

Change of Extracellular Polymeric Substances Composition of *Thiobacillus thioparus* in Presence of Sulfur and Steel

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Abstract

The process of microbially influenced corrosion (MIC) of metallic underground constructions is often related with biofilm formation and metabolic activity of sulfur cycle bacteria. The extracellular polymeric substances (EPS) produced by these bacteria in biofilms on metallic surfaces establish the interfacial reaction space in which relevant corrosion processes occur. The EPS composition of the sulfur oxidizing bacterium *Thiobacillus thioparus* DSM 505 varies according to growth conditions. Presence of elemental sulfur and mild steel are demonstrated here as stimulants for variation of the EPS composition. The distribution of sugar moieties in the EPS of planktonic and biofilm grown cells of *Thiobacillus thioparus* DSM 505 was observed by fluorescently labeled lectin binding assays. The strongest signal was found with the PWM lectin (Pokeweed, *Phytolacca americana*) specific for Poly-N-Acetylglucosamine (PNAG). Cell associated proteins, visualized by Thiazine Red staining, were observed in the planktonic growth mode. Low amounts of EPS proteins from planktonic were detected. The observed influence of elemental sulfur and mild steel on the EPS composition in biofilm forming cells can be suggested to have a pivotal role in interfacial processes such as (bio) corrosion. Understanding factors that mediate changes in the composition and surface associated structural features of EPS as a consequence of working materials may help to develop a new strategy for biocorrosion prevention.

Keywords: Biofilm; Extracellular polymeric substances (EPS); Lectin; Biocorrosion; Biodeterioration; Ferrosphere

Introduction

Bacteria can form biofilms on almost every natural and artificial surface. Biogenic substrata, such as cover tissues of plants, animal skin, other bacteria and abiotic surfaces or interfaces such as surfaces of minerals or phase boundaries of water/air or air/solid materials harbor biofilms [1-4]. Unwanted biofilm formation can cause damages to constructions as well as to industrial plants and transport systems [5]. The process of microbially influenced corrosion (MIC) is an example of biofilm formation where microorganisms become corrosion-relevant [6]. Iron, manganese and sulfur cycle bacteria are often found connected with these corrosion cases since the redox reactions carried out by these microorganisms drive materials deterioration. At metallic gas pipeline constructions, soil bacteria such as *Thiobacillus thioparus* appear in close contact with metal surfaces. This interface between the metal surfaces of the construction in contact with soil is termed ferrosphere [7]. The strictly autotrophic sulfur bacterium *T. thioparus* is a relevant part of a corrosion favoring community, which has been found to be present in the ferrosphere, and has the ability to oxidize sulfur compounds such as elemental sulfur, thiosulfate, thiocyanate and polythionates. These compounds occur in industrial processes such as extraction of sulfur compounds from natural gas or the anaerobic treatment of sulfate-containing waste water [8-10]. During the processing of natural gas the wastewater generated contains up to 3.0 g l⁻¹ thiosulfate [11]. Due to oxidation of reduced sulfur compounds by sulfur oxidizing bacteria elemental sulfur and sulfuric acid are generated. Corrosion relevant sulfate reducing prokaryotes may establish a closed sulfur cycle in microaerophilic and anaerobic niches in the ferrosphere [7]. Acidification in some microniches may promote the development of biofilms of extremely acidophilic bacteria like *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans*.

The role of elemental sulfur (S⁰) in biocorrosion processes is

established [12]. According to the unified electron-transfer hypothesis, elemental sulfur particles immobilized by extracellular polymeric substances (EPS) on the mild steel surface in bacterial biofilms could act as an anode [6,7]. As mentioned [13], EPS localize both, cells and metabolic products of the biofilm, and retain them in close proximity. The EPS components are polysaccharides, proteins, lipopolysaccharides and extracellular DNA. The polysaccharides are often the major fraction of the EPS matrix and fluorescently labeled lectins can be used as probes to investigate carbohydrate distribution within a biofilm structure [13]. Also carboxyl group residues present in the EPS-matrix can complex metal ions. Metal cations are therefore removed from the solubility equilibrium favoring further material dissolution processes [6]. EPS may additionally be corrosive as complexed Fe³⁺ or manganese oxides may act as electron scavengers catalyzing cathodic reactions on metal or mineral surfaces. The importance of EPS in the biocorrosion processes has not been well determined yet, however many aspects suggest their pivotal role for interfacial processes such as (bio) corrosion [6,14,15].

