon et al. 2003). However, **the molecular details of the microbe-minerals interactions and the cascade involved in exopolysaccharide production are still unclear**, even if several reports related to biofilm regulation by acidophilic bacteria have been released. Mineral adhering cells show variations in their gene expression profiles at different time points after attachment as studied in acidophilic filamentous streamers containing *Leptospirillum* ssp. (Moreno-Paz 2010). Within the biofilm community several genes for biofilm formation and maintenance were up-regulated compared planktonic cells, including genes for QS, the peptidoglycan synthesis, and mixed acid fermentation. Due to a specific transcriptomic fingerprint the authors proposed a different behaviour in terms of motility: a "swimming motility" in planktonic and "swarming motility" in biofilm cells. However, the identified genes corresponded to others known to be regulated during biofilm formation in other bacteria. Mamani et al. (2016) gave first insight into QS regulated genes during the initial steps of *At. ferrooxidans* mineral attachment by help of transcriptome microarray assays. Besides the determination of the gene expression levels, functional classification of genes can decipher information on biofilm biogenesis. To get a comprehensive picture of molecular functions and metabolic activity during early and late biofilm formation on minerals, data obtained by transcriptomics need to be linked with protein profiling data (proteomics), which are also limited for metal-oxidizing bacteria. Proteomics on *At. ferrooxidans* primarily reviewed physiological adaptations and stress responses (Varela and Jerez 1992), phosphate starvation (Vera et al. 2003), or adaptations on different energy sources (Bouchal et al. 2006; Ramirez et al. 2004). A more recent shotgun proteomic study on early pyrite colonization has predicted metabolic adaptions occurring within the biofilm subpopulation (Vera et al. 2013). However**, except of the aforementioned studies "-omic" data on metal-oxidizing biofilms are scarce**. To understand the molecular mechanisms of metamorphosis from planktonic to sessile lifestyle, mapping of differentially expressed genes and proteins into pathways is important for understanding the developing biofilm stages. The molecular processes of biofilm formation on sulfide minerals (others than pyrite) and with gram-positive and hetero- or mixotrophic metal-oxidizing bacteria need closer investigation.

The aims of the proposed project are the *in-vivo* imaging of surface attachment and biofilm formation of metal-oxidizing bacteria and investigations of the

