Max Buchner Fellowship Report

EPS proteomics in biofilms of *Acidithiobacillus ferrooxidans*. "A new approach to elucidate biofilm related functions and electron transfer pathways"

Natascha Teubner[1], Christian Trötschel[2], Sören Bellenberg[1], Wolfgang Sand[1], Ansgar Poetsch[3] and Mario Vera[1].

[1] Biofilm Centre, Aquatische Biotechnologie. Universität Duisburg-Essen, Germany
[2] Plant Biochemistry, Ruhr Universität Bochum, Germany
[3] Max Planck Institut für Lungen und Herz Forschung, Bad Neuheim, Germany

1. Abstract

Acidithiobacillus ferrooxidans species are present in microbial communities involved in bioleaching of metal sulfides, and are widely used in biomining, biotechnological process well established for copper recovery. Biofilm formation and the presence of Extracellular polymeric substances (EPS) mediate an efficient leaching. In this project we have used microscopy and high throughput proteomics in order to characterize the biofilm formation and the EPS proteome *At. ferrooxidans* under different growth conditions.

2. Introduction

Biofilms are surface associated colonies of microorganisms embedded in extracellular polymeric substances (EPS) [1]. In bioleaching bacteria, the electrochemical processes resulting in the dissolution of metal sulfides take place at the interface between the bacterial cell and the metal sulfide surface. In the case of *At. ferrooxidans*, EPS contain iron(III) ions, which allow an electrostatic attachment to the negatively charged pyrite surface, while their second function is the oxidative dissolution of the metal sulfide [2]. In both cases, the electrons extracted from the metal sulfide reduce molecular oxygen via a redox chain connecting both outer and inner membranes [3].

In this bacterium we have shown by Confocal Laser Scanning Microscopy (CLSM) that the induction of EPS polysaccharide biosynthesis occurs simultaneously with the formation of microcolonies on pyrite grains (Fig. 1). Interestingly, EPS biosynthesis of seems to be regulated at several levels [4]. No studies have been done to date in order to characterize EPS proteomes in *Acidithiobacilli.*

3. Tasks and objectives

The main goal on this project is to identify the proteins present in the EPS of biofilms formed by *Acidithiobacillus ferrooxidans* species, in order to further elucidate **i)** their ontribution to biofilm formation on metal sulfides and sulfur surfaces, and **ii)** their potential involvement in electron transport processes occurring during bioleaching.

4. Materials and Methods

Strains and media. The strains *At. ferrooxidans* ATCC 53993 and *At. ferrivorans* SS3 (DSM 17398) were used. Precultivation was done with MAC medium [5] and ferrous iron as energy source in 5-L flasks and air supply as previously described [6]. For cultivation with

pyrite (2 %, w/v) modified MAC medium with 1 mM KH_2PO_4 and 1 mM sodium glucuronate were used. Cultures were grown under orbital shaking at 120 rpm in 1-L Erlenmeyer flasks containing 500 mL MAC medium. For all experiments initial pH was adjusted to 1.8 for *At. ferrooxidans* and 2.5 for *At ferrivorans*. Pyrite grown cultures were inoculated with approximately 5 x 10^8 cells/mL.

Mineral preparation. Pyrite from BRGM, France with grain sizes between 50 and 200 µm were used. The grains were treated as described [4].

Cell staining and fluorescence microscopy. Pyrite grains and sulfur prills were washed with sterile double distilled water (ddH_2O) to remove planktonic cells and medium. Afterwards, the minerals were covered with 6 µM SYTO9® (Invitrogen©, L13152 LIVE/DEAD® BacLight) for 10 min. Then samples were washed two times with ddH_2O and covered with an anti-fading agent (Citifluor, Ltd. AF2) and 22×22-mm cover slides. Samples were examined by confocal laser scanning microscopy (CLSM) with a laser scanning module (LSM 510 Carl Zeiss® Jena) coupled to an Axiovert-100 MBP microscope (Zeiss®). The operating software was LSM 510 release 3.2 (Zeiss®).

EPS extraction. Extraction of tightly bound EPS (tb EPS) was done as described [6] with a cation exchange resin (DOWEX™ Marathon C, Sigma). The dialyzed solutions were freeze-dried and remaining dry EPS were solved in 2 mL of double distilled H_2O.