EPS produced by bacteria determine the first step of biofilm formation, the attachment of single cells [16,17]. EPS can be considered

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initially as a part of cell capsules which develop further in the biofilm formation process. Thus, EPS can be associated with surface attached biofilm cells or free-floating planktonic cells.

Structure and composition of EPS depend on many different factors. Structural components of EPS are biopolymers and biogenic minerals such as carbonates and sulfides, elemental sulfur as well as divalent cations such as Ca^{2+} or Mg^{2+} . In the ferrosphere and in case of biofilms of acidophilic leaching bacteria on metal sulfides Fe^{2+} and Fe^{3+} have an important function [6,18].

Due to the complex nature of EPS, there is no universal method for its chemical characterization. The latter also requires the application of different extraction methods regarding the type of EPS (capsular or loosely bound) and the physicochemical characteristics of the biofilm. There are two basic approaches for studying EPS. Chemical and physical extraction and analysis of the polymeric compounds from the bulk solution and cell surfaces or capsules can be distinguished from non-destructive probing of native EPS components. The latter can be analyzed by non invasive microscopical observations using specific probes [19]. Confocal Laser Scanning Microscopy (CLSM) allows the non-destructive three-dimensional investigation of fully hydrated and living structures and has been proven to be a very useful tool for the observation of biofilms and the distribution of EPS and cells on material surfaces [20].

The factors that mediate changes in the composition and distribution of the EPS directly impact material deterioration [18]. The influence of the substratum material itself on these processes may help to understand the role of EPS in MIC and help to develop new strategies for biocorrosion prevention. The objective of this study is the elucidation of the role of biocorrosion-inclined model surfaces, such as elemental sulfur or mild steel, on the EPS composition of *Thiobacillus thioparus* DSM 505 cells in either the planktonic or in the biofilm state.

Materials and Methods

Bacterial strain and growth conditions

Thiobacillus thioparus DSM 505 was cultivated using DSM 36 medium (Deutsche Sammlung von Mikroorganismen und Zellkulturen). 300 mL Erlenmeyer flasks with 100 mL medium were inoculated with 1×10^8 cells/mL and cultivated for 3 d or 10 d at 30°C with 150 rpm shaking.

Substrate preparation

Elemental sulfur as well as mild steel coupons were used as biocorrosion-inclined model surfaces. Elemental sulfur coupons were obtained by melting powdered elemental sulfur (Merck) on glass slide surfaces [21]. Afterwards, they were sterilized by autoclaving for 90 min at 110°C , at 1.5 bar. Mild steel coupons (S235J2G3) were treated by degreasing solution as described [22]. Before inoculation treated for 30 s with 6 N H_2SO_4 and added to *T. thioparus* cultures.

Cell staining procedures and lectin binding assays

Nucleic acids of planktonic and biofilm cells were stained with 0.01 w/v % 4,6-diamidino-2-phenylindole (DAPI, Sigma) containing 2% v/v formaldehyde for 10 min. The carbohydrate moieties present in EPS was visualized using lectins from *Erythrina cristagalli* (ECA), *Canavalia ensiformis* (ConA), *Phytolacca americana* (Pokeweed) (PWN), *Glycine max* (soybean) (SBA) and *Arachis hypogaea* (PNA) (Sigma) labeled with either tetramethylrhodamine isothiocyanate