underlying molecular mechanisms by comparative genomics, transcriptomics, and proteomics. The model bacterium used for investigations is *Acidibacillus ferrooxidans*, an obligate acidophilic bacterium which, according to recent results, frequently occurs in mining-affected sites word-wide. The genus *Acidibacillus* has been postulated recently by Holanda et al. (2015) and constitutes a distinct firmicute clade well separated from the other acidophilic genera *Alicyclobacilli* and *Sulfobacilli*. *Acidibacilli* have an interesting metabolism, being both dependent on organic C-sources and either iron (*Ab. ferrooxidans*) or sulfur (*Ab. thiooxidans*) as energy source (mixotrophic metabolism). From waters of the Freiberg water drainage system "Hüttenrösche" the isolation of a pure culture of *Ab. ferrooxidans* Huett 2 was successful (Vogel 2016, Schopf et al. 2017). It is deficient in fixing inorganic carbon and requires an organic source, as experimental data and bioinformatics analysis of genome data revealed (unpublished data). Given the novelty of the genus and its metabolic features *Ab. ferrooxidans* Huett2 is an ideal candidate for comprehensive molecular biofilm investigations: **Due to its mixotrophic lifestyle, it produces more biomass** compared to chemolithoautotrophic iron-oxidizers, which are faced with the thermodynamic constraints of energy generation at low pH: Ferrous iron oxidation is a low energy yielding reaction and only oxygen can act as electron acceptor (Hedrich et al. 2011) and additionally, electrons have to be pumped "uphill" by revers electron transfer for the regeneration of reducing equivalents (NADH) for CO_2 fixation.
Preliminary work on mineral colonization by *Ab. ferrooxidans* Huett2 has been carried out with polished sections of the metal sulfides sphalerite, chalcopyrite, chalcocite, and pyrite by epifluorescence microscopy, CLSM, and SEM. The results showed that *Ab. ferrooxidans* Huett2 colonized all sulfide minerals with the tendency to form three-dimensional microcolonies (Schieferbein 2017; Schieferbein et al. 2017).
To get first insights into the pathways of mineral colonization the genome of *Ab. Ferrooxidans* was de-novo sequenced by using the in-house Illumina MiSeq platform (Schopf et al. 2017). The whole genome shotgun project was deposited at GenBank under the accession number MWPS00000000. **Preliminary comparative genome analyses** showed large consensus with other recently published *Ab. ferrooxidans* genome sequences and allowed identification of genes involved in EPS production, chemotaxis, and putative QS signaling by using the Mauve and Artemis genome comparison tools (unpublished data). Whole-genome comparisons with more distantly related genomes will generate more comprehensive data sets serving as basis RNA sequencing and gene expression profiling. For mapping of transcriptome data to various biofilm conditions, proteomics need to be carried out as well.
The applicant has funded know-how on gene expression studies including the isolation of RNA, reverse transcription, and northern blotting (Näther-Schindler et al. 2014 The applicants experience includes enrichment, isolation, and cultivation of iron-oxidizing bacteria, **next generation genome sequencing**, and **bioinformatic analysis of genome raw data sets** (Klink et al. 2016; Eisen et al. 2017; Schopf et al. 2017). The applicant has a proven track record in **monitoring growth and bioleaching performance of iron-oxidizing bacteria** (Giebner et al. 2015, 2017a; Mühling et al. 2016), and in **the visualization of bacterial cells attached onto metal sufides by confocal microscopy using of fluorescent dyes** (Schopf 2016; Schieferbein 2017; Schieferbein et al. 2017; Hüttel 2017). The applicant has also a profound experience in the investigation of surface colonization by extremophilic microorganisms using various microscopic techniques (Schopf et al. 2008; Rachel et al. 2010; Wirth et al. 2011; Weiner et al. 2012; Orell et al. 2017) and in PCR-techniques, gene cloning, protein expression, purification, and identification (Schopf 2011).

2 Project and Habilitation Relevant Publications and Activities

2.1 Publications with Peer Review Published during Funding Period

1. **Eisen N, Straube F, Schopf S, Schlömann M** (2017) Adhesion studies of microorganisms on natural ore material. Solid State Phenomena 262:398-402
2. **Giebner F, Eisen S, Schlömann M, Schopf S** (2017a) Measurements of dissolved oxygen in bioleaching reactors by optode application. J Hydromet 168:64-68
3. **Giebner F, Rolle J, Helmich H, Schlömann M, Schopf S** (2017b) Influence of citrate on metal dissolution and respiration activity of microbial leaching cultures. Solid State Phenomena:262:172-176
4. **Orell A, Schopf S, Randau L, Vera M** (2017) Biofilm lifestyle of thermophile and acidophile archaea. (2017) In: Witzany G (ed.) Biocommunication of Archaea. Springer International Publishing, pp. 133-145
5. **Schieferbein F, Bauer M, Klingl A, Schopf S** (2017) Mineral specific biofilm formation of "*Acidibacillus ferrooxidans*" Huett2. Solid State Phenomena 262: 334-338
6. **Schopf S, Ullrich S, Heine T, Schlömann M** (2017) Draft genome of the heterotrophic iron-oxidizing bacterium "*Acidibacillus ferrooxidans*" strain Huett2, isolated from a mining site drainage ditch in Freiberg, Germany. Genome Announc 5:e00323-17