Protein electrophoresis, digestion and Mass Spectrometry Analysis. Proteins were precipitated with 10% trichloroacetic acid (100%). SDS-PAGE was done as described [7]. Prior to the MS analysis, Gel lines were cut into four pieces and every gel slices was diced into little cubes. Gel cubes were immediately transferred into 50% v/v methanol. Gel pieces were washed 3 to 4 times at 37°C with gel washing solution (NH_4CO_3 0.025 M, ACN 50 %(v/v)) until total destaining. Cubes were dried in an evaporator (SpeedVac, Eppendorf). Following an in-gel digestion was performed with 100 µL trypsin solution (12.5ng/µL in 40 mM NH_4HCO_3, Sequencing Grade modified Trypsin, Promega) over night at 37 °C and orbital shaking. 100 µL of eluent solution (50 % (v/v) acetonitrile, 0.5% (v/v) trifluoroacetic acid (TFA)) were added and incubated for 20 min in an ultrasonic bath. Protein containing supernatant was evaporated (SpeedVac, Eppendorf) in glass vials (Waters, USA) and remaining protins were solved in buffer A (0.1% formic acid (FA) in 2 % acetonitrile (ACN)). ESI-MS/MS analysis was performed on a nanoAcquity UPLC (Waters, USA) coupled with an LTQ Orbitrap Elite (Thermo Fisher Scientific). The peptide separation was obtained by a trap column (Symmetry C18, 5µm particle size, 180 µm × 20 mm (Waters, USA)) and an analytical column (HSS T3, 1.8 µm particle size, 75 µm x 150 mm (Waters, USA)) at a flow rate of 0.4µL/min coupled to a PicoTip emitter (SilicaTip, 10µm, New Objective). Gradient settings: 90-min gradient at 55°C; 0–5 min 98% bufferA (0.1% FA) and 2% buffer B (0.1% FA in ACN), 5–10 min 98–95% A, 10–71 min 95–70% A, 71–76.5 min 70–15% A, 76.5–90 min 15–98% A.

Protein identification. Searching of RAW-files were performed with Proteome Discoverer (1.4.1.14, Thermo Fisher Scientific) using SEQUEST algorithm, with an *At. ferrooxidans* ATCC 53993 (Gene bank ID: NC_011206; containing 2747 protein sequences) and an *At. ferrivorans* SS3 (Gene bank ID: NC_015942; containing 3067 protein sequences) database were accepted. Maximum of two missed cleavages for tryptic peptides were accepted. Methionine was permitted as dynamic modification. Mass tolerance for precursor ions was 8ppm, while fragment mass tolerance was set to 0.6 Da. The presence of

secretion/export signals was analyzed with the software packages LipoP/SecretomeP/SignalP available at http://www.cbs.dtu.dk/services/.

5. Results

An example of biofilm formation on pyrite by both strains is shown in Fig 1. Biofilm development on pyrite surfaces was not homogeneous. After 7 days of biofilm formation EPS was extracted and the EPS proteome fractions were separated and analyzed as described in materials and methods.