(TRITC) or fluorescein isothiocyanate (FITC) as shown in Table 1. A solution of 50 $\mu\text{g}/\text{mL}$ of each lectin was applied to the samples and they were incubated for 40 min. Proteins were stained with a solution of 0.1 $\mu\text{g}/\text{mL}$ Thiazine Red R (Sigma) containing 0.1% v/v acetic acid and applied by covering the samples for 30 min. A laser scanning module (LSM 510 Carl Zeiss, Jena) coupled to an inverted Axiovert 100 MBP microscope (Zeiss) was used. All images were obtained with the plan-apochromatic 100X 0.79 oil DIC objective. For prolongation of the fluorescence of dyes the antifading agent CitiFluorTM AF2 was used. The microscope was operated with the software LSM 510 Release 3.2 (Carl Zeiss, Jena).

Preparation of cells for microscopic observation

Planktonic cells were quantified by cell counting with a Thoma chamber. For their microscopic observation 1 mL of culture medium was filtered through a polycarbonate filter (GTTB, diameter 0.25 cm, 0.22 μm pore size, Millipore). Subsequently, cells on the filters were washed, air dried and stained with fluorochrome dyes. After incubation of the coupons in the bacterial culture they were taken out, carefully washed with fresh medium to remove planktonic cells and particles for visualization of the biofilms. Afterwards, coupons were air dried and stained immediately by application of fluorochrome dyes, as described above.

Extraction and analysis of Capsular EPS

Cultures were centrifuged at 7000 rpm for 10 min and cell pellets were diluted with 20 mL of PBS buffer (2 mM Na_2PO_4 , 4 mM NaH_2PO_4 , 9 mM NaCl, 1 mM KCl; pH 7). Extractions were done in PBS buffer for 4 h at 4°C and 100 rpm using equivalent amounts of diluted cell pellets and cation exchange (CER) resin (DOWEXTM Marathon C, Sigma) previously treated with PBS buffer for 1 h. Afterwards the aqueous phase was filtered through a glass filter funnel (Simax) and the filter was washed with 10 mL of PBS buffer to obtain EPS extract trapped within DOWEX beads. This EPS containing extract was centrifuged at 6000 rpm for 5 min, filtered through a 0.2 μm pore size filter membrane and dialyzed against ddH_2O using a 3.5 kDa cutoff membrane tube (Sigma). Contamination of the extract by cells was checked by 2-keto-3-deoxyoctonate analysis indicating cell membrane fragments [23]. The total carbohydrate content was measured using a phenol-sulfuric method according to Dubois [24]. Capsular EPS proteins were precipitated with trichloroacetic acid (TCA) [25] and analyzed by SDS-PAGE (15%), performed according to Laemmle [26].

Verification of EPS detachment after extraction

Verification of detachment of capsular EPS after cationic resin extraction treatment was done by transmission electron microscopy

Lectin	Origin and specification	Fluorescent label
ECA	<i>Erythrina cristagalli</i> lectin Galactose residues, the highest binding activity toward galactosyl (β -1,4)-N-acetylglucosamine	FITC (Sigma)
ConA	<i>Canavalia ensiformis</i> (Jack bean) seeds (β -1,3) glycosidic bonds	TRITC/FITC (Sigma)
PWM	<i>Phytolacca americana</i> (Pokeweed) N-acetyl- β -D-glucosamine oligomers	FITC (Sigma)
SBA	<i>Glycine max</i> (soybean) seeds terminal α - or β -linked N-acetylglactosamine	FITC (Sigma)
PNA	<i>Arachis hypogaea</i> specific for terminal β -galactose, galactosyl (β -1,3) N-acetylglactosamine	TRITC (Sigma)

Table 1: Specificity of lectins.