2.2 Other Publications with Relevance for the Project

1. **Giebner F, Kaschabek S, Schopf S, Schlömann M** (2015) Three adapted methods to quantify biomass and activity of microbial leaching cultures. Min Eng 79:116-125
2. **Giebner F, Rolle J, Helmich H, Schlömann M, Schopf S** (2017b) Influence of citrate on metal dissolution and respiration activity of microbial leaching cultures. Solid State Phenomena:262:172-176
3. **Klink C, Heim J, Daus B, Eisen S, Schlömann M, Schopf S** (2016) Investigation of *Acidithiobacillus ferrooxidans* in pure and mixed-species culture for bioleaching of Theisen sludge from former copper smelting. J Applied Microbiol 120:1520-1530
4. **Mühling M, Schopf S, Tischler D, Schlömann M** (2016) Biohydrometallurgie – ein für Freiberg neues Arbeitsgebiet mit Bedeutung für Metallgewinnung und für die Reinigung von Bergbauwässern. In: Groß U (ed.) Glanzlichter der Forschung an der TU Bergakademie Freiberg 250 Jahre nach ihrer Gründung. Chemnitzerverlag, pp 135-15
5. **Näther-Schinder DJ, Schopf S, Bellack A, Rachel R, Wirth R** (2014) *Pyrococcus furiosus* flagella: biochemical and transcriptional analysis identify the newly detected gene to encode the major flagellin. Front Microbiol 5:695-705
6. **Schopf S** (2016) Untersuchungen der Biofilmbildung des Laugungsbakteriums *Acidibacillus ferrooxidans* auf sulfidischen Mineralen. ACAMONTA – Zeitschrift der Freunde und Förderer der Technischen Universität Bergakademie Freiberg 23:39-41
7. **Schopf S, Wanner G, Rachel R, Wirth R** (2008) An archaeal bi-species biofilm formed by *Pyrococcus furiosus* and *Methanopyrus kandleri*. Arch Microbiol 190:371-377

2.3 Graduate Theses Supervised during Funding Period with Relevance for the Project

1. **Leiser R** (2017) Establishment of reductive bioleaching technologies for iron-rich sludges from waste water treatment. Project thesis, Institute of Biosciences, TU Bergakademie Freiberg (Supervisor S. Schopf)
2. **Hüttel SV** (2017) Establishment of the continous observation of *Acidibacillus ferrooxidans* biofilms in a flow chamber. Bachelor thesis, Institute of Biosciences, TU Bergakademie Freiberg (Supervisor S. Schopf)
3. **Schieferbein F** (2017) Investigation of biofilm formation of *Acidibacillus ferrooxidans* and *Acidithiobacillus ferrooxidans* on various minerals. Master thesis, Institute of Biosciences, TU Bergakademie Freiberg (Supervisor S. Schopf)

2.4 Further Professional Activities during Funding Period with Relevance for Habilitation

Invited Talks/Lectures:

1. Invited speaker at SysMetEx Summer School in Biomining (organized by Uni Duisburg-Essen and TU Freiberg, August 2016) Applications for biomining – an academic point of view.
2. Invited speaker at University of Exeter, Environment and Sustainable Institute and Camborn School of Mines (Falmouth, UK, Februar 2017): In-situ bioleaching for the winning of strategic elements with Indium as example
3. Invited (by DAAD) short term lecturer for “Biotechnology in Mining” at German Mongolian Institute for Resource Technology in Ulaanbaatar, Mongolia (November 2016)

Conference Participation:

1. Annual Conference of the “Vereinigung für Allgemeine und Angewandte Mikrobiologie” (March 2017) in Würzburg
2. Synmiko Symposium “Biofilms in Nature, Technology, and Medicine” (May 2017) in Marburg

Conference Organization:

Member of the local organizing committee of the 22nd International Biohydro-metallurgy Symposium at TU Bergakademie Freiberg (September 2017)

Third party funding proposal:

Funding for a **DFG Research Grant** (Sachbeihilfe) from the individual research grant program is requested with the following modules:

Basic Module

- Funding for research employee with master´s degree and student assistants
- Direct project costs: Consumables and equipment up to €10,000
- Travel expenses and assignments to third parties for analytics

Module Temporary Position

The proposal will finally be submitted by the end of November 2017.