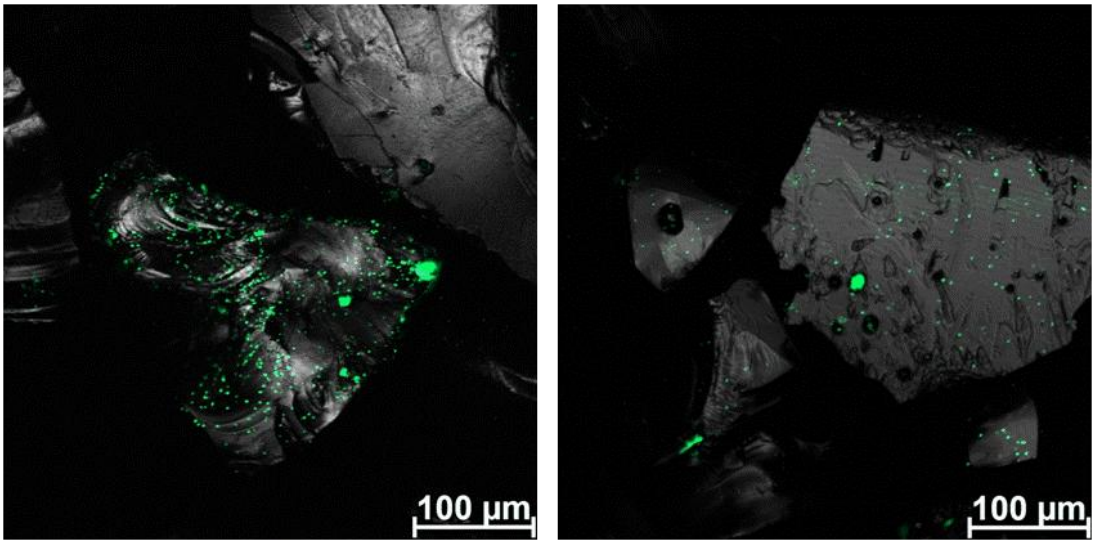

Figure 1. Biofilm formation of *Acidithiobacillus* species on pyrite. Confocal Laser Scanning Microscopy image of A) *At. ferrivorans* and B) *At. ferrooxidans* stained with SYTO®9. Images were taken after 7 days of cultivation.

The EPS proteome analysis for both species after 7 days of biofilm formation showed high protein numbers and diversity (Table 1). In total, 450 and 1238 proteins were found in *At. ferrivorans* and *At. ferrooxidans* EPS, respectively. The proteins were analyzed using clusters of orthologous goups. Several known cytoplasmic proteins as well as proteins involved in metabolic processes such as aminoacid, nucleotide and carbohydrate metabolism were found in EPS. In addition, the presence of housekeeping functions such as translation, transcription and replication suggests a strong presence of cytoplasmic proteins in EPS fractions of both species. Interestingly several proteins found showed presence of export/ secretion signals, , which suggest a periplasmic/extracellular destination.

Currently we are testing: **i)** changes in our EPS extraction protocols, in order to minimize cell breakdown as well as **ii)** to perform EPS extraction and proteome analysis of younger biofilms (i.e. 2-5 days) as well as biofilms of iron oxidizing *acidithiobacilli* on other metal sulfides and sulfur surfaces.

Table 1. Cluster of orthologous (COG) group classification of the proteins found in EPS fractions of 7 days old biofilms of *At. ferrivorans* and *At. ferrooxidans.*

		At. ferrivorans	*At. ferrooxidans*
Total amount of proteins		**450**	**1238**
Proteins found in both biological duplicates		277	849
Clusters of Orthologous Groups of proteins (COGs)			
A	RNA processing and modification	0	1
B	Chromatin Structure and dynamics	0	0
C	Energy production an conversion	27	77
D	Cell cycle control and mitosis	4	14
E	Amino Acid metabolism and transport	41	95
F	Nucleotide metabolism and transport	11	41
G	Carbohydrate metabolism and transport	24	49
H	Coenzyme metabolism	12	51
I	Lipid metabolism	4	21
J	Translation	53	108
K	Transcription	13	29
L	Replication and repair	8	30
M	Cell wall/membrane/envelop biogenesis	19	58
N	Cell motility	3	4
O	Post-translational modification, protein turnover, chaperone functions	19	54
P	Inorganic ion transport and metabolism	8	30
Q	Secondary Structure	5	15
R	General Functional Prediction only	14	73
S	Function Unknown	7	61
T	Signal Transduction	0	20
U	Intracellular trafficing and secretion	5	9
V	Defense mechanisms	0	9
Y	Nuclear structure	0	0
Z	Cytoskeleton	0	0

6. References

1. Flemming, H.-C. and J. Wingender, *The biofilm matrix.* Nature Reviews: Microbiology, 2010. **8**(9): p. 623-633.
2. Schippers, A., Rohwerder, T., and Sand, W., *Intermediary sulfur compounds in pyrite oxidation: implications for bioleaching and biodepyritization of coal.* Applied Microbiology and Biotechnology, 1999. **52**: p. 104-110.
3. Vera, M., A. Schippers, and W. Sand, *Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation-part A.* Appl Microbiol Biotechnol, 2013. **97**(17): p. 7529-7541.
4. Bellenberg, S., et al., *Visualization of capsular polysaccharide induction in Acidithiobacillus ferrooxidans.* Hydrometallurgy, 2012. **129-130**: p. 82-89.
5. Mackintosh, M., *Nitrogen fixation by Thiobacillus ferrooxidans.* Journal of General Microbiology, 1978. **105**: p. 215-218.
6. Bellenberg, S., et al., *Manipulation of pyrite colonization and leaching by iron-oxidizing Acidithiobacillus species.* Appl Microbiol Biotechnol, 2015. **99**(3): p. 1435-1449.
7. Vera, M., et al., *Shotgun proteomics study of early biofilm formation process of Acidithiobacillus ferrooxidans ATCC 23270 on pyrite.* Proteomics, 2013. **13**(7): p. 1133-1144.