(TEM). The floating drop method [27] was used with modifications for sample preparation. 1 mL of cell suspension before and after CER resin treatment was centrifuged (5 s, 2000 rpm) to remove sulfur particles. Subsequently, 10 μ L drops were placed on Parafilm[®] sheets. Formvar[®] copper coated grids were put on these drops. Cells were allowed to adhere for 10 min; afterwards the grids were air-dried. Samples were observed using a TEM (Jeol 1400, Japan) at an accelerating voltage of 80 kV. For each sample 20 images were acquired.

Results and Discussion

Two experimental *Corrosion scenarios* were assayed using elemental sulfur (I) and mild steel surfaces (II). Their EPS properties were compared to features of cells and EPS from cells cultivated without these material surfaces. The ability of cells of *T. thioparus* DSM 505 to modify the composition of EPS in the planktonic and the biofilm state was analyzed.

Corrosion scenario I (elemental sulfur coupon surface)

Visualization of EPS from biofilms on the elemental sulfur coupon surface showed cell surface polysaccharide binding with three lectins on 3 d and 10 d after inoculation. PWM, ECA and SBA-lectin signals were observed indicating the presence of poly-N-acetyl-glucosamine (PNAG) residues, galactosyl (β -1,4)-poly-N-acetylglucosamine and terminal α - or β -linked N-acetyl galactosamine residues, respectively (Figure 1a). In contrast to sessile cells, planktonic cells cultivated for a period of 3 d in the presence of elemental sulfur coupons produced EPS, which reacted only with the PWM lectin (Figure 1b) with comparable signal intensity 10 d after inoculation. The diversity of carbohydrate moieties present in biofilm-derived EPS, which are not present in EPS of planktonic cells, including the presence of galactose in biofilm EPS is in agreement with previous data obtained by a GC-MS method [15].

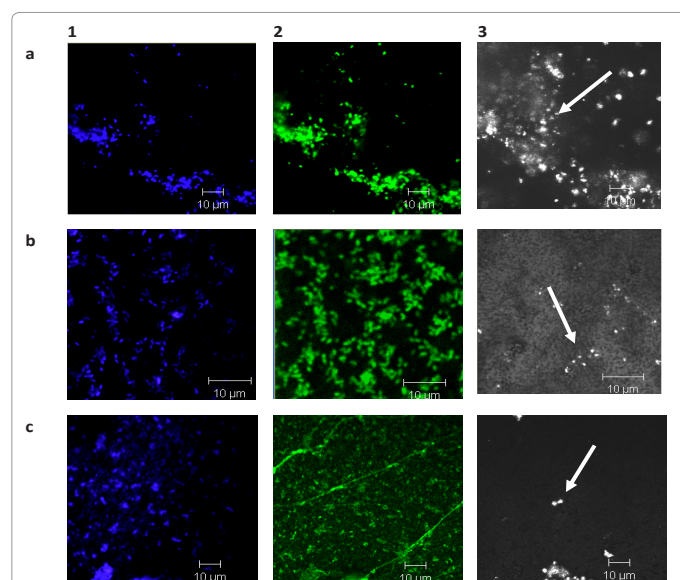


Figure 1: PWM-lectin reveals presence of poly-N-acetyl-glucosamine (PNAG) moieties in EPS of *Thiobacillus thioparus* DSM 505 after growth in presence of sulfur or steel surfaces. Cells were grown at 28°C with shaking at 120 rpm. A biofilm structure formed on a sulfur coupon after 3 d (a), planktonic cells from the same culture filtered on polycarbonate filters (b) and a biofilm structure formed on a mild steel surface after 10 d (c) are shown. Fluorescence signal of the nucleic acid specific fluorochrome DAPI (1) is shown separately from FITC-labeled PWM lectin signal (2) and surface reflection signal (3) of sulfur particles (arrows). Size bars represent 10 μ m.

Thus, applied lectins revealed differences in the EPS compositions corresponding with alternative growth modes of *T. thioparus*.