3 Objectives of the Habilitation Project and Time Line for Working Program

3.1 Objectives

The majority of attachment studies have been performed with *Acidithiobacillus ferrooxidans* and some acidophilic Archaea attaching onto pyrite and chalcopyrite. More comprehensive colonization studies using other mineral surfaces and bacteria have rarely been undertaken leading to a lack of understanding the molecular mechanisms behind cell attachment and mineral colonization. Considering those aspects, less investigated metal sulfides are especially interesting for colonization studies. Surface colonization experiments should mainly focus on investigations with sphalerite (ZnS), arsenopyrite (FeAsS), molbydenite (MoS_2), and skutterudite ($CoAs_3$). The project proposal focuses on mineral colonization of the newly described mixotrophic isolate *Ab. ferrooxidans* Huett2. The studies should be carried out both as microscopic imaging studies and as molecular studies by using comparative genomics, transcriptomics, and proteomics.
Ab. ferrooxidans Huett2 can be considered as gram-positive model organism for the investigation of bacterial colonization of minerals because of several remarkable characteristics: Compared to the well-studied chemolithoautotrophic species it i) achieves higher cell densities and produces more biomass for investigation on RNA and protein level, ii) develops, according to preliminary results, three-dimensional microcolonies as prerequisite for multi-layered biofilms instead of monolayers, iii) grows in a continuous flow chamber system for in-vivo imaging of developing mineral colonization; iv) contains, according to first bioinformatic insights, “biofilm genes” in its genome as targets for transcriptome studies; iv) has not been investigated in terms of metal sulfide colonization (neither in imaging based studies nor in molecular studies) and therefore offers great potential for new findings.

The objectives to be achieved in this project comprise:

1. The investigation of *Ab. ferrooxidans* biofilm development and architecture in dependence of the pre-cultivation conditions, mineral composition, and surface topography by imaging techniques and quantification of biofilm growth (**WP 1 and WP 2**).
2. *In-vivo* imaging of mineral colonization over time by use in a continuous running hydrated flow chamber (Fig. 2) by using fluorescent dyes. The dyes are targeted against specific cell components (e.g. sugars in the EPS) in order to investigate their spatially and temporally distribution (**WP 3**). The flow chamber enables live cell imaging of a developing biofilm and at varying cultivation conditions (temperature, O_2-, and CO_2-concentration).

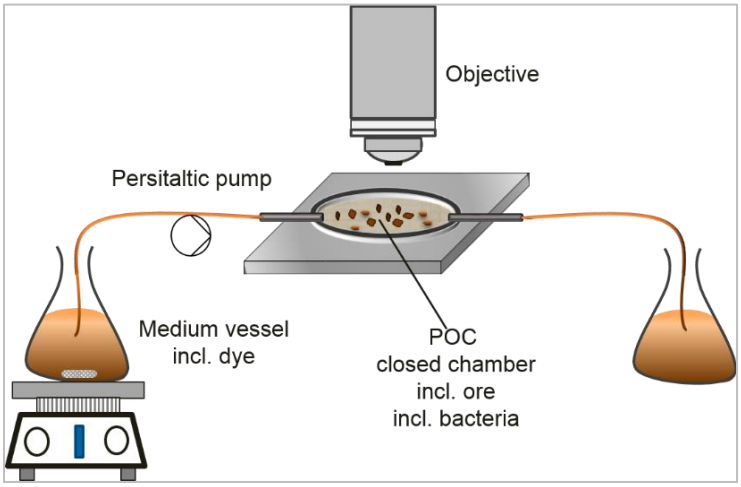

Fig. 2: Schematic drawing of an experimental flow chamber set-up for *in-vivo* observation of biofilm formation on minerals. Minerals can be fixed within the chamber in either as grains or as thick sections.

3. The investigation of the influence of quorum sensing signaling peptides, derived from gram-positive acidophiles, on the development of the biofilm on minerals. Signaling molecules of gram-positives are mainly oligopeptides, which can be cloned and expressed in established bacterial host systems (**WP 4**).

4. Comparative genomics for the identification of genes involved in EPS production, quorum sensing, and biofilm development (**WP 5**).

5. RNA-sequencing for gene expression studies from cells in early colonization state to late mature biofilm state under consideration of various cultivation conditions. The data obtained from gene expression studies will be mapped to the corresponding proteome data (**WP 6**).

3.2 Work program with Time Line

	Time Line [month]											
	1-3	4-6	7-9	10-12	13-15	16-18	19-21	22-24	25-27	28-30	31-33	34-36
WP 1: Visualization Quantification												
WP 2: Pre/Co-colonization												
WP 3: Flow Chamber Experiments												
WP 4: Cloning Application of Signal Peptides												
WP 5: Comparative Genomics												
WP 6: RNA-Sequencing, Transcriptome												
Publications												

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