Corrosion scenario II (mild steel coupon surface)

EPS of biofilms on mild steel surfaces showed binding only of the PWM and SBA lectins after 3 d and 10 d of cultivation. Structural features visible as strands of EPS from biofilms on the steel surface rich in PNAG were observed after 10 d of cultivation using the PWM lectin (Figure 1c). PNAG is a major factor of adhesion to hydrophobic polystyrene surfaces by *Aggregatibacter actinomycetemcomitans* [28]. Thus, we can suggest a similar attachment mechanism that determines adhesion to hydrophobic sulfur particles in soil. The EPS of planktonic cells cultivated for a period of 3 d and 10 d in the presence of steel coupons could only be stained and visualized with the PWM-lectin.

It should be noted that in the presence of elemental sulfur the synthesis of galactosyl (β -1,4)-poly-N-acetylglucosamine was stimulated. Obviously, this is related with a metabolic change of *T. thioparus* in the presence of elemental sulfur surfaces.

Control scenario (absence of sulfur or steel surface)

Planktonic cells cultivated without any additional materials surface showed the presence of glucose or mannose residues in the EPS as revealed by the lectin ConA binding to β -1,3 glycosidic bonds in mannose and glucose rich capsule material (Figure 2). Binding of this lectin was not observed in case of planktonic or biofilm cells in model corrosion scenarios. This finding suggests specific modifications of the EPS polysaccharide composition in the presence of iron ions or sulfur compounds. In our *Control scenario* the process of thiosulfate oxidation provides energy for growth and the carbohydrate expression seems to be regulated in a different way in absence of a solid substratum. PWM-, ECA- and SBA-lectin binding signals were not detectable for capsule polysaccharides of such planktonic cells.

The PNA lectin, which binds specifically to terminal β -galactose or galactosyl (β -1,3) N-acetylgalactosamine did not bind to the EPS under any of the described conditions. This strongly indicates a lack of this sugar moiety in the EPS produced by *T. thioparus* DSM 505 under these conditions.

It is shown that corrosive metabolic activity influences the EPS composition, which can be observed after lectin staining of biofilms grown on sulfur and steel surfaces. Thus, the presence of elemental sulfur can stimulate the capsular EPS and PNAG production in *T. thioparus* DSM 505. The regulatory mechanisms presumably exerted by soluble corrosion products affecting the compositional modification of EPS remain to be elucidated in detail. However, this switch may play an important role in subsequent attachment of planktonic cells to surfaces, presumably promoting corrosion relevant reactions on the material surface.

EPS compounds belong to different classes of macromolecules [16,29,30]. According to these authors, the microbial attachment to solid surfaces is affected by the protein structure and the molecular interactions between conjugative pili and surfaces. Elimination of the outer protein layer from cells caused significant (even 100 times) reduction of *Streptococcus* spp. and *Bacillus* spp. adhesion to the stainless steel surfaces. For observing proteins produced by *T. thioparus* DSM 505 Thiazine Red staining was used. The protein distribution was visualized with planktonic cells cultivated in the presence of mild steel and elemental sulfur (Figure 3).

As capsular exopolymers of planktonic cells are determining the initial attachment stage of biofilm formation [31], these compounds were examined more closely by extraction and chemical analysis. The effectiveness of extraction of such capsular material was visualized by electron microscopy (Figure 4). Microscopic observation revealed a capsule surrounding planktonic cells. After treatment with CER the extracellular capsule layer was obviously removed from the cells. This treatment was therefore considered sufficient for detachment of capsular material from cells and suitable for its subsequent chemical analysis.

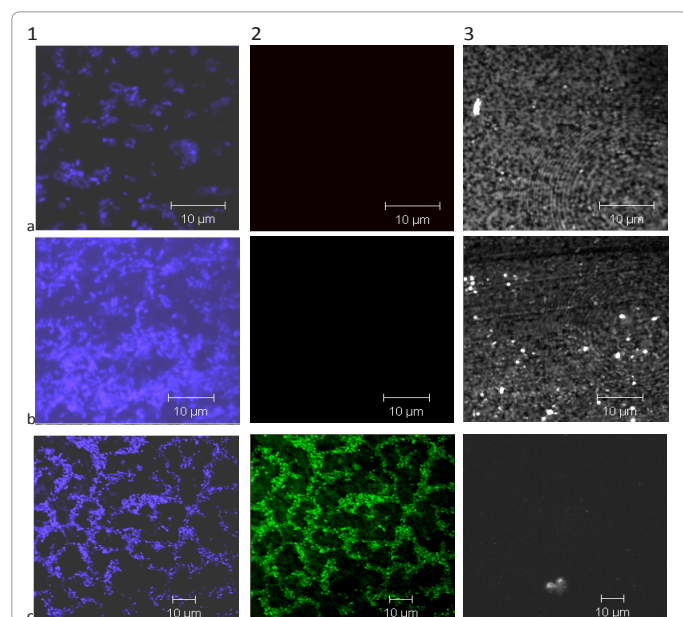


Figure 2: ConA lectin does not bind to cells of *Thiobacillus thioeparus* DSM 505 grown in presence of mild steel or elemental sulfur. Cells were grown at 28°C with shaking at 120 rpm. Planktonic cells cultivated for 10 days in presence of mild steel (a), elemental sulfur (b) and planktonic cells incubated without steel or sulfur coupons (c) are shown. Cells were filtered on a polycarbonate membrane and stained with the nucleic acid specific fluorochrome DAPI (1) and FITC-labeled ConA lectin (2). Surface light reflection signal is shown in (3). Size bars represent 10 µm.

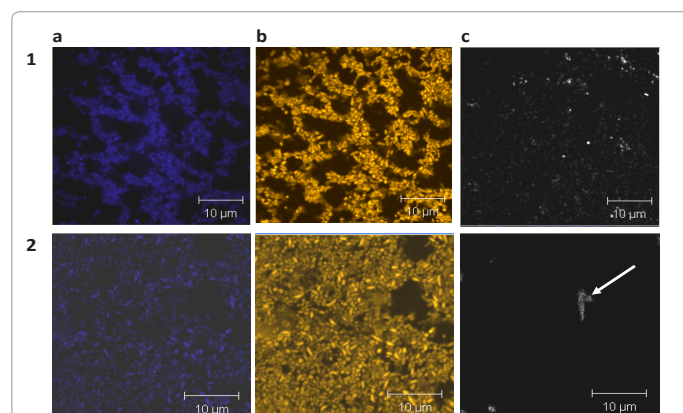


Figure 3: Protein visualization of planktonic cells of *Thiobacillus thioeparus* DSM 505 in presence of mild steel (1) and elemental sulfur coupons (2) after 3 d. Planktonic cells were transferred on a polycarbonate filter (0.22 µm poresize) and subsequently stained with the nucleic acid specific dye DAPI (a) and the protein stain Thiazine Red (b). Reflection signal from e.g. sulfur particles (arrow) are shown in c. Size bars represent 10 µm.

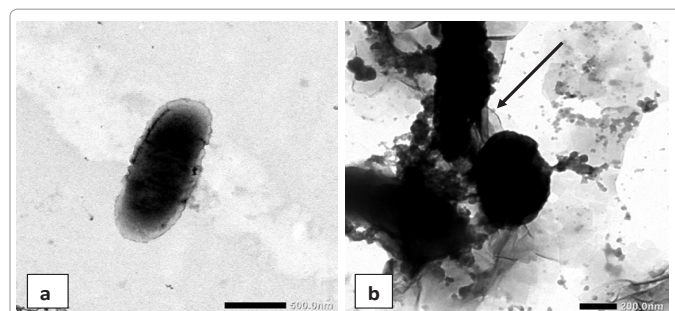


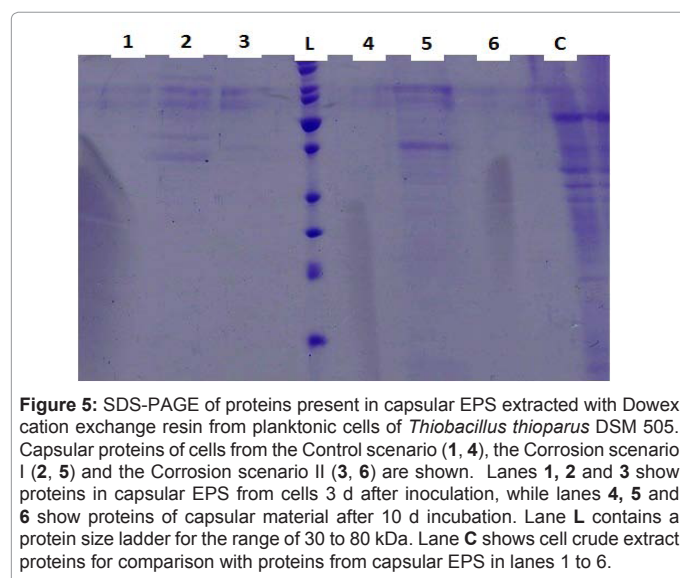
Figure 4: Removal of capsular exopolymeric substances from planktonic cells of *Thiobacillus thioeparus* DSM 505. 100 µL drop of sample was applied to the copper grid with Formvar® layer (A). The removal of the capsular polymer layer was demonstrated after cationic exchange resin treatment (B) by TEM.

The carbohydrate amount of capsular EPS from planktonic cells cultivated in the presence of different surfaces was analyzed (Table 2). The maximum amount of total carbohydrates (174 µg/10⁸ cells) in capsular EPS 3 d after inoculation was detected in the Control scenario, where reactive steel or sulfur surfaces were absent. According to our previous data [32], which correlated with lectin binding tests, such kind of polysaccharides as a major fraction of the EPS consisted mostly of glucose (34% from total neutral carbohydrates). Planktonic cells in the Control scenario and Corrosion scenario I on the 3rd and 10th day after inoculation were equal (2×10⁸ cells/mL). This may be explained by a presence of elemental sulfur as it may occur as an intermediate during thiosulfate oxidation. In the same time the amount of planktonic cells in the Corrosion scenario II significantly declined (from 0.5 ± 0.1×10⁸ to 0.1 ± 0.2×10⁸ cells / mL). This could be connected with metal surface colonization and active solubilization of metal ions, which would correlate with our previous data [22]. The highest amount of total carbohydrates in the capsular EPS in this sample after 10 d of cultivation could be related with a chemotaxis process. The cells were attaching efficiently to the steel coupon. The attraction due to solubilized metals from the steel surface is presumably followed by a synthesis of a capsule containing required adhesins and metal complexing carboxyl group residues [6]. Electrostatic interactions mediated by complexed metal ions in EPS is a described initial attachment mechanism [6,16].

Extracellular secreted proteins with molecular masses between 10 and 200 kDa contain 40 to 60% of hydrophobic amino acids [33]. For determining the molecular weight of the proteins in the capsular EPS of *T. thioeparus* DSM 505 SDS-PAGE was used. Medium weight proteins of 60 to 80 kDa were detected in EPS extracts from planktonic cells (Figure 5). Two distinct bands of proteins were detected in capsular EPS from planktonic cells originating from the Control scenario, detected by SDS-PAGE, after 3 and 10 days. Besides of increasing planktonic cell numbers during the cultivation period the amount of protein in the SDS-PAGE gel after 10 days of cultivation did not differ from those observed after 3 days. In presence of elemental sulfur coupon surfaces in the Corrosion scenario I higher amounts and a more complex pattern of EPS proteins compared to those observed in the Control scenario were detected. Also with the proceeding cultivation period after 10 days the pattern differed from the one observed after 3 days. Hence, an increase in the planktonic cell number correlated with higher amounts of protein after 10 d. Further investigations concerning these protein bands and the planktonic and the biofilm cell subpopulations will presumably provide further insights in the

Type of surface	Cells number during experiment ($\times 10^8$)	Total carbohydrates in capsular EPS ($\mu\text{g} / 10^8$ cells)
3 d after inoculation		
Corrosion scenario I (sulfur)	1,06 \pm 0,23	148
Corrosion scenario II (steel)	0,54 \pm 0,12	164
Control scenario (without of surface)	0,73 \pm 0,08	174
10 d after inoculation		
Corrosion scenario I (sulfur)	2,21 \pm 0,14	142
Corrosion scenario II (steel)	0,14 \pm 0,2	155
Control scenario (without of surface)	2,03 \pm 0,18	136

Table 2: Characterization of capsular exopolymeric substances of *Thiobacillus thioparus* DSM 505 in planktonic cells.



molecular processes that mediate attachment to elemental sulfur. We can suggest that presumably due to attachment and biofilm formation those cells remain in the free-swimming, planktonic state which lack effectual proteinaceous capsular material that mediates attachment to hydrophobic substrata such as elemental sulfur. In general, low amounts of EPS proteins were detected in the Control scenario, were a reactive surface, such as elemental sulfur or mild steel was not present. Analogously, in the Corrosion scenario II (mild steel) low amounts of proteins were detected and a decrement of the planktonic cell number was attended by a decrease of the amounts of capsular proteins observed after 3 and 10 d, respectively. The variety and the abundance of capsular proteins detected by SDS-Page in presence of elemental sulfur is significantly different from those observed in the other assays. These proteins will be studied further to determine their annotated functions and to relate their abundance. Further on these functions can be related to processes such as attachment and electron transfer, to elucidate their role in the biocorrosion process. The latter of special importance will be those EPS proteins which are expressed in the biofilm cell subpopulation.

Concluding Remarks

In this study the influence of steel and elemental sulfur surfaces on the EPS composition of *T. thioparus* is shown. Sulfur compounds present in soil habitats and in the ferrosphere are hydrogen sulfide, polysulfides, elemental sulfur and sulfate [34]. These compounds are formed due to metabolic activity of sulfur cycle bacteria. The *Thiobacillus/Acidithiobacillus* genera are widespread in soil, rock, mine and water econiches and according to [7], geochemical activity of these thionic bacteria is closely linked with sulfur generation during the oxidation of biogenic hydrogen sulfide. The latter is produced by sulfate reducing bacteria. The contribution of sulfur cycle bacteria to the corrosion of underground constructions has been shown already in the 1930s by C.H. Kühr and L.S. van Der Vlugt [7]. The corrosive activity of elemental sulfur is established in corrosion research and its influence on biofilms of *T. thioparus* and their EPS properties has been addressed in this study. However, not only elemental sulfur, but also generation of sulfuric acid is responsible for the corrosive metabolic activity of *T. thioparus*. As mentioned [8], sulfuric acid increases the solubility of metal ions and other corrosion products. These corrosion products, such as iron ions seem to mediate a specific modification of the capsular EPS composition, enabling cells to attach efficiently to metallic surfaces. Structural features rich in PNAG residues in biofilms on steel could be visualized and their presence suggests a specific function in the biocorrosion process, as these structures and the sugar moieties were not observed in biofilms on elemental sulfur. Thus, our comparative corrosive scenario model allows us to elucidate interrelationships between biominerals such as elemental sulfur and biopolymer compounds in EPS, which are present in the ferrosphere near pipelines or other metal constructions. Our results indicate that the EPS composition is changing after exposure of cells to elemental sulfur or to mild steel surfaces. A more close investigation in the future regarding sulfur content and a determination of the soil aggressiveness regarding underground metal constructions is suggested. Thus biogenic sulfur and metal ions released during initial corrosion processes can be suggested to act as an intrinsic stimulant of EPS matrix modification of corrosion-relevant bacteria.